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

[1]M.C. Tejeda-Agredano[a], [2]S. Gallego[b], [2]J. Vila, [2]M. Grifoll, [1]J.J. Ortega-Calvo, [1*]M. Cantos

[1]*Instituto de Recursos Naturales y Agrobiología, CSIC, Avda. Reina Mercedes, 10, 41012, Sevilla, Spain*

[2]*Departament de Microbiologia, Universitat de Barcelona, Avda. Diagonal, 645, 08028, Barcelona, Spain*

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

32   improving the availability of the contaminant to be degraded. We characterized the sunflower

33   exudates in vitro to determine the total organic carbon (TOC) and its chemical composition. Our

34   results indicate that the rhizosphere promotes the degradation of PAHs by increasing the

35   biodegradation of the pollutants and the number and diversity of PAH degraders. We propose that

36   the biostimulation exerted by the plants is based on the chemical composition of the exudates.

37

40

41   [*]Corresponding author tel: (+34) 5-4624711; fax: (+34) 5-4624002; e-mail: cantos@irnase.csic.es

42   a,b Both of these authors contributed equally to this work.

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. Bioaccessibility 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 $mg kg^{-1}$ 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

288 shaker operating at 80 rpm. The production of $^{14}CO_2$ was measured as the radioactivity appearing

289 in an alkali trap. The trap consisted of a 5 mL vial suspended from the Teflon-lined stopper; the

290 vial contained 1 mL of NaOH (0.5 M). Periodically, the solution was removed from the trap and

291 replaced with fresh alkali. The NaOH solution was mixed with 5 ml of a liquid scintillation

292 cocktail (Ready Safe; Beckman Instruments), and the mixture was maintained in darkness for

293 approximately 8 h to allow dissipation of chemiluminescence. Radioactivity was then measured

294 with a liquid scintillation counter (model LS5000TD; Beckman Instruments).

295   To determine the biodegradation of the native PAHs present in the soil, separate duplicate

296 flasks (with and without exudates) were incubated under the same conditions, but without

297 addition of the ^{14}C-labeled compound. At the end of the incubation period (250 h), extraction and

298 analysis of the PAHs present in the soil mixture suspension were conducted by Soxhlet and then

299 by HPLC (residual contents in aqueous phase are under the detection limit) by the same method

300 as described in section 2.2.2. Analysis of microbial communities from cultures with and without

301 exudates was performed as described previously in sections 2.2.3.2. and 2.2.3.3.

302

303 *2.4. Statistical methods*

304   Analysis of variance (ANOVA) and Tukey honest significant differences (HDS) were used to

305 assess the significance of means, and Student's t-test was used to determine the significance of

306 percentages. These statistical analyses were performed using SPSS v. 19 software. Differences

307 obtained at the p≤0.05 level were considered to be significant.

308

309 **3. Results**

310

311 *3.1. Greenhouse experiment*

312

313 *3.1.1. Plant response*

314   All of the sunflower seeds germinated in both contaminated and uncontaminated soils within

315 15 days of the beginning of the experiment. However, after 90 days, the average stem height (67

316 cm) and dry weight of whole plants (6.51 g) were significantly higher (p≤0.05) in plants grown in

317 contaminated soil than in those developing in uncontaminated soil (57.9 cm and 4.46 g,

318 respectively). These differences may be related to the autoclaving procedure used for the

319   uncontaminated soil. The activity of microorganisms introduced into the soil mixture with the

320   creosote-polluted soil may have been beneficial for the plants due to detoxifying contaminants

321   and mobilizing soil nutrients. Therefore, the good development of plants in contaminated soil is

322   an indirect indicator of the origin of the microbial populations developed during the greenhouse

323   experiment.

324

325   *3.1.2. Dissipation of PAHs in pots with polluted soil*

326   Measurement of residual PAH concentrations showed the promoting effect of planting *H.*

327   *annuus* on the dissipation of these chemicals from soil (Table 1). The concentrations of

328   anthracene, fluoranthene, pyrene and crysene in planted soils decreased significantly below the

329   levels detected in the unplanted controls after 45 and 90 d. A positive effect of planting on the

330   dissipation of fluorene was only observed after 45 d, and its concentration remained below the

331   detection limit in both planted and unplanted soils after 90 days. The presence of sunflower

332   plants had no significant effect on the dissipation of phenanthrene in any of the sampling periods.

333   The absence of effect may be connected to the higher solubility in water of this compound (1.8

334   mg L^{-1}), as compared with anthracene (0.045 mg L^{-1}), fluoranthene (0.206 mg L^{-1}), pyrene (0.13

335   mg L^{-1}) and chrysene ($1.8 \cdot 10^{-3}$ mg L^{-1}), what may have caused less bioavailability restrictions to

336   biodegradation of phenanthrene. The increased dissipation was reflected in the significantly

337   lower (P≤0.05) concentration of total PAHs in planted pots compared to the unplanted controls,

338   which resulted in a 60% additional decrease in the total PAH content in both sampling periods.

339   With the exception of fluorene, extending the experimental period to 90 days did not result in a

340   significantly lower residual concentration of any of the PAHs in the soils. The chemical analysis

341   of major soil characteristics (e.g., pH, texture) did not reveal significant differences after planting

342   with sunflowers, with the only difference being found in the content of total organic carbon,

343   which increased in the planted soils from 0.9 to 2.1% after 90 days.

344

345   *3.1.3. Analysis of the autochthonous microbiota and its population dynamics*

346   Microbial counts indicated that the soil used in this study was highly enriched in PAH

347   degraders (Fig. 1). The heterotrophic microbial populations increased more than two orders of

348   magnitude between days 0 and 45 under all the conditions. The treatments with plants did not

349   seem to produce an additional enhancement of the growth of the heterotrophs in comparison to

350 the untreated soil. These populations decreased slightly between days 45 and 90, except in the

351 treatment with plants, where they remained at similar levels. This finding could be explained by

352 general depletion of the available carbon sources, which would be compensated for by the

353 rhizosphere in the plant treatment. The LMW PAH-degrading populations also increased in size

354 by approximately two orders of magnitude between 0 and 45 days under all conditions but

355 decreased thereafter in the control soil, while remaining approximately constant in the plant

356 treatment. Interestingly, HMW PAH degraders experienced a substantial increase by 45 d,

357 especially in the plant treatment, and remained at high levels until the end of the experiment.

358 Because at 90 days, the ratio between the HMW PAH degraders and total heterotrophic

359 populations was substantially higher in the treated than in the untreated soil, it could be

360 concluded that in addition to stimulating the general growth of the heterotrophic populations

361 (including that of PAH degraders), the rhizosphere treatment had an additional selective effect of

362 enhancing the growth of the HMW PAH-degrading populations. In addition, these results show

363 that an increase in microbial growth can be obtained by supplementing soil with carbon sources

364 and nutrients (present in exudates) and by improving the biodegradation of PAHs, possibly by

365 increasing their bioaccessibility.

366 It is known that the microbial communities in the rhizosphere can be considerably different

367 than those in nearby soil that grow without the direct influence of roots. As a first step in

368 understanding whether the increase in PAH degradation observed in the treated soil containing

369 plants could be related to specific changes in the microbial community structure, we used DGGE

370 and clone library analysis. The DGGE fingerprints obtained during the incubation period from

371 replicate samples for each treatment showed very similar banding profiles (Fig. 2), indicating

372 strong homogeneity within the pots for each condition. In general, the DGGE analysis revealed

373 an initially diverse microbial community, with specific populations increasing in relative

374 abundance throughout the incubation period in both the non-treated and the rhizosphere soil. A

375 number of the bands obtained coincided in the two treatments, but their relative intensities

376 differed, indicating that the shift in community structure induced by the rhizosphere was different

377 than that induced by the simple potting and watering of the polluted soil.

378 To gain insight into which microbial groups were selectively promoted by the rhizosphere in

379 comparison to the non-treated soil, corresponding 16S rRNA gene libraries were obtained from

380 samples taken at 90 days, and a total of 84 clones were analyzed. Table 2 indicates the relative

381 abundance and phylogenetic affiliation of each of the eubacterial populations detected, while Fig.

382 3 summarizes the importance of the different bacterial phyla in the non-treated and sunflower

383 rhizosphere soils. Approximately two-thirds (60 and 68%) of the bacteria detected under both

384 conditions belonged to the α-, β-, and γ-*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and

385 *Chloroflexi* phyla. However, with the exception of the *Actinobacteria*, the relative abundances of

386 these phyla and their compositions varied substantially with the treatment applied, confirming

387 that the plants caused a dramatic shift in the community structure. The rhizosphere promoted the

388 appearance of new populations within the three *Proteobacteria* subphyla, including

389 representatives with a well-known capacity to degrade PAHs (Kanaly and Harayama, 2010).

390 Within the α-*Proteobacteria,* the increase of the *Sphingomonas* group was interesting because

391 this group included numerous members isolated from plant root systems and members with a

392 versatile degrading capability allowing them to attack 2-, 3- and 4-ring PAHs (Fernández-

393 Luqueño et al., 2011). There was also a noticeable increase in the β-*Proteobacteria* (from 9% to

394 27%), as the rhizosphere promoted the appearance of members of the *Commamonas* group

395 showing high similarity matches to members in the database isolated from PAH-contaminated

396 soil or xenobiotic degraders (i.e., *Variovorax).*

397   In the non-planted soil, 40% of the detected microorganisms belonged to seven phylogenetic

398 groups not detected in the sunflower planted soil. Interestingly, among these microbes, we found

399 members of the Candidate divisions OD1, OP11, TM7 and WS6, which are lineages of

400 prokaryotic organisms for which there are no reported cultivated representatives but which

401 present sufficiently well-represented environmental sequences to conclude that they represent

402 major bacterial groups (Chouari et al., 2005). In addition to these sequences, the sequences

403 retrieved from non-treated soil revealed a relatively high abundance of *Firmicutes*, while the

404 *Planctomycetes* and *Deinococcus* groups were represented with lower proportions. In contrast,

405 the sunflower rhizosphere soil promoted the presence of four phylotypes (*Acidobacteria,*

406 *Gemmatimonadetes,* δ-*Proteobacteria* and *Cyanobacteria*) that were not detected in the non-

407 treated soil. The most abundant, the *Acidobacteria* (14.6%) and *Gemmatimonadete*s (7.3%),

408 constitute recently described new phyla (Ludwig et al., 1997; Zhang et al., 2003) and are broadly

409 distributed in soils but poorly represented in cultures, which makes it difficult to ascertain their

410 role in nature. The *Acidobacteria* have been observed previously in planted soil (Yrjala et al.,

411 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≤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

627

628

629 **References**
630
631 Alexander, M., 2000. Aging, bioavailability, and overestimation of risk from environmental
632 pollutants. Environmental Science and Technology, pp. 4259-4265.
633
634 Anderson, T.A., Coats, J.R., 1995. An overview of microbial degradation in the rhizosphere and
635 its implications for bioremediation, Bioremediation: Science and Applications, pp. 135-143.
636
637 Bengtsson, G., Zerhouni, P., 2003. Effects of carbon substrate enrichment and DOC
638 concentration on biodegradation of PAHs in soil. Journal of Applied Microbiology 94, 608-617.
639
640 Bernard, L., Mougel, C., Maron, P.A., Nowak, V., Leveque, J., Henault, C., Haichar, F.e.Z.,
641 Berge, O., Marol, C., Balesdent, J., Gibiat, F., Lemanceau, P., Ranjard, L., 2007. Dynamics and
642 identification of soil microbial populations actively assimilating carbon from C-13-labelled wheat
643 residue as estimated by DNA- and RNA-SIP techniques. Environmental Microbiology 9, 752-
644 764.
645
646 Bertin, C., Yang, X.H., Weston, L.A., 2003. The role of root exudates and allelochemicals in the
647 rhizosphere. Plant and Soil 256, 67-83.
648
649 Bueno-Montes, M., Springael, D., Ortega-Calvo, J.J., 2011. Effect of a non-ionic surfactant on
650 biodegradation of slowly desorbing PAHs in contaminated soils. Environmental Science and
651 Technology 45, 3019-3026.
652
653 Corgie, S.C., Beguiristain, T., Leyval, C., 2004. Spatial distribution of bacterial communities and
654 phenanthrene degradation in the rhizosphere of *Lolium perenne* L. Applied and Environmental
655 Microbiology 70, 3552-3557.
656
657 Chistoserdova, L., Kalyuzhnaya, M.G., Lidstrom, M.E., 2009. The Expanding World of
658 Methylotrophic Metabolism, Annual Review of Microbiology, pp. 477-499.
659
660 Chouari, R., Le Paslier, D., Dauga, C., Daegelen, P., Weissenbach, J., Sghir, A., 2005. Novel
661 major bacterial candidate division within a municipal anaerobic sludge digester. Applied and
662 Environmental Microbiology 71, 2145-2153.
663
664 Degryse, F., Smolders, E.,Merckx, R., 2006. Labile Cd Complexes Increase Cd Availability to
665 Plants. Environmental Science and Technology 40, 830-836.
666
667 Fernández-Luqueño, F., Valenzuela-Encinas, C., Marsch, R., Martínez-Suarez, C., Vázquez-
668 Núñez, E., Dendooven, L., 2011. Microbial communities to mitigate contamination of PAHs in
669 soil-possibilities and challenges: a review. Environmental Science and Pollution Research 18, 12-
670 30.
671
672 García-Gil, J.C., Plaza, C., Senesi, N., Brunetti, G., Polo, A., 2004. Effects of sewage sludge
673 amendment on humic acids and microbiological properties of a semiarid Mediterranean soil.
674 Biology and Fertility of Soils 39, 320-328.
675

676 Gong, Z.Q., Wang, X.G., Tu, Y., Wu, J.B., Sun, Y.F., Li, P., 2010. Polycyclic aromatic
677 hydrocarbon removal from contaminated soils using fatty acid methyl esters. Chemosphere 79,
678 138-143.

680 Grayston, S.J., Vaughan, D., Jones, D., 1996. Rhizosphere carbon flow in trees, in comparison
681 with annual plants: the importance of root exudation and its impact on microbial activity and
682 nutrient availability. Applied Soil Ecology 5, 29-56.

684 Green, S.J., Michel, F.C., Hadar, Y., Minz, D., 2007. Contrasting patterns of seed and root
685 colonization by bacteria from the genus Chryseobacterium and from the family
686 Oxalobacteraceae. Isme Journal 1, 291-299.

688 Grifoll, M., Selifonov, S.A., Gatlin, C.V., Chapman, P.J., 1995. Actions of a versatile fluorene-
689 degrading bacterial isolate on polycyclic aromatic-compounds. Applied and Environmental
690 Microbiology 61, 3711-3723.

692 Hao, L.H., He, P.Q., Liu, C.Y., Chen, K.S., Li, G.Y., 2004. Physiological effects of taurine on the
693 growth of wheat (Triticum aestivum L.) seedlings. J Plant Physiol Mol Biol. 30:595–598.

695 Haftka, J.J.H., Parsons, J.R., Govers, H.A.J., Ortega-Calvo, J.J., 2008. Enhanced kinetics of
696 solid-phase microextraction and biodegradation of polycyclic aromatic hydrocarbons in the
697 presence of dissolved organic matter. Environmental Toxicology and Chemistry 27, 1526-1532.

699 Kalamaki, M.S., Merkouropoulos, G., Kanellis, A.K., 2009. Can ornithine accumulation
700 modulate abiotic stress tolerance in Arabidopsis? Plant Signal Behav 4:1099–1101.

702 Kamilova, F., Kravchenko, L.V., Shaposhnikov, A.I., Azarova, T., Makarova, N., Lugtenberg,
703 B., 2006. Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on
704 stonewool and their effects on activities of rhizosphere bacteria. Molecular Plant-Microbe
705 Interactions 19, 250-256.

707 Kanaly, R.A.,Harayama, S., 2010. Advances in the field of high-molecular-weight polycyclic
708 aromatic hydrocarbon biodegradation by bacteria. Microbial Biotechnology 3, 136-164.

710 Kuiper, I., Kravchenko, L.V., Bloemberg, G.V., Lugtenberg, B.J.J., 2002. *Pseudomonas putida*
711 strain PCL1444, selected for efficient root colonization and naphtalene degradation, effectively
712 utilizes root exudate components. Molecular Plant-Microbe Interactions 15, 734-741.

714 Lane, D. J., 1991. 16S/23S rRNA sequencing, p. 115–175. In E. Stackebrandt and M.
715 Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons,
716 Chichester, United Kingdom.

718 López, Z., Vila, J., Ortega-Calvo, J.J., Grifoll, M., 2008. Simultaneous biodegradation of
719 creosote-polycyclic aromatic hydrocarbons by a pyrene-degrading *Mycobacterium*. Applied
720 Microbiology and Biotechnology 78, 165-172.

721

722 Ludwig, W., Bauer, S.H., Bauer, M., Held, I., Kirchhof, G., Schulze, R., Huber, I., Spring, S.,
723 Hartmann, A., Schleifer, K.H., 1997. Detection and in situ identification of representatives of a
724 widely distributed new bacterial phylum. Fems Microbiology Letters 153, 181-190.

726 Maliszewska-Kordybach, B., Smreczak, B., 2000. Ecotoxicological activity of soils polluted with
727 polycyclic aromatic hydrocarbons (PAHS) - Effect on plants. Environmental Technology 21,
728 1099-1110.

730 Miya, R.K., Firestone, M.K., 2000. Phenanthrene-degrader community dynamics in rhizosphere
731 soil from a common annual grass. Journal of Environmental Quality 29, 584-592.

733 Miya, R.K., Firestone, M.K., 2001. Enhanced phenanthrene biodegradation in soil by slender oat
734 root exudates and root debris. Journal of Environmental Quality 30, 1911-1918.

736 Nayak, A.S., Sanganal, S.K., Mudde, S.K., Oblesha, A., Karegoudar, T.B., 2011. A catabolic
737 pathway for the degradation of chrysene by Pseudoxanthomonas sp PNK-04. Fems Microbiology
738 Letters 320, 128-134.

740 Newman, L.A., Reynolds, C.M., 2004. Phytodegradation of organic compounds. Current Opinion
741 in Biotechnology 15, 225-230.

743 Ofosubudu, K.G., Fujita, K., Ogata, S., 1990. Excretion of ureide and other nitrogenous
744 compounds by the root-system of soybean at different growth-stages Plant and Soil 128, 135-142.

746 Olson, P.E., Castro, A., Joern, M., DuTeau, N.M., Pilon-Smits, E.A.H., Reardon, K.F., 2007.
747 Comparison of plant families in a greenhouse phytoremediation study on an aged polycyclic
748 aromatic hydrocarbon-contaminated soil. Journal of Environmental Quality 36, 1461-1469.

750 Ortega-Calvo, J.J., Marchenko, A.I., Vorobyov, A.V., Borovick, R.V., 2003. Chemotaxis in
751 polycyclic aromatic hydrocarbon-degrading bacteria isolated from coal-tar- and oil-polluted
752 rhizospheres. Fems Microbiology Ecology 44, 373-381.

754 Ortega-Calvo, J.J., Saiz-Jimenez, C., 1998. Effect of humic fractions and clay on biodegradation
755 of phenanthrene by a *Pseudomonas fluorescens* strain isolated from soil. Applied and
756 Environmental Microbiology 64, 3123-3126.

758 Pandya, S., Iyer, P., Gaitonde, V., Parekh, T., Desai, A., 1999. Chemotaxis of *Rhizobium* SP.S2
759 towards Cajanus cajan root exudate and its major components. Current Microbiology 38, 205-
760 209.

762 Parrish, Z.D., Banks, M.K., Schwab, A.P., 2005. Effect of root death and decay on dissipation of
763 polycyclic aromatic hydrocarbons in the rhizosphere of yellow sweet clover and tall fescue.
764 Journal of Environmental Quality 34, 207-216.

766 Rentz, J.A., Alvarez, P.J.J., Schnoor, J.L., 2005. Benzo[a]pyrene co-metabolism in the presence
767 of plant root extracts and exudates: Implications for phytoremediation. Environmental Pollution
768 136, 477-484.

769
770 Smith, K.E.C., Thullner, M., Wick, L.Y., Harms, H., 2009. Sorption to Humic Acids Enhances
771 Polycyclic Aromatic Hydrocarbon Biodegradation. Environmental Science & Technology 43,
772 7205-7211.
773
774 Tejeda-Agredano, M.C., Gallego, S., Niqui-Arroyo, J.L., Vila, J., Grifoll, M., Ortega-Calvo, J.J.,
775 2011. Effect of interface fertilization on biodegradation of polycyclic aromatic hydrocarbons
776 present in nonaqueous-phase liquids. Environmental Science & Technology 45, 1074-1081.
777
778 Vila, J., Nieto, J.M., Mertens, J., Springael, D., Grifoll, M., 2010. Microbial community structure
779 of a heavy fuel oil-degrading marine consortium: linking microbial dynamics with polycyclic
780 aromatic hydrocarbon utilization. Fems Microbiology Ecology 73, 349-362.
781
782 Viñas, M., Sabate, J., Espuny, M.J., Solanas, A.M., 2005. Bacterial community dynamics and
783 polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-
784 contaminated soil. Applied and Environmental Microbiology 71, 7008-7018.
785
786 Wrenn, B.A., Venosa, A.D., 1996. Selective enumeration of aromatic and aliphatic hydrocarbon
787 degrading bacteria by a most-probable-number procedure. Canadian Journal of Microbiology 42,
788 252-258.
789
790 Yi, H., Crowley, D.E., 2007. Biostimulation of PAH degradation with plants containing high
791 concentrations of linoleic acid. Environmental Science & Technology 41, 4382-4388.
792
793 Yrjala, K., Keskinen, A.K., Akerman, M.L., Fortelius, C., Sipila, T.P., 2010. The rhizosphere and
794 PAH amendment mediate impacts on functional and structural bacterial diversity in sandy peat
795 soil. Environmental Pollution 158, 1680-1688.
796
797 Zhang, H., Sekiguchi, Y., Hanada, S., Hugenholtz, P., Kim, H., Kamagata, Y., Nakamura, K.,
798 2003. Gemmatimonas aurantiaca gen. nov., sp nov., a gram-negative, aerobic, polyphosphate-
799 accumulating micro-organism, the first cultured representative of the new bacterial phylum
800 Gemmatimonadetes phyl. nov. International Journal of Systematic and Evolutionary
801 Microbiology 53, 1155-1163.
802
803 Zheng, X.Y., Sinclair, J.B., 1996. Chemotactic response of *Bacillus megaterium* strain B153-2-2
804 to soybean root and seed exudates. Physiological and Molecular Plant Pathology 48, 21-35.
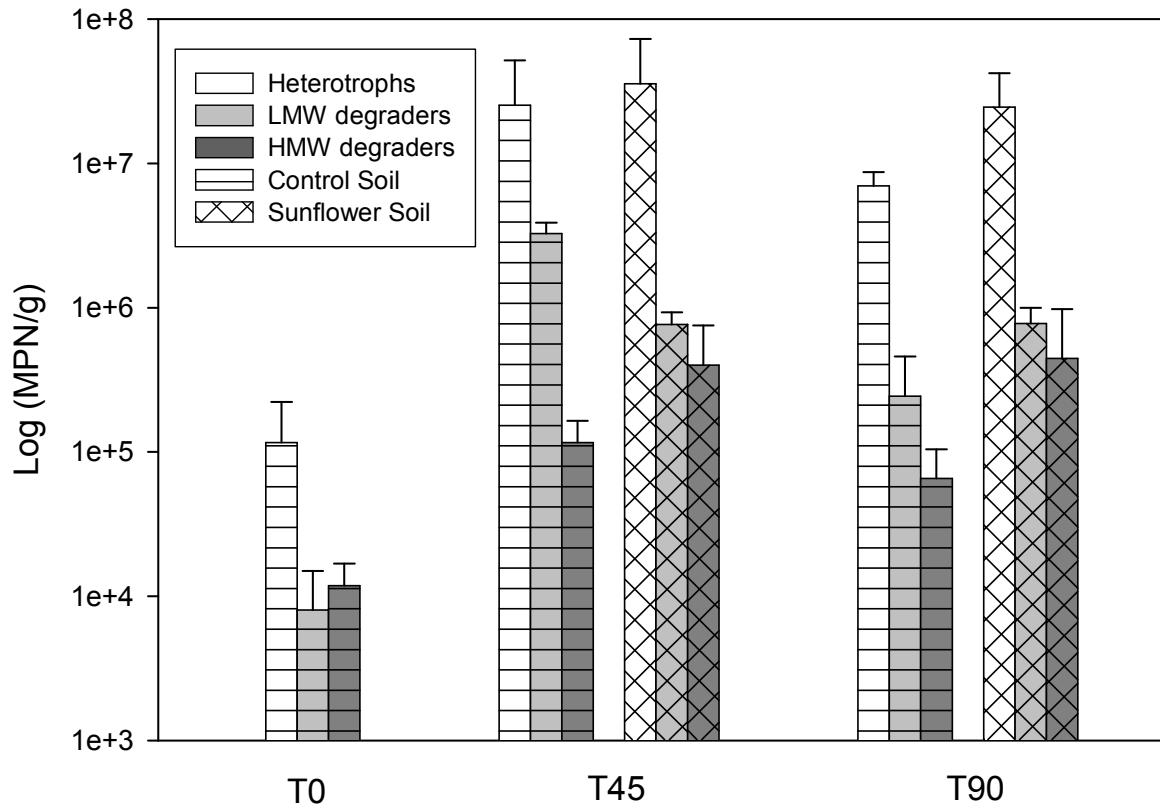
Fig. 1. Counts of heterotrophic and PAH-degrading microbial populations in the soil under the different treatments applied in the greenhouse experiment. MPN, most probable number. LMW, low-molecular-weight PAHs. HMW, high-molecular-weight PAHs. T0, at the beginning of the experiment; T45, at 45 days after; T90 at 90 days after. Error bars represent the standard deviation of triplicates.
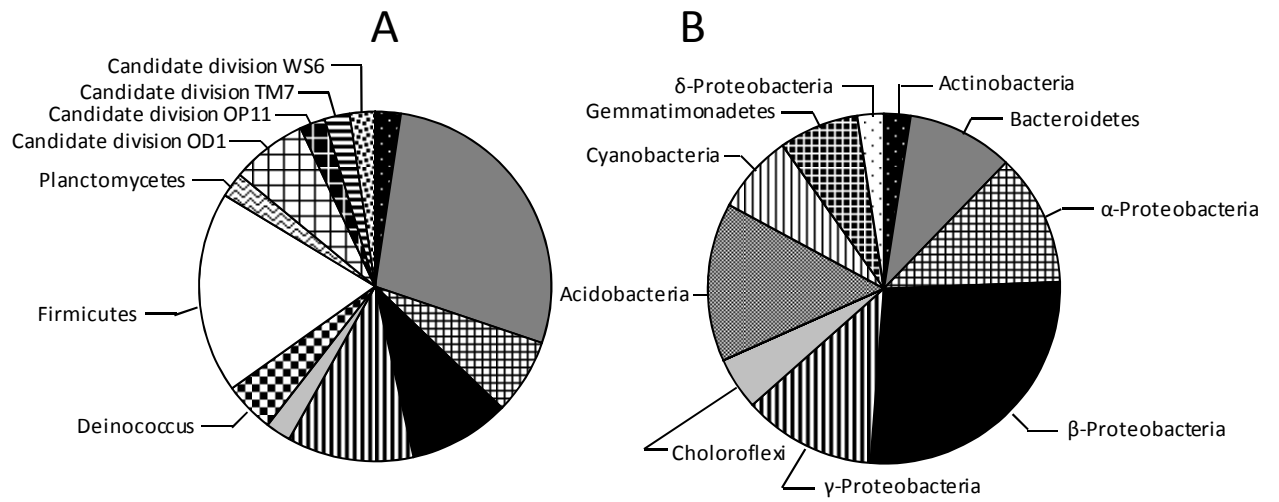
830

831

832

833

834

835

836
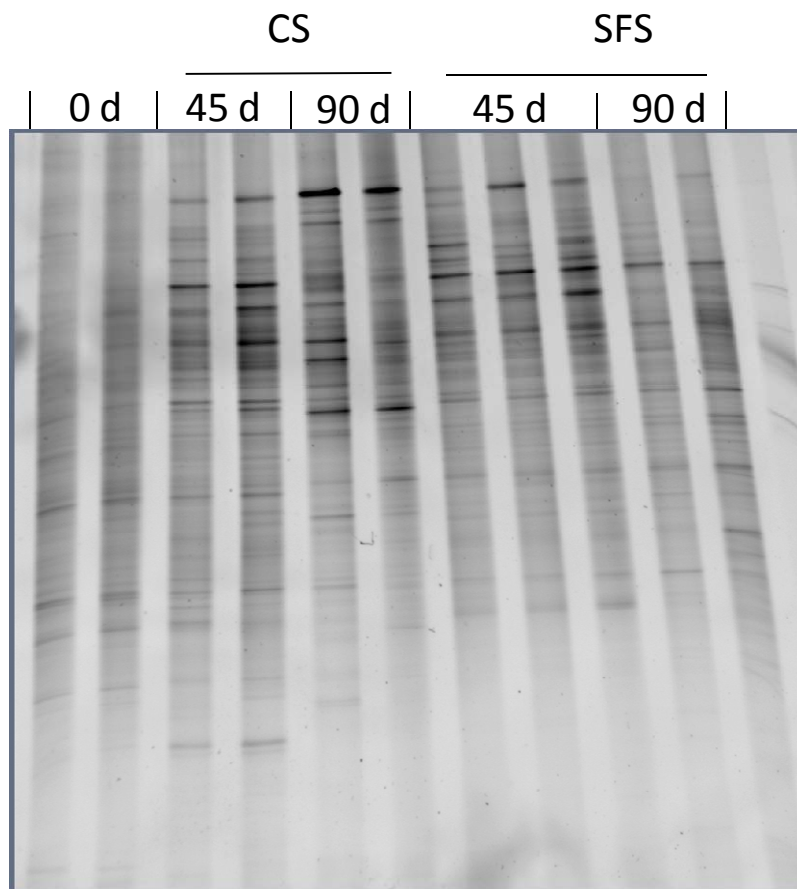
Fig. 2. DGGE profile of PCR-amplified 16S rRNA gene fragments from independent
replicate samples from control soil (CS) and rhizosphere sunflower soil (SFS) samples
after 0, 45 and 90 days. Each lane was loaded with an identical amount of DNA.

844

845

846

847

848

Fig. 3. Relative abundance of eubacterial phylogenetic groups identified in control soil (A) and sunflower rhizosphere soil (B) samples after 90 days of incubation in the greenhouse experiment.
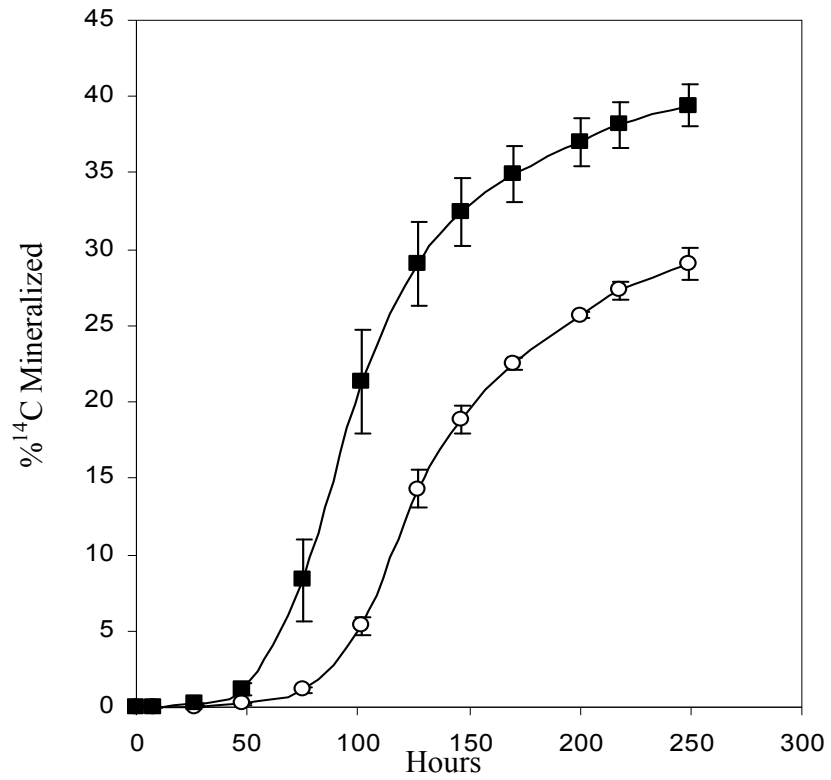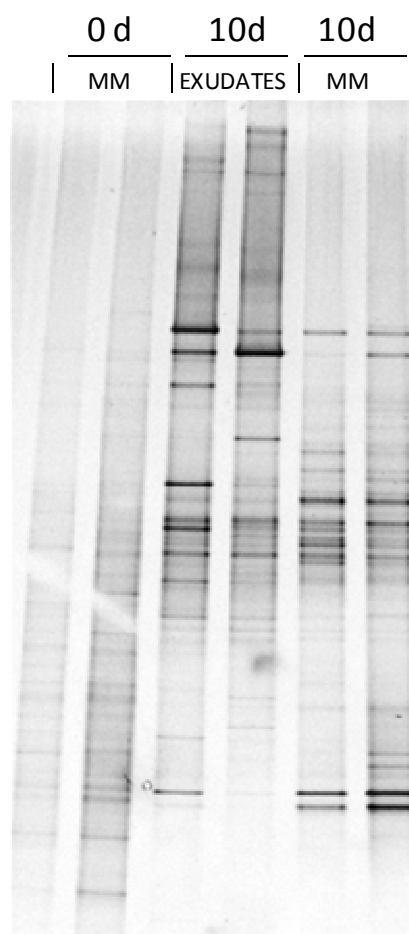
Fig. 4. Mineralization of pyrene in soil suspensions in the absence (o) and presence (■) of sunflower root exudates. Error bars represent the standard deviation of duplicates.

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≤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

892

893 [a] Frequencies in clone libraries obtained from CS (control soil) and SFS (sunflower soil). [b]In sequences showing an

894 identical match to uncultured and to isolated strains, only the latter are listed. Sequences with more than 94% identity

895 are grouped. α, β, γ, δ correspond to *alpha-*, *beta-*, *gamma-* and *deltaproteobacteria*, respectively. Clones with

896 sequences belonging to the same phyla or subphyla (in case of *Proteobacteria*) are grouped and marked with

897 alternatively shade and non shaded backgrounds

898

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.

904

905

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≤0.05).