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# Water Toxicity Monitoring using Optical Oxygen Sensing and Respirometry

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## 1. Introduction

Approximately one third of available freshwater is currently used for agricultural, industrial or domestic purposes. This results in contamination of the water with a wide range of pollutants originating from ~300 million tons of compounds used in industrial and consumer products, ~140 million tons of fertilizers, several million tons of pesticides, 0.4 million tons from oil and gasoline spillages (1). To tackle the emerging threat of contamination and depletion of freshwater stocks, large initiatives such as the EU Water Framework Directive (WFD) (2) have been established. The WFD is concerned with “scope of water protection to include all waters, to set clear objectives in order that a “good status” be achieved.”

Successful realization of such projects, and of the other environmental monitoring tasks, is linked to the availability of techniques for detailed toxicological assessment, screening and monitoring of large number of chemical and environmental samples, plus validation and wide deployment of such techniques.

Conventional toxicity tests with higher animal models such as rodents or primates based on the determination of lethal doses of toxicants (3) have limited use, due to their ethical constraints, low speed and high costs. Other systems include bioluminescent test for the presence of toxic compounds using freeze dried luminescent bacteria *Vibrio fischeri* (formerly called *Photobacterium phosphoreum* (4)) found in the marine environments (5) and functioning via an endogenous flavin monooxygenase enzyme luciferase. *V. fischeri* provided the basis for several commercial kits such as Microtox® (Azur Environmental, Carlsbad, CA), Mutatox® (with dark mutant of *V. fischeri*) (6), Deltatox® (portable, without temperature control), which have been extensively validated (7, 8) and accepted as a standard method by International Standard Organization (ISO) (9). Although providing good sensitivity, short assay time and simplicity, these tests are limited to just one strain of simple prokaryotic test organism and to samples that do not interfere with luminescent measurements. Samples that are turbid, absorb light or quench luminescent reaction can interfere the assay and cause measurement problems and invalid results.

The need to find alternatives to expensive, space, time and labour consuming toxicity tests using aquatic and terrestrial species has led to the development of alternative methods. Thus, ethical (10) and regulatory issues (11) are favouring the use of animal models such as bacteria (12), small vertebrates (zebrafish *Danio rerio*) (13), invertebrates (the fruit fly *Drosophila melanogaster* (14), and brine shrimp *Artemia salina* (15). Daphnids, particularly *D.*

*magna*, show widespread occurrence, ecological significance (broad distribution and important link in pelagic food chains), parthenogenetic reproduction, short life cycle and sensitivity to a broad range of chemicals and environmental pollutants. As a result, daphnids are regarded as general representative of freshwater zooplankton species (16). Due to the ease of laboratory culture, discrete growth, small size, high fecundity, low cost and minimal equipment required for bioassays, they have been accepted as standard invertebrates for aquatic toxicologists for testing chemicals (17, 18), surface water and effluents (19) (for example standard EPA toxicity test using *D. magna* (20)). Rapid tests for acute toxicity have been described based on the assessment of immobilization (or mortality) of *D. magna* (17), however they show reduced sensitivity.

*Danio rerio* (zebrafish) is another widely used test organism which relates to vertebrate animals. Zebrafish embryos are transparent and develop externally. During early phases of development they readily absorb chemicals, thus permitting the *in vivo* assessment of toxic effects of the latter on internal organs and tissues (21). The fish is easy to maintain and breed, its fecundity is high (each female can produce 100 - 200 eggs per mating) providing large numbers of animals for high throughput screening (HTS) applications (21). Small size makes zebrafish one of the few vertebrates that can be analysed in 96- or even 384-well plates, which is essential for HTS of compound libraries (21). Application of potential toxins and drugs to zebrafish is simple: through skin and gills by simply diluting low molecular weight compounds in the surrounding media, or highly hydrophilic compounds can be injected directly into the embryos. Again, most of toxicity tests using zebrafish (and *D. magna*) rely on simple mortality assessment ( $LD_{50}$ ), thus being subjective, prone to false-positives and providing limited information and specificity. They are not very adequate for predicting toxic effects in humans and higher animals.

Monitoring the rate of oxygen consumption - a sensitive metabolic biomarker of aerobic organisms - has high potential for toxicity testing. Early respirometric studies with daphnids employed Strathkelvin respirometer (22), calibrated oxygen electrode in BOD bottles (23) or in a through-flow system (24), or chemical Winkler method (25) where the amount of dissolved oxygen reflects the biological activity of water masses. However, these techniques are rather labour-intensive and slow, require high numbers of test organisms, and have limited sample throughput. In contrast, optical oxygen respirometry employs a fluorescence/phosphorescence based oxygen sensing probe - a soluble reagent which is added to the sample (26). Probe fluorescence is quenched (reversibly) by dissolved oxygen, and depletion of the latter due to animal respiration causes an increase in probe signal, thus allowing continuous monitoring and real-time quantification of dissolved oxygen. Fluorescent signal of the probe relates to oxygen concentration as (27):  $[O_2] = (I_0 - I) / I \cdot K_{s-v}$ , where  $I_0$  and  $I$  emission intensities of the oxygen probe in the absence and presence of oxygen concentration  $[O_2]$ , and  $K_{s-v}$  = Stern-Volmer quenching constant.

Measurement of probe signal in respiring samples on a fluorescence reader allows monitoring of oxygen concentration, e.g. in a standard 96 well plate (WP). From these data, respiration rates can be obtained for each sample, and changes in animal respiration (fold-increase or decrease relative to the untreated organisms) determined, thus reflecting the effect of the toxicant on the metabolism. This approach has been demonstrated with different prokaryotic and eukaryotic cell cultures and model animals including *Artemia salina* (brine shrimp) *Danio rerio*, *C.elegans* (28),(26). Optical micro-respirometry provides simple, high throughput toxicity testing of various compounds and their effects on test organisms.

In this study, we describe the application of optical oxygen micro-respirometry to the assessment of toxicity of chemical and environmental samples, using *V. fischeri* (prokaryote), *D. magna* (invertebrate), and *Danio rerio* (vertebrate) as test organisms. Representative toxicants were heavy metal ions, organic solvents, marine toxins microcystins (MCs) and WWS. The marine toxin microcystin-LR relates to a group of cyclic heptapeptides produced by cyanobacterial species such as *Microcystis aeruginosa*. MCs are associated with poisoning of animals and humans during cyanobacterial and algal blooms (29). Due to their widespread distribution, high toxicity and threat to public health, MC levels have become an important parameter in water quality control, environmental monitoring and toxicology. A deeper understanding of the toxic action of MC on cells and higher organisms and development of techniques for their detection in environmental samples are important for ecotoxicology. We describe new methods of analysis of environmental samples for MC-LR type of toxicity using optical oxygen micro-respirometry and *Danio rerio* as test organisms. These tests were subsequently validated with a panel of contaminated water samples. The toxicants were examined for their dose-, time- and organism-dependent patterns of response emanating from such respirometric experiments performed in a simple and convenient 96 WP format. This was aimed to achieve a more detailed toxicological assessment and profiling have a deeper insight into the modes of toxicity.

## 2. Materials and methods

### 2.1 Materials

Phosphorescent oxygen sensing probe, MitoXpress™ (excitable at 340-400 nm and emitting at 630-690 nm (30)) and sealing oil were obtained from Luxcel Biosciences (Cork, Ireland). Analytical grade  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{CdCl}_2$ ,  $\text{K}_2\text{Cr}_2\text{O}_7$ , sodium lauryl sulfate (SLS), DMSO and MC-LR were from Sigma-Aldrich (Ireland). Solutions of chemicals were prepared using Millipore grade water. The components for nutrient broth medium were supplied from Sigma-Aldrich (Ireland). Standard flat bottom 96 WP and 384 WP were made from clear polystyrene were from Sarstedt (Ireland). The low-volume sealable 96-well plates, type MPU96-U1 were from Luxcel Biosciences (Ireland).

The gram-negative marine luminescent bacterium *V. fischeri* (strain NRRLB-11177, freeze-dried), reconstitution solution (ultrapure water) and diluents (2% NaCl solution to provide osmotic protection for the organism) were obtained from Strategic Biosolutions (USA). *D. magna* stock was collected from continuous culture at the Shannon Aquatic Toxicology Laboratory (Shannon, Co. Clare, Ireland). *Danio rerio* were obtained from Murray Aquatics, UK.

Effluent samples collected from different sites (EPA license classification) were obtained from the Shannon Aquatic Toxicology Lab. Samples of drinking water contaminated with MCs from reservoirs, lakes, fish ponds (more than 300 samples from over 100 localities) were collected during 2007 summer season within the National monitoring program on toxic cyanobacteria, Czech Republic (31).

#### *V. fischeri* culture and exposure to toxicants

The lyophilized bacteria were rehydrated in 10 mL and then cultivated in nutrient medium containing: NaCl (23 g),  $\text{Na}_2\text{HPO}_4$  (15.5 g), nutrient broth 2 (10 g),  $\text{NaH}_2\text{PO}_4$  (2 g), glycerol per 1 L deionised water (32). 100 mL cultures were grown in 500 mL flask at room temperature (20°C) and shaken at 200 rpm after inoculation with 1 mL of *V. fischeri* culture. Bacteria proliferation was monitored by measuring the increase of optical density in the culture

suspension at 600 nm ( $OD_{600}$ ). When the culture reached  $OD_{600} \sim 0.5$ , it was used in toxicity assays. Cells were enumerated by light microscopy using standard Neubauer haemocytometer (Assistant) and light microscope Alphaphot-2 YS2 (Nikon). Stock of bacteria was used in the experiments at different dilutions or stored at  $+4^{\circ}\text{C}$  for up to 1 week.

In a toxicity assay, 135  $\mu\text{L}$  of *V.fischeri* in nutrient broth containing 0.1  $\mu\text{M}$  of MitoXpress™ probe were pipetted directly into the wells of standard 96 WP, and 15  $\mu\text{L}$  of toxicant stock were added to each well to give the desired final concentration. Each concentration of the toxicant was prepared and analysed in 4 replicates on the 96 WP. For the 24 h incubation, 9 mL of LB inoculated with bacteria were added to 50 mL reagent tubes (Sarstedt) containing 1 mL of test compound at the required concentration, and incubated at  $30^{\circ}\text{C}$ . After incubation, samples were diluted to a concentration of  $10^6$  cells/mL, mixed with the oxygen sensitive probe and transferred in 150  $\mu\text{L}$  aliquots to the 96 WP. In the 1 h incubation assay, 135  $\mu\text{L}$  of *V.fischeri* in LB broth ( $10^6$  cell/mL) containing 100 nM of the oxygen probe were pipetted directly in the wells of standard 96 WP, and 15  $\mu\text{L}$  of toxicant stock were added to each well to give the required concentration.

#### ***D.magna* culture and exposure to toxicants/effluents**

*D.magna* was maintained in continuous culture under semi-static conditions at  $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$  in 1 L beakers in de-chlorinated water, using 16h light/18h dark photoperiod and a density of 20 adults per litre. Dilution water (total hardness  $250 \pm 25$  mg/L ( $\text{CaCO}_3$ ), pH  $7.8 \pm 0.2$ , Ca/Mg molar ratio of about 4:1 and dissolved oxygen concentration of above 7 mg/L (33)) was used as both culture and test medium. It 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). The algal culture was cultivated continuously using freshwater Algal culture medium (34). 3-weeks old offsprings of *D.magna* were separated from cultures at regular intervals and used for the production of juveniles ( $\leq 24$  h), which were then used in toxicity tests.

For acute toxicity testing, 20 juveniles ( $\leq 24$  h) were randomly selected and placed in 50 mL glass beakers or plastic tubes (Sarstedt) containing 40 mL of de-chlorinated (fresh) water with different concentrations of toxicants/effluents and without (untreated controls). As in the standard test (33), *D.magna* were not fed during the incubation. Following 24h or 48h incubation, individual organisms were transferred by Pasteur pipette into microplate wells containing medium and the toxicant.

Effluent samples were initially analyzed undiluted using 24 h exposure and a procedure similar to the chemicals (see above). Subsequently, highly toxic samples were analyzed at several different dilutions. In parallel with respirometric measurements, standard toxicity tests (33) were also conducted to determine the percentage of *D.magna*, which become immobilized after the exposure to different effluent concentrations. Corresponding  $EC_{50-24}$  h values were calculated and compared with the respirometric values.

#### ***Danio rerio* culture and exposure to toxicants/effluents**

*Danio rerio* were raised and kept in a 10 L freshwater tank at  $28^{\circ}\text{C}$ , on a 14 h light/10 h dark photoperiod (35). *Danio rerio* were fed daily with live *Artemia nauplii* and Tropical Flake® food. Spawning and fertilization of unexposed parent fish was stimulated by the onset of first light. Marbles were used to cover the bottom of the spawning tank to protect newly laid eggs and facilitate their retrieval for study. Fertilized eggs were collected from the bottom of the tank by siphoning with disposable pipette, transferred into a 6-well plate (Sarstedt) with



5 mL of water and kept at 28 °C (for 48 h). For toxicity assays, hatched *Danio rerio* (48 hpf) (36) were transferred into the wells of 6 WP containing 5mL of water to which toxicants and oxygen probe were added at the required concentrations. Following incubation (1 or 24 h), individual animals were transferred into wells of a low-volume 96-well plate (Luxcel Biosciences) - one animal in 10 µL of assay medium per well. The plate was then sealed and analyzed in the same way as described above for *D.magna*.

### Respirometric Measurements

The MitoXpress™ probe was reconstituted in 1 mL of MilliQ water to give 1 µM stock. This probe stock was added to the media used in the corresponding toxicity assay at the following working concentrations: 0.1 µM for the 96WP and 0.5 µM for Luxcel plates. Respirometric measurements with *D. magna* and *Danio rerio* were conducted in low-volume sealable Luxcel plates using sample volume 10 µL, and with cells - in 96WP using sample volume 150 µL. Optical measurements were carried out on a fluorescence 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* were transferred with a Pasteur pipette into each assay well containing medium with probe. To initiate the respirometric assay, samples were sealed with adhesive tape in Luxcel plates or with mineral oil in 96 or 384 WP (100 µL or 40 µL per well). The plate was then placed in the fluorescent reader set at 25 °C (for *D. magna*) or at 30°C (for *V. fischeri*) and measured in kinetic mode.

For animal based assays fluorescent readings in each assay well were taken every 2 min over 0.5-2 h. Measured time profiles of probe fluorescence for each sample were used to determine changes in respiration for each samples relative to control (wells with untreated test organisms). For that, the initial slopes of probe fluorescent signal, which reflects oxygen consumption rate, were calculated for each well and normalized for their initial intensity signal. These slopes were compared to those of the untreated organisms (positive controls, 100 % respiration) and to those without organisms (negative controls, 0 % respiration). Relative changes in animal respiration and EC<sub>50</sub> values for the toxicants were determined using sigmoidal fits with logged data fit function as logistic dose response and error bars weighting in OriginPro 7.5G software. 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 4 (*V.fischeri*) or 8 (*D.magna*, *Danio rerio*) repeats, and each experiment was repeated 2-3 times to ensure consistent results. Concentrations which caused significant change in respiration, (Cmin) were identified by T test with confidence limits of >99 %.

For the *V.fischeri* assay, readings were taken every 10 minutes over 12 h. Calibration curve for *V.fischeri* was produced by plotting the time required to reach threshold intensity versus seeding density of *V.fischeri* in range from 10-10<sup>8</sup> cell/mL. Threshold intensity was defined as half maximum signal reached by an average respiration-growth profile (37). Calibration was used to determine the reasonable concentration of *V.fischeri* used in toxicity test afterwards.

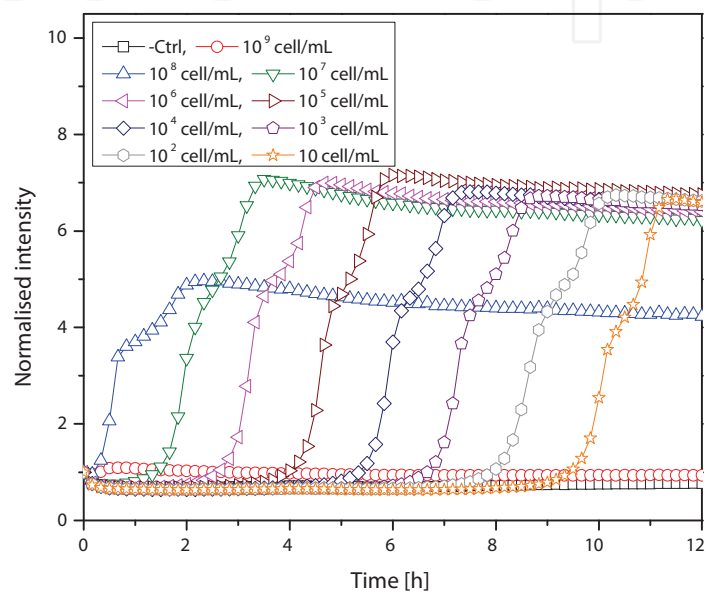
### Optical Density (OD<sub>600</sub>) Analysis of *V.fischeri*

Measurement setup was the same as for the respirometric assay (see above), but no oxygen probe was added to the samples. The microplate was monitored on the Tecan Genious Pro plate reader, measuring absorbance in each well at 620 nm over 8 h periods. Corresponding profiles were then compared with calibration generated with different cell numbers.

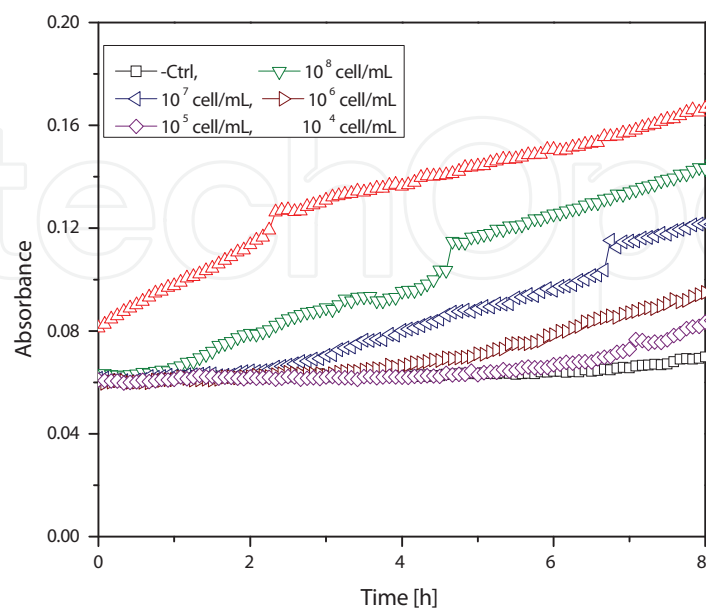
3. Results

Respirometric analysis of model toxicants using *Vibrio fischeri* and *D. magna*

*V. fischeri* culture was used for toxicity assessment of several types of known toxicants by optical respirometry. For reliable and reproducible measurement of respiration of *V. fischeri* in 96WP, exclusion of ambient air oxygen by sealing the samples with a layer of mineral oil (creates barrier for oxygen diffusion) was used. Respiration profiles of *V.fischeri* seeded at different concentrations in nutrient media containing MitoXpress™ probe and monitored at 20°C are shown in Fig 1a. Profiles of probe fluorescence reflect the process of de-oxygenation of test sample, which is dependent on the initial number of bacteria, their



(a)



(b)

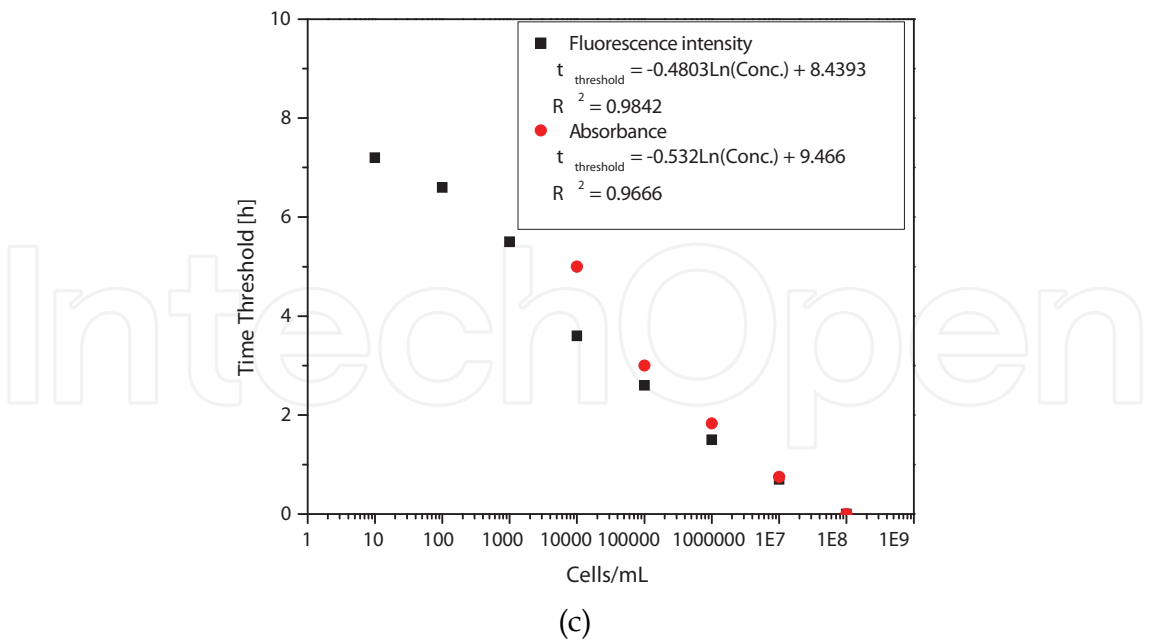


Fig. 1. Growth profiles of *V.fischeri* seeded at the indicated concentrations in nutrient medium 2 at room temperature (~20°C) and measured on Tecan Genius Pro reader: (a) by oxygen respirometry in time resolved fluorescence mode, (b) by turbidometry in absorbance mode. (c) Calibration curves for quantification of *V.fischeri* by fluorescence intensity (■) and absorbance (●) measurements.

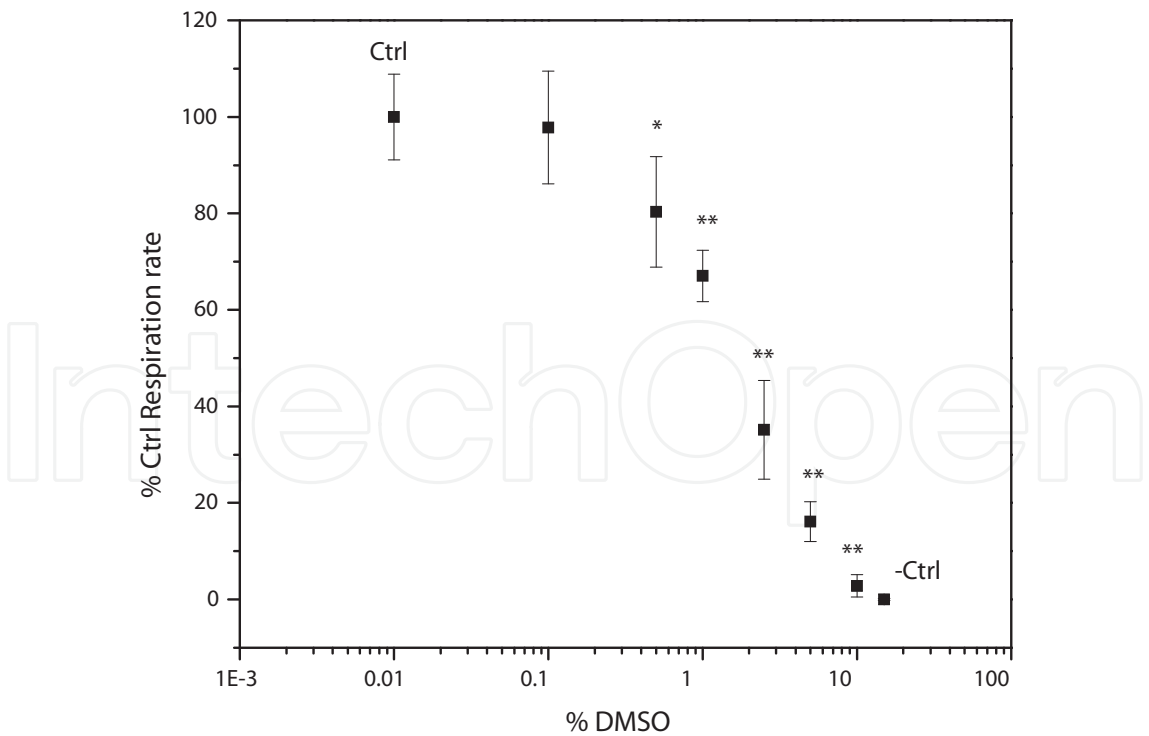


Fig. 2. Processed data (dose response curves) for *V. fischeri* respiration in the presence of DMSO. From such dependence, parameters of toxicity 50 % inhibition values ( $EC_{50}$ ) were determined, which correspond to the range of toxicant concentrations tested.



proliferation rate and toxicity of the sample. As a result of cellular respiration, dissolved oxygen levels are changing in a sigmoidal fashion from air-saturated at the start of the assay to almost anoxic at long monitoring time. Sample deoxygenation due to bacterial growth is evident as rapid increase of probe signal at high cell concentrations, while low cell concentrations require certain time to induce measurable deoxygenation. Negative samples produce flat signal profiles staying at the baseline level.

Growth profiles of *V. fischeri* were also measured by turbidometric assay (OD<sub>600</sub>) – the results are shown in Fig 1b. Signal threshold time for *V.fischeri* obtained from fluorescence intensity and absorbance is shown in calibration curve on figure 1c.

For *D. magna*, due to superior performance and greater sensitivity, Luxcel plate with single organism per well were selected for toxicity testing experiments with reference chemicals and effluents. This platform, coupled with a standard fluorescent reader provides low volume and hence more optimal organism to sample ratio giving higher sensitivity of respirometric measurements, and low probe consumption. Other parameters such as temperature (20 ±2 °C) and the age of *D.magna* (≤24h old juveniles) were the same as in the standard method (33). The chemicals chosen for testing were classical reference toxicants. The effect of the toxicants on probe signal (at 0.5 μM) was tested and no interference was observed (data not shown). Following a 24 h exposure, SLS surfactant found in many personal care products (soaps, shampoos etc.) reduced *D.magna* respiration at concentrations of 60 mg/L (p = 1.1×10<sup>-5</sup>) with EC<sub>50</sub>-24 h value 33.37 ± 8.72 mg/L (Table 1).

The inorganic toxicant K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> is widely used as an oxidizing agent in various laboratory and industrial applications, for cleaning glassware and etching materials commonly used in aquatic toxicity assays (33). After 24 h exposure at 1 mg/L concentration, K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> reduced *D.magna* respiration significantly (p=4×10<sup>-4</sup>) compared to positive controls (see Figure 2). Calculated EC<sub>50</sub>-24 h value was 0.90±0.11 mg/L, which correlates well with literature data, although being slightly lower (Table 1). The respirometric assay also met the criteria of EC<sub>50</sub>-24 h 0.6 to 2.1 mg/L required for the validation of the conventional test (33).

Toxicant	Standard Assay EC <sub>50</sub> -24h [mg/L]	Respirometric Assay EC <sub>50</sub> -24h, (c <sub>min.</sub> ) [mg/L]	Standard Assay EC <sub>50</sub> -48h [mg/L]	Respirometric Assay EC <sub>50</sub> -48h, (c <sub>min.</sub> ) [mg/L]
K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	1.12 (33), 3.9 (43)	0.899±0.11, (0.8)	—	—
Sodium lauryl sulfate	50 (43)	64.9±8.28, (60)	—	—
Zn <sup>2+</sup>	—	4.52±0.58, (4)	1.83±0.07 (44)	1.49±0.14, (0.9)
Cd <sup>2+</sup>	4.66 (45)	0.63±0.23, (0.3)	1.88 (45), (0.615±0.03) (46)	0.16±0.06, (0.08)

C<sub>min</sub>: the lowest concentration giving a significant effect (p<0.01).

Table 1. Medium effective concentrations (EC<sub>50</sub>-24 h/48 h) for different chemicals obtained with *D. magna*.

Exposure to heavy metal ion Zn<sup>2+</sup> for 24 h had no significant effect on *D.magna* respiration at concentrations 2.2 mg/L (p=0.9) and lower (Fig. 3). However, at 4.4 mg/L and higher it was reduced (p=7×10<sup>-4</sup>) in a dose-dependent manner. 48 h exposure enhanced the toxic effect, which became significant at 0.88 mg/L (p=1×10<sup>-3</sup>) and gave almost complete inhibition at 2

mg/mL. Cd<sup>2+</sup> ions bind to free sulfhydryl residues, displace zinc co-factors, and generate reactive oxygen species, and exposure to Cd<sup>2+</sup> results in cellular damage (38). *D.magna* exposed to different Cd<sup>2+</sup> concentrations after 24 h incubation showed a significant reduction in respiration at 0.3 mg/L (p=4x10<sup>-3</sup>) and 0.6 mg/L (p<0.001) (Fig. 3). For 48 h incubation time, significant reduction in respiration was seen at 0.24 mg/L (p=0.003). EC<sub>50-24 h</sub> and EC<sub>50-48 h</sub> values for Cd<sup>2+</sup> and Zn<sup>2+</sup> 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.

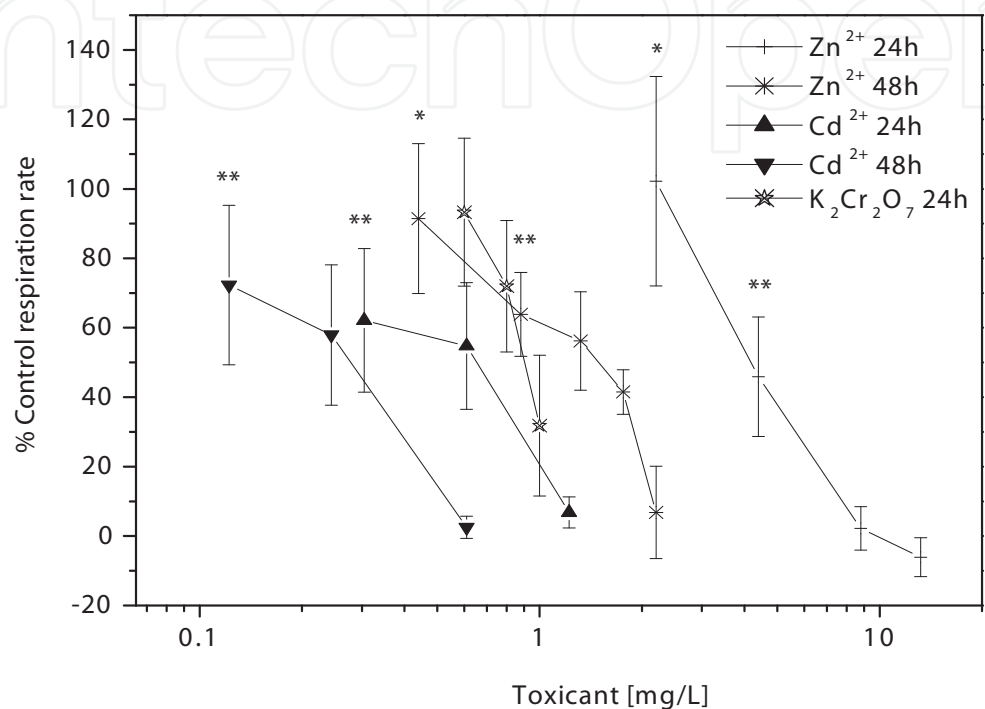


Fig. 3. Dose dependence of toxic effects on *D.magna* respiration of: Zn<sup>2+</sup> and Cd<sup>2+</sup> at 24 and 48 h exposure and K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> at 24 h, measured in Luxcel plate. T=22°C, N=8.

Analysis of MC-LR toxicity using zebrafish embryos

For animal-based toxicity testing of samples spiked with MC-LR, 48-72 hpf old *Danio rerio* were selected, for which the sensitivity to toxicants and respiration rates appear to be optimal (26). For these fish embryos the culturing procedure is simple and does not require feeding, thus eliminating ethical issues associated with using them in such tests. *Danio rerio* embryos showed very pronounced toxicity to MC-LR at concentrations 0.1-50 nM (Figure 4). Remarkably, after 3h incubation with MC-LR embryos showed a moderate decrease in O<sub>2</sub> consumption, with only those treated with 10 nM MC-LR had their respiration significantly decreased. The toxic effect on respiration was enhanced after 24 hour incubation, with significant drop in oxygen consumption observed at concentrations above 1nM, respectively.

Although *Danio rerio* embryos were not as sensitive to MC-LR as mammalian cells (39), they showed relatively strong susceptibility to MC-LR treatment, with clear time and dose dependent response. This can be explained by the fact that at this stage of development embryos already have a functional liver (40) with cells possessing OATP transporters at their membrane. Freshly isolated fish hepatocytes have shown similar response to MC-LR treatment as rat hepatocytes (41).

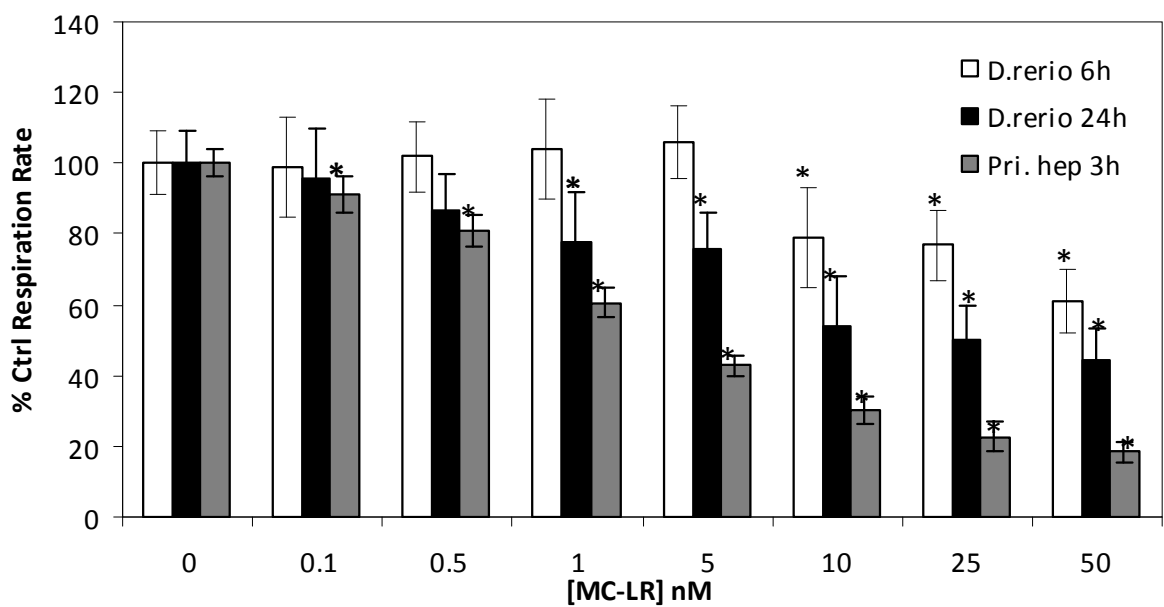


Fig. 4. Respiration of zebrafish embryos and freshly isolated rat hepatocytes (39) in response to MC-LR treatment (relative changes). \* -  $p < 0.05$ ; \*\* -  $p < 0.001$

Analysis of environmental water samples by optical respirometry

To test the efficiency of the respirometric toxicity test with *D.magna* and compare it with standard method, we analysed 10 industrial wastewater samples that were initially examined for their residual toxicity. Initially, samples were analysed undiluted and in a blind manner, i.e. without knowing their source, composition and toxicity in the conventional assay. Thus, a number of toxic samples were identified and subsequently analysed at different dilutions to determine their  $EC_{50}$  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. A summary of effects of all 10 effluents on *D.magna* respiration at different incubation times is shown in see Table 2. The analysis of the samples by the standard test showed toxicity in samples 2-9, which were mainly effluents with elements of metals, pesticides, and pharmaceuticals. Samples 2 and 6 gave  $EC_{50}$  values similar to standard test: 6.5% (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  $EC_{50}$  values of 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. The inter-assay variation for three independent experiments was in the region of 15-30%. 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). Overall, these results show that the respirometric toxicity assay with *D.magna* provides comparable sensitivity and performance with wastewater samples.

The respirometric toxicity assay with *Danio rerio* was also applied to the analysis of water samples contaminated with MCs. 44 hpf *Danio rerio* were incubated for 24h in undiluted field water samples, and then analysed as described above. Two samples were used as a positive (Millipore water), and negative (Millipore water spiked with 100 nM MC-LR) controls, to which the respiration of 17 other unknown samples was compared. The results

Effluent No.	Standard assay <i>D.magna</i>	Respirometric <i>D.magna</i> EC <sub>50</sub> -24h [% vol/vol]	EPA Class	Industrial Activity Description
1	>100	ER	5	The use of a chemical or biological process for the production of basic pharmaceutical products
2	6.5	14.03± 4.97	8	The manufacture of paper pulp, paper or board
3	27.7	85.60±37.39	3	The production, recovery, processing or use of ferrous metals in foundries having melting installations
4	7.5	19.85± 3.82	12	The surface treatment of metals and plastic materials using an electrolytic or chemical process.
5	7.4	4.01±0.47	5	The manufacture by way of chemical reaction processes of organic or organo-metallic chemical products
6	14.3	14.54±0.74	5	The manufacture of pesticides, pharmaceuticals or veterinary products and their intermediates.
7	41.1	14.19± 6.05	5	The use of a chemical or biological process for the production of basic pharmaceutical products.
8	20	ER	7	Commercial brewing, distilling, and malting installations.
9	76.9	ND	12	The manufacture or use of coating materials in processes.
10	>100	ND	5	The use of a chemical or biological process for the production of basic pharmaceutical products

ER: Enhanced respiration

ND: Not detectable

Table 2. Toxic effects of industrial effluents on *D.magna*.

are show in Figure 5. We found that the results obtained with fish embryos were similar to those with primary rat hepatocytes (39), although sensitivity of the latter was several times higher. Thus, samples 1, 2, 9, 10, 11, and 12 showed high toxicity. For sample 5 no result was obtained as the embryos were all dead after 24 h incubation. Samples 8, 13, 14, 15, 16 showed moderate to low toxicity, and samples 6, 7, 17 showed no toxicity.

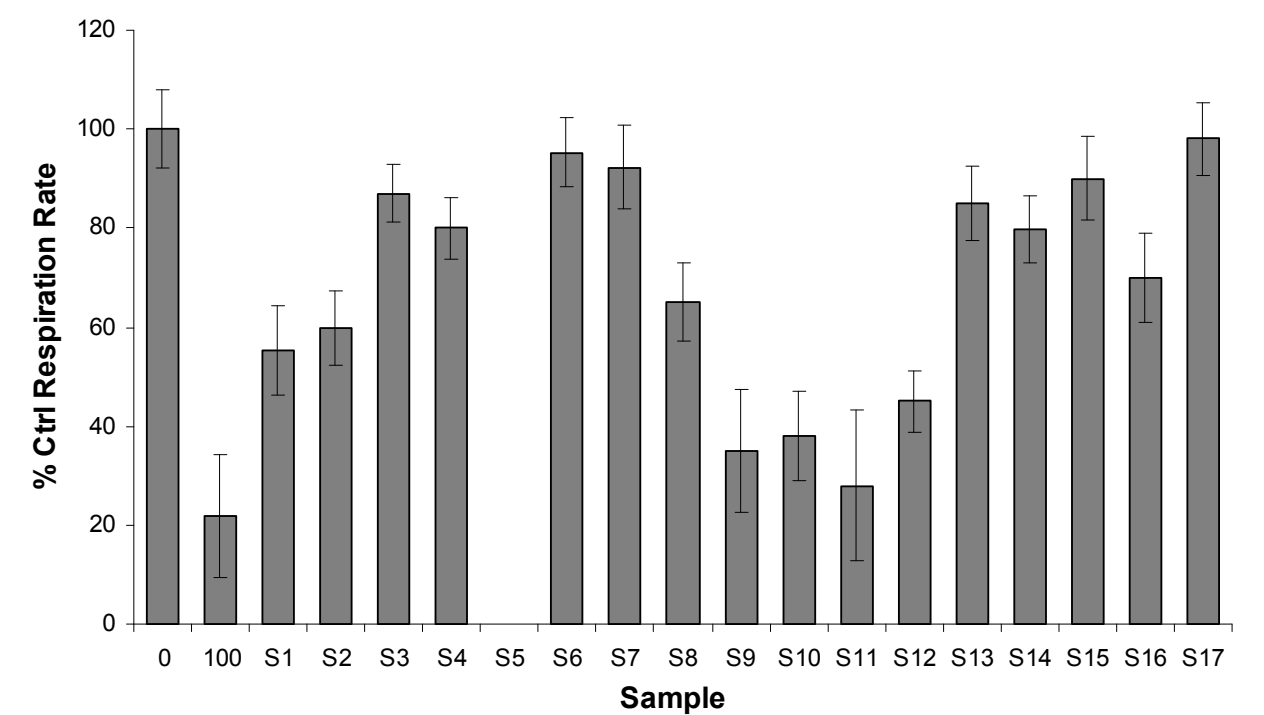


Fig. 5. Changes in respiration of zebrafish embryos treated for 24h with environmental water samples contaminated with MCs (S1-S17). The first two columns correspond to the respiration of embryos incubated with 0 nM and 100 nM MC-LR respectively.

4. Discussion

The results show that fluorescence based oxygen micro-respirometry provides a useful tool for toxicological assessment and screening of water samples. The generic nature of oxygen consumption allows its use as an indicator of viability, metabolic status for various model organisms including *V.fischeri*, *D.magna*, and *Danio rerio*, and sub-lethal toxic effects. Due to its short life cycle and robust culturing conditions, *D.magna* is a good model organism for rapid preliminary toxicity studies, and such assay can be easily set up even in a small lab. For the analysis of *D.magna* low-volume sealable Luxcel plates specially developed for respirometry are used which can work with one animal per well. Respiration profiles were reproducible and unambiguous. The variation of measured parameters, i.e. respiration rates and EC<sub>50</sub>-24 h values (see Table 1) is largely attributed to the variation in respiration between individual animals (in line with the variation observed for the other individually tested multicellular metazoans (26)). The results of respirometric tests are comparable with conventional acute toxicity tests. *Danio rerio* which has similar organs found in mammals, it is another useful model for toxicity assessment, well established in environmental studies. It is also relatively easy to



breed, maintain and produce in high numbers for screening assays. Their size also allows the respirometric assays in Luxcel plates with individual embryos. The assay was used to assess their sensitivity to microcystin-LR, for which susceptibility of *Danio rerio* embryos appeared to be relatively high. However, this assay showed a relatively large variation compared e.g. cell based assays (42), due to significant variation in animal size and embryonic development of organs (MC-LR may influence liver cells differently at different development stages, and liver can metabolise the toxin differently). To generate statistically reliable data, we therefore used higher number of replicates (8-12). Loading *Danio rerio* in Luxcel plates was also a bit cumbersome. Nevertheless, this assay has the potential for the analysis of water samples suspected for contamination with MCs.

Overall, this methodology shows similar sensitivity to standard tests (e.g. Microtox®), and a number of advantages - sample throughput, automation simultaneous measurements, miniaturisation and general simplicity. High flexibility of this platform allows the user to choose test organisms and customize the assay with respect to availability of culturing facilities, the type of samples and toxicants analysed, instrumentation and personnel skills. The possibility to screen large number of chemical and environmental samples highlights the power of this approach. Even with manual liquid handling, one operator can easily generate 100-200 data points per day. The assay is robust and works reliably with complex samples such as effluents or environmental.

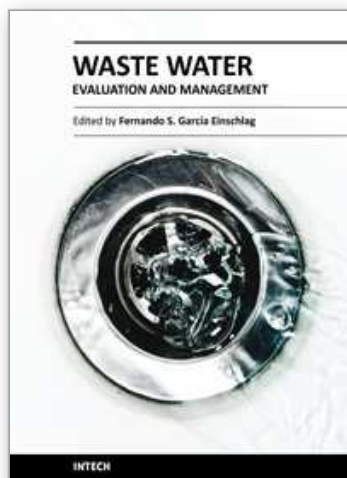
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Fresh water resources are under serious stress throughout the globe. Water supply and water quality degradation are global concerns. Many natural water bodies receive a varied range of waste water from point and/or non point sources. Hence, there is an increasing need for better tools to assess the effects of pollution sources and prevent the contamination of aquatic ecosystems. The book covers a wide spectrum of issues related to waste water monitoring, the evaluation of waste water effect on different natural environments and the management of water resources.

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