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Laboratory analysis of soil respiration using oxygen-sensitive microplates

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ABSTRACT

Soil respiration is usually monitored by measuring CO_2 efflux. Most techniques available for such kind of analyses are inconvenient and difficult to adapt to micro-method format. The present study evaluates a new microplate-based method for studying soil respiration in the laboratory. Second-generation oxygen-sensitive microplates (OSM - containing a fluorescent probe attached to the bottom of the well which provides time-resolved fluorescence data) were used to measure soil respiration either in microcosm assays or in soil water extracts. The latter procedure (water extracts) was the least cumbersome, hence it was selected for further experiments. Soil respiration curves generally showed no lag phase, starting with an exponential oxygen consumption phase, followed by a period where respiration became stable after 8–10 h of incubation at 25 °C. Once the procedure for measurement of oxygen consumption in soil was established, the acute toxic effect of diverse chemicals on soil was analysed with OSM. Streptomycin and penicillin failed to reduce soil respiration. Kanamycin plus neomycin, trimethoprim and 5-fluorocytosine exhibited limited inhibitory effects. In contrast, some chemicals (copper sulphate and amphotericin B) and fungicides (such as dodine and fosetyl) noticeably reduced fluorescence readings, showing concentrations to give half-maximal inhibition of respiration (ICR_{50}) ranging from 0.0064 to 0.082 g/L. Finally, some insecticides and soil amendments assayed were either neutral or increased respiration.

It is concluded that OSM are reliable, convenient, and yield quantitative results. Moreover, the system is relatively inexpensive and amenable to automation. However, results obtained using soil water extracts may be different from those derived from undisturbed soil aggregates, clods or slurries studied under field conditions.

1. Introduction

Inadequate use of pesticides and fertilizers causes worldwide concern. Monitoring environmental changes in terrestrial ecosystems caused by chemical pollution requires a better understanding of soil respiration. The latter is defined as the production of carbon dioxide by heterotrophic soil microorganisms (mainly bacteria and fungi - Sandor, 2010). However, the CO_2 efflux is not always an accurate measurement of the respiration rate, since under field conditions carbon dioxide may migrate with soil water, be part in a carbonate dissolution reaction or in plant root uptake (Angert et al., 2015). In addition, CO_2 efflux is often measured with equipment obtaining data in situ, which show great differences in accuracy, spatial and temporal resolution and applicability (Janssens et al., 2000). On the other hand, in a closed environmental system, CO_2 measurement is hampered in alkaline (calcareous) soils for artefacts due to $CaCO_3$–CO_2–H_2O equilibria (Oren and Steinberg, 2008). One of the best options available for laboratory studies on soil CO_2 efflux are Microresp® microplates, an indirect system, amenable to automation, which measures respiration as a halochromic indicator changes colour when CO_2 reacts with bicarbonate (Campbell et al., 2003).

The CO_2 production/O_2 consumption ratio is another parameter that can be used in respiratory studies, but it varies with the kind of substrate consumed and may be biased when [O_2] drops below 0.5% due to partial anaerobiosis. Therefore, the best alternative to CO_2 determination under aerobic conditions is the analysis of oxygen consumption. There are diverse methods for measuring dissolved oxygen. Jorge et al. (2004) described an approach based on a device using fluorophores and optical fibres to determine [O_2] in water. In edaphology, a number of studies based on the use of electrodes and microelectrodes for oxygen detection have been published (Reddy et al., 1980; Sexstone et al., 1985; Pang et al., 2007; Fan et al., 2014; Angert et al., 2015). Unfortunately, the methods used by these authors showed low sensitivity or proved uneconomic or cumbersome. In contrast, Garland et al. (2003) performed their soil respiration analyses choosing a different approach, based on the BD oxygen microsensor

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system®. The method consisted of an oxygen-sensitive fluorophore (tris 4,7-diphenyl-1,10-phenanthroline ruthenium (II) chloride) included in a silicone rubber matrix in the bottom of each well of a standard microplate. These plates may be read with most commercial fluorescence plate readers available on the market, with optimal results obtained when an excitation filter at 485 nm was used in conjunction with an emission filter at 590–630 nm (Rolo et al., 2009). However, this first-generation system relying on direct fluorescence readings became obsolete with the arrival of microplates based on time-resolved fluorescence, much more sensitive to [O_2] variations, hence being the BD microplates supply discontinued a few years ago. Second-generation microplates (namely Oxoprobics® OSM) are now available and capable of performing a highly-sensitive measurement of oxygen content. These new plates have functionalized Pt(II) complexes, acting as long-decay oxygen-sensitive photoluminescent probes (excitation at 340 nm/emission at 642 nm). Furthermore, the fluorophore is covalently bound to the bottom of their wells to prevent probe leakage and, hence, cytotoxicity problems, allowing the assessment of time-resolved fluorescence instead of direct fluorescence. These microplates have been successfully used in our laboratory in previous respiration and viability studies in *Acanthamoeba* cultures (Heredero-Bermejo et al., 2015). Here, we report the application of this new approach as a novel method for monitoring growth of diverse microorganisms in pure cultures. Once this approach proved successful, microplates were explored as a tool for measuring soil respiration. In addition, diverse chemical products (drugs, pesticides, fertilizers and a pollutant) were tested for their acute toxic effect on soil samples in order to confirm that the OSM model system provides similar or better results than other methods currently being used in soil biology studies.

2. Materials and methods

2.1. Soil samples

Soil samples were obtained from an on-campus *Quercus coccifera* stand near our laboratory (40° 30′ 50.951 N; 3° 20′ 27.344 W) in the period of March – June 2016. According to the WRB system, the site's brown soil belongs to the Reference Soil Group rhodic-chromic luvisols (Table 1). Samples were obtained at three different places in a digging depth of 5–10 cm. After collection, samples were pooled and kept in an incubator for one day at either 105 °C (to determine soil composition and structure) or 30 °C (for biological purposes). In the latter case, final soil sample humidity was always below 5%. Soil was passed through a 1 mm mesh sieve and kept in the dark at 4 °C (for < 45 days) until used, in agreement with the OECD guidelines for the preservation of soil biota integrity (OECD, 2000). Controls of sterile soil were prepared by incubating sifted soil at 180 °C for 18 h.

2.2. Microorganisms and culture conditions

Control microorganisms used in this study and their respective culture conditions are shown in Table 2. All strains were purchased from the Spanish Type Culture Collection (CECT-Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain).

Table 1
Characteristics of the soil used in the present study.

Composition/parameter	Percentage/pH units
Sand	35%
Silt	50.24%
Clay	14.76%
pH (1:3 in water)	7.72 ± 0.23
Carbon (%)	3.19 ± 0.33
Hydrogen (%)	1.06 ± 0.13
Nitrogen (%)	1.11 ± 0.18
Sulphur (%)	0.34 ± 0.25

Table 2
Microorganisms and culture conditions employed in the present study.

Species	Origin/ reference	Temperature of growth	Growth media used
Bacillus atrophaeus	CECT 38	32 °C	Luria-Bertoni (USB corp – ref. US 75852)
Pseudomonas aeruginosa	CECT 108	32 °C	Luria-Bertoni (USB corp – ref. US 75852)
Candida albicans	CECT 1002	32 °C	Sabouraud (Scharlau, ref. 02–165-500)
Aspergillus niger	CECT 2090	25 °C	Malt extract (Scharlau, ref. 07–080-500)

2.3. Measurement of microbial growth based on optical density analysis

This procedure was used as a standard method to assess microbial growth and its correlation with the new OCR assay. Basically, microorganisms were grown in microplates at their optimal temperature for 20–24 h and optical density was measured at 630 nm every hour on an ELX 808 IU microplate reader (BioTek, VT, USA).

2.4. Measurement of oxygen consumption in microbial pure cultures and soil

Oxygen consumption was measured on OSM (LT-96 plates from Oxoprobics Biosciences, Madrid, Spain). Positive (oxygen-saturated) and negative (oxygen-free) control reagents, as well as mineral oil (necessary to avoid any oxygen exchange between medium and air), were supplied with the commercial plates. Attempts to measure oxygen content either without adding mineral oil or using an adhesive plastic top (Titer-tops®, Diversified Biotech, Dedham, MA, USA) were made as well. Further details on microplate-related features, reagents and procedures may be found elsewhere (Heredero-Bermejo et al., 2015). The oxygen content of the medium (OC) or the oxygen consumption rate (OCR) were both calculated by converting differences in fluorescence intensity into changes in dissolved oxygen concentration using the Stern-Volmer equation as described by Olry et al. (2007). Most respiration data in this study are presented as $1/[O_2]$ (expressed as nM^{-1}) for the fact that normalized relative fluorescence is difficult to correlate with results based on [CO_2], the most common parameter used in soil respiration studies. Alternatively, data are also expressed as OCR in nmol h^{-1} mL^{-1}.

For respiration assays, microbial overnight cultures were diluted 100-fold into fresh medium, except for *Aspergillus niger*, where a concentrated spore suspension prepared from solid cultures was used following the same procedure as for bacteria. Assays were started by inoculating each well with 100 μL of diluted microbial suspension containing approximately 0.1–1 × 10^8 colony forming units depending on the microorganism. Control wells with 100 μL of either sterile culture medium or the positive or negative controls supplied by the manufacturer were always included in each experiment. Sterile mineral oil was added (100 μL per well) to seal all the wells, as recommended by the manufacturer. The microplate was covered with its lid to avoid contamination and then placed in a plate reader (VICTOR® - Perkin Elmer, Waltham, MA, USA), which was programmed to obtain two readings per well and hour (for up to 24 h). Time-resolved fluorescence (excitation at 340 nm/emission at 642 nm) was measured at delay times of 30 and 70 microseconds. Experiments were carried out at 25 °C or 32 °C depending on the microorganism (Table 2).

Table 3
Chemicals used in toxicity tests in this study. Abbreviations and notes: NA: not applicable. (1) Dilutions of liquid commercial products were always prepared in water. (2) Excipient or solvent used for pesticide solubilisation not specified by the manufacturer.

Chemicals	Commercial name	Supplier	Target organism(s)	Solvent or excipient (1)	Concentrations tested
1) Penicillin + streptomycin	NA	Sigma, St Louis MI, USA	Bacteria	Water	1 + 2 g/L; 0.1 + 0.2 g/L and 0.01 + 0.02 g/L
2) Kanamycin + neomycin	NA	Sigma, St Louis MI, USA	Bacteria	Water	both at 1 g/L, 0.1 g/L and 0.01 g/L
3) Trimethoprim	NA	Sigma, St Louis MI, USA	Bacteria	Dimethyl sulfoxide	1 g/L1 g/L, 0.1 g/L and 0.01 g/L
4) Chlorhexidine	NA	Sigma, St Louis MI, USA	Microorganisms	Dimethyl sulfoxide	1 g/L, 0.1 g/L and 0.01 g/L
5) Chlorhexidine digluconate	NA	Sigma, St Louis MI, USA	Microorganisms	Water	1 g/L, 0.1 g/L and 0.01 g/L
6) Amphotericin B	NA	Sigma, St Louis MI, USA	Fungi	Dimethyl sulfoxide	1 g/L, 0.1 g/L and 0.01 g/L
7) 5-Fluorocytosine	NA	Sigma, St Louis MI, USA	Yeast and some fungi	Water	1 g/L, 0.1 g/L and 0.01 g/L
8) Levamisole	NA	Sigma, St Louis MI, USA	Nematodes	Water	1 g/L, 0.1 g/L and 0.01 g/L
9) Dodine	Syllit flow®	Bayer Cropscience, Spain	Fungi and other microorganisms	Unknown (2) + water	1 g/L, 0.1 g/L and 0.01 g/L
10) Fosetyl	Aliette WG®	Bayer Cropscience, Spain	Fungi	Water	1 g/L, 0.1 g/L and 0.01 g/L
11) Acetamiprid + triticonazole	Roseclear Ultra RTU®	Masso SA, Spain	Fungi and insects	Unknown (2) + water	0.05 + 0.15 g/L, 0.05 + 0.015 g/L and 0.005 + 0.0015 g/L
12) Thiacloprid	Calypso®	Bayer Cropscience, Spain	Insects	Unknown (2) + water	0.015 g/L, 0.0015 g/L and 0.00015 g/L
13) Pyrethrin + abamectin	Fazilo®	Compo SL, Spain	Insects and Acari	Unknown (2) + water	0.02 + 1.5 g/L, 0.002 + 0.15 g/L and 0.0002 + 0.0015 g/L
14) Copper sulphate	NA	Sigma, St Louis MI, USA	Fungi, bacteria	Water	0.1 g/L, 0.01 g/L and 0.001 g/L
15) Diesel	Local petrol station	CEPSA Spain	NA	NA	1/1 (w/w)

Soil respiration assays were run following two different procedures. In microcosm assays, diverse amounts of soil were tested (namely 10, 20 and 30 mg) in microplate wells along with 100 μL of sterile water. Alternatively, soil water extracts at different concentrations were prepared separately by shaking 20 mL of sterile water containing soil (10, 20 or 30% w/v) for 10 min at 50 rpm and subsequent centrifugation at 500 × *g* for 1 min to remove coarse materials. Usually 12–15 mL of supernatant (soil water extract) were recovered to fill up the wells available in a microplate after preparing the control wells. Due to the type of material employed in soil studies, these were carried out under non-sterile conditions.

Control wells containing previously sterilized soil or soil water extracts were always included to ensure that the fluorescence background is not significant, when soil microorganisms were killed by heat.

Fluorescence readings were performed as described above for pure cultures, but microplates were incubated at 25 °C and measurements were taken hourly over 24 to 72 h.

2.5. Effect of diverse chemicals on soil respiration

Chemicals tested as inhibitors in respiration assays (Table 3) were used separately and some of them in combination. Except for streptomycin, copper sulphate and three commercial pesticides (Roseclear Ultra®, Calypso® and Fazilo® - see Table 3 for details), most chemicals were tested at 1, 0.1 and 0.01 g/L. In addition, the effect of diesel fuel on soil respiration was evaluated by placing 1 g of soil on a circular-shaped sterile Whatman filter paper in a Petri dish. The soil sample was drenched with 1 g of diesel fuel applied in droplets (proportion diesel/soil = 1:1 w/w), so that it was imbibed but not submerged, exposing the soil sample to the pollutant for 2, 24 or 48 h. After exposure, the soil sample was carefully scraped from the filter paper and processed to produce a soil water extract as above, but in a final volume of 3 mL. Diesel was purchased at a local petrol station.

For water soluble drugs, 10 μL of the chemical product was added to 90 μL of soil water extract per well. Water-insoluble drugs were dissolved in 50% (v/v) dimethyl sulfoxide (DMSO), as recommended by El-Jay (1996) for acute toxicity tests, thus running the assays in the presence of a final concentration of 5% (v/v) DMSO. Sterilized soil, as well as chemicals in their appropriate solvents were included as negative controls to ensure that there is no significant fluorescence background. Negative controls of those chemical solutions showing no fluorescence background, were omitted as controls in subsequent experiments, whereas those exhibiting significant fluorescent background were run along with the assay and the obtained readings transformed to make them comparable with the positive control assay. Microplates were incubated at 25 °C for at least 20 h, a time lapse sufficient to reach stabilization in OCR in soil control wells. The toxic/stimulant effect of chemicals on oxygen consumption was assessed after approximately 8–10 h of incubation, since readings at shorter incubation times were less accurate hampering the calculation of ICR_{50} for differences between the soil samples in reaching a fluorescence peak.

The ICR_{50} (concentration to give half-maximal inhibition of respiration) was only determined for chemicals showing a high toxic effect (namely copper sulphate, dodine, fosetyl and amphotericin B). Inhibitor concentrations used in such experiments ranged from 1 g/L to 0.0015 g/L in a series of two-fold dilutions.

Finally, the effect of some nutritive substrates/soil amendments was tested by addition of either glucose (at 1 g/L, purchased from SIGMA, St Louis, MI, USA), a mineral mix for soil bacteria (as proposed by Nwankwo et al., 2014) or a commercial plant fertilizer (Hortrilon®, COMPO IBERICA, Barcelona, Spain) at 1, 0.1, 0.01 and 0.001 g/L. All these products were dissolved in water and added as 10% (v/v) of the final volume assay, as described above for inhibitors. The glucose solution was filter-sterilized (0.22 μm) and the mineral mix of Nwankwo et al. (2014) autoclaved. The commercial fertilizer was used without previous sterilization.

2.6. Statistical analysis

Experiments were run in triplicate and results are provided as mean ± SD of data obtained from at least two independent experiments. The statistical significance of the results (either differences in respiration between microcosm and water extract models or studies on inhibition/stimulation of soil respiration caused by chemicals, based on the comparison between treated and control samples) was determined using the paired Student's *t*-test. The concentrations to give half-maximal inhibition of respiration (ICR_{50}) were determined by non-linear regression. For such purposes, at least seven different concentrations of chemicals were tested and measurements of respiration were made after reaching maximal fluorescence, i.e. after 8–10 h of incubation, as stated in section 2.5. All statistical analyses were performed using the GraphPad Prism 5® software package (GraphPad Software, San Diego CA, USA). The statistical significance was defined at $P < 0.05$.

3. Results

3.1. Growth of microbial pure cultures assessed by optical density (OD) or OSM

Pseudomonas aeruginosa growth (using high-inoculum) was first evaluated in OSM (Fig. 1). Both, raw fluorescence and $1/[O_2]$ graphs, showed a similar shape (exponential growth with no lag phase), whereas the OCR graph evidenced exponential decay. Since $1/[O_2]$ graphs must correlate well with the standard CO_2 efflux curves commonly used in edaphology, such kind of graph was selected for further studies on oxygen consumption. In general, growth curves of the assayed microorganisms (monitored by either OD or OSM) were similar in shape, showing lag, exponential and stabilization phase when using moderate inocula (Fig. 2). However, growth was much earlier detectable when assessed by oxygen consumption than by the increase in OD.

Oxygen consumption by *P. aeruginosa* cultures was also tested in microplates either covered with adhesive tops or without adding mineral oil, decreasing fluorescence to 48.75 ± 9.11% and 55.03 ± 12.46% ($P < 0.0001$ in both cases), respectively, when compared with the fluorescence observed in control wells (with oil) measured at the beginning of the stabilization phase of respiration. As such alternative procedures proved less performant, the procedure recommended by the microplate supplier was adopted and mineral oil was henceforth used throughout all the experiments.

3.2. Comparison of respiration between soil (microcosm) and soil water extract assays

In microcosm assays, respiration was already readable by the plate reader using 10 mg of soil (Fig. 3). Larger amounts of soil led to a significant decrease in fluorescent signal, probably due to the mechanic barrier effect caused by soil particles, as the instrument employed in this study was only capable of reading upper fluorescence. In soil water extract assays, wells containing 30% (w/v) soil (equivalent to 30 mg of soil in microcosm assays) yielded best results (Fig. 3). Since both procedures seemed equivalent in performance (water extracts yielded 104.64 ± 15.01% of the fluorescent signal obtained by the microcosm method, a non-significant difference), assays were henceforth conducted with 30% (w/v) soil water extracts. Dimethyl sulfoxide at 5% did not cause significant inhibition (97.88 + 12.37% of the fluorescent signal in the control). Soil respiration showed exponential curves similar to those obtained for *Pseudomonas aeruginosa* at high inoculum, with no lag phase and reaching an OCR-stabilized phase at approximately 8–10 h at 25 °C. Albeit fluorescence readings continued up to 72 h, no additional fluorescence peaks could be observed later in most cases, so that in further experiments fluorometric readings were limited to 24 h.

3.3. Effect of chemicals on soil respiration

Most of the chemicals included in respiration inhibition assays caused no background fluorescence, with the only exception of chlorhexidine DMSO at 1 g/L (data not shown).

The strongest inhibitors for soil respiration were (in decreasing order of activity): copper sulphate > dodine > fosetyl > amphoter-

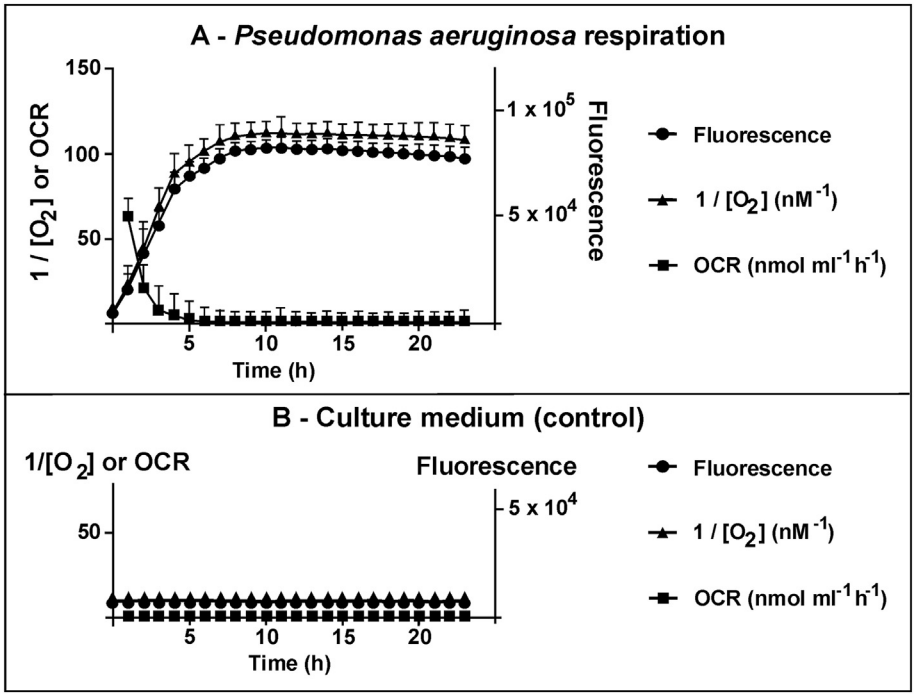

Fig. 1. A. Growth curves of *Pseudomonas aeruginosa* (high inoculum – 5×10^8 cfu) obtained using oxygen-sensitive microplates (OSM). **B.** The same kind of graphs as in **A** but for the control samples (culture medium). Three different parameters are included in both graphs: raw fluorescence (measured in fluorescence units), $1/[O_2]$ (nM^{-1}) and OCR (nmol h^{-1} mL^{-1}). Standard deviation error bars are plotted on points of the line graph.

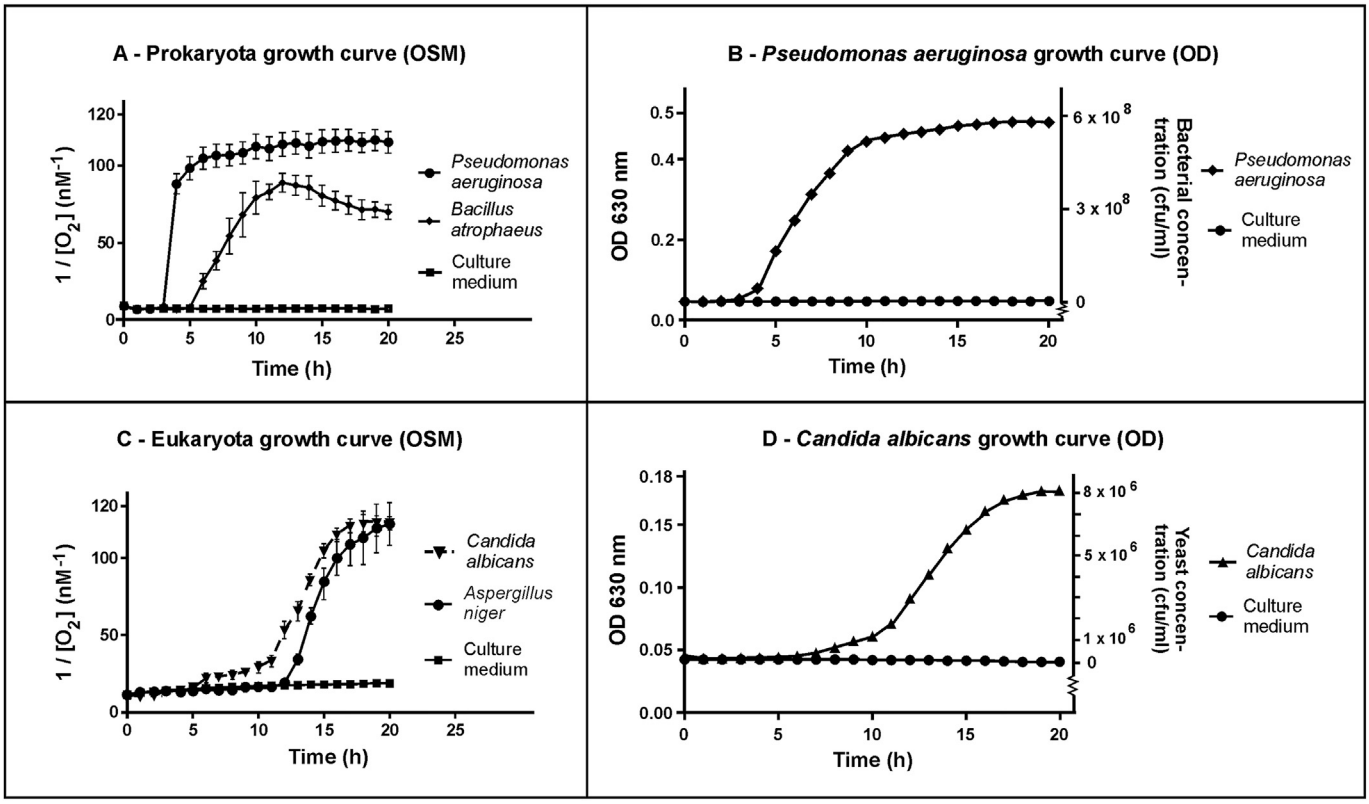

Fig. 2. Growth curves based on OSM ($1/[O_2]$) in two prokaryotic (**A**) and two eukaryotic (**C**) microorganisms. Standard growth curve based on OD_{630nm} for *Pseudomonas aeruginosa* (**B**) and for *Candida albicans* (**D**). Standard deviation error bars are plotted on points of the line graph.

icin B > chlorhexidine (Fig. 4). Chlorhexidine digluconate and chlorhexidine (dissolved in 50% v/v DMSO) returned equivalent results, which suggests that neither the slight fluorescence interference observed at a concentration of 1 g/L in 5% v/v DMSO, nor the use of DMSO as solvent did cause any distortion when measuring the inhibitory effect of chemicals. Some antibiotics (neomycin plus kanamycin and trimethoprim) and the anti-yeast agent 5-fluorocytosine showed only moderate inhibitory activity at a moderate concentration. Levamisole, insecticides and diesel fuel were either neutral or stimulating soil respiration at high or moderate concentrations.

ICR_{50} was only calculated for strong OC inhibitors (Table 4). In agreement with previous results (Fig. 4), copper sulphate was the most toxic chemical, followed by dodine, amphotericin B and fosetyl. Some chemicals (copper sulphate and fosetyl) yielded more reliable and reproducible results (as judged by standard deviation and R^2 figures), in contrast to dodine and amphotericin B, which showed much more deviations when they were tested at low concentrations.

Addition of glucose to OC assays increased respiration by 300 ± 26.87% ($P < 0.0005$). The mineral mix of Nwankwo et al. (2014) did not produce significant effects (107 ± 23%), whereas the commercial mix Hortrilon® showed toxicity at 1 g/L (35 ± 6% $P < 0.0005$), was neutral at 0.1 or 0.01 g/L (99 ± 44% and 146 ± 96%, respectively) and stimulated respiration at 0.001 g/L (186 ± 132%, $P < 0.05$).

4. Discussion

4.1. OSM as a tool to measure growth of microorganisms in pure cultures

The addition of oil to microplate wells (as recommended by the supplier) was strongly advisable, as it prevented oxygen replenishment from air, an otherwise observed problem. The oxygen reloading in the absence of oil decreases fluorescence readings, hence negatively affecting the sensitivity of respiration measurements. In other respects, the four microorganisms assayed in the present contribution showed respiration curves strongly similar to standard exponential growth curves obtained by measuring OD at 630 nm. This classical absorbance method has been previously used to evaluate microbial growth yielding good results with *Pseudomonas aeruginosa* (Ribbons, 1969), *Saccharomyces cerevisiae* (Rosenfeld et al., 2003) and *Aspergillus fumigatus* (Meleatiadis et al., 2001). In contrast, OC measurement has been scantly used to measure microbial growth and viability (Furtado et al., 2012; Heredero-Bermejo et al., 2015). In spite of this, currently available data suggest that cellular growth and respiration seem to be comparable parameters, provided that there are no limiting nutrients, as expected under the conditions described herein. Optical density values reflect the microbial replication rate, while oxygen-based analysis reflects both, growth and alterations in cell metabolism. Hence, OC analysis is a method generally suitable for viability assessment in aerobic microorganisms. In contrast, some alternative methods developed to assess cellular growth, based on substrate degradation producing coloured substances [such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT, Alamar Blue®, etc.] may lack correlation between colour development and growth in some cases. Such discrepancies are caused by differences in mitochondrial metabolism, culture medium components, cell density, pH and miscellaneous factors (Rampersad, 2012; Heredero-Bermejo et al., 2013).

4.2. Soil respiration features

The BD oxygen biosensor system was employed by Garland et al. (2003) in studies on physiological profiling of rhizosphere samples treated with surfactants with apparent good results. However, the system is considered outdated and these microplates are no longer on the market. This is due to problems associated with the formerly employed system, namely:

1) Leakage of the ruthenium probe causing toxicity to the microorganisms deposited in microplate wells (Han et al., 2015; Turel et al.,

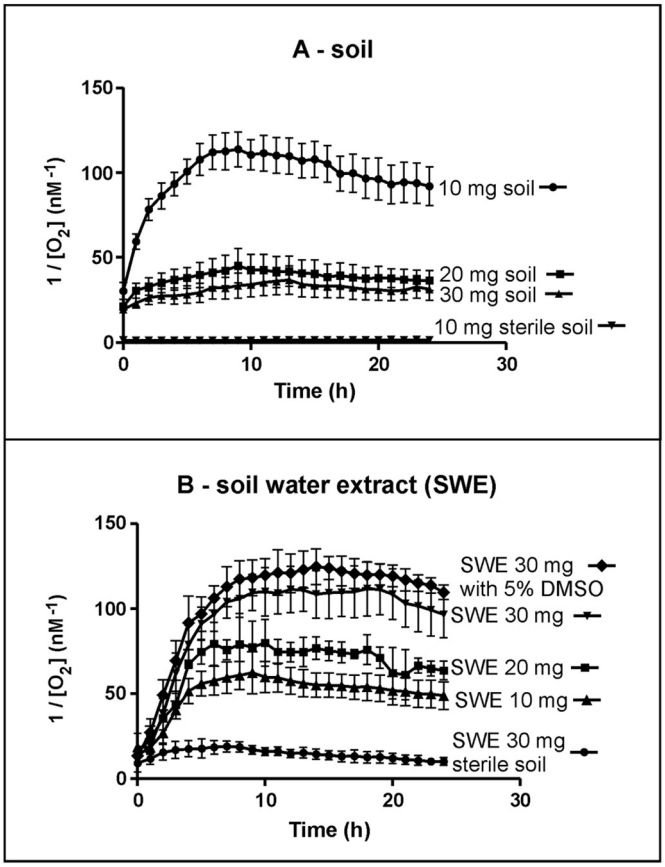

Fig. 3. A. Respiration curves ($1/[O_2]$) obtained with different amounts of soil (untreated or sterilized) directly deposited in microplate wells. **B.** Respiration curves ($1/[O_2]$) obtained with water extracts derived from untreated soil (SWE) (at 10, 20 or 30% w/v) or sterile soil (30% w/v). DMSO was included in the experiment to prove no effects on respiration and, hence, its suitability as solvent for further drug toxicity assays. Standard deviation error bars are plotted on points of the line graph.

2015).

2) Use of direct fluorescence, and therefore, noticeable autofluorescence derived from diverse soil components, such as phenols or tannins (Hiranoa and Brunnera, 2006), to be expected severely limiting the sensitivity of the assay.

These drawbacks are overcome with the second-generation Oxoprobics® OSM microplates that use a probe covalently linked to the bottom of the well and measure time-resolved fluorescence. In fact, respiration curves for soil samples obtained in the present study were similar to those by Creamer et al. (2014), who reported that in samples from Hungary (analysed as well under laboratory conditions), carbon dioxide production showed an exponential increase, reaching a stabilization period after 6–8 h of incubation at 15 °C. Although in our hands both laboratory methods (microcosm or water extracts) yielded similar respiration patterns, it has been shown that under field conditions (using initially dry soil aggregates), OCR decreases and stabilizes in 1–7 days to a non-zero value, as oxygen content in soil does not decrease so sharply in Nature (Sierra and Renault, 1995). Moreover, the water extract model may not exactly correlate with results obtained when using undisturbed soil aggregates, clods or slurries. The addition of water causes great changes in the biogeochemical conditions surrounding microorganisms, while the low proportions of solids relative to water may decrease their impact on chemical immobilization with regard to real soil conditions. For those who consider that water extraction procedures may be biased towards microorganisms that are readily recovered from soil (Campbell et al., 2003), the Oxoprobics® microplates can be directly used without water extraction, as demonstrated in the present contribution. Moreover, it should be also mentioned that the problem observed in the microcosm model, when larger amounts than 10 mg soil samples are loaded per well, might be circumvented by using a bottom-reading VICTOR instrument, which was not available in our facilities. Finally, although some micromethods employed in soil research used a buffer solution to measure enzymatic activities in soil water extracts (Garland et al., 2003; Stefanowicz, 2006) or emitted CO_2 (Campbell et al., 2003), we preferred to employ just distilled water, as this exactly mimics what occurs in Nature when rainfall reaches the soil.

4.3. Soil oxygen consumption as a tool to evaluate toxic/stimulant effects of chemicals on soil microbiota

It is difficult to assess with certainty the effects caused by chemicals on soil respiration. Some products are inhibitors for certain microorganisms, whereas others may use the same chemical as a nutrient or energy source. In addition, commercial pesticides are made up of ingredients including non-pesticide materials and excipients which are often not disclosed by manufacturers (Ekelund, 1999). In spite of this, a review of the published literature on the effect of diverse chemicals on soil respiration (either in the field or in the laboratory) reveals that there is a clear correspondence between results obtained by measuring CO_2 efflux and oxygen consumption by OSM. In general, previous reports found that penicillin, streptomycin and neomycin have a neutral effect on soil respiration (Ingham and Coleman, 1984; Zhang and Dick, 2014). In contrast, both kanamycin and trimethoprim showed partial inhibitory effects on CO_2 efflux (Liu et al., 2009; Wickham and Atlas, 1988). However, Rousk et al. (2009) indicated that such observations may be equivocal, since they found that streptomycin seemed not to affect the respiration rate in soil, yet high inhibition of protein synthesis (evaluated by incorporation of ^{14}C leucine) occurred. Therefore, the use of inhibitors must be evaluated with caution whenever studies on the composition of the decomposer community are considered.

Many antifungal drugs seem to present toxic effects on multiple organisms, as previously pointed out in the literature (Cabral, 1992; Ingham and Coleman, 1984; Olayinkaa and Babalobab, 2001; Rousk et al., 2009; Solel and Siegel, 1984; Spader et al., 2011; Steinfeld et al., 1979; Sukarno et al., 1998). The same is true for some oxygen consumption experiments performed in this contribution, where total inhibition of respiration was observed for copper sulphate and dodine. Yet, other anti-yeast/antifungal drugs like 5-fluorocytosine and triticonazole showed a much more limited inhibitory effect on soil respiration, as previously pointed out by other authors (Niewiamdomska et al., 2011; Spader et al., 2011; Steinfeld et al., 1979; Yao et al., 2006).

The application of levamisole or insecticides/acaricides to soil samples from Spain was neutral or even caused an increase of respiration in most cases, in agreement with the literature (Ma et al., 2002; Rombola et al., 2014; Vig et al., 2008). Nevertheless, in some studies, a negative impact of these chemicals has been reported (Martínez-Toledo et al., 1992). The stimulant effect of insecticides is usually mild and transient (Latif et al., 2008). The reason for this stimulation may be due to the degradation of pesticides by some resistant microorganisms. In addition, some chemicals employed in this study were commercial preparations which contained undetermined solvents. These may have been used as nutritive substrate by some microorganisms, contributing to exacerbate fluorescence readings. Such explanation seems very likely in the case of acetamiprid + triticonazole, which produced a similar level of stimulation of respiration to glucose. The same is true for diesel, a hydrocarbon substrate that some soil microorganisms are capable of degrading, thus enhancing their growth (Dadrasnia and Agamuthu, 2013; Labud et al., 2007). However, it must be remarked that in some cases, a decrease in respiration in diesel-polluted soils has been reported (Lapinskiene et al., 2006), hence not all soils responding in the same way.

Finally, the soil in our location is relatively well balanced in mineral content, since the fertilizing mix proposed by Nwankwo et al. (2014)

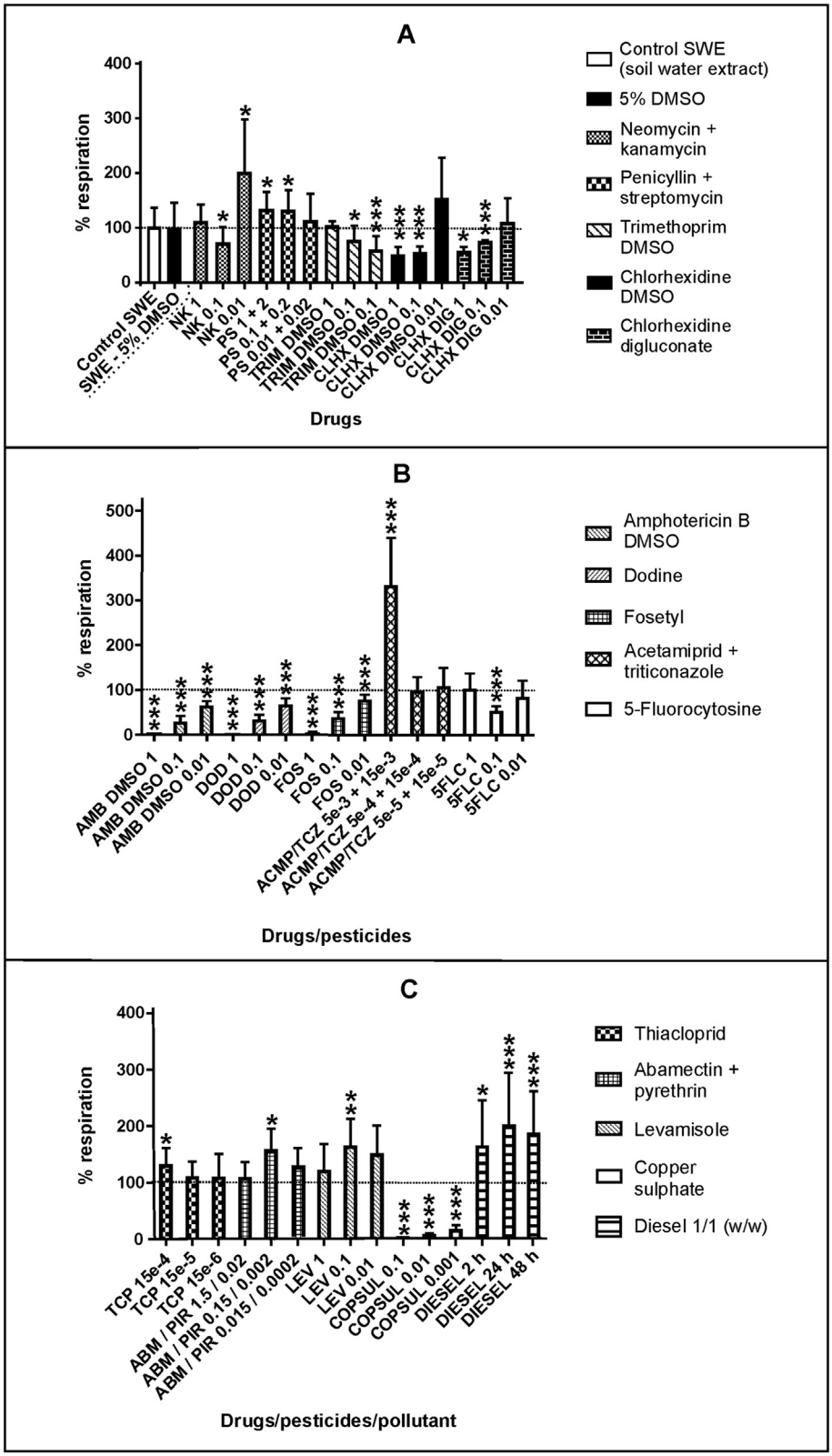

Fig. 4. Toxic effect of chemicals and diesel fuel on soil respiration of soil water extracts (30% w/v) measured with OSM. Differences in oxygen consumption between control and treated samples were compared at approximately 8–10 h after the beginning of the experiment. **A.** Controls, antibiotics and chlorhexidine. **B**. Fungicides. **C**. Pesticides, levamisole and diesel. Concentrations used (g/L) are indicated under the abscissa axis. Significance: * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.0005$. In graphics **A**, **B** and **C**, the line at 100% of activity is drawn to ease the comparison of respiration between control and treated soil water extracts. Standard deviation error bars are plotted on points of the line graph.

Table 4
ICR_{50} obtained for some inhibitors of soil respiration and calculated by non-linear regression. Abbreviation: n = number of experiments.

Drug	ICR_{50} (mean + SD)/n	Range for R^2
Copper sulphate	0.0064 ± 0.0054/3	0.93–0.94
Dodine	0.019 ± 0.014/3	0.83–0.84
Amphotericin B	0.076 ± 0.051/2	0.87–0.92
Fosetyl	0.082 ± 0.041/2	0.87–0.94

did not improve soil oxygen consumption. However, the commercial fertilizer Hortrilon® enhanced soil respiration only when employed at the right proportion, which was much lower than that recommended by the manufacturer (5 g/L). This may be explained by the fact that this amendment is mainly destined to promote plant growth and therefore it does not necessarily cause beneficial effects on soil microbiota at the suggested dosage.

It is important to underline that, although only acute toxicity was measured in the present work, long term experiments are planned in the future to evaluate if chronic chemical toxicity may be assessed with this micro-method. In fact, this should be feasible as OSM (namely, the Oxoprobics® LT microplates) contain a sufficient amount of probe as to endure one week or longer.

5. Conclusions

The OSM system used for respiration analyses in soil can be considered successful, as it confirmed previous results on chemical toxicity in soil published by other authors applying different methods (either in the field or in the laboratory) where no water is added to soil, following the standard procedure for respiration analyses. The coincidence of results with those rendered by the OSM system in this contribution may be explained in terms of the relatively short incubation times used in our assays. In this sense, possible problems with OSM caused by total oxygen depletion cannot be ruled out for long term experiments. However, it must be reminded that the OSM system does not always reflect what occurs in Nature, especially when soil water extracts are used for respiration analyses.

On the other hand, the new model-system requires only limited laboratory space compared to some cumbersome mesocosmos assays currently in use for measuring soil respiration. Although there is one commercial micromethod available for CO_2 efflux determination, such system is expensive, especially when compared to the commercially available 384-well OCR plates.

Finally, it is important to remark that OCR cannot evaluate the growth of anaerobic organisms. Therefore, alternative procedures must be applied for such purposes (Abed et al., 2013).

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