Influence of the sunflower rhizosphere on the biodegradation of PAHs in soil

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Abstract

Reduced bioavailability to soil microorganisms is probably the most limiting factor in the bioremediation of polycyclic aromatic hydrocarbons PAH-polluted soils. We used sunflowers planted in pots containing soil to determine the influence of the rhizosphere on the ability of soil microbiota to reduce PAH levels. The concentration of total PAHs decreased by 93% in 90 days when the contaminated soil was cultivated with sunflowers, representing an improvement of 16% compared to contaminated soil without plants. This greater extent of PAH degradation was consistent with the positive effect of the rhizosphere in selectively stimulating the growth of PAH-degrading populations. Molecular analysis revealed that the increase in the number of degraders was accompanied by a dramatic shift in the structure of the bacterial soil community favoring groups with a well-known PAH-degrading capacity, such as *Sphingomonas* (α-*Proteobacteria*), *Commamonas* and *Oxalobacteria* (β-*Proteobacteria*), and *Xhanthomonas* (γ-*Proteobacteria*). Other groups that were promoted for which degrading activity has not been reported included *Methylophyllus* (β-*Proteobacteria*) and the recently described phyla *Acidobacteria* and *Gemmatimonadetes*. We also conducted mineralization experiments on creosote-polluted soil in the presence and absence of sunflower root exudates to advance our understanding of the ability of these exudates to serve as bio-stimulants in the degradation of PAHs. By conducting greenhouse and mineralization experiments, we separated the chemical impact of the root exudates from any root surface phenomena, as sorption of contaminants to the roots, indicating that sunflower root exudates have the potential to increase the degradation of xenobiotics due to its influence on the soil microorganisms, where sunflower root exudates act

improving the availability of the contaminant to be degraded. We characterized the sunflower exudates in vitro to determine the total organic carbon (TOC) and its chemical composition. Our results indicate that the rhizosphere promotes the degradation of PAHs by increasing the biodegradation of the pollutants and the number and diversity of PAH degraders. We propose that the biostimulation exerted by the plants is based on the chemical composition of the exudates.

Keywords: rhizosphere, microbial community structure, bioremediation, PAHs, sunflower root exudates, biodegradation

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1. Introduction

Bioremediation techniques are routinely applied to recover soils polluted by polycyclic aromatic hydrocarbons (PAHs). These techniques are based on the well-established capability of soil microorganisms to degrade PAHs through growth-linked or co-metabolic reactions (Kanaly and Harayama, 2010). However, a major limiting factor in the bioremediation of PAH-polluted soils is the reduced bioaccessibility that is often exhibited by these pollutants, which results in difficulty in predicting whether an acceptable end-point decontamination level can be achieved. Bioacessibility can be defined as the fraction of a pollutant that is potentially biodegradable over time in the absence of limitations to biodegradation other than restricted phase exchanges. Microorganisms can potentially overcome bioaccessibility restrictions through a variety of mechanisms, including biosurfactant production, attachment and chemotaxis (Tejeda-Agredano et al., 2011). Bioaccessibility can also be increased in the soil externally, for example, by adding surfactants (Bueno-Montes et al., 2011).

Rhizoremediation, i.e., the use of ecosystem services provided by the plant rhizosphere to decontaminate polluted soils, has recently gained attention in relation to organic pollutants, such as PAHs. Translocation of dissolved contaminants in the rhizosphere and the microbial utilization of root exudates as co-substrates in the biodegradation of PAHs have been proposed as mechanisms through which plants contribute to the elimination of PAHs (Newman and Reynolds, 2004). The sunflower (*Helianthus annuus*, L) has been used as a pilot system in phytoremediation assays for PAHs. The sunflower rhizosphere removes a greater quantity of fluorene, anthracene and pyrene from contaminated soil than the rhizospheres of other plant species, such as wheat, oat and maize and exhibits a better response to seed germination and root elongation in the presence of these PAHs (Maliszewska-Kordybach and Smreczak, 2000). Olson et al. (2007) reported the sunflower as the best plant among 11 dicotyledonous species to use in assays of PAH bioavailability. Further advantages of focusing on the sunflower as a model plant for use in PAH rhizoremediation studies are related to the importance of this species as an edible oil producer. The ability to investigate the root exudation process and the role of the exudates under natural conditions has been hampered by a number of significant quantification problems, due to interference by microbial metabolites and components of the soil (Grayston et al., 1996). These problems can be overcome through the development of appropriate in vitro techniques to obtain root exudates that allow analysis of the products secreted by the plant roots.

74 The research approach applied in the present study was to generate soils polluted with aged
75 PAHs at concentrations that would be realistic for polluted soils that had undergone extensive
76 bioremediation, and we used these samples to test the hypothesis that the germination and
77 development of sunflower plants would enhance the bioaccessibility and biodegradation of PAHs
78 in the soil. We used both culture-dependent and culture-independent (i.e., based on DNA)
79 techniques to determine the effects of planting on the dissipation of the chemicals from the soil
80 under greenhouse conditions and on the structure of the soil microbial communities. We also
81 developed a method to produce sunflower root exudates, which were chemically characterized
82 and tested for possible effects on biodegradation by soil microorganisms through a dual
83 radiorespirometry/residue analysis method that allowed precise estimation of compound
84 biodegradation.
85
86 **2. Materials and methods**
87
88 *2.1. Soil*
89 Two soils were used in this study: a creosote-polluted clay soil and an agricultural soil. The
90 polluted soil (Calcaric Fluvisol) constituted the source of aged contaminants as well as PAH-
91 degrading microorganisms for our greenhouse and laboratory experiments. This soil was
92 provided by EMGRISA (Madrid, Spain) from a wood-treating facility in southern Spain that had
93 a record of creosote pollution exceeding 100 years. The agricultural, non-polluted soil was a
94 loamy sand soil from Coria del Río, Seville, Spain (Typic Xerochrepts). A PAH-containing soil
95 mixture was obtained from these soils in two steps. First, the agricultural soil was mixed (67:33
96 w/w) with washed sand (Aquarama), and subsequently autoclaved. Next, 6 Kg of this mixture
97 (referred to as uncontaminated soil) was homogenized with polluted soil (1:1 w/w) in a cement
98 mixer for seven days (9 hours per day), with regular changes in the direction of rotation. This
99 homogenization period was necessary to allow reproducible results to be obtained. The mixture
100 was then dried for 18 hours at 30°C, ground and sieved (2 mm mesh). The resulting material was
101 used in all experiments as a source of polluted soil with the following composition: pH 8.1;
102 15.9% $CaCO_3$; 0.9 % total organic carbon (TOC); 0.055% organic nitrogen (Kjeldahl); 7 mg kg^{-1}
103 available phosphorus; 461 mg kg^{-1} potassium; particle size distribution 46.6% coarse-grained
104 sand, 4.3% fine-grained sand, 15.8% silt, and 33.2% clay and 21.75 mgkg^{-1} of total PAHs (as the

105 sum of 6 PAHs: fluorene, phenanthrene, anthracene, fluoranthene, pyrene and chrysene; Table 1).
106 The resulting profile of PAH concentrations was consistent with soils that have undergone
107 extensive bioremediation (Bueno-Montes et al., 2011).
108
109 *2.2. Greenhouse experiments*
110
111 *2.2.1. Experimental design*
112 For this study, we used sunflower (*Helianthus annuus L.* cv. *PR 63A90*) seeds from the
113 University of California that were certified for agronomic crop production. The greenhouse
114 experimental design consisted of 5 pots with 2 kg of soil per treatment. The treatments included
115 uncontaminated soil planted with seeds (as a positive control for plant growth) and contaminated
116 soil with or without seeds. Five seeds were used per planted pot. The experiment was carried out
117 in a greenhouse at 23±1 ºC and 20% field capacity. After 45 and 90 days, soil samples were
118 collected in each of three randomly chosen pots for each treatment for measurements of residual
119 PAH contents and microbiological determinations. Soil samples (20 g) were carefully extracted
120 from the rhizosphere zone with the aid of a glass tube (150x25 mm) used as a bore. Care was
121 taken to avoid damaging the plants. Samples for the PAH analyses were stored at -20ºC, and
122 samples for the microbiological analyses were stored at 4ºC. In both cases the samples were
123 analyzed separately. At the end of the experimental period, the percentage of germination was
124 evaluated for each treatment, and the fresh and dry weights of stems and roots were determined
125 separately. Dried stems and roots were generated by incubating the separated plant materials in a
126 desiccation oven (70 ºC) for 72 hours.
127
128 *2.2.2. PAH Analysis*
129 Triplicate soil samples (1 g of soil per sample) from the initial polluted soil (1:1 w/w) and
130 from the three pots with differet treatment at 45 days and 90 days after in the greenhouse
131 experiment, were dried completely using anhydrous sodium sulfate to grind the mixture in a
132 mortar and pestle. Samples were extracted in a Soxhlet with 100 mL dichloromethane for 8 h.
133 Once the extract was obtained, the organic solvent was evaporated in a vacuum to nearly
134 complete dryness, and the residue was dissolved in 5 mL dichloromethane and cleaned by
135 passing through a Sep-Pak Fluorisil cartridge. The purified extracts were evaporated with N_2, and

136 the residues were dissolved in 2 mL of acetonitrile. Finally, the samples were filtered through a
137 nylon syringe filter (0.45 µm, 13 mm Ø, Teknokroma, Barcelona, Spain). Quantification of PAHs
138 was performed using a Waters HPLC system (2690 separations module, 474 scanning
139 fluorescence detector, Nova-Pak C_{18} Waters PAH column, 5 µm particle size and 4.6 x 250 mm,
140 1 mL min^{-1} flow and mobile phase with an acetonitrile-water gradient). The column was installed
141 in a thermostatic oven maintained at 30ºC.
142
143 *2.2.3. Autochthonous microbiota*

144 *2.2.3.1. Quantification of heterotrophic and hydrocarbon-degrading microbial populations*
145 Bacterial counts from triplicate soil samples were performed using the miniaturized most
146 probable number (MPN) method in 96-well microtiter plates with 8 replicate wells per dilution
147 (Wrenn and Venosa, 1996). Total heterotrophs were counted in diluted (1:10) Luria-Bertani
148 medium; low molecular weight (LMW) PAH-degraders were counted in mineral medium (Grifoll
149 et al., 1995) containing a mixture of phenanthrene (0.5 g L^{-1}), fluorene, anthracene, and
150 dibenzothiophene (each at a final concentration of 0.05 g L^{-1}); and high molecular weight
151 (HMW) PAH-degraders were counted in mineral medium containing pyrene at a final
152 concentration of 0.5 g L^{-1}. Hydrocarbon was added to the plates dissolved in pentane, and
153 medium was added after solvent evaporation. MPN plates were incubated at room temperature
154 (25ºC±2ºC) for 30 days. Positive wells were detected based on turbidity (heterotrophs) and
155 observable coloration (brownish/yellow) for PAH degraders.

156 *2.2.3.2. DNA extraction and PCR amplification of eubacterial 16S rRNA genes*
157 Total DNA from soil and rhizosphere samples was extracted using a Power Soil DNA
158 isolation kit (Mobio, Carlsbad, USA). Eubacterial 16S rRNA gene fragments were amplified
159 from the extracted total DNA through PCR using pureTaq^{TM}Ready-To-Go^{TM} PCR bead tubes
160 (GE healthcare, United Kingdom) in a final volume of 25 µL containing 1 µl of DNA extract as
161 the template and 25 pmol of each primer (Sigma-Aldrich, Steinheim, Germany). To obtain clone
162 libraries, we used the primers 27f and 1492r (Weisburg et al., 1991), and for the denaturing
163 gradient gel electrophoresis (DGGE) fingerprinting analysis, we used GC40-63f and 518r. After
164 10 min of initial denaturation at 94°C, 30 cycles of amplification were carried out, each
165 consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 56°C and 1 min (DGGE) or 2

166 min (clone libraries) of primer extension at 72°C followed by a final primer extension step of 10
167 min at 72°C. All of the PCR amplifications were performed in an Eppendorf Mastercycler.
168
169 *2.2.3.3. DGGE analysis*
170 The 16S rRNA PCR amplification products were purified using the Wizard®SV Gel and
171 PCR Clean-Up system (Promega, Madison, USA) and quantified in a NanoDrop®
172 Spectrophotometer ND-1000 prior to DGGE analysis. Identical amounts of PCR products were
173 loaded in 6% polyacrylamide gels with denaturing gradients ranging from 45% to 70% (100%
174 denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at a constant
175 voltage of 100 V for 16 h in 1x TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA,
176 pH 7.4) at 60°C in a DGGE-2001 System (CBS Scientific, Del Mar, CA, USA) machine. The
177 gels were stained for 30 min with 1x SYBR Gold nucleic acid gel stain (Molecular Probes,
178 Eugene, OR, USA) and photographed under UV light using a Bio-Rad molecular imager FX Pro
179 Plus multi-imaging system (Bio-Rad Laboratories, Hercules, CA, USA) in the DNA stain gel
180 mode for SYBRGold at medium sample intensity. DGGE bands were processed using Quantity-
181 one version 4.5.1 image analysis software (Bio-Rad Laboratories) and corrected manually.
182
183 *2.2.3.4. Construction, sequencing and phylogenetic analysis of 16S rRNA gene clone libraries.*
184 Amplified 16S rRNA gene fragments were purified as described above and were cloned using
185 the pGEM®-T Easy Vector System (Promega, Madison, USA). Transformants were selected
186 through PCR amplification using vector PCR primers. The PCR mixture contained 1.25 U of *Taq*
187 DNA polymerase (Biotools B&M Labs, Madrid, Spain), 25 pmol of each primer (Sigma-Aldrich,
188 Steinheim, Germany), 5 nmol of each dNTP (Fermentas, Hanover, MD) and 1x PCR buffer
189 (Biotools B&M Labs) in a total volume of 25 μL. The obtained PCR products were purified, and
190 inserts were sequenced using the ABI Prism Bigdye Terminator cycle-sequencing reaction kit
191 (version 3.1) with the amplification primers 27f and 1492r and the internal primers 357f and
192 1087r (Lane, 1991). The sequencing reactions were performed using an ABI prism 3700 Applied
193 Biosystems automated sequencer at Scientific-Technical Services of the University of Barcelona.
194 DNA sequencing runs were assembled using BioEdit Software. Sequences were aligned using the
195 BioEdit software package and manually adjusted. The resulting DNA sequence was examined
196 and compared with BLAST alignment tool comparison software and the classifier tool of the

197 Ribosomal Database Project II at http://rdp.cme.msu.edu/. The 16S rRNA gene sequences
198 obtained for the bacterial clones were deposited in the GenBank database with accession
199 numbers (JQ771957-JQ772014).
200
201 *2.3. Experiments with exudates*
202
203 *2.3.1. In vitro production*
204 In vitro production of sunflower root exudates was performed by placing 50 seeds in an
205 inorganic salt solution (MM, pH 5.7) described elsewhere (Tejeda-Agredano et al., 2011). To
206 avoid the introduction of alternative sources of organic carbon in the biodegradation experiments,
207 the solution did not contain sucrose, vitamins or plant growth regulators. The medium was
208 prepared using ultrapure water (MILLIPORE). We transferred 500 mL of MM to glass jars
209 (1,000 mL capacity, 28 x 11.5 cm) previously sterilized for 20 min. (121 ºC, 1 atm. of pressure).
210 Inside these glass jars, we installed a square piece of stainless steel wire cloth (0.98 mm light and
211 0.40 mm in diameter), held in place by four stainless steel wires extending from the edge of each
212 jar. The length of these wires was calculated such that the seeds on the mesh were in contact with
213 the surface of the MM without sinking into the solution to avoid producing anoxia. The jars were
214 closed firmly with a pressure system using a glass lid.
215 To sterilize the seeds, a batch of 50 seeds was surface-sterilized in 250 mL of absolute
216 ethanol for 3 minutes in sterilized Erlenmeyer flasks at 550 rpm. The ethanol was subsequently
217 removed, and 250 mL of a solution of 57% sodium hypochlorite (14% active chlorine) was added
218 for 25 min. Finally, the hypochlorite was eliminated, and the seeds were rinsed 3 times with
219 sterilized distilled water for 5 min each time, working in a laminar flow biosafety cabinet. Next,
220 the sterilized seeds were distributed on square cloth mesh. The size of the mesh allowed root
221 growth to occur and kept the seeds in place. The jar was closed, sealed with Parafilm and placed
222 in a culture room at 25 ± 1ºC, 65.24 $\mu Em^{-2}s^{-1}$ and with an 18-hour photoperiod for 30 days. After
223 this period, the MM with the excreted exudates was collected under sterile conditions and
224 centrifuged for 3 h at 31,000 x g to obtain a solution that included the organic matter present,
225 removing the pellet as the method of Haftka et al., 2008. These samples were stored at -20ºC until
226 further use in the mineralization experiment. It is of particular note that the seeds were situated on

227 the medium surface and never submerged such that only the developed roots were responsible for
228 exudate production.
229 In vitro exudate extraction was repeated 6 times, and at the end of each repetition, we
230 quantified the number of plants, determined the fresh and dry weight of the roots and assessed the
231 relative growth rate (RGR) of whole plants calculated according to the equation RGR = (ln Bf -
232 ln Bi) D^{-1} (Merckx et al., 1987), where Bf is the final dry biomass; Bi is the initial dry biomass
233 (average of 5 seedlings dried 3 days after germination of seeds); and D is the number of days of
234 the experiment. The plants acquired at the beginning and end of the greenhouse experiment were
235 dried by placing the plant material in the desiccation oven at 70 ºC for 72 hours.
236

237 *2.3.2. Chemical analyses of sunflower root exudates*
238 Total organic (TOC) and inorganic carbon estimations were carried out at IRNAS-CSIC
239 based on measurements performed in a TOC Analyzer (TOC, model TOC-V CPH, Shimadzu,
240 Japan) using a non-purgeable organic carbon (NPOC) analysis. The analyses of amino acids,
241 organic acids and sugar were carried out at Scientific-Technical Services of the University of
242 Barcelona. Prior to analysis, the exudate sample was concentrated by freeze drying. The amino
243 acid content was analyzed through cationic exchange chromatography (Amino acids analyzer,
244 Biochrom 30, Biochrom, UK) and post-column derivatization with ninhydrin. The
245 chromatograph was equipped with a polysterene-divinylbenzene sulphonate column (200x4 mm)
246 with a 5 μm film thickness. Elution was carried out using lithium citrate buffer with a pH and
247 ionic strength according to the manufacturer's instructions.
248 Low-molecular-weight organic acids were analyzed using a Water Alliance 2695
249 chromatograph coupled to a PE SCIEX API 365 triple quadruple mass spectrometer. The column
250 was an Aminex HPX-87H (300x7.8 mm) column (Bio-Rad, CA). The oven temperature was held
251 at 40°C. The sample (100 μL) was injected with a flow rate of 0.8 mL min^{-1} of water acidified
252 with acetic acid (0.1%) and subjected to a post-column addition of methanol acidified in the same
253 manner. The analyses were performed using a Turbo Ion spray ionization source in negative
254 polarity with the following parameters: capillary voltage −3500 V, nebulizer gas (N_2) 10
255 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), declustering potential -60 V, focusing
256 potential −200 V, entrance potential 10 V. The drying gas (N_2) was heated to 350°C and

257 introduced at a flow-rate of 7000 mL min^{-1}. The results were analyzed in both Full Scan (40-400
258 Da) and SIM (selected ion monitoring) modes.
259 The sugar content was analyzed in a Waters Alliance 2695 chromatograph equipped with
260 Aminex HPX-87P (300 x 7.8 mm) and Aminex HPX-87C (300 x 7.8 mm) columns (BioRad,
261 CA) connected to a refraction index detector (Waters 2414) at a temperature of 37°C. The solvent
262 system consisted of purified water at a flow rate of 0.6 mL min^{-1}. The oven temperature was held
263 at 85°C.
264 Aromatic carboxylic acids and fatty acids were detected using GC-MS analysis based on
265 methylated derivatives. After acidification with 1 M HCl (pH 2), 50 mL of the exudates was
266 extracted with ethyl acetate (5 x 20 mL), and the extracts were concentrated under vacuum to 1
267 mL and derivatized *via* treatment with ethereal diazomethane. Analyses were performed on a
268 Hewlett Packard HP5890 Series II gas chromatograph coupled to an HP 5989 mass spectrometer
269 using a DB5 (J&W Scientific, Folsom, CA) capillary column (30 x 0.25 mm i.d.) with a 0.25-µm
270 film thickness. The column temperature was held at 50°C for 1 min and increased to 310°C at
271 10°C min^{-1}, and this final temperature was maintained for 10 min. The mass spectrometer was
272 operated at a 70 eV electron ionization energy. The injector and analyzer temperatures were set at
273 290°C and 315°C, respectively. The samples (1 µL) were injected in splitless mode using helium
274 as the carrier gas at a flow rate of 1.1 mL min^{-1}. When possible, products were identified and
275 quantified through comparison of their MS spectra and GC retention times with those obtained
276 for authentic commercial standards. When authentic products were not available, identification
277 was suggested on the basis of data in databases (National Institute of Standards and Technology).
278
279 *2.3.3. Bioaccessibility experiments with exudates*
280 The bioaccessibility estimations relied on the determination of residual concentrations of
281 native PAHs when ^{14}C-tracer biodegradation decreased in radiorespirometry assays performed in
282 parallel (Bueno-Montes et al., 2011). To measure pyrene mineralization by indigenous bacteria in
283 the presence or absence of sunflower root exudates, 1 g of soil was suspended in 70 mL of MM
284 or sunflower root exudates. The suspensions were placed in 250 mL Erlenmeyer flasks under
285 sterile conditions, and each treatment was performed in duplicate. Each of the flasks contained
286 30000 dpm of radiolabeled pyrene (58.7 mCi·$mmol^{-1}$, radiochemical purity >98%) in 1 mL of
287 MM. The flasks were sealed with Teflon-lined stoppers and were maintained at 25ºC on a rotary

shaker operating at 80 rpm. The production of $^{14}CO_2$ was measured as the radioactivity appearing in an alkali trap. The trap consisted of a 5 mL vial suspended from the Teflon-lined stopper; the vial contained 1 mL of NaOH (0.5 M). Periodically, the solution was removed from the trap and replaced with fresh alkali. The NaOH solution was mixed with 5 ml of a liquid scintillation cocktail (Ready Safe; Beckman Instruments), and the mixture was maintained in darkness for approximately 8 h to allow dissipation of chemiluminescence. Radioactivity was then measured with a liquid scintillation counter (model LS5000TD; Beckman Instruments).

To determine the biodegradation of the native PAHs present in the soil, separate duplicate flasks (with and without exudates) were incubated under the same conditions, but without addition of the ^{14}C-labeled compound. At the end of the incubation period (250 h), extraction and analysis of the PAHs present in the soil mixture suspension were conducted by Soxhlet and then by HPLC (residual contents in aqueous phase are under the detection limit) by the same method as described in section 2.2.2. Analysis of microbial communities from cultures with and without exudates was performed as described previously in sections 2.2.3.2. and 2.2.3.3.

2.4. Statistical methods

Analysis of variance (ANOVA) and Tukey honest significant differences (HDS) were used to assess the significance of means, and Student's t-test was used to determine the significance of percentages. These statistical analyses were performed using SPSS v. 19 software. Differences obtained at the $p \leq 0.05$ level were considered to be significant.

3. Results

3.1. Greenhouse experiment

3.1.1. Plant response

All of the sunflower seeds germinated in both contaminated and uncontaminated soils within 15 days of the beginning of the experiment. However, after 90 days, the average stem height (67 cm) and dry weight of whole plants (6.51 g) were significantly higher ($p \leq 0.05$) in plants grown in contaminated soil than in those developing in uncontaminated soil (57.9 cm and 4.46 g, respectively). These differences may be related to the autoclaving procedure used for the

uncontaminated soil. The activity of microorganisms introduced into the soil mixture with the creosote-polluted soil may have been beneficial for the plants due to detoxifying contaminants and mobilizing soil nutrients. Therefore, the good development of plants in contaminated soil is an indirect indicator of the origin of the microbial populations developed during the greenhouse experiment.

3.1.2. Dissipation of PAHs in pots with polluted soil

Measurement of residual PAH concentrations showed the promoting effect of planting *H. annuus* on the dissipation of these chemicals from soil (Table 1). The concentrations of anthracene, fluoranthene, pyrene and crysene in planted soils decreased significantly below the levels detected in the unplanted controls after 45 and 90 d. A positive effect of planting on the dissipation of fluorene was only observed after 45 d, and its concentration remained below the detection limit in both planted and unplanted soils after 90 days. The presence of sunflower plants had no significant effect on the dissipation of phenanthrene in any of the sampling periods. The absence of effect may be connected to the higher solubility in water of this compound (1.8 mg L^{-1}), as compared with anthracene (0.045 mg L^{-1}), fluoranthene (0.206 mg L^{-1}), pyrene (0.13 mg L^{-1}) and chrysene ($1.8 \cdot 10^{-3}$ mg L^{-1}), what may have caused less bioavailability restrictions to biodegradation of phenanthrene. The increased dissipation was reflected in the significantly lower ($P \leq 0.05$) concentration of total PAHs in planted pots compared to the unplanted controls, which resulted in a 60% additional decrease in the total PAH content in both sampling periods. With the exception of fluorene, extending the experimental period to 90 days did not result in a significantly lower residual concentration of any of the PAHs in the soils. The chemical analysis of major soil characteristics (e.g., pH, texture) did not reveal significant differences after planting with sunflowers, with the only difference being found in the content of total organic carbon, which increased in the planted soils from 0.9 to 2.1% after 90 days.

3.1.3. Analysis of the autochthonous microbiota and its population dynamics

Microbial counts indicated that the soil used in this study was highly enriched in PAH degraders (Fig. 1). The heterotrophic microbial populations increased more than two orders of magnitude between days 0 and 45 under all the conditions. The treatments with plants did not seem to produce an additional enhancement of the growth of the heterotrophs in comparison to

the untreated soil. These populations decreased slightly between days 45 and 90, except in the treatment with plants, where they remained at similar levels. This finding could be explained by general depletion of the available carbon sources, which would be compensated for by the rhizosphere in the plant treatment. The LMW PAH-degrading populations also increased in size by approximately two orders of magnitude between 0 and 45 days under all conditions but decreased thereafter in the control soil, while remaining approximately constant in the plant treatment. Interestingly, HMW PAH degraders experienced a substantial increase by 45 d, especially in the plant treatment, and remained at high levels until the end of the experiment. Because at 90 days, the ratio between the HMW PAH degraders and total heterotrophic populations was substantially higher in the treated than in the untreated soil, it could be concluded that in addition to stimulating the general growth of the heterotrophic populations (including that of PAH degraders), the rhizosphere treatment had an additional selective effect of enhancing the growth of the HMW PAH-degrading populations. In addition, these results show that an increase in microbial growth can be obtained by supplementing soil with carbon sources and nutrients (present in exudates) and by improving the biodegradation of PAHs, possibly by increasing their bioaccessibility.

It is known that the microbial communities in the rhizosphere can be considerably different than those in nearby soil that grow without the direct influence of roots. As a first step in understanding whether the increase in PAH degradation observed in the treated soil containing plants could be related to specific changes in the microbial community structure, we used DGGE and clone library analysis. The DGGE fingerprints obtained during the incubation period from replicate samples for each treatment showed very similar banding profiles (Fig. 2), indicating strong homogeneity within the pots for each condition. In general, the DGGE analysis revealed an initially diverse microbial community, with specific populations increasing in relative abundance throughout the incubation period in both the non-treated and the rhizosphere soil. A number of the bands obtained coincided in the two treatments, but their relative intensities differed, indicating that the shift in community structure induced by the rhizosphere was different than that induced by the simple potting and watering of the polluted soil.

To gain insight into which microbial groups were selectively promoted by the rhizosphere in comparison to the non-treated soil, corresponding 16S rRNA gene libraries were obtained from samples taken at 90 days, and a total of 84 clones were analyzed. Table 2 indicates the relative

abundance and phylogenetic affiliation of each of the eubacterial populations detected, while Fig. 3 summarizes the importance of the different bacterial phyla in the non-treated and sunflower rhizosphere soils. Approximately two-thirds (60 and 68%) of the bacteria detected under both conditions belonged to the α-, β-, and γ-*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Chloroflexi* phyla. However, with the exception of the *Actinobacteria*, the relative abundances of these phyla and their compositions varied substantially with the treatment applied, confirming that the plants caused a dramatic shift in the community structure. The rhizosphere promoted the appearance of new populations within the three *Proteobacteria* subphyla, including representatives with a well-known capacity to degrade PAHs (Kanaly and Harayama, 2010). Within the α-*Proteobacteria,* the increase of the *Sphingomonas* group was interesting because this group included numerous members isolated from plant root systems and members with a versatile degrading capability allowing them to attack 2-, 3- and 4-ring PAHs (Fernández-Luqueño et al., 2011). There was also a noticeable increase in the β-*Proteobacteria* (from 9% to 27%), as the rhizosphere promoted the appearance of members of the *Commamonas* group showing high similarity matches to members in the database isolated from PAH-contaminated soil or xenobiotic degraders (i.e., *Variovorax)*.

In the non-planted soil, 40% of the detected microorganisms belonged to seven phylogenetic groups not detected in the sunflower planted soil. Interestingly, among these microbes, we found members of the Candidate divisions OD1, OP11, TM7 and WS6, which are lineages of prokaryotic organisms for which there are no reported cultivated representatives but which present sufficiently well-represented environmental sequences to conclude that they represent major bacterial groups (Chouari et al., 2005). In addition to these sequences, the sequences retrieved from non-treated soil revealed a relatively high abundance of *Firmicutes*, while the *Planctomycetes* and *Deinococcus* groups were represented with lower proportions. In contrast, the sunflower rhizosphere soil promoted the presence of four phylotypes (*Acidobacteria, Gemmatimonadetes,* δ-*Proteobacteria* and *Cyanobacteria*) that were not detected in the non-treated soil. The most abundant, the *Acidobacteria* (14.6%) and *Gemmatimonadete*s (7.3%), constitute recently described new phyla (Ludwig et al., 1997; Zhang et al., 2003) and are broadly distributed in soils but poorly represented in cultures, which makes it difficult to ascertain their role in nature. The *Acidobacteria* have been observed previously in planted soil (Yrjala et al., 2010), are usually found in non-polluted environments, and generally decrease in the presence of

412 pollutants. Therefore, their higher abundance here after 90 days of treatment may be explained by
413 both the rhizosphere effect and the high degree of removal of PAHs attained in this condition.
414
415 *3.2. In vitro production of exudates*
416
417 *3.2.1. Production*
418 After 30 days, the average of germination rate was 55.33%, with the growth per day in terms
419 of weight being 74 mg. The average fresh and dry weights of roots were 12.04 and 0.965 g,
420 respectively, and the levels of TOC produced by the exudates were between 54.4 and 339 mgL^{-1},
421 with an average of 129.73 mg L^{-1}. A direct significant linear correlation (R=0.9125) ($p \leq 0.05$) was
422 established between the RGR (74 mg per day) and fresh weight (12.04 g). There was also a direct
423 linear correlation found between TOC (129.73 mg L^{-1}) with RGR (R=0.7293) and TOC with
424 fresh weight (R=0.6366), although these correlations were not statistically significant.
425
426 *3.2.2. Exudate Composition*
427 Table 3 shows the compounds identified in the sunflower root exudates using different
428 analytical techniques, including carbohydrates, amino acids, fatty acids, aromatic acids and
429 certain secondary metabolites. As major carbohydrates, we identified fructose (2.44 ppm) and
430 galactose (1.16 ppm); however, the chromatogram also showed a major unidentified peak that
431 would have interfered with the detection of glucose if it had been present. Previous studies
432 addressing exudate composition in tomato, sweet pepper, cucumber and Barmultra grass showed
433 that fructose was one of most dominant sugars (Kuiper et al., 2002; Kamilova et al., 2006).
434 Galactose is also present in root exudates, providing a favorable environment for the growth of
435 microorganisms (Bertin et al., 2003), and has been detected in the root exudates of different
436 species of Eucalyptus (Grayston et al., 1996). Amino acids were detected in a wide range of
437 concentrations, among which asparagine (0.593 ppm) and glutamine (0.301 ppm) were the most
438 abundant, while methionine, tryptophan, proline, glutamic acid and valine were not detected.
439 Phosphoethanolamine was also detected at a relatively high concentration (0.571 ppm) and has
440 been reported to be abundant in the cell membrane (Ofosubudu et al., 1990). The main fatty acids
441 present were palmitic and estearic acids, whereas others, including the most abundant component
442 of sunflower oil, linoleic acid, were detected at lower concentrations and could not be quantified.

443 Several aromatic acids were identified, the most abundant of which were phthalic and
444 protocatechuic acids. This result is of particular note, given that these compounds are typical
445 intermediates in the metabolism of PAHs by bacteria (Kanaly and Harayama, 2010). The HPLC-
446 MS analysis of organic acids revealed several products. The most intense signal corresponded to
447 a compound with a mass compatible with gluconic acid. Other products were tentatively
448 identified as caffeic, isocitric, butiric, pyruvic, propionic, fumaric, malic, and malonic acids, all
449 of which are typically found in root exudates (Bertin et al., 2003). Abietic acid and the
450 sesquiterpene tomentosin were identified as the methyl derivatives of organic acids in the GC-MS
451 analysis, and in addition to having structures analogous to some PAHs, they exhibit different
452 functions in the plant-microbe interaction.
453 In vitro, the sunflower root exudates showed a surface tension close to the surface tension of
454 the mineralization medium (MM), showing either an absence or a low concentration of
455 surfactants that could improve accessibility.
456
457
458 *3.2.3. Effects of exudates on the bioaccessibility of PAHs and on soil microbial populations*
459 Bioaccessibility experiments showed that the maximum rate of pyrene mineralization was
460 enhanced twofold by the presence of exudates (from 0.024 ± 0.002 ng mL^{-1} h^{-1} to 0.052 ± 0.008
461 ng mL^{-1} h^{-1}, Figure 4). The maximum extent of pyrene C mineralization was also enhanced (from
462 $29 \pm 1.01\%$ to $40 \pm 1.41\%$), and the acclimation phase for pyrene mineralization was shortened
463 from 75 h to 30 h. Interestingly, the results showed that the concentrations of total PAHs
464 decreased to significantly lower values in the presence of exudates (Table 4), thereby
465 demonstrating the positive influence of exudates on biodegradation for native chemicals.
466 Furthermore, the residual contents of total PAHs, both with and without exudates, were not
467 significantly different than those reached in the corresponding treatments in greenhouse
468 experiments after 90 days ($P \leq 0.05$). Therefore, we can conclude that the degradation-promoting
469 effect of sunflower plants on the dissipation of PAHs from soil that occurred in the greenhouse
470 experiment could be reproduced through laboratory incubation of the soil with shaking and the
471 addition of root exudates. The shorter time period needed in the slurries to reach residual
472 concentrations (10 days), as compared to greenhouse conditions (90 days), can be attributed to an
473 enhanced mass transfer of the pollutants. However, because the controls and the exudate-

474 containing slurries were treated exactly in the same way, the observed relative differences still
475 suggest an effect of root exudates on bioaccessibility.
476 Soil suspensions were sampled at the end of the experimental period (10 d) to determine the
477 evolution of autochthonous microbiota using DGGE (Fig. 5). The DGGE profiles from cultures
478 in the mineral medium with or without exudates indicated an increase in the number of
479 microorganisms during the 10 days of the experiment in both conditions. In the absence of
480 exudates, duplicate cultures showed similar banding profiles with slight differences in the relative
481 intensity of each band. The banding profile changed as a result of exposure to exudates, which
482 indicates that the enhanced PAH degradation was accompanied by the growth of specific
483 microbial populations.
484
485 **4. Discussion**
486
487 Our data indicate that the development of sunflower plants enhanced the biodegradation of
488 PAHs in the soil, and due to the ability of microbes to reduce the phytotoxicity of the pollutants,
489 PAHs included, sunflower plants were able to grow in polluted soil even better than those grown
490 in uncontaminated soil. The slowly degrading compounds remaining in the soil at the end of
491 greenhouse and bioaccessibility assays probably exhibited slow desorption, which usually limits
492 biodegradation of these compounds by microorganisms (Bueno-Montes et al., 2011). This
493 restriction on biodegradation would explain the absence of further decreases in the PAH
494 concentrations in unplanted soils from 45 d to 90 d in the greenhouse experiment and the good
495 agreement between the residual PAH concentrations in the greenhouse and bioaccessibility
496 assays. The bioaccessibility experiments were designed to test the disappearance of the chemicals
497 under laboratory conditions. These assays specifically addressed biodegradation using an excess
498 of nutrients, radiorespirometry determinations with ^{14}C-pyrene and analysis of residual
499 concentrations of native PAHs. This method had been applied previously to determine the
500 efficiency of bioremediation approaches designed to increase the bioaccessibility of aged PAHs
501 (Bueno-Montes et al., 2011) . Despite the inherent difficulties in performing bioaccessibility
502 estimations related to the specific the time period and/or target organisms considered (Alexander,
503 2000), this approach was very useful in the present study for reproducing the greenhouse results
504 in the presence of root exudates produced in vitro, which indicates that the exudates played an

505 important role in the effectiveness of the plants in promoting the bioaccessibility of PAHs. To our
506 knowledge, the direct comparison of pollutant losses in the presence of plants with those caused
507 by the addition of exudates had not been included in previous studies of PAH rhizoremediation.
508 The TOC content observed in the sunflower root extracts in this study, 129.73 mg L^{-1}, was in
509 agreement with TOC values reported in other studies on the promoting effects of root extracts on
510 PAH-degrading microorganisms. For example, Rentz et al. (2005) reported TOC concentrations
511 of 84.2, 175.0 and 51.7 mg L^{-1} from root extracts of hybrid willow (*Salix alba x matsudana*), kou
512 (*Cordia subcordata*) and milo (*Thespesia populnea*), respectively, whereas Miya and Firestone
513 (2001) reported a TOC concentration of 54 mg L^{-1} for slender oat root exudates. In the present
514 study, it is possible that the organic carbon in the exudates enhanced the bioaccessibility of PAHs
515 through a mechanism related to the carbon's capacity to mobilize PAHs that are initially
516 absorbed in the soil. Indeed, addition of DOM to contaminated soils results in enhanced
517 biodegradation of PAHs, probably as a result of enhanced desorption (Bengtsson and Zerhouni,
518 2003). DOM-mediated enhancement of biodegradation can also be caused by direct access to
519 DOM-sorbed PAHs due to the physical association of bacteria and DOM (Ortega-Calvo and
520 Saiz-Jimenez, 1998) and an increased diffusive flux toward bacterial cells (Haftka et al., 2008;
521 Smith et al., 2009). The latter mechanism would be analogous to that described for the enhanced
522 uptake of metals by plants in the presence of labile metal complexes, which is caused by an
523 increased diffusional flux through unstirred boundary layers around roots (Degryse et al., 2006).
524 The occurrence of DOM-mediated enhancement of bioaccessibility through root exudation would
525 also explain the greater extent of biodegradation observed under greenhouse conditions, despite
526 the significant increase of total organic carbon in the planted soils.
527 The chemical characterization of exudates also identified specific substances with the
528 potential to directly enhance bioaccessibility. These substances include chemicals that are able to
529 induce chemotaxis, which constitutes a relevant mobilization mechanism for motile
530 microorganisms in the soil (Ortega-Calvo et al., 2003). For example, sugars such as fructose have
531 a well-known positive chemotactic effect on soil microorganisms. Amino acids, such as
532 glutamine, aspartic acid and isoleucine, which were also found in this study as components of
533 sunflower root exudates, are powerful chemoattractants for *Rhizobium* and *Bradyrhizobium*
534 *japonicum* (Pandya et al., 1999). Zheng and Sinclair (1996) indicated that alanine, asparagine,
535 glutamine, serine, and threonine in soybean root exudates may serve as chemoattractants to

536 *Bacillus megaterium* strain B153-2-2. Finally, we detected fatty acids, such as palmitic acid and
537 stearic acid, which are plant components with a known potential to enhance the bioaccessibility
538 of PAHs in soil by acting as surfactants (Yi and Crowley, 2007). Vegetable oils have also been
539 widely used as natural surfactants (Gong et al., 2010), resulting in the dissolution of PAHs and
540 consequently, in the enhancement of biodegradation. Therefore, the presence of these compounds
541 may explain the greater decrease in PAHs observed in the sunflower soil treatments.
542 Furthermore, it is also possible that the preferential growth of rhizosphere microorganisms
543 observed on the exudate components at specific sites inside soil aggregates may have caused
544 colony growth in the vicinity of pollutant sources and may have modified the structure of the soil
545 aggregates to promote bioaccessibility through the excretion of extracellular polymeric
546 substances and biosurfactants.
547 Therefore, the results obtained associated with root exudates indicated a role for promoting
548 the bioaccessibility of PAHs. However, the present study may not allow complete discrimination
549 between the effects on bioaccessibility from the enhanced biodegradation activity of
550 microorganisms caused by the chemical components of exudates. The evolution of the
551 heterotrophic bacterial population in the soil during the greenhouse experiment indicates that
552 homogenization, aeration and watering had a general activation effect on this population, but
553 planting sunflowers had a further positive impact due to maintaining their viability (Fig. 1). The
554 chemical analysis of exudates reflected the presence of organic compounds in the root exudates
555 with the potential to cause this effect. For example, fructose and galactose are known to provide a
556 favorable environment for the growth of rhizosphere microorganisms (Grayston et al., 1996;
557 Bertin et al., 2003); amino acids are a source of easily degradable N compounds, inducing
558 protease synthesis (García-Gil et al., 2004); and ornithine is considered to be non-protein amino
559 acids showing a protective function against stress to cell membranes (Kalamaki et al., 2009).
560 Furthermore, the presence of plants had also a profound impact on the relative abundance of
561 specific groups of bacteria in the soil, thereby increasing their biodiversity. This consideration is
562 consistent with the results obtained in the PAH analysis and confirm the results obtained by other
563 authors (Miya and Firestone, 2000; Parrish et al., 2005). The proportion of gram-negative
564 bacteria increased in planted soils compared with unplanted controls, which is in agreement with
565 previous observations (Anderson and Coats, 1995). For example, we observed better
566 development of β-*Proteobacteria* in planted soils, which can be explained by the capability of

567 this group of bacteria to readily assimilate the C present in sugars and residues of plant origin
568 (Bernard et al., 2007). In the same way, the rhizophere promoted the appearance of members of
569 the *Oxalobacteriaceae*, a recently described but uncharacterized family with root colonizing
570 members (Green et al., 2007) that are closely related to the *Burkholderia*, which include
571 important soil PAH degraders of both single compounds and creosote mixtures (Grifoll et al.,
572 1995). The increase observed in members of the *Methylophillus* group was interesting because in
573 a recent study, a methylotrophic bacterial species was identified as one of the most abundant
574 components of a heavy fuel-degrading consortium (Vila et al., 2010). Methylotrophic bacteria are
575 more widely distributed than previously thought, but their roles in natural habitats remain
576 unknown (Lidstrom, 2006; Chistoserdova et al., 2009). The *Xanthomonas* group within the γ-
577 *Proteobacteria* was also favored by the rhizosphere, with several of the detected representatives
578 of this group corresponding to bacteria previously detected in polluted sites and identified as
579 PAH degraders. For example, a *Pseudoxhantomonas* strain was recently described as being able
580 to degrade the 4-ring PAH chrysene (Nayak et al., 2011). The reduction in the abundance of
581 Bacteroidetes in the rhizosphere soil could be a direct consequence of the presence of nutrients
582 from the exudates because this phylum has often been associated with non-nutrient environments
583 (Viñas et al., 2005).
584 Interestingly, certain aromatic organic acids were detected in the root exudates, such as
585 phthalic and protocatecuic acids, that are intermediate metabolites in the degradation of PAHs
586 (Lopez et al., 2008). These secondary plant metabolites may stimulate PAH degradation by
587 rhizosphere microorganisms and broaden the spectrum of their activity by inducing and
588 promoting the development of organic pollutant-degrading enzymes or acting as cosubstrates in
589 cometabolic reactions. Indeed, the population of high-molecular-weight (HMW) PAH degraders
590 increased in number in the planted soils compared with the unplanted controls, demonstrating the
591 selective influence of the sunflower rhizosphere on these populations. These results agree with
592 those from Parrish et al. (2005), who observed that after 12 months of plant development, the
593 PAH degrader population was multiplied 100-fold in comparison with unplanted soil. Corgie et
594 al. (2004) also found that the number of HMW PAH degraders decreased inversely with the
595 distance from roots. Consistent with this selective effect on the PAH-degrading populations, there
596 was a demonstrated increase in the relative abundance of bacterial groups with a know PAH-
597 degrading capability or that were previously detected as key components in PAH-degrading

598 microbial consortia, including *Sphingomonas* (within the α-*Proteobacteria*), *Comamonas*,
599 *Oxalobacteria* and *Methylophillus* (β-*Proteobacteria*), and *Xanthomonas* (Kanaly and Harayama,
600 2010). Although the relative abundance of the *Actinobacteria* group does not change in the
601 presence of sunflowers, it is known that this a group characterized by its ability to degrade
602 recalcitrant organic compounds. Other microbes that are able to degrade recalcitrant organic
603 compounds include *Actinomycetes,* which are able to compete with fungi for lignin degradation,
604 and *Mycobacteria*, which can degrade a variety of PAHs either as individual compounds or
605 within fuel (Vila et al., 2010) and creosote mixtures (López et al., 2008), particularly at sites
606 where there is a low level of nutrients. Other bacterial phyla favored by the rhizosphere,
607 including *Acidobacteria* and the *Gemmatimonadetes*, are recently described groups with few
608 culturable representatives, and more research is needed to understand their potential role in
609 polluted soils (Ludwig et al., 1997; Zhang et al., 2003).
610 Considering the advantages of this plant species in relation to its agronomic interest and
611 potential as a biofuel producer, this strategy represents a promising alternative for increasing
612 bioaccessibility in a sustainable and low-risk manner. Our results demonstrate that the
613 rhizosphere caused a substantial shift in the structure of the autochthonous microbial populations
614 in the soil that selectively favored the development of PAH degraders. Most of the literature
615 discussed herein involves recent work on the effect of the rhizosphere on selected microbial
616 PAH-degrading populations in artificially PAH-spiked soils. This study is the first to analyze the
617 effect of the rhizosphere on autochthonous bacterial community structure from a real PAH-
618 polluted soil. The exact contribution of the direct effects of the sunflower exudates and the effects
619 related to the ecology of soil microorganisms will be the subject of future research.
620
621 **Acknowledgments**
622 Support for this research was provided by the Spanish Ministry of the Economy and
623 Competitiveness (grants CGL2007-64199, CGL2010-22068-C02-01 and CGL2010-22068-C02-
624 02) and the FPI Programme (M.C. Tejeda-Agredano) and FPU Programme (S. Gallego), M.
625 Grifoll and J. Vila are members of the Xarxa de Referència de R+D+I en Biotecnologia (XRB),
626 wich receives funding from the Generalitat de Catalunya.
627
628

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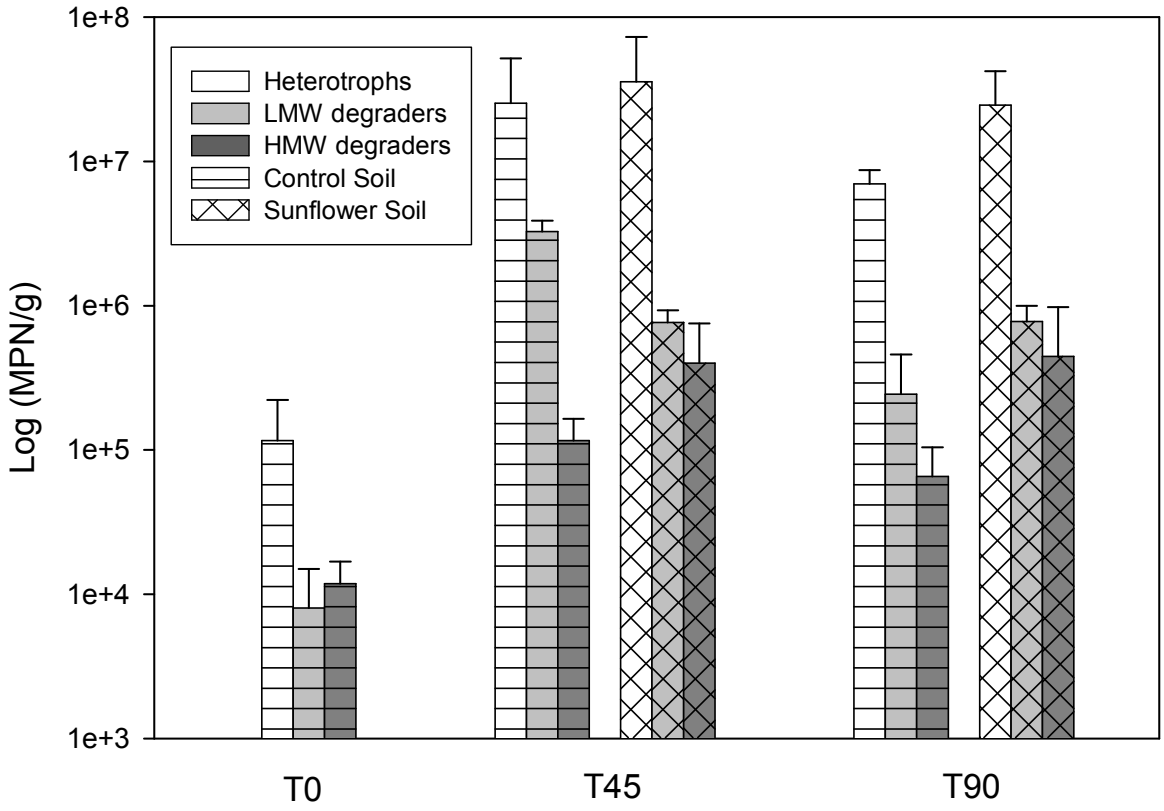

Fig. 1. Counts of heterotrophic and PAH-degrading microbial populations in the soil under the different treatments applied in the greenhouse experiment. MPN, most probable number. LMW, low-molecular-weight PAHs. HMW, high-molecular-weight PAHs. T0, at the beginning of the experiment; T45, at 45 days after; T90 at 90 days after. Error bars represent the standard deviation of triplicates.

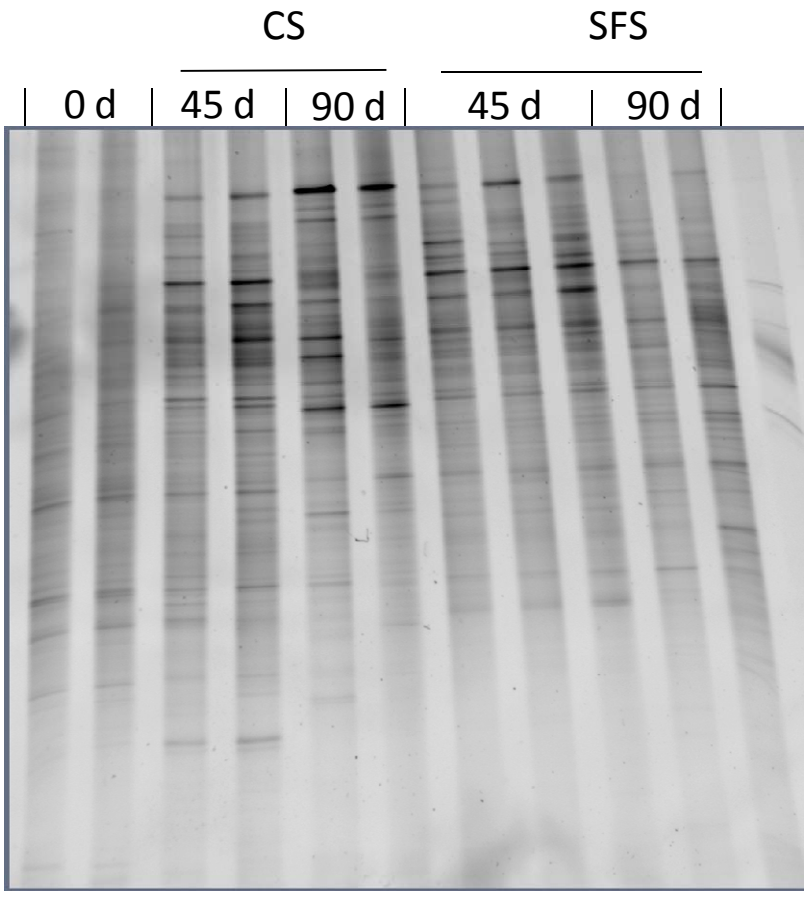

Fig. 2. DGGE profile of PCR-amplified 16S rRNA gene fragments from independent replicate samples from control soil (CS) and rhizosphere sunflower soil (SFS) samples after 0, 45 and 90 days. Each lane was loaded with an identical amount of DNA.

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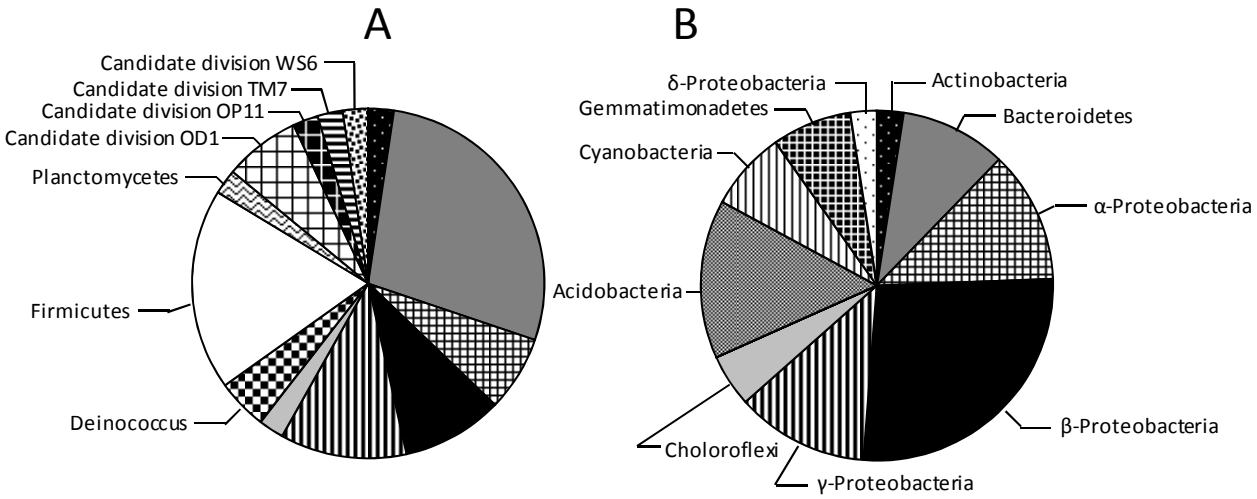

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852 Fig. 3. Relative abundance of eubacterial phylogenetic groups identified in control soil (A) and
853 sunflower rhizosphere soil (B) samples after 90 days of incubation in the greenhouse experiment.

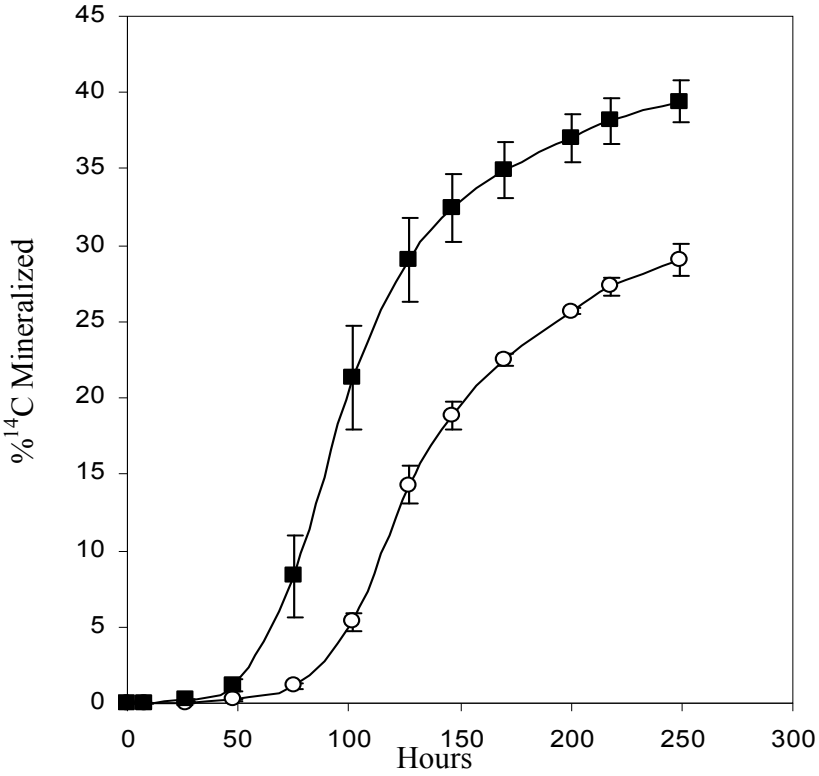

Fig. 4. Mineralization of pyrene in soil suspensions in the absence (o) and presence (■) of sunflower root exudates. Error bars represent the standard deviation of duplicates.

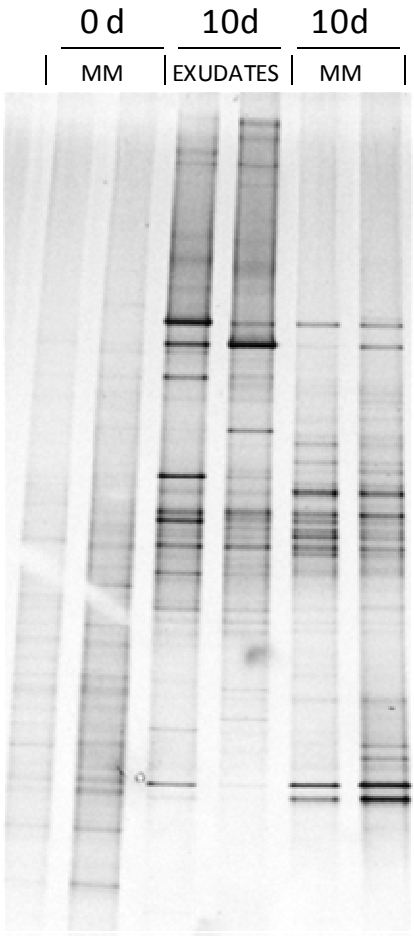

Fig. 5. DGGE profile of PCR-amplified 16S rRNA gene fragments in samples from soil suspensions in the bioaccessibility experiment presented in Figure 4 at the beginning (0 d) and at the end of the experimental period (10 d).

882 Table 1

883 Effect of planting with sunflowers on residual PAH contents (mg kg^{-1}) in soil under greenhouse
884 conditions after 45 and 90 days.

PAHs	C_0	45 days		90 days	
		Control	Planted	Control	Planted
Fluorene	1.23 ± 0.08A	0.18 ± 0.03B	0.04 ± 0.01C	< 0.00C	< 0.00C
Phenanthrene	3.98 ± 0.29A	0.77 ± 0.06B	0.39 ± 0.03B	0.54 ± 0.05B	0.12 ± 0.05B
Anthracene	8.14 ± 0.39A	2.14 ± 0.45B	0.67 ± 0.14C	1.63 ± 0.37B	0.36 ± 0.13C
Fluoranthene	5.10 ± 0.07A	1.51 ± 0.53B	0.46 ± 0.01C	1.47 ± 0.14B	0.37 ± 0.11C
Pyrene	2.04 ± 0.05A	0.39 ± 0.13B	0.15 ± 0.001C	0.34 ± 0.03B	0.14 ± 0.05C
Chrysene	1.26 ± 0.05A	1.16 ± 0.08A	0.68 ± 0.12B	1.02 ± 0.07A	0.51 ± 0.16B
Total PAHs	**21.75 ± 0.9A**	**6.15 ± 1.26B**	**2.39 ± 0.30C**	**4.99 ± 0.66B**	**1.50 ± 0.5C**

885

886 C_o, initial concentration of PAHs in the soil. The values shown are the mean ± standard deviation
887 of triplicates. Values in a row followed by the same capital letter are not significantly different
888 ($P \leq 0.05$).

889 Table 2

890 Sequence analysis of the 16S rRNA gene clone libraries from the PAH-polluted control soil (without plants)

891 and sunflower rhizosphere soil at the end of the greenhouse experiment (90 days).

Clone	Frequency (%)[a]		Fragment legth (bp)	Sim %	Closest relative in GenBank database[b] (accession no)	Phylogenetic group
	CS	SFS				
CS1		2.4	1364	99	Uncultured *Acidobacteria* bacterium clone HEG_08_216 (HQ597545)	*Acidobacteriaceae (Acidobacteria)*
CS2		2.4	1373	98	Uncultured bacterium clone 60C1 (EU676416)	*Acidobacteriaceae (Acidobacteria)*
CS3		2.4	1422	99	Uncultured *Acidobacteria* bacterium clone SEG_08_603 (HQ729829)	*Acidobacteriaceae (Acidobacteria)*
CS4		2.4	1404	97	Uncultured *Acidobacteria* bacterium clone HG-J02120 (JN409027)	*Acidobacteriaceae (Acidobacteria)*
CS5		2.4	1421	99	Uncultured bacterium clone p6h2ok (FJ478980)	*Acidobacteriaceae (Acidobacteria*
CS6		2.4	1386	97	*Acidobacteria* bacterium IGE-018 (GU187039)	*Acidobacteriaceae (Acidobacteria)*
CS7	2.3		1466	99	Uncultured bacterium clone p27d24ok (FJ478675)	*(Actinobacteria)*
CS8		2.4	1384	99	*Lentzea waywayandensis* strain 173629 (EU570362)	*Pseudonocardiaceae (Actinobacteria)*
CS9	4.7		1444	98	Uncultured bacterium clone 125 (FM209343)	*Cytophagaceae (Bacteroidetes)*
CS10	7.0		1453	99	Uncultured bacterium clone p8c07ok (FJ479495)	*Chitinophagaceae (Bacteroidetes)*
CS11	2.3		1225	98	Uncultured bacterium isolate 1112864242247 (HQ120332)	*Ohtaekwangia (Bacteroidetes)*
CS12	2.3		1314	98	Unculured *Bacteroidetes* bacterium clone HG-J01164 (JN408934)	*Ohtaekwangia (Bacteroidetes)*
CS13	2.3		1443	95	Uncultured bacterium clone TX2_4C19 (JN178178)	*Rhodothermaceae (Bacteroidetes)*
CS14	2.3		949	99	Uncultured soil bacterium clone BJ-287 (EU365214)	*Ohtaekwangia (Bacteroidetes)*
CS15	4.7	4.9	1445	98	Uncultured *Bacteroidetes* bacterium clone g31 (EU979040)	*Ohtaekwangia (Bacteroidetes)*
CS16	2.3	2.4	1393	99	Uncultured bacterium clone 224T (EU676412)	*Chitinophagaceae (Bacteroidetes)*
CS17		2.4	1394	98	Uncultured soil bacterium clone UA2 (DQ298006)	*Chitinophagaceae (Bacteroidetes)*
CS18	2.3		1410	99	Uncultured *Anaerolinae* bacterium clone AMAG11 (AM935836)	*(Chloroflexi)*
CS19		2.4	1353	99	Uncultured bacterium clone S-Rwb_62 (DQ017911)	*(Chloroflexi)*
CS20		2.4	1353	99	Uncultured bacterium clone H3-26 (JF703479)	*(Chloroflexi)*
CS21		4.9	1373	99	*Phormidium autumnale* CCALA 143 (FN813344)	*(Cyanobacteria)*
CS22		2.4	1324	99	Uncultured diatom clone H-101 (HM565019)	*Bacillariophyta (Cyanobacteria)*
CS23	4.7		1455	96	Uncultured bacterum isolate 1112864242286 (HQ120393)	*Trueperaceae (Deinococcus)*
CS24	2.3		1423	99	*Bacillus* sp. M71_D96 (FM992837)	*Bacillaceae (Firmicutes)*
CS25	9.3		1440	99	*Bacillus* sp. R-36493 (FR682744)	*Bacillaceae (Firmicutes)*
CS26	2.3		1486	98	*Virgibacillus carmonensis* (T) LMG 20964 (NR_025481)	*Bacillaceae (Firmicutes)*
CS27	4.7		1438	99	*Bacillus* sp. BF149 (AM934692)	*Bacillaceae* 2 *(Firmicutes)*
CS28		4.9	1399	96	Uncultured bacterium clone 15-4-139 (JN609373)	*Gemmatimonadaceae(Gemmatimonadetes)*
CS29		2.4	1429	98	Uncultured bacterium clone TX5A_120 (FJ152828)	*(Gemmatimonadetes)*
CS30	2.3		1368	94	Uncultured bacterium clone B6 (FJ660498)	*Planctomycetaceae (Planctomycetes)*
CS31	2.3		1285	99	*Chelatococcus asaccharovorans* CP141b (AJ871433)	*Methylobacteriaceae (α)*
CS32	2.3		1276	99	Uncultured bacterium clone HDB_SIOP800 (HM186473)	*Bradyrhizobiaceae (α)*
CS33	2.3	2.4	1410	97	Uncultured soil bacterium clone F6-154 (EF688392)	*Sphingomonadaceae (α)*
CS34		2.4	1342	99	*Rhizobium* sp. AC93c (JF970343)	*Rhizobiaceae (α)*
CS35		2.4	1355	97	Uncultured bacterium clone FCPS478 (EF516121)	*Rhizobiales (α)*
CS36		2.4	1353	99	Uncultured alpha proteobacterium clone QZ-J4 (JF776915)	*Sphingomonadaceae (α)*
CS37		2.4	1355	99	*Altererythrobacter* sp. JM27 (GU166344)	*Sphingomonadales (α)*
CS38	2.3		1425	98	Uncultured bacterium clone SNR65 (AB608675)	*Burkholderiales (β)*
CS39	4.7	7.3	1418	99	Uncultured bacterium clone HC18-11B13 (JF417848)	*Commamonadaceae (β)*
CS40	2.3	4.9	1410	99	*Naxibacter suwonensis* (T) 5414S-25 (FJ969487)	*Oxalobacteraceae (β)*
CS41		2.4	1389	99	*Variovorax* sp. RA8 (AB513921)	*Commamonadaceae (β)*
CS42		2.4	1396	99	Uncultured beta proteobacterium clone E2006TS6.19 (GU983311)	*(β)*
CS43		2.4	1394	99	Uncultured beta proteobacterium clone C173 (JF833705)	*Methylophilaceae (β)*
CS44		2.4	1400	99	Uncultured beta proteobacterium clone C173 (JF833705)	*Methylophilaceae (β)*
CS45		2.4	1391	99	Uncultured beta proteobacterium clone G2-50 (JF703344)	*(β)*
CS46		2.4	1391	99	Uncultured ammonia-oxidizing bacterium clone FQ-13C-HF-1 (HQ678202)	*Nitrosomonadaceae (β)*
CS47	2.3		1434	97	Uncultured bacterium clone 0-99 (GU444064)	*Xanthomonadaceae (γ)*
CS48	9.3		1458	99	*Pseudomonas* sp. JQR2-5 (DQ124297)	*Pseudomonadaceae (γ)*
CS49		2.4	1412	99	*Lysobacter niabensis* (AB682414)	*Xanthomonadaceae (γ)*
CS50		2.4	1391	99	Uncultured bacterium clone Kas172B (EF203204)	*Sinobacteraceae (γ)*
CS51		2.4	1409	99	*Pseudoxanthomonas* sp. XC21-2 (JN247803)	*Xanthomonadaceae (γ)*
CS52		2.4	1410	99	Uncultured *Lysobacter* sp. clone T302B2 (HM438520)	*Xanthomonadaceae (γ)*
CS53		2.4	1399	99	Uncultured bacterium clone BR121 (HQ190468)	*Pseudomonadaceae (γ)*
CS54		2.4	1419	97	Uncultured bacterium clone RH1020 (AB511013)	*(δ)*

CS55	7.0	1050	87	Uncultured candidate division OD1 bacterium clone AKYH1067 (AY922093)	Candidate division OD1
CS56	2.3	1396	94	Uncultured bacterium clone B03-05G (FJ542974)	Candidate división OP11
CS57	2.3	1368	97	Uncultured bacterium clone N1903_34 (EU104291)	Candidate división TM7
CS58	2.3	1250	93	Uncultured bacterium clone FF_-aag84c04 (EU469637)	Candidate division WS6

[a] Frequencies in clone libraries obtained from CS (control soil) and SFS (sunflower soil). [b]In sequences showing an identical match to uncultured and to isolated strains, only the latter are listed. Sequences with more than 94% identity are grouped. α, β, γ, δ correspond to *alpha*-, *beta*-, *gamma*- and *deltaproteobacteria*, respectively. Clones with sequences belonging to the same phyla or subphyla (in case of *Proteobacteria*) are grouped and marked with alternatively shade and non shaded backgrounds

899 Table 3.

900 Organic compounds identified in the sunflower root exudates obtained in vitro after 30 days of
901 culture.

Class of compounds	Single compounds	Concentration (ppm)
Sugars[1]	Galactose	1.16
	Fructose	2.44
Amino acids[1]	Phenyl serine	0.423
	Taurine	0.083
	Phosphoethanolamine	0.571
	Aspartic acid	0.035
	Threonine	0.034
	Serine	0.069
	Asparagine	0.593
	Glutamine	0.301
	Glycine	0.016
	Alanine	0.017
	Cysteine	0.003
	Isoleucine	0.025
	Leucine	0.026
	Tyrosine	0.029
	Phenylalanine	0.012
	Ornithine	0.008
	Lysine	0.024
	Histidine	0.074
	Arginine	0.078
Fatty acids[3]	Azelaic acid[2]	nq
	Myristic acid[2]	nq
	Palmitic acid[1]	0.0353
	Linoleic acid[2]	nq
	Stearic acid[1]	0.0425

Aromatic organic acids[3]	Phtalic acid[1]	0.04358
	Paraben[2]	nq
	Protochatechuic[1]	0.00388
	Gallic acid[2]	nq
	5-Acetylsalicylic acid[2]	nq
	Abietic acid[2]	nq
	Hydroxydehydroabietic acid[2]	nq
Terpenoids[3]	Tomentosin[2]	Nq

902 *Identification was based on analysis of authentic standards[1] or on a match higher than 90% with the
903 NIST library[2]. Identified as their methylated derivates[3] (diazomethane); nq= not quantified.

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906
907 Table 4. Effect of incubation with sunflower root exudates on the residual PAH
908 content (mg kg^{-1}) in soil suspensions under laboratory conditions after 10 days.
909

PAHs	***C_0***	***Control***	***Exudates***
Fluorene	0.47 ± 0.21A	0.02 ± 0.009B	0.09 ± 0.07B
Phenanthrene	4.55 ± 0.18A	1.55 ± 0.06B	1.02 ± 0.16C
Anthracene	5.83 ± 2.43A	0.10 ± 0.02B	0.09 ± 0.02B
Fluoranthene	4.73 ± 0.44A	1.73 ± 0.85B	0.69 ± 0.19B
Pyrene	0.98 ± 0.12A	0.53 ± 0.09B	0.15 ± 0.007C
Chrysene	1.54 ± 0.20A	0.79 ± 0.15B	0.47 ± 0.02C
Total PAHs	**19.28 ± 1.40A**	**4.77 ± 0.55B**	**2.50 ± 0.16C**

910
911 C_0, initial concentration. The values shown are the mean ± standard deviation of
912 duplicates. Values in rows with the same capital letter are not significantly different
913 ($P \leq 0.05$).