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A new *P. putida* instrumental toxicity bioassay

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Abstract Here, we present a new toxicity bioassay (CO_2-TOX), able to detect toxic or inhibitory compounds in water samples, based on the quantification of *Pseudomonas putida* KT2440 CO_2 production. The metabolically produced CO_2 was measured continuously and directly in the liquid assay media, with a potentiometric gas electrode. The optimization studies were performed using as a model toxicant 3,5-DCP (3,5-dichlorophenol); later, heavy metals (Pb^{2+}, Cu^{2+}, or Zn^{2+}) and a metalloid (As^{5+}) were assayed. The response to toxics was evident after 15 min of incubation and at relatively low concentrations (e.g., 1.1 mg/L of 3,5-DCP), showing that the CO_2-TOX bioassay is fast and sensitive. The EC_{50} values obtained were 4.93, 0.12, 6.05, 32.17, and 37.81 mg/L for 3,5-DCP, Cu^{2+}, Zn^{2+}, As^{5+}, and Pb^{2+}, respectively, at neutral pH. Additionally, the effect of the pH of the sample and the use of lyophilized bacteria were also analyzed showing that the bioassay can be implemented in different conditions. Moreover, highly turbid samples and samples with very low oxygen levels were measured successfully with the new instrumental bioassay described here. Finally, simulated samples containing 3,5-DCP or a heavy metal mixture were tested using the proposed bioassay and a standard ISO bioassay, showing that our test is more sensible to the phenol but less sensible to the metal mixtures. Therefore, we propose CO_2-TOX as a rapid, sensitive, low-cost, and robust instrumental bioassay that could perform as an industrial wastewater-process monitor among other applications.

Keywords Acute toxicity · Lyophilized bacteria · Respirometry · CO_2 potentiometric electrode

Introduction

The pollution with xenobiotics is an important environmental problem worldwide, since metal cations in general and some xenobiotics in particular are persistent substances with relatively low mobility (mostly nonvolatile). These pollutants can affect water quality and therefore jeopardize the health of ecosystems. Surface-contaminated water could become an inacceptable risk and increase the cost of water production for human and animal consumption, among others. For these reasons, the assessment of toxicity in water, including wastewater and effluent water, is becoming indispensable.

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Toxic compounds may have their origin by direct or indirect discharges of pollutants to water bodies, since atmospheric and soil contaminants frequently are washed away by precipitations, reaching eventually superficial or subterraneous water bodies. Therefore, water resources should be monitored frequently to detect any change in the water quality. In fact, to have simple and inexpensive automatic equipment could help to minimize the effects of any possible contamination problem which can affect humans and the environment.

Since the potential hazard of toxicants cannot be determined directly through chemical analysis (Durrieu and Tran-Minh 2002; Fulladosa et al. 2005; Lopez-Roldan et al. 2012), biological sensors (e.g., microorganisms, invertebrates, or plants) were historically implemented for the detection of any toxic compound or mixtures contained in water. Microbial bioassays use different mechanisms to measure metabolic and physiological disturbance. They are mainly based on the following: (a) carbon, sulfur, or nitrogen metabolisms; (b) enzymatic activity; (c) growth rate; (d) metabolic heat production; (e) respiration rate; and (f) bioluminescence output (Kilroy and Gray 1995; Plata et al. 2009; Ricco et al. 2004; Tothill and Turner 1996).

In general, bioluminescence tests (ISO 11348–3 2007), including Microtox as the commercial and standardized toxicity test system, have been widely adopted for toxicity testing because they allow the rapid detection of toxic compounds in water through the interference of chemicals in cellular metabolism (that gives as result a diminution of light emission by the bacteria) of a single strain of microorganisms.

However, other tests based on the respiration rate are also commonly implemented, as the denominated activated sludge respiration inhibition test (ASRIT) which is based on the measurement of changes in O_2 concentration in an enclosed chamber containing an O_2 electrode (OECD Method 209 2010).

In particular, bioluminescent toxicity tests as Microtox and respirometric tests as ASRIT are useful for drinking water and wastewater toxicity characterization in comparison with other microbial bioassays because of their relatively high reproducibility (Gutiérrez et al. 2002; Ricco et al. 2004; Rizzo 2011).

Although they exhibit relatively high sensitivities to a wide variety of toxic compounds, each method has its own limitations. For example, ASRIT (microbial consortium, 3 h of exposure) is slower than the luminescent bacteria test (ISO 11348–3 2007) and Microtox (one microbial strain, 30 min of exposure). However, luminescent tests as Microtox require additional steps, including the use of an osmotic adjusting solution (22 % NaCl, to be used in one part each 10 parts of sample) and refrigeration as part of the protocol. Moreover, colored or turbid samples, and specially samples where the optical interfering parameters change during the time of analysis, are cumbersome or impossible to measure using optical detection, as luminescence- or absorbance-based methods.

Even though some authors have proposed modifications to overcome this problem without pretreatment (Lappalainen et al. 1999), the presented solution is not easy to apply, given that involves kinetics measurement at the samples and very short exposition times (30 s). The elimination of turbidity and color, if possible, involves pretreatments, which are not only expensive and time consuming, but that can also alter the sample and therefore the quality of the obtained data. Most of the tests used actually, including the ASRIT, based on light inhibition of luminescent bacteria, and many others, are designed to work exclusively with oxygenated (oxic) samples. This is expected, given that they were designed to assess the possible damage of effluents or other contaminated liquids over natural or artificial continental freshwater environments. These types of aquatic environments are only anoxic in rare conditions, as extreme organic contamination. Only few assays, as the Rantox, have been designed to be used in anoxic conditions. Rantox is basically an anaerobic reactor, inoculated with sludge, where the production of biogas is monitored (Pollice et al. 2001).

The main drawback that ASRIT and Rantox bioassays share is the use of a nondefined mixed microbial community as inoculum; therefore, the results obtained are hard or impossible to compare with measurements made at different places or times.

The aim of our study has been to develop a respiration inhibition bioassay, simpler and with superior performance than the currently available systems, to be unaffected by colored or turbid samples, at any level, and useful in saturated and low oxygen level conditions. In this way, the bioassay presented here can be used to process control in wastewater treatment and other industrial processes. This new analytical bioassay, basically a CO_2 respirometric inhibition bioassay (denominated CO_2-TOX), is based on the continuous measurement of the CO_2 gas produced by the catabolic metabolism of microorganisms, through a CO_2

potentiometric electrode (Campanella et al. 1997; Chiappini et al. 2010; Zosel et al. 2011). As part of the bioassay design, a single microbial strain, *Pseudomonas putida* KT2440, was chosen as the test organism, since it is a nonpathogenic soil bacterium able to grow in oxic conditions but capable to tolerate low oxygen levels as we showed in our experiments, and previously in other *P. putida* strains (Mahendran et al. 2006). Also, given *P. putida* is commonly employed in toxicity tests, including the *Pseudomonas* cell multiplication inhibition test (PCMIT) (ISO 10712 1995), allowed us to compare this established method (as a control) with the test proposed here.

Following this goal, different conditions were tested for the proposed bioassay, including exposure time, cell densities, and pH, among others. Besides, considering that long-term storage cultures (lyophilized) could represent an advantage to the development of any direct toxicity assay (Bonetto et al. 2012), and in particular to be employed with the CO_2-TOX bioassay, we evaluated the respirometric signal and the viability of lyophilized cells over time (Miyamoto-Shinohara et al. 2006; Muñoz-Rojas et al. 2006).

Due to its characteristics, we propose the CO_2-TOX as a simple, low cost, and fast instrumental bioassay, which may have application as a warning system in freshwater management and as a valuable sensor in wastewater industrial processes.

Materials and methods

Test organism and microbiological methods

P. putida KT2440 was kindly provided by Dr. S. Ruzal (Universidad de Buenos Aires, Argentina). The microorganisms were maintained on Petri dishes (4 °C) containing nutrient agar and were replicated every 15 days to ensure viability. To start bioassays based on fresh cultures, *P. putida* was inoculated (two or three colonies) into sterile tryptone soya broth (23 g/L, Laboratorios Britania S.A., Argentina) and was grown aerobically on an orbital shaker at 32 °C. Cell growth was monitored at 600 nm (OD_{600}) and determined using a spectrophotometer (Shimadzu UV-160A). Colony-forming units per mL (CFU/mL) were assessed by dilution plating on Luria-Bertani (LB) agar medium, and colonies were counted after 24 h of growth at 32 °C.

In order to harvest microbial biomass, the cultures were grown until the early exponential phase was reached ($OD_{600}=1\pm0.2$, which corresponds to 3.0×10^8 CFU/mL). *P. putida* cells were then harvested by centrifugation with a microcentrifuge (Cavour VT-1216) for 0.5 min at 14,000 *g*, and the pellet was washed twice with sterile saline solution (NaCl 8 g/L). In case of harvested cells to be immediately used (fresh culture experiments), pellets were resuspended in sterile NaCl solution (8 g/L). Bioassays performed in anaerobic conditions uses *P. putida* grown and harvested in the same conditions, with the only exception that the liquid culture was bubbled with sterile N_2 30 min before placing it in the shaker, and tightly closed.

If the harvested cells were to be lyophilized, a sterile lyoprotectant solution (0.1 M trehalose, 8 g/L NaCl) was used for resuspension (Muñoz-Rojas et al. 2006). After that, the suspended cells were left for 15 min at room temperature, frozen at −20 °C for 2 h, and then placed in the freeze-dryer chamber at −40 °C (Rificor L-05 freeze-dryer, Buenos Aires, Argentina). The vacuum was applied from 10 up to 0.1 mbar, and the final temperature after 12 h of freeze-drying was 25 °C.

CO_2 measurement and signal processing

The transducer of the CO_2-TOX bioassay is a potentiometric sensor, basically a modified Severinghaus CO_2 electrode (Zosel et al. 2011; Cortón et al. 2000). The CO_2 electrodes (usually we use five simultaneously) were connected to an impedance adapter (Fig. 1), and the signal was digitized by a National Instrument board (NI USB-6210). The digital data was read by a Vi Logger NI-DAQmx software (Apache Software Foundation 4.1.0.3001, National Instrument), and the potentiometric signal was plotted in real time.

CO_2-TOX bioassay

The assay medium was prepared with different concentrations of the selected toxicant, or without it (control), a buffer, and glucose in 50-mL Falcon tubes (see section "Optimization of the CO_2-TOX bioassay"). After mixing, a bacterial suspension of *P. putida* cells was incorporated, and the assay volume was adjusted with double osmosis water to 8 mL (final volume). The Falcon tubes were immediately included in a water bath and bubbled with air; this time was set to be the beginning of the bioassay (exposure time corresponding to

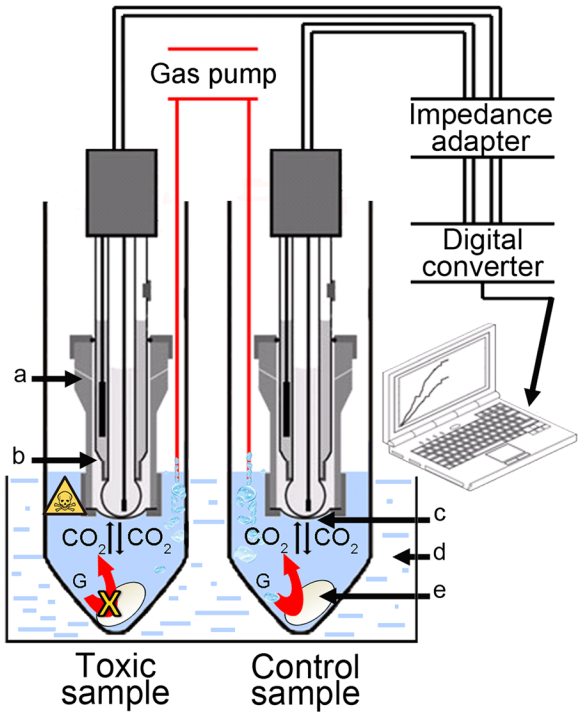

Fig. 1 Main components and mode of operation of the CO_2-TOX bioassay. **a** Acrylic cell, **b** chamber solution, **c** gas permeable film, **d** thermostatic bath, and **e** *P. putida*. Note that G means glucose. Air pump was shut off after 10-min incubation time

0 min). The incubation was at 32 °C; air was bubbled during the first 10 min (with the exception of the anaerobic tests, in which N_2 was bubbled 10 min before and after incorporating the *P. putida* cells). Thereafter, the CO_2 electrodes were introduced into the Falcon tubes and closed with Parafilm. The endpoint selected was carbon dioxide production; therefore, EC_{50} was defined as the toxicant concentration where the CO_2 production rate was half of the controls.

Preparation of standards and samples

The toxic substances used in this study were of analytical grade and prepared with double osmosis water. Glassware and plastic material used were previously cleaned with warm nitric acid solution 1 % (*v*/*v*) and then rinsed with double osmosis water three times. The toxicants assayed were 3,5-dichlorophenol (3,5-DCP, Supelco), heavy metals as Cd^{2+} (from $CdCl_2 \cdot H_2O$), Cr^{6+} (from $K_2Cr_2O_7$), Cu^{2+} (from $CuCl_2 \cdot 2H_2O$), Hg^{2+} (from $HgCl_2$), Mn^{2+} (from $MnCl_2 \cdot 4H_2O$), Ni^{2+} (from $NiSO_4 \cdot 6H_2O$), Pb^{2+} (from $Pb(NO_3)_2$), and Zn^{2+} (from $ZnSO_4 \cdot 7H_2O$), and a metalloid, As^{5+} (from K_3AsO_4). Stock solutions were composed of a single toxicant, 225 mg/L of 3,5-DCP, or 1 g/L of the metal cations (Cd^{2+}, Cr^{6+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, or As^{5+}). A simulated sample (SS) was prepared according to the metal composition of water channel sediments and tailings that normally or eventually could reach Pilcomayo River (PR) waters (PR basin is shared by Bolivia, Argentina and Paraguay). These values derive from several chemical analyses found in bibliography (Hudson-Edwards et al. 2001; Smolders et al. 2003; Miller et al. 2007) but at higher concentration levels, looking to simulate surface water characteristics after a mine-tailing spill, as a possible accident at the mining district, near Potosí, Bolivia (e.g., 235,000 m^3 of tailings, fluid, and mine waste were released, and then, transported downstream in a reported PR basin tailings accident in 1996), as reported previously (Hudson-Edwards et al. 2001; Macklin et al. 1996). SS was composed by Cd^{2+}, Cr^{6+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} at a fixed concentrations and defined as 1X (being X the concentration factor of the entire solution, Table 1). Also, to explore the bioassay as the core of an early warning system, a surface water sample (SWS) has been collected from PR at Misión La Paz village (S 22° 22′ 41.2″, W 62° 31′ 6.9″) in 2011, and it did not receive any treatment before it was tested. The absorbance (OD_{600}) of homogenized SWS samples was ca. 1.8, decreasing to 0.5 in 5 min, given the precipitation of particulate material. Inductively coupled plasma mass spectroscopy (ICP-MS, NexION 300Q ICP-MS, Perkin-Elmer), as per EPA 3051 digestion procedure, was used to determine the total metal concentration (Cd^{2+}, Cr^{6+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}) of the SWS. The toxicant solutions and water samples were stored in 50-mL Falcon tubes at 4 °C.

Optimization of the CO_2-TOX bioassay

Optimal cell density experiments were done in an OD_{600} range from 0.05 to 0.8 (final concentration in the assay tube), being exposed to 5.5 mg/L of 3,5-DCP. Optimal exposure and measurement time experiments were done with different concentrations of 3,5-DCP, ranging from 1.1 to 11 mg/L. The CO_2-TOX bioassay was also evaluated using two different buffers, acetate (0.02 M, pH 5.5), and phosphate buffer (0.1 M, pH 7.4). The

Table 1 Metal composition of SS (simulated sample) and RP SWS (surface water sample); the former concentrations were obtained by ICP-MS analysis after the EPA 3051 digestion routine, and only filtrated (that estimate soluble concentrations)

Metal cation	SS, μg/L (1X)[a]	SWS, μg/L (microwave digested)	SWS, μg/L (filtrated, 0.45 μm)
Cr^{6+}	2500	232±12	37±1
Mn^{2+}	4450	2107±105	985±49
Ni^{2+}	1.55	146±7	126±6
Cu^{2+}	1275	105±5	500±25
Zn^{2+}	5800	490±25	240±12
Cd^{2+}	30	<1	<1
Pb^{2+}	1635	129±6	28±1
Hg^{2+}	2	<0.04	<0.04

[a] X is the concentration factor of the simulated sample

optimized conditions used in this paper (otherwise stated) were as follows: exposure time 50 min, cell densities equivalent to an OD_{600} of 0.2 in the assay media, assay media containing phosphate buffer (0.1 M, pH 7.4), and glucose (10 g/L), fresh harvested bacterial biomass, aerobic conditions, and performed as described in the section "CO_2-TOX bioassay."

Survival rate and respiration response of freeze-drying cultures

Survival rates after freeze-drying were estimated by plate counting (LB solid medium and triplicates for each dilution) and expressed as percentages. The CFU/mL after freeze-drying, $(CFU)_A$, was compared with the CFU/mL observed before freeze-drying, $(CFU)_B$, (Miyamoto-Shinohara et al. 2006) and has also been calculated as follows:

$$\text{Survival rate}(\%) = (CFU)_A/(CFU)_B \times 100$$

Furthermore, the yield of the freeze-drying process was calculated as the bacterial survival ratio (*BSR*) as described previously (Muñoz-Rojas et al. 2006) and expressed as follows:

$$BSR(\%) = \left[\text{Log}(CFU)_A/\text{Log}(CFU)_B\right] \times 100$$

To evaluate the possibility to use lyophilized *P. putida* cells (replacing the fresh cultures), the CO_2 respiration rate was measured up to 55 days after lyophilization. The results of CO_2 respiration rate were compared with those obtained with fresh liquid cultures (using a culture aliquot, just before the cells were freeze-dried). To this end, a parameter was defined according to the following:

$$Rr(\%) = R_A/R_B \times 100$$

where Rr is the respiration response, R_A is the respiration rate of the culture after freeze-drying at the time $t=i$, and R_B is the respiration rate of the culture before freeze-drying when $t=0$.

Detection of toxicity with CO_2-TOX bioassay

Optimized bioassay conditions (see section "Optimization of the CO_2-TOX bioassay") were used to perform toxicity tests. Six different concentrations of the 3,5-DCP were used, ranging from 0.55 to 22 mg/L or without the toxicant for the control groups. Metal cations Cu^{2+}, Pb^{2+}, Zn^{2+}, or the metalloid cation As^{5+} were used at a defined concentration range from 0.05 to 1, 10 to 150, 1 to 20, and 0.1 to 100 mg/L, respectively; this range was set after exploratory experiments were made to find the minimal concentration that exhibit a differential response with respect to the nontoxic control.

A limited series of experiments were performed using 3,5-DCP, SS, and SWS as toxicants, replacing the phosphate for acetate buffer (0.02 M, pH 5.5), all the other assay conditions were the denominated optimized conditions, as described in section "Optimization of the CO_2-TOX bioassay." Also, some experiments were performed under very low oxygen condition, by bubbling ultra pure N_2 (O_2<10 ppm) instead of air at the beginning of the experiments. Most of the results obtained here using the CO_2-TOX proposed method (including SWS and SS results) were compared with a slightly modified well-established acute toxicity method as the PCMIT (ISO 10712 1995).

Data analysis

The percentage inhibition of the respiration rate was calculated with the following equation:

$$\text{Inhibition rate}(\%) = ((R_C - R_T)/R_C) \times 100$$

where R_T is the respiration rate of the bacteria strain exposed to the toxic sample and R_C is the respiration rate of the control sample.

EC_{50} was estimated by either a linear or nonlinear iterative curve fitting procedures. Regression curves

from the concentration-response values were calculated using statistics software. For all data, Log transformation on the concentration axis was used when necessary in order to fit the most convenient curve model taking into account an r^2>0.90. ANOVA analyses were made with the purpose of providing the basis for doing a lack of fit test of the subsequent linear and nonlinear regression curve. The regression curves and the analysis results obtained for all toxicant assayed are available in Supplementary Data (SD). Averaged data are presented, including standard deviations.

Reference method, the *Pseudomonas* cell multiplication inhibition test (PCMIT)

The denominated *Pseudomonas* cell multiplication inhibition test (ISO 10712 1995) was modified (mPCMIT) to allow rapid and simultaneous measurements. Unlike the standard method, the bioassay was performed in a filter-based absorbance microplate reader (FLUOstar OPTIMA, BMG LABTECH) at 32 °C in a final test volume of 0.2 mL/well. Optical density was measured in periods of 4 min at 595 nm after 30 s of orbital shaking movement with amplitude of 1 mm and frequency of 600 rev/min. Since the reference standard method was modified (final volume, temperature, and OD wavelength), a validation procedure with 3,5-DCP according to the ISO 10712 standard was made.

Results and discussion

CO_2-TOX bioassay optimization

Exploratory experiments showed that reproducibility was better if the first 30-min incubation data was not used to calculate the respiration rate. Probably, this is due to slight differences in the CO_2 transducers, as for example the effect of temperature in the nernstian response curve (the transducers were maintained at room temperature until used), or other effects related to initial manipulation and setup at the beginning of the experiment. Therefore, carbon dioxide production slope from 30 to 50 min (see Fig. 2) was used for EC_{50} calculations. It is possible to employ longer incubation times, which will probably increase the sensitivity, but they were not selected because we focus this work in the development of a rapid toxicity bioassay, which usually represents a methodological advantage. The respirometric curves of

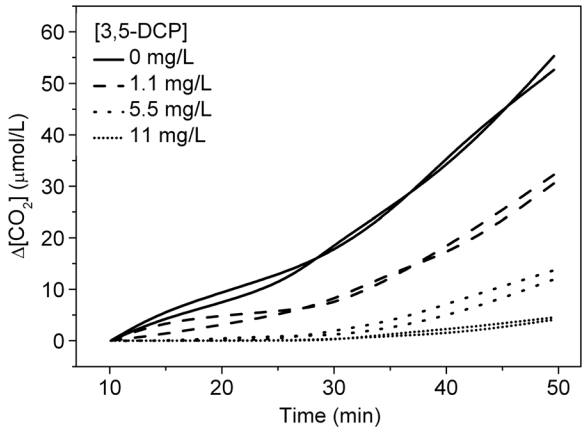

Fig. 2 Potentiometric response of the CO_2 electrodes in the CO_2-TOX bioassay at pH 7.4 with different concentrations of 3,5-DCP. *Duplicate curves* are shown (control and treatment groups, 1.1, 5.5, and 11 mg/L)

the CO_2 production of *P. putida* (OD_{600} of 0.2) for control groups and exposed to different concentrations of 3,5-DCP are shown in Fig. 2.

To obtain the optimum cell density value in our experiments, the inhibition of the respiration rate was calculated using 5.5 mg/L of 3,5-DCP and cell densities ranging from 0.05 to 0.8 OD_{600} values (final concentration in the bioassay). As shown in Fig. 3, the respirometric signal (CO_2) produced in the range between 30 and 50 min of exposure time increases from OD_{600} values of 0.05 to 0.4 and then tends to be constant. Furthermore, if the cell density increases, the amount of available toxicant per cell decreases, which can

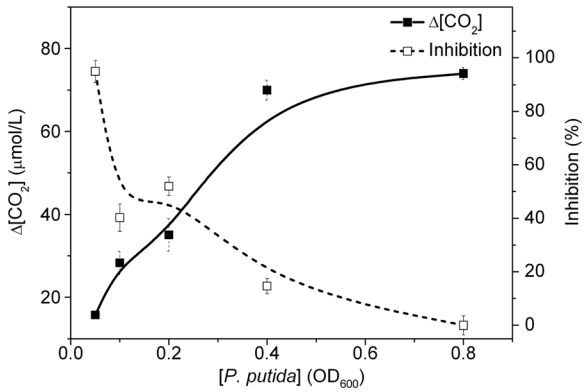

Fig. 3 Relationship between bacterial concentration in the assay media (expressed as OD_{600}) and inhibition at a fixed toxicant concentration (5.5 mg/L of 3,5-DCP). ΔCO_2 was calculated between exposure times from 30 to 50 min, pH 7.4. *Vertical bars* represent±standard deviation of the media (n=3)

explain the lower inhibition we found at high absorbance experiments (Fig. 3). At OD_{600} equal to 0.05, the inhibition of the respiration rate was close to 100 % showing high biological sensitivity, although the quantity of CO_2 produced was relatively low (which is a limit for the transducer sensitivity) and therefore could be an inconvenient to detect the low respirometric signal considering a short incubation time. For higher cell densities (0.1, 0.2, 0.4, and 0.8), the inhibition was over the range of ~52–0 %. Intermediate inhibitions (51.95 and 40.30 %) and respirometric signals (ΔCO_2) easily measurable with potentiometric CO_2 electrodes were found with bacterial concentrations around 0.1–0.2 (OD_{600}), respectively. Cell densities corresponding to OD_{600} values of 0.2 were determined to be optimal for the bioassay, taking into account the balance between biological sensitivity (that is better, in the analytical sense, when the bacterial concentration is minimal) and the CO_2 transducer sensitivity (even though is really fixed, performs better at higher bacterial concentrations).

Toxicity of 3,5-DCP at different pH values

3,5-DCP was assayed, and the concentration-response data obtained at a pH value of 7.4 (phosphate buffer, 0.1 M) is shown in Fig. 4. Additionally, to evaluate the feasibility to carry out the CO_2-TOX bioassay under lower pH conditions, we performed a complementary experiment using acetate buffer (0.02 M, pH 5.5) in the assay media (Fig. 4). The EC_{50} values obtained were calculated after making a Log transformation and a lineal regression from the data (Fig. S1, SD). The calculated EC_{50} values at pH 5.5 and 7.4 were of 19.14± 1.56 and 4.93±1.09 mg/L, respectively (Table 2). The results obtained could be explained by the 3,5-DCP's pKa of 8.14, which indicates that at lower pH, the ionized forms of 3,5-DCP will be predominant in comparison with the neutral form (Penttinen 1995). Taking into account that the cellular lipid membrane is impermeable to charged molecules (at least without considering any particular transport process), it is possible that the 3,5-DCP at a pH value of 5.5 could not easily reach the intracellular space of the cell, affecting slightly the microbial metabolism, when compared with higher pH values, where the neutral chemical species is predominant. Other authors have shown the importance of the pH on the toxic effect of the organic or inorganic compounds to be assayed (Van Beelen and Fleuren-Kemilä 1997) and in luminescent toxicity test (Fulladosa et al. 2004). The relevance of the pH conditions in the toxicity assays is sometimes underestimated, and the drastic

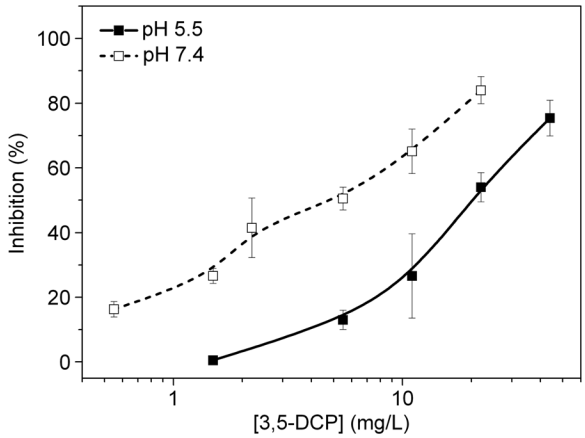

Fig. 4 Inhibition of the respiration rate of *P. putida exposed* to 3,5-DCP. The concentration-response data was obtained by performing the CO_2-TOX bioassay at neutral and acidic conditions (pH 7.4 and 5.5). *Vertical bars* represent±standard deviation of the media (n=3)

Table 2 EC_{50} values for 3,5-DCP reported in the literature for different toxicity bioassays, and in this paper

Bioassay (exposure time)	EC_{50} (mg/L)	CV%	Reference
CO_2-TOX-*P. putida* pH 7.4 (50 min)	4.93	22.11	This paper
CO_2-TOX-*P. putida* pH 5.5 (50 min)	19.14	8.15	This paper
mPCMIT-*P. putida* (16 h)	10.06	12.73	This paper
FM-TOX-*P. putida* (60 min)	9.2	28.26	Yong et al. (2011)
FM-TOX-*E. coli* (60 min)	4.88	n.a.	Catterall et al. (2010)
Baroxymeter-*P. putida* (5 min)	25[a]	10	Tzoris et al. (2005)
Micredox-*P. putida* (60 min)	8.5	n.a.	Tizzard et al. (2004)
Microtox-*V. fischeri* (30 min)	3.39[b]	11.25[b]	Dalzell et al. (2002)
ASRIT (3 h)	12.97	30.06	Gutiérrez et al. (2002)
PCMIT-*P. putida* (16 h)	21.4	23	ISO 10712 (1995)

[a] 48 % of inhibition

[b] Calculated from presented results

n.a. not available

effect that it has in membrane transport is not fully considered.

Detection of metal and metalloid toxicity at pH 7.4

The CO_2-TOX bioassay was tested with some heavy metals and a metalloid, most of them being of great relevance for water quality assessment. The concentration-response data obtained when *P. putida* was exposed to metal cations is shown in Fig. 5. For As^{5+} and Zn^{2+}, it was possible to see a rapid increase in the inhibition below the concentration values of 10 and 5 mg/L, respectively, whereas above these concentrations, the inhibition increased slowly. Instead, concentration-response data of Cu^{2+} showed a rapid increase of the inhibition over two short intervals of concentration (0.05 to 0.1 and 0.9 to 1 mg/L). For Pb^{2+}, the inhibition increased steadily in all the range of concentrations analyzed.

In addition, the concentration-response data obtained with As^{5+} shows that the inhibition of the respiration rate was produced over a wide range of concentrations whereas for Cu^{2+}, Pb^{2+}, and Zn^{2+}, the inhibition of the respiration was achieved only over a small range of concentrations. Furthermore, when the concentration-response data of *P. putida* was analyzed (Fig. S3, SD), an EC_{50} of 0.12, 6.05, 32.17, and 37.81 mg/L for Cu^{2+}, Zn^{2+}, As^{5+}, and Pb^{2+}, respectively, was obtained, showing that Cu^{2+} was the most toxic metal assayed here.

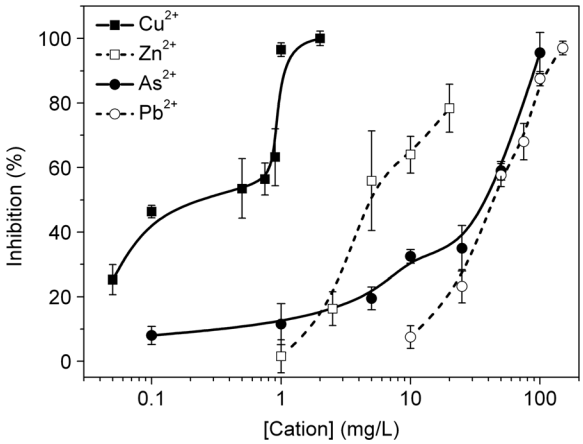

Fig. 5 Concentration-response data for *P. putida* exposed to As^{5+}, Cu^{2+}, Pb^{2+}, and Zn^{2+} and determined with CO_2-TOX bioassay al pH 7.4. *Vertical bars* represent±standard deviation of the media (n=3)

Evaluation of the bioassay using lyophilized cells

The respiration response (*Rr*) of the lyophilized cultures decreased over time, as expected. After 33 days from the freeze-drying process, the *Rr* value was ~37 %, showing an important but not critical decay (in order to use this material as part of a bioassay) over the period of time studied (Fig. 6).

When the survival rate and the respiration response were compared, we observed that after the freeze-drying process, the CFU/mL decreased rapidly whereas the respiration response decreased at a slower pace, showing that even though the microorganisms maintained high respiratory activity, this is not directly related to the cellular division capacity (measured as CFU forming capacity).

Additionally, the bacterial survival ratio of *P. putida* was calculated in order to compare the obtained value with the results published by other authors, using the same lyoprotectant and microbial strain. We found that both values were very similar being 88.7 % (this paper) and 83.72 % (Muñoz-Rojas et al. 2006).

In order to establish the performance of the optimized CO_2-TOX bioassay with lyophilized bacteria, a single concentration of 3,5-DCP (1.49 mg/L) was assessed. The inhibition of the respiration rate achieved with the lyophilized *P. putida* was 44.85 % (CV% 11.12) and with the fresh bacterial cultures was 31.52 % (CV% 3.12) showing that the lyophilized cells were slightly more sensitive to 3,5-DCP at the used level (1.49 mg/L). These phenomena could be explained by the fact that the freeze-drying process may produce cell damage, in-

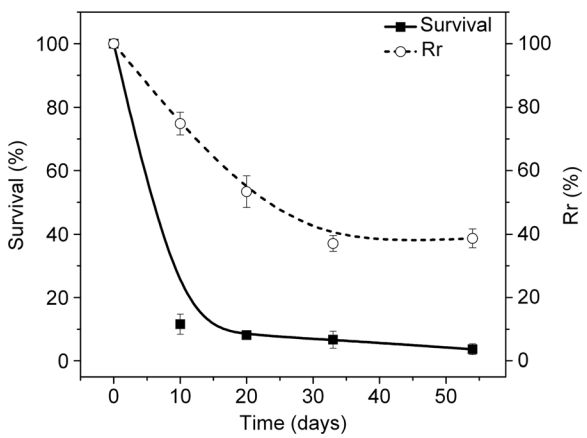

Fig. 6 Survival rate and respiration response (*Rr*) curves of *P. putida* after the freeze-drying process. *Vertical bars* represent ±standard deviation of the media (n=3)

creasing the diffusive transport of this compound inside the microbial cells (Tan and Lim 2005; Wenfeng et al. 2013).

Results obtained using simulated (SS) and surface water (SWS) samples

The CO_2-TOX bioassay at pH 5.5 and mPCMIT at pH 7.4 were also performed with SS and SWS samples. The proposed bioassay was done at pH 5.5 in order to increase the metal cation solubility and therefore achieve higher sensitivities, which is especially important when natural water samples are to be tested because they can be acidic (as when a mine drainage contamination or acid spill accident occurs). The relationship between the inhibition and the concentration factor (X) of SS is shown in Fig. 7. Our data showed an inhibition of the respiration rate by using the CO_2-TOX bioassay in the range between 0.5X and 6X, being the EC_{50} value equal to 1.21X (CV% 8.18). Moreover, mPCMIT was able to detect inhibitions at lower concentration levels (1×10^{-3} to 1X) displaying an EC_{50} value of 6.6×10^{-2}X (CV% 32.83).

In addition, the SWS sample from PR (Misión la Paz, Argentina) was assayed. An EC_{50} value of 0.88X (CV% 46.20) was obtained when the SWS was tested with the mPCMIT (pH 7.4). The concentration-response curve was obtained with four dilutions tested from 1:16 to 1:2 which showed inhibitions between the 10 to 80 % range. On the other hand, when the CO_2-TOX bioassay was tested with SWS, an inhibition on *P. putida* respiration rate was not detectable, in agreement with other

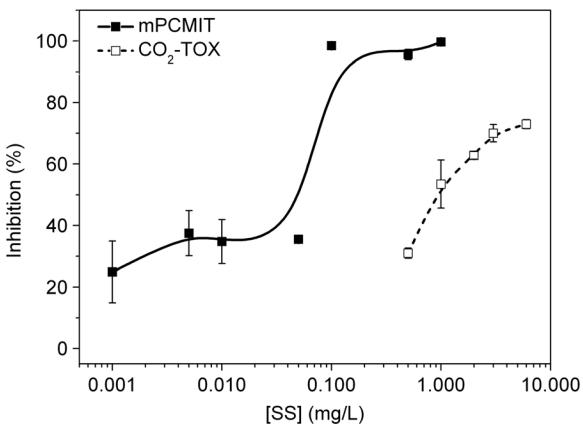

Fig. 7 Concentration-response data obtained with a simulated sample, by using the mPCMIT and the CO_2-TOX bioassays. *Vertical bars* represent±standard deviation of the media (n=3)

experiments presented here, which showed higher sensitivity when the mPCMIT was used.

Performance of the proposed test in samples with very low oxygen levels

Samples with low oxygen levels (pH 7.4) were inoculated with *P. putida* growing in very low oxygen conditions; it was expected slow growth using these conditions which was in fact observed (ca. 23 and 90 h with oxygen and with very low oxygen levels, respectively, to reach O.D.=1). Also, a lower CO_2 production rate was observed, because of that, the assay incubation time was increased to 100 min, and the time interval used for inhibition calculations was the CO_2 production rate between 70 and 100 min. When 3,5-DCP was used as a toxicant, a EC_{50}=11.65±2.31 was obtained. Even though the observed toxicity of the phenol used was lower in this condition, these results show that the CO_2-TOX can be employed when samples with very low oxygen levels need to be analyzed.

Comparison with a standard toxicity bioassay

The ISO standard PCMIT method was modified and assayed with 3,5-DCP. The EC_{50} obtained of 10.06 mg/L (CV%=12.73) was within 10–30 mg/L range of concentrations of 3,5-DCP stipulated by ISO 10712; therefore, the test was validated (Table 2; for more information, see Fig. S4, SD). As the mPCMIT was performed using the same *P. putida* strain and culture/harvesting conditions, it is interesting to note that the test we propose here, at similar pH (7.4), is more sensitive with a EC_{50}= 4.93 mg/L (CV%=22.1).

Comparison between CO_2-TOX and other toxicity tests in the literature

In order to compare the results obtained in this study, we examined the toxicity of 3,5-DCP, As^{5+}, Cu^{2+}, Pb^{2+}, and Zn^{2+} reported with other bioassays found in the literature. The comparison between several tests and CO_2-TOX are summarized in Table 2 and Table 3.

As it can be seen, the EC_{50} value determined using the CO_2-TOX bioassay for 3,5-DCP (pH 7.4) (Table 2) was within the same order of magnitude than for Microtox and Micredox, both commercial

Table 3 EC_{50} values for As^{5+}, Cu^{2+}, Pb^{2+}, and Zn^{2+} reported in the literature for different toxicity bioassays and our results

Bioassay (exposure time)	EC_{50} (CV%), mg/L				Reference
	As^{5+}	Cu^{2+}	Pb^{2+}	Zn^{2+}	
CO_2-TOX-*P. putida* pH 7.4 (50 min)	32.17 (17.19)	0.12 (16.41)	37.81 (17.45)	6.05 (30.73)	This paper
ToxTell-*Psychrobacter* sp. (30 min)	———	2.6 (n.a.)	110.1 (n.a.)	10.9 (n.a.)	Wang et al. (2013)
FM-TOX-*E. coli* (60 min)	———	3.71 (n.a.)	20.4 (n.a.)	7.5 (n.a.)	Catterall et al. (2010)
Microtox-*V. fischeri* (15 min)	———	0.39 (7.55)	34.6 (33.81)	10.5 (1.73)	Rosen et al. (2008)
Microtox-*V. fischeri* (15 min)	20.28 (7.5)	———	———	———	Fulladosa et al. (2004)
Microtox-*V. fischeri* (15 min)	43.6 (n.a.)	0.8 (n.a.)	———	———	Hsieh et al. (2004)
Microtox-*V. fischeri* (30 min)	———	———	———	0.62 (30.87)	Dalzell et al. (2002)
Microtox-*V. fischeri* (30 min)	———	0.19 (10.52)	———	0.76 (26.31)	Gutiérrez et al. (2002)
ASRIT (3 h)	———	32.07 (8.01)	———	55.79 (9.14)	Gutiérrez et al. (2002)
MGIT-*P. putida* (16 h)	———	0.2 (n.a.)	11.7 (n.a.)	8.2 (n.a.)	Schmitz et al. (1998)

n.a. not available

toxicity tests, the first based on marine luminescent bacteria, the second uses the same strain and pH of the bioassay developed in the present work (*P. putida* and neutral pH) (Tizzard et al. 2004). CO_2-TOX do not suffer of light attenuation because of turbid or colored samples, neither is needed to add salt to the media as Microtox; also, electrochemically active substances or electrode fouling is not a problem, nor the dispose of toxic waste as ferricyanide, as in Micredox or other kind of ferricyanide-mediated respiration bioassays as FM-TOX.

Evaluation of the EC_{50} value of 3,5-DCP reported for ASRIT and CO_2-TOX was similar (Table 2), although exposure times were very different. PCMIT and respirometry using Baroxymeter was the less sensitive bioassay. In addition, it is important to take into account that exposure times and bioassay principles are considerably different between PCMIT and respirometry using a Baroxymeter. 3,5-DCP toxicity results obtained with mPCMIT were more sensitive than those reported by the PCMIT standard ISO, which may be related to the higher incubation temperature (32 vs 23 °C) of the modified test used in this work.

The EC_{50} values for As^{5+}, Cu^{2+}, Pb^{2+}, and Zn^{2+} determined by CO_2-TOX, ToxTell, FM-TOX, Microtox, ASRIT, and the miniaturized growth inhibition test (MGIT) are shown in Table 3. From the comparison between bioassays, CO_2-TOX is the most sensitive bioassay to detect Cu^{2+} toxicity, with lower sensitivity toward Zn^{2+}, As^{5+}, and Pb^{2+} in this order. Similar sensitivity ranking was obtained by other toxicity bioassays found in literature (Schmitz et al. 1998; Catterall et al. 2010), see Table 3.

In particular, the highest sensitivities (exposure time of 30 min) to Zn^{2+} were reported for the Microtox (Gutiérrez et al. 2002; Dalzell et al. 2002), followed (decreased sensitivity) by the CO_2-TOX bioassay, FM-TOX, MGIT, ToxTell, and ASRIT (Table 3). However, the toxic effect of Zn^{2+} detected by our bioassay was also similar to ToxTell and FM-TOX, both toxicity tests with exposure times less than 60 min. In fact, Van Beelen and Fleuren-Kemilä (1997) showed the toxic effect of Zn^{2+} on glucose mineralization of *P. putida* MT-2. This experiment was developed for exposure times of 110 min, and the EC_{50} was derived from the determination of [$^{14}CO_2$] production from [^{14}C]glucose. The EC_{50} value obtained for Zn^{2+} was 1 mg/L, which is more sensitive, but still in the same order of magnitude than the obtained in the present work.

We compared our results with other relevant work at Tables 2 and 3 (Hsieh et al. 2004; Rosen et al. 2008; Tzoris et al. 2005; Wang et al. 2013; Yong et al. 2011), to avoid unnecessary repetition some of them are not discussed in the text.

Conclusions

We present here a new toxicity bioassay, based on the quantification of CO_2 (by means of a potentiometric gas

electrode) and *P. putida* as the biological component. The comparison with a control allows the calculation of the inhibition responses for each toxic used, and the obtaining of the corresponding EC_{50} values. This method can estimate the possible toxic effect of an unknown sample.

The optimization experiments allowed us to found the best conditions to perform the bioassay, with an exposure time of 50 min, data analysis of the figures obtained in the 30–50-min period after the incubation starts, pH of 7.4 and bacterial concentration of 0.2 (OD_{600} units), and similar figures using very low oxygen level experiments. In general, the toxicity detected for 3,5-DCP at pH 7.4 was in the same order of magnitude of other tests, as Microtox and ASRIT. In the case of single metal or metalloid toxicities, our results show good sensitivity among several recognized toxicity bioassays. Moreover, we show that the sensitivity of the method can be easily tunable by changing the microbial biomass available in the bioassay; more biomass make less toxicant available per cell; therefore, the bioassay becomes less sensitive.

Some experiments were performed using acetate buffer (pH 5.5) instead of phosphate buffer (pH 7.4); at low pH levels, the bioassay was less sensitive to 3,5-DCP, which is probably related to ionized prevalence of this compound at pH 5.5, which limits the free transport across the cellular lipid membrane. This is not trivial and reveals the importance of pH regulation in any toxicity study, related to bioavailability and transport of the toxic substances.

We showed that lyophilized bacteria can be used in this bioassay, an important factor to be considered to design portable and simple bioassays, with long shelf life, and easy to use. Even though the potentiometric electrode used here is perhaps not the simpler and reliable way to measure this gas, other equipment as NDIR CO_2 sensors have evolved very fast, reaching high performance and lower cost, which could boost new designs or devices using the principle we show here.

Some relevant advantages of CO_2-TOX bioassay with respect to other methods relays in the detection principle, which is not based on optical detection; therefore, the proposed system could be used for toxicity determination of highly colored or turbid water, frequently found not only in large, turbulent rivers, but also when flooding or mine spills occurs and in water-processing industries. We also show that samples containing very low oxygen levels can be measured using this bioassay, a characteristic that will make this system unique and useful not only in environmental conditions, but also in industrial wastewater processing industry.

Finally, we demonstrate that the CO_2-TOX bioassay is a sensitive, simple, rapid, and low cost bioassay, which can produce reliable data using low volume samples, with low risk (hazardous substances or pathogenic bacteria were avoided), and prone to the design of automatic systems, as early warning systems.

Therefore, the proposed assay may be added to others, to complete a wider battery of bioassays, as no one bioassay alone can provide comprehensive information of water toxicity.

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