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Influence of a mixture of metals on PAHs biodegradation processes in soils

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19

20 Abstract

21

22 In order to assess the effect of mixed pollutants, the influence of different concentration levels of a
23 mixture of metals (Cr, Co, Pb, Mn, Ni, Cu, Zn) on the biodegradation of some PAHs (phenanthrene,
24 fluoranthene, pyrene, benzo[*b*]fluoranthene and benzo[*a*]pyrene) in soil samples was evaluated. To do
25 so, groups of microcosms of a natural soil from the region of Sabadell (Barcelona, Spain) were
26 prepared as a reproduction of the native environment at laboratory scale, under controlled conditions.
27 Mixtures of PAHs and metals were carefully selected, according to soil characterization and
28 microbiological growth preliminary assays, and were added to microcosms. These microcosms were
29 analyzed at various times, along two months, to obtain PAHs dissipation time-courses. A first-order
30 kinetic modelling allowed obtaining different rate constants and DT50 values as a function of the
31 metal levels introduced in microcosms. As a general observation, the higher the concentration of
32 metals, the lower the biodegradation of PAHs of 3-4 rings (phenanthrene, fluoranthene and pyrene).
33 On the other hand, no important effect on the biodegradation of higher molecular weight PAHs
34 (benzo[*b*]fluoranthene and benzo[*a*]pyrene) was observed at the different concentration levels of
35 metals tested.

36

37 Keywords

38 Polycyclic aromatic hydrocarbons, metals, co-contamination, microcosms, biodegradation, soils.

39

40

41 1 Introduction

42

43 Nowadays it is well-known that harmful toxic pollutants such as polycyclic aromatic hydrocarbons
44 (PAHs) and heavy metals contribute to the pollution of the biosphere, which has been dramatically
45 accelerated since the industrial revolution (Chen et al., 2015). PAHs are a wide group of organic
46 pollutants produced by the incomplete combustion of organic matter at high temperatures (Wilson and
47 Jones, 1993). They are carcinogenic and mutagenic. They are constituted of two or more fused
48 benzene rings and are found in water, air, soils, food, etc. PAHs accumulate in soils principally after
49 atmospheric deposition mechanisms (Tobiszewski and Namienik, 2012). Metals can be found in the
50 earth's crust, soils and vegetation, and many of them are essential for the development of living
51 organisms but can become toxic if they exceed certain thresholds (Huertos and Baena, 2008; Zehetner
52 et al., 2009).

53 PAHs in soils are known to degrade into metabolites through different paths. In general,
54 biodegradation of organic substances in soil involves a complex community of bacteria and fungi with
55 numerous enzymatic pathways (Deary et al., 2016). The main mechanism of PAHs degradation in soils
56 is naturally controlled by microorganisms like *Pseudomonas*, *Burkholderia cepacia*, *Sphingomonas*,
57 *Flavobacterium*, *Acinetobacter* (Siddiqi et al., 2002; Watanabe, 2001; Zhou et al., 2016; Janbandhu
58 and Fulekar, 2011), other groups such as actinomycetes (Samanta et al., 2002), white-rot fungi (Boyle
59 et al., 1998; Fernández-Luqueño et al. 2011), an acid-metal-tolerant *Trabulsiella*, among others
60 (Kuppusamy et al. 2016a). A native microbial consortium (instead of a single degrader) has been also
61 checked recently (Biswas et al., 2015; Kuppusamy et al., 2016b). Furthermore, photooxidation and
62 other chemical reactions (such as Fenton-like reactions) can also take place (Jonsson et al., 2007; Tam
63 et al., 2008).

64 There are studies which showed that metals can have an effect on microbial communities, inducing
65 changes on the size, growth and activity (Giller et al., 1998) as well as reducing the availability of
66 substrates used for respiration or causing acute toxicity leading to their death (Landi et al., 2000).
67 Therefore, scientists agreed that these pollutants could have a negative effect on the biodegradation of
68 organic compounds (such as PAHs) through the inhibition of the enzymatic activity involved in these
69 processes.

70 There are several reported studies which highlight the problem of the mixed contamination of PAHs
71 and heavy metals (Subashchandrabose et al., 2015). The toxicity of mixtures of PAHs and metals can
72 show synergistic or antagonist effects on toxicity and/or enzymatic activity, depending on the nature
73 and relative concentration, of these pollutants (Moreau et al., 1999; Shen et al., 2006, 2005;
74 Thavamani et al., 2012; Biswas et al., 2015). About the effect of PAH/metal mixtures, some authors
75 reported an absence of biodegradation of anthracene in the presence of Pb (Fualkowska et al., 1998) or
76 a decrease in the mineralization of Phe when communities were exposed to Cu (Sokhn et al., 2001).
77 Others, reported higher degradation rates for Pyr/Pb mixtures (30 and 300 mg kg⁻¹, respectively) than
78 for isolated Pyr (30 mg kg⁻¹), suggesting that Pb promotes bacterial growth through the detoxification
79 of Pyr, resulting in a higher degradation of this PAH (Khan et al., 2009). Some authors had also seen
80 that Zn could enhance the mineralization of Phe (Moreau et al., 1999), while other studies indicated
81 that the presence of Zn (50-1,000 mg kg⁻¹) and Cu (50-100 mg kg⁻¹) did not induce any significant
82 effects on its degradation, but higher amounts of Cu caused a decrease in the biodegradation capability
83 via reduction of dehydrogenase activity (Obuekwe and Semple, 2013). Most of these effects were
84 observed in studies conducted under *in vitro* conditions, which is useful for the evaluation of specific
85 effects, for example, on enzymatic activities. However, in such cases, interactions with the
86 environmental matrices where biodegradation processes take place were not considered and could be
87 certainly significant. Consequently, other authors have studied the effect of metals such as Cd, Hg, Pb,
88 Zn, Cu, Ni on the degradation of mixtures of PAHs in the corresponding environmental matrices,

89 finding significant differences depending on the experimental conditions tested (Baldrian et al., 2000;
90 Ke et al., 2010; Khan et al., 2009; Biswas et al., 2015; Deary et al., 2016). Other studies, show the
91 biodegradation evaluation of one PAH in the presence of a mixture of heavy metal solutions at
92 different pH (Kuppusamy et al. 2016b) and the comparison of the simultaneous biodegradation of two
93 PAHs in the presence of individual metal solutions of Zn, Pb, Cu and Cd (Kuppusamy et al. 2016a).
94 The main objective of the present work is to study the influence of different concentrations of a
95 mixture of metals on the biodegradation of some PAHs, under controlled conditions (i.e. temperature,
96 light exposure, humidity, etc.). Contrary to evaluating specific single-metal/PAH interactions, focusing
97 on mixtures allows to have a global picture of the mixed pollutants impact in the native soil. Hence, a
98 group of soil based microcosms were prepared, spiked with a selected mixture of PAHs and different
99 concentration levels of a mixture of metals. First, the selected soil was characterized in order to decide
100 the concentration of PAHs to be added to the microcosms and to determine the original bioavailable
101 metal content. Secondly, bacterial growth assays were performed in order to choose the range of metal
102 concentration levels to be studied. Finally, microcosms were prepared and analyzed to carry out the
103 biodegradation experiments to accomplish the aforementioned main objective.

104

105 2 Experimental

106

107 2.1 Chemicals

108

109 Toluene, acetone (HPLC grade) and nitric acid (69.5 %) were obtained from Sigma-Aldrich
110 (Barcelona, Spain) and hydrochloric acid (37 %) from Panreac (Barcelona, Spain). Water was purified
111 and deionized using an Elga Classic system from Veolia Water Solutions and Technology (Madrid,
112 Spain). The internal standard, perdeuterated phenanthrene (Phe-D₁₀) and five PAHs used to spike
113 microcosms (phenanthrene, fluoranthene, pyrene, benzo[*a*]pyrene, benzo[*b*]fluoranthene) were

114 purchased from Sigma-Aldrich (Madrid, Spain). A stock solution of the PAHs was prepared in
115 acetone. Nitrate metal salts (cobalt, chromium, manganese, lead, zinc), copper sulfate and magnesium
116 chloride were acquired from Merck (Madrid, Spain). All reagents were of analytical grade. Stocks
117 solutions were prepared in diluted nitric acid in ultrapure deionized water. Ringer Oxoid BR52 was
118 purchased from ThermoFisher Scientific (Barcelona, Spain) and Tryptic Soy Agar from Scharlab
119 (Barcelona, Spain), used for bacterial culture assays.

120

121 2.2 Collection and characterization of soil samples

122

123 Soil samples were collected in March 2013 in Sabadell (Catalonia, Spain). The winter rainfall was of
124 less than 25 mm (official data by *Servei Meteorològic de Catalunya, Generalitat de Catalunya*, Spain).
125 Sampling point was nearby the area of Ripoll's river (41°31'57.62"N, 2°07'35.06"E), an area of
126 sparsely populated scrubland with irregular cover and mainly surrounded by textile industries (Figure
127 1). About 10 kg of soil were collected from the upper horizontal layer (0-25 cm). The sample was
128 passed through a 2 mm stainless steel sieve to remove large debris and 1 kg was separated and used for
129 characterization experiments. A nested column of sieves with wire mesh cloth of different diameters
130 was used to assess soil's particle size distribution. According to the Soil Taxonomy (U. S. Department
131 of Agriculture, 1999), the soil is an Inceptisol (Typic Haplustepts), with coarse sandy soil containing
132 94 % of sand (24 % very coarse sand, 21 % coarse sand, 20 % medium sand, 1 % fine sand and 28 %
133 very fine sand) and 6 % of silt and clay (5 % coarse silt and 1 % smaller particles) (Wentworth, 1922).
134 Moisture content was 1.9 ± 0.2 %, determined as the relative weight difference after drying 3.5 g of
135 sample at 115 °C for 24 h, until constant weight was reached (less than 0.1 % weight difference
136 between two successive weightings within a 4 h time interval). Similarly, organic matter (0.9 ± 0.2 %)
137 was determined as the relative weight difference after calcination of 2.5 g of dry sample at 500 °C
138 during 4 h until constant weight was reached. The pH was 7.8 ± 0.1 , obtained from the potentiometric

139 measure of an extract of soil:water (1:2.5), after 30 min of mechanical shaking. The maximum water
140 holding capacity (MWHC) was 32 ± 1 %, according to humidity determination of a 250 g water-
141 saturated soil column. Carbonate ions content reached 6 ± 1 %, calculated as the amount of CO_2
142 generated in a calcimeter, after the addition of hydrochloric acid. Electrical conductivity (194 ± 7 μS
143 cm^{-1}) was obtained by the conductometric measurement of an extract of soil:water (1:10), after 2 h of
144 mechanical shaking. The total concentration of bioavailable metals was 1.5 ± 0.1 mg kg^{-1} (see section
145 2.7), the total background amount of the five selected PAHs was 264 ± 12 $\mu\text{g kg}^{-1}$ (see section 2.5), and
146 the number of colony forming units (CFU) was $(1.5\pm 0.4)\times 10^4$ CFU g^{-1} . CFU were determined by
147 preparation of a series of dilutions of an aqueous soil extract (in Ringer solution) inoculated with a
148 Digrafsky spreader in tryptic soy agar growth media poured into Petri dishes. Then, incubation was
149 performed at 30 °C during 48 h, previous to CFU counting. All the material employed was previously
150 sterilized in an autoclave.

151 The rest of the sample was stored at 20 °C for one week (under humidity control). Then, 10 % of the
152 collected soil was sterilized by autoclaving, dried one night at 35 °C and softly crushed. This fraction
153 was used to spike PAHs in the preparation of the microcosms.

154 The soil of Sabadell was selected after characterization and preliminary biodegradation assays of three
155 different soils collected from two other villages on the same day. The soil of Sabadell was located
156 nearby an industrial area and had been stable for more than 20 years, whereas the others had been
157 potentially impacted by nearby highway construction and agricultural activities. Initial tests involving
158 the preparation of some microcosms revealed that the number of colony forming units was more
159 abundant in Sabadell's soil and the ability of microorganisms to degrade PAHs was also more
160 efficient. Furthermore, Sabadell's soil is drier in comparison to the other soils, and humidity can be a
161 limiting factor for PAHs desorption. In this regard, due to the hydrophobic nature of PAH and poor

162 mass transfer of PAH to bacterial cells (Zhang et al., 2006; Kobayashi et al., 2009), less PAH
163 bioavailability was expected for the more humid soils.

164 All this information contributed to the decision of choosing the soil of Sabadell as the more suitable
165 for this work.

166

167 2.3 Selection of spiking concentrations for PAHs and metals

168

169 To select the metals and PAHs of the study, an initial screening of their content in the sample was
170 performed by ICP/MS and GC/MS, respectively. Then, they were selected according to the total
171 concentration found in the soil, and its representativeness in other contaminated soil studies (Blum et
172 al., 2009; Kabata-Pendias, 2010; Wuana and Okieimen, 2011; Crampon et al., 2014). Metals were also
173 selected according to the concentration found in the exchangeable fraction (bioavailable) (sections 2.7
174 and 3.1), and PAHs to have different molecular weights also represented (3, 4 and 5rings).

175 Five representative PAHs were selected to conduct the biodegradation studies: phenanthrene (Phe)
176 (three rings), fluoranthene (Fluo) and pyrene (Pyr) (four rings) and benzo[*b*]fluoranthene (BbF) and
177 benzo[*a*]pyrene (BaP) (five rings). The concentration of PAHs which was spiked into microcosms was
178 selected according to the previously PAH content found in soil, following other related biodegradation
179 studies (Baltrons et al., 2013; Niepceron et al., 2013) where soils were typically spiked at a
180 concentration of 100-10,000 times to that found in soil.

181 To select the spiking concentrations of metals, microbiological assays were carried out to assess
182 possible impacts on bacterial growth. To do so, nine portions of 100 g of fresh native collected soil
183 were prepared. Eight of them were spiked to reach a concentration of 2, 5, 10, 50, 100, 250, 500 and
184 1,000 times the bioavailable metals concentration found in soil. The ratio of the bioavailable metals
185 concentration found in the native soil was preserved. Metals were introduced in 22.1 mL of deionized
186 sterile water. The ninth portion was not spiked (in order to observe microorganisms growth under the

187 native bioavailable concentration of the metals found in soil), and 22.1 mL of sterilized water was also
188 added. After this, samples were kept under temperature and humidity control during five days for
189 stabilization. Then, fifty-four incubation experiments (including duplicates) in Petri dishes were
190 performed. To do so, bacteria from 1.0 g of each of the nine portions of soil (containing the different
191 concentrations of metals) was extracted with 100 mL of sterile deionized water for 30 min, under
192 stirring. Then, series of dilutions (1:10; 1:100 and 1:1,000) of the main extract were prepared in Ringer
193 solution and 0.1 mL was poured into nutrient agar and incubated at 30 °C for 48 h. The nutrient agar
194 was prepared by dissolving 40.0 g of Tryptic Soy Agar (TSA) in 1 L of water. All the material used
195 was sterilized by autoclaving at 120 °C during 20 min.

196

197 2.4 Preparation of microcosms

198

199 Microcosms were prepared in 200 mL sterilized glass flasks. Each one was filled with 10 g of dry
200 sterile soil and spiked to reach a total concentration of 1,000 mg kg⁻¹ of the five selected PAHs (200
201 mg kg⁻¹ of each PAH). They were shaken under an extractor hood for 48 h to better distribute PAHs
202 and evaporate acetone (solvent used to dissolve PAHs). After a week of stabilization, dry sterilized
203 soils enriched with PAHs were mixed with 91.7 g wet native soil (equivalent to 90 g of dry soil) and
204 homogenized to obtain microcosms. Therefore, final PAHs concentration in microcosms was 100 mg
205 kg⁻¹. Then, metals were introduced at the desired concentration dissolved in 22.1 mL of sterile
206 deionized water to reach the 60 % of MWHC, and the system was homogenized again (see Figure 2, a
207 scheme of microcosms preparation). Finally, the glass flasks were hermetically closed and incubated at
208 20°C in static mode in a dark room with aeration (opening the flasks 1 h per day). Microcosms were
209 divided into five different groups depending on the metal content added. Each group consisted of eight
210 microcosms (one for each time of analysis: 0, 3, 7, 10, 15, 21, 30 and 60 days), prepared in duplicates.
211 The first group of microcosms was not spiked with metals (corresponding to the native bioavailable

212 concentration of metals found in soil). Three more groups of microcosms were prepared by spiking
213 different concentrations of metals to reach a final concentration of 10, 250 and 500 times the
214 bioavailable concentration of metals naturally found in the soil. The last group was used as blank,
215 consisting of 100 g of dry sterile soil spiked with PAHs to reach a final concentration of 100 mg kg⁻¹
216 and 22.1 mL of sterile deionized water with the intermediate level of metals (x250).

217

218 2.5 Microwave assisted extraction of PAHs from soil

219

220 Microwave assisted extraction (MAE) was performed using a MARS X equipment (CEM Corporation,
221 Matthews, USA). Wet soils from microcosms were previously dried one night at 35°C and then
222 crushed. Before the extraction, perdeuterated phenanthrene (Phe-D₁₀) was added as internal standard to
223 reach a final concentration of 1.0 mg L⁻¹. A sample size of 1.5 g of crushed dry soil was extracted
224 using 25 mL of acetone:toluene (1:1) during 30 minutes at 140 °C and 1200 W. Then, extracts were
225 filtered through 0.22 µm PVDF filters (Tianjin Heiaon Technology, Tianjin, China) in order to remove
226 soil particles prior to the analysis. PAHs recoveries were found between 95-102 %.

227

228 2.6 GC/MS analysis of PAHs

229

230 After MAE, 1 µL of the filtered extract was injected (pulsed splitless injection at 275 °C) by an
231 autosampler (Triplus) in a gas chromatographer (Trace GC Ultra) coupled to a mass spectrometer
232 (DSQ II) from ThermoFisher Scientific (Barcelona, Spain). The column used was a TRACE TR-5MS
233 5 % poly(phenylsilphenylen)siloxane (30 m × 0.25 mm × 0.25 µm) from ThermoFisher Scientific
234 (Barcelona, Spain) . The oven program started at 60 °C (5 min isothermal) increasing to 290 °C, at 8 °C
235 min⁻¹ (2 min isothermal), under a constant carrier gas flow (He) of 1.5 mL min⁻¹. Ionization source

236 temperature was set at 225 °C (electron impact, 70 eV) and the transfer line at 300 °C. The detection of
237 the analytes was conducted in selected ion monitoring (SIM), with the following selected masses: Phe-
238 D₁₀ (188) as internal standard, Phe (178,179,176), Fluo (202, 201, 203), Pyr (202, 200, 203) and BbF
239 and BaP (252, 253, 125). The detection and quantification limits of the complete method (MAE-
240 GC/MS) were respectively 3.0/10.0 ng g⁻¹ for Phe, 4.9/16.1 ng g⁻¹ for Fluo and Pyr, 50.0/165.0 ng g⁻¹
241 for BbF and 94.2/314.0 ng g⁻¹ for BaP. They were calculated as three and ten times the standard
242 deviation of the blank sample noise area, respectively, after the extraction and analysis. The software
243 used for data treatment was Xcalibur v.2.6.2.

244

245 2.7 Metal extraction from soil and ICP/MS analysis

246

247 The bioavailable fraction of metals in the native soil was determined using MgCl₂ as a single
248 extracting solution. The extract, representing the exchangeable fraction, includes the metals adsorbed
249 in the solid surfaces by weak electrostatic interactions and metals that can be released by ion exchange
250 processes. It is accepted that the use of MgCl₂ in single extractions constitutes a quick method to
251 estimate the bioavailable metal content in soil (Bakircioglu et al., 2011). Hence, a mixture of
252 soil:MgCl₂ 0.1 mol L⁻¹ (1:8) was agitated for 1 h, at room temperature, centrifuged for 15 min at 2,500
253 rpm and filtered through 0.22 µm PVDF filters. The supernatant was later analyzed by ICP/MS
254 (Elemental X Series 2), with an autosampler (CETAC ASX520), from ThermoFisher Scientific
255 (Barcelona, Spain). Monoelemental metal solutions were used to prepare the standards in 2 % nitric
256 acid for the instrument calibration. Total dissolved solids (TDS) were fixed under 0.5 % to minimize
257 depositions on skimmer and sampling cones. Sc, Ga, In and Tl were used as internal standards at a
258 concentration of 5 µg L⁻¹. Determinations were done in triplicate and lines were rinsed during 1 min at
259 2.5 mL min⁻¹ with 2 % nitric acid between samples. An auxiliary gas flow of He/H₂ (at 4.5 mL min⁻¹)
260 was used in the collision cell to reduce interferences, principally formed as a result of species

261 recombination (*e.g.* $^{40}\text{Ar}^{12}\text{C}^+$ interferes with ^{52}Cr). The detection and quantification limits of the
262 method (extraction-ICP/MS) were respectively 0.8/2.6 ng g⁻¹ for Cr, Co and Pb, 3.2/10.6 ng g⁻¹ for Mn,
263 2.4/7.9 ng g⁻¹ for Ni, 4.0/13.2 ng g⁻¹ for Cu and 7.2/23.8 ng g⁻¹ for Zn. They were calculated as three
264 and ten times the standard deviation of the blank sample noise counts, respectively, after extraction
265 and analysis. The software used for data treatment was PlasmaLab v.2.6.2.

266

267 2.8 Quality Assurance and Quality control (QA/QC)

268

269 For GC/MS and ICP/MS analysis, control samples were used to evaluate analysis performance.
270 Standard solutions were analyzed between soil samples to verify that calibration curves were valid
271 throughout the analysis and that the instrument was not affected by the matrix of the samples
272 (Continuing Calibration Verification). For GC/MS and ICP/MS analysis, internal standard calibration
273 was used. In both cases, recoveries of the compounds from the standard solutions were accepted within
274 80-120 % of the nominal concentrations. Furthermore, one blank (sterile soil) was also included for
275 each day of microcosms' analysis to control that the spiked PAHs concentration remained constant
276 until the completion of the experiments.

277

278 3 Results and discussion

279

280 3.1 Determination of bioavailable fraction of metals in the soil

281

282 The analysis of the exchangeable fraction (accepted as the bioavailable fraction) in the native soil
283 revealed a concentration of 0.15±0.01 µg g⁻¹ of Cr, 0.50±0.05 µg g⁻¹ of Mn, 0.010±0.001 µg g⁻¹ of Co,
284 0.05±0.01 µg g⁻¹ of Ni, 0.25±0.01 µg g⁻¹ of Cu, 0.50±0.07 µg g⁻¹ of Zn and 0.150±0.003 µg g⁻¹ of Pb.
285 The total content of bioavailable metals was 1.61±0.05 µg g⁻¹. Therefore, these seven metals were

286 selected for the present study. Zn and Mn are the most concentrated metals in this fraction, whereas Ni
287 and Co are the less concentrated, with a difference of one order of magnitude.

288

289 3.2 Microbiological growth assessment at different concentrations of metals

290

291 Microbiological assays were prepared, as indicated in section 2.3, with the concentrations specified in
292 Table 1 considering the bioavailable concentrations found in soil. After incubation, the number of
293 colony forming units (CFU) g^{-1} was determined in each soil (Table 1). The number of CFU did not
294 vary significantly after exposing bacterial communities to the eight different total concentrations of
295 metals ($p < 0.05$). This indicates that the growth and abundance of tolerant species were not affected
296 either by the metals introduced or by the nitrate/sulfate content (considering that these results do not
297 give any information about bacterial diversity). Therefore, 10, 250 and 500 times the bioavailable
298 metals found in soil (named x10, x250 and x500, respectively) were selected as the three metal
299 contamination levels to conduct the experiments of PAHs biodegradation. Lower spiking limits were
300 omitted because, in previous experiments, no significant influence on PAHs biodegradation was
301 observed. The higher spiking limit was also omitted because it contained amounts of some metals
302 above the maximum allowed concentration in soils according to the current Spanish legislation
303 (Spanish Government, 2013). After spiking the soil with metals, pH was verified and adjusted to the
304 original value if necessary.

305

306 3.3 PAHs biodegradation at different concentrations of metals

307

308 The results of PAHs biodegradation at different concentrations of metals are shown in Figure 3. First,
309 it can be observed that standard deviation values are very low (relative standard deviations - RSD -
310 below 5 %), which means that the homogenization of microcosms was optimally performed. Secondly,

311 the analysis of the blank (sterile soil spiked with PAHs and x250 level of metals) demonstrates that the
312 initial concentration of PAHs found in microcosms after spiking remained constant throughout the
313 sixty days of the experiment, proving that the degradation observed in the other microcosms (not
314 sterilized) was mainly due to biological activity of the native soil and not to other kind of chemical
315 reactions, volatilization or photooxidation (abiotic losses). On the other hand, the analysis of duplicates
316 of some microcosms (at the intermediate level of metals) did not show significant differences ($p < 0.05$)
317 (RSD below 8 %), which indicates that the preparation of microcosms was also reproducible.

318 Regarding PAH biodegradation kinetics, zero order, first order and second order were tested (Thiele-
319 Bruhn and Brümmer, 2005). The best fit was generally found with equations of first order for all PAHs
320 ($0.782 \leq R^2 \leq 0.956$) at all metals concentrations, with the exception of BbF and BaP which fit better
321 with the zero-order kinetics model ($0.672 \leq R^2 \leq 0.960$), with a quite linear and very slow dissipation.

322 The correlation coefficients with the logistic fitting and first order model, degradation rates and
323 calculated and experimental DT50 values (time required for 50 % dissipation) are shown in Table 2.

324 Figure 3 shows different degradation profiles which can be divided into two groups: Phe, Fluo and Pyr
325 were together in the first group and BbF and BaP in the second one. The variation of the degradation at
326 the distinct levels of metals is more important for the first three PAHs than for BbF and BaP.

327 For Phe, Fluo and Pyr, in general, less degradation of PAHs was observed when the concentration of
328 metals increased (Figure 3a, 3b and 3c). This is also deduced from data shown in Table 2 with
329 decreasing degradation rate constants from the non-spiked to x500 microcosms for Phe (from 0.1383
330 day^{-1} to 0.0118 day^{-1}), Fluo (from 0.1211 day^{-1} to 0.0008 day^{-1}) and Pyr (from 0.1021 day^{-1} to 0.0003
331 day^{-1}) and increasing DT50 values. Also, in the case of these three compounds, no significant
332 differences between the degradation in the microcosms non-spiked with metals and the lower level of
333 metal concentration (x10) were observed in almost all days of the study (means were compared using
334 Student's t-test, $p < 0.05$). Possibly, the metal concentration introduced did not represent a high toxicity
335 for the living microorganisms in the soil. Degradation rates were quite similar in the non-spiked and

336 x10 microcosms for Phe ($k=0.1383 \text{ day}^{-1}/0.1442 \text{ day}^{-1}$, respectively), Fluo ($k=0.1211 \text{ day}^{-1}/0.1174 \text{ day}^{-1}$, respectively) and Pyr ($k=0.1021 \text{ day}^{-1}/0.1051 \text{ day}^{-1}$, respectively). Nonetheless, after 10 days, 77 and 79 % of Phe was degraded in the non-spiked and x10 microcosms, respectively while Pyr (22 and 25 %) and Fluo (16 and 24 %) were degraded more slowly, as can be also observed from the experimental DT50 values for Phe (8 days) and Fluo and Pyr (12 days). For Fluo and Pyr, degradation percentages after 15 days were higher, between 84-90 %, for both compounds in both microcosms (Phe was degraded up to a 95-96 %, already). Contrary to the biodegradation of PAHs at low levels of metals, stronger differences can be seen at the higher metals concentration levels (x250 and x500). In x250 microcosms, the degradation of Phe was much slower than the observed before ($k=0.040 \text{ days}^{-1}$; DT50=17 days), arriving only to 80 % degradation after 21 days of incubation, whereas at lower levels of metals this value was achieved after just 10 days (at 21 days, only 19 % of Phe has been degraded at x250). Nevertheless, after 60 days, 87 % of Phe was metabolized, which is significantly a higher degradation percentage than the observed for Fluo (60 %) and Pyr (53 %) at the same time ($p<0.05$). Fluo and Pyr degrade similarly at x250 microcosms, with $k=0.0169 \text{ days}^{-1}/0.0146 \text{ days}^{-1}$ and theoretical DT50=41/47 days, respectively. Finally, non-significant variation of the concentration of Fluo and Pyr was detected after two months in x500 microcosms in relation to the initial level ($p<0.05$) and only 30 % of Phe was eliminated. This also explains the low correlation coefficient found for Fluo and Pyr at this higher amount of metals, for any of the kinetic models tested ($R^2<0.624$ for Fluo, and $R^2<0.128$ for Pyr).

355 All these results agree with the known ability of many microorganisms to degrade low weight PAH (LWPAH) faster than heavy weight PAHs (HWPAH). PAHs in sediments are rather immobile as a result of their hydrophobic nature which inhibits them from dissolving in water. PAHs solubility is inversely related to their molecular weights, which make lighter PAHs more soluble and consequently more bioavailable (Thorsen et al., 2004). The fact that Phe has been observed to degrade faster than Fluo and Pyr could be related to differences in solubility and, according to some authors, to the

361 existence of a “K” and “bay” regions in the Phe structure. This would confer Phe with an optimum
362 conformation for the anchorage of multiple enzymes involved in the oxidation of this kind of
363 compounds (Zhang et al., 2006; Kuppusamy, 2016b).

364 Therefore, the degradation rates of Phe, Fluo and Pyr decreased when the concentration of metals
365 increased, showing a significant effect when mixed pollutants are present in soil (PAHs and metals),
366 affecting negatively the microorganisms’ activity present in soils. Previous studies found in the
367 literature show that this effect is dependent on the native soil microorganisms’ composition
368 (Kuppusamy et al., 2016b). Also, Fluo and Pyr had a comparable behavior at all levels of metals
369 concentration and showed strong correlated kinetic parameters (Table 2). Other authors saw this
370 parallel behavior between the former compounds in biodegradation studies involving mixtures of
371 PAHs and five different soils (without metal consideration) (Crampon et al., 2014), and also for Pyr
372 biodegradation in soils with different microorganism’ mixtures content and in presence of metals
373 (Kuppusamy et al., 2016b).

374 On the opposite, the biodegradation of BbF and BaP (HWPAs) seems not to be affected by the
375 different concentrations of metals introduced (Figure 3d and 3e), probably due to the little ability of
376 the bacterial consortia to efficiently degrade them under any circumstances (compared to other
377 previous studies, Deary et al., 2016; Kuppusamy et al., 2016a). After 60 days, only 29, 22, 16 and 19
378 % of BbF was degraded in the non-spiked x10, x250 and x500 microcosms, respectively, and the
379 results were not much different for BaP either (32, 30, 27 and 36 %, respectively) at the same time.
380 After 60 days, no significant differences in the concentration of BaP were found either between the
381 non-spiked and x10 or between x10 and x250 microcosms ($p < 0.05$). Although the concentration of
382 BaP in x500 was statistically different from the other microcosms after 60 days, the biodegradation
383 profile of BaP in each level of metals introduced was not as sharp as it was for Phe, Fluo and Pyr.
384 Analogous judgment could be used to describe the biodegradation profile of BbF in each level of
385 metals, where no significant differences were observed either between x250 and x500 or between x10

386 and x500 microcosms. These conclusions can also be drawn from the comparable degradation rates
387 and theoretical DT50 values for BbF (mean $k=0.0039$ days⁻¹, mean DT50=191 days) and BaP (mean
388 $k=0.0055$ days⁻¹, mean DT50=126 days). These low degradation rates confirm the recalcitrant nature
389 of these heavier PAHs. This is principally explained by the progressive decrease in solubility and
390 increase in hydrophobicity (therefore, less bioavailability) of PAHs as their molecular weight
391 increases. Also, it is widely accepted that the higher the molecular weight of PAHs the lower the
392 degradation ability of microorganisms, because other simpler organic forms, including LWPAHs, are
393 more suitable to be used as a sole carbon energy source. This competitive inhibition is particularly
394 important when microorganisms such as bacteria use enzymes with non-specific active sites for PAHs
395 breakdown (Stroud et al., 2007; Wang et al., 2009; Abdel-Shafy and Mansour, 2016). HWPAHs can
396 also represent a carbon source for microorganisms but only a few can efficiently degrade them, and
397 often by co-metabolism. Another reason of the poor degradation of HWPAHs is the low amounts of
398 bacteria in soils able to degrade them (Kästner et al., 1994) and the lack of enzymatic induction, which
399 is more complicated due to their bigger size and more complex conformation. This makes PAHs less
400 accessible for the active centers of the enzymes involved in the metabolism of many microorganisms.
401 In fact, BaP and BbF show better correlation with a zero order kinetics model, typically observed in
402 biodegradations profile which undergo through the co-metabolism phenomena.

403 Co-metabolism can be defined as a non-specific enzymatic reaction between a new substrate that
404 competes with a primary substrate of similar structure for the active site of the enzyme (Stroud et al.,
405 2007). This phenomenon explains that degradation of certain HWPAHs can be enhanced by the
406 presence of simpler carbon source structures such as LWPAHs (*e.g.* Phe), which would not occur (or
407 would be lower) in their absence. It was reported that the presence of Phe enhanced the biodegradation
408 of Anth, Flu and Pyr (Yuan et al., 2001) and also contributed to the increase in biomass when acting as
409 a co-substrate for the co-metabolism of Chry, Fluo and Pyr (Hwang and Cutright, 2003; Iqwo-Ezipke,
410 2010), as could have happened in this study. It can be seen in Figure 3 that the biodegradation of Fluo

411 and Pyr started always later than Phe (at any of the concentrations of metals where degradation is
412 observed). On the other hand, the degradation rates of Fluo and Pyr seem to decrease when Phe was
413 nearly completely metabolized (easy to observe in x250 microcosms after 30 days or in non-spiked or
414 x10 microcosms after 15 days). However, more consistent data should be collected if the objective of
415 the study were to support a co-metabolism effect on the biodegradation of BaP and BbF.

416 About the degradation profiles (for those cases where degradation is significantly observed; *i.e.* non-
417 spiked, x10 and x250 microcosms), three different phases can be clearly distinguished. The first one,
418 generally comprising the first 7 days of the study, shows latent or very low microbiological activity
419 and probably corresponds to an adaptation phase (lag-phase) of the microorganisms in response to the
420 stress caused by the introduced contamination (metals and PAHs), which agrees with previous
421 observations by other authors (Wen et al., 2011). It can be noted that the initial lag-phase of concerned
422 compounds was not excluded from the kinetic models described previously because this phase was
423 relatively short and did not have any significant impact on the first order modelling. Bacterial
424 communities exhibit different tolerance to contamination and those which are resistant evolve to a
425 second phase, where biodegradation takes place very quickly (7-30 days). Finally, the third phase
426 consists in the achievement of a *plateau* state, where biodegradation rates stabilize or decrease very
427 slowly. This slow biodegradation of residual PAHs is generally assigned to a lower PAH
428 bioavailability, depending on the contact time between lipophilic contaminants and soils particles
429 (especially for HWPAs, as previously seen by Deary et al, 2016). Indeed, at this final stage,
430 degrading microorganisms are present in soils and have shown an important degrading activity on
431 certain PAHs, but strong and almost irreversible interactions take place between residual PAHs and
432 soil organic matter which prevent contaminants from desorption and subsequent absorption by
433 microorganisms.

434 These biodegradation profiles were also observed by some authors who stated a dependency between
435 the adaptation time required by bacterial communities and the concentration of PAHs supplemented,

436 establishing a direct relationship between the number of CFU g⁻¹ and the degradation percentage of
437 these compounds (Khan et al., 2009; Wen et al., 2011.). In our study, the number of CFU g⁻¹ was
438 neither correlated with the lag-phase duration nor with the level of metals introduced in the
439 microcosms. In fact, the spiked metal level had no influence on the total number of soil
440 microorganisms in the concentration range studied (Table 1), but probably had more influence on the
441 relative abundance of specific PAH-degrading strains, which represents generally less than 1% of the
442 cultivable soil bacteria (Crampon et al., 2014).

443

444 In the present work, there is no evidence that the mixtures of metals enhance PAHs biodegradation but
445 the opposite. Nonetheless, the purpose of the research cited above was the evaluation of metal/PAH
446 interactions individually, and not to follow their behavior as multiple mixtures, which is the present
447 purpose. To study all contaminants together is certainly difficult since soils contain high numbers of
448 other compounds at many different concentrations, leading to complex interactions between them.
449 Furthermore, PAHs' partition between the aqueous phase in contact with the soil phases, each
450 possessing a different degree of bioaccessibility, can also affect their biodegradation (Deary et al.,
451 2016).

452 The present work demonstrates that studies, even if performed under controlled conditions, must
453 enforce to be more representative of real conditions in order to better understand in-field
454 bioremediation processes by natural attenuation.

455

456 4 Conclusions

457

458 A study about mixed pollutants has been performed to assess the influence of different concentrations
459 of seven metals on the biodegradation of five PAHs during sixty days by means of the analysis of soil-
460 based microcosms.

461 The study of the interaction of mixed contaminants in a complex environmental matrix has shown that
462 the different levels of metals caused a significant negative effect on the biodegradation capability of
463 the 3-4 rings PAHs Phe, Fluo and Pyr, with decreased dissipation constant rates and increased DT50
464 values. No effect was detected on the 5-rings PAHs BbF and BaP, which are recalcitrant PAHs
465 (HWP AHs) and showed very low dissipation rates and the highest DT50 values even in the absence of
466 metals. Also, it has been seen that degradation rates in the first order kinetics approach vary
467 significantly depending on the nature of PAHs and the concentration of metals introduced in the
468 microcosms. Dissipation profiles of biodegraded PAHs consisted of few phases, starting with a latency
469 period and then consistently being degraded until reaching a *plateau* state.

470 It would be interesting to conduct further studies to evaluate whether the impact on PAH
471 biodegradation was caused by one single metal or by combinations of them.

472 These results show the importance of the decontamination of some metal polluted areas if PAHs
473 bioremediation activities are to be performed in natural soils, particularly those carried out by
474 microbiological processes.

475

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477

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481

482 References

483

484 Bakircioglu, D., Bakircioglu-Kurtulus, Y.B., Ibar, H., 2011. Comparison of extraction procedures for
485 assessing soil metal bioavailability of two wheat grains. *Clean-Soil, Air, Water* 39, 728-734.

487 Baldrian, P., Wiesche, C., Gabriel, J., Nerud, F., Zadrazil, F., 2000. Influence of cadmium and mercury
488 on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by
489 *Pleurotus ostreatus* in soil. Appl. Environ. Microbiol. 66, 2471-2478.

491 Baltrons, O., López-Mesas, M., Palet, C., Le Derf, F., Portet-Koltalo, F., 2013. Molecularly imprinted
492 polymer - liquid chromatography/fluorescence for the selective clean-up of hydroxylated polycyclic
493 aromatic hydrocarbons in soils. Anal. Methods 5, 6297-6305.

495 Blum, W.E.H., Horak, O., Mentler, A., Puschenreiter, M., 2009. Trace metals, in: Sabljic, A. (Ed.),
496 Environmental and Ecological Chemistry, Volume 2, EOLSS Publishers Company Ltd., Oxford, pp.
497 156-164.

499 Biswas, B., Sarkar, B., Mandal, A., Naidu, R., 2015. Heavy metal-immobilizing organoclay facilitates
500 polycyclic aromatic hydrocarbon biodegradation in mixed-contaminated soil. J. Hazard. Mater. 298,
501 129-137.

503 Boyle, D., Wiesner, C., Richardson, A., 1998. Factors affecting the degradation of polyaromatic
504 hydrocarbons in soil by white-rot fungi. Soil. Biol. Biochem. 30, 873-882.

506 Chen, M., Xu, P., Zeng, G., Yang, C., Huang, D., Zhang, J., 2015. Bioremediation of soils
507 contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy
508 metals by composting: Applications, microbes and future research needs. Biotechnol. Adv. 33, 745-
509 755.

511 Crampon, M., Bureau, F., Akpa-Vinceslas, M., Bodilis, J., Machour, N., Le Derf, F., Portet-Koltalo,
512 F., 2014. Correlations between PAH bioavailability, degrading bacteria, and soil characteristics during
513 PAH biodegradation in five diffusely contaminated dissimilar soils. *Environ. Sci. Pollut. Res. Int.* 21,
514 8133-8145.

515

516 Deary, M.E., Ekumankama, C.C., Cummings, S.P., 2016. Development of a novel kinetic model for
517 the analysis of PAH biodegradation in the presence of lead and cadmium co-contaminants. *J. Hazard.*
518 *Mater.* 307, 240-252.

519

520 Fernández-Luqueño, F., Valenzuela-Encinas, C., Marsch, R., Martínez-Suárez, C., Vázquez-Núñez, E.,
521 Dendooven, L., 2011. Microbial communities to mitigate contamination of PAHs in soil - possibilities
522 and challenges: a review. *Environ Sci Pollut Res.* 18, 12-30.

523

524 Fijałkowska, S., Lisowska, K., Długoński, J., 1998. Bacterial elimination of polycyclic aromatic
525 hydrocarbons and heavy metals. *J. Basic. Microbiol.* 38, 361-369.

526

527 Giller, K.E., Witter, E., McGrath, S.P., 1998. Toxicity of heavy metals to microorganisms and
528 microbial processes in agricultural soils: A review. *Soil. Biol. Biochem.* 30, 1389-1414.

529

530 Huertos, E.G., Baena, A.R., 2008. Contaminación de suelos por metales pesados. *Macla* 10, 48-60.

531

532 Abdel-Shafy, H.I., Mansour, M.S.M., 2016. A review on polycyclic aromatic hydrocarbons: Source,
533 environmental impact, effect on human health and remediation. *Egypt. J. Pet.* 25, 107-123.

534

535 Hwang, S., Cutright, T.J., 2003. Effect of expandable clays and cometabolism on PAH
536 biodegradability. Environ. Sci. Pollut. Int. 10, 277-280.

537

538 Igwo-Ezikpe, M.N., Gbenle, O.G., Ilori, M.O., Okpuzor, J., Osuntoki, A.A., 2010. Mixture of high
539 molecular weight polycyclic aromatic hydrocarbons biodegradation by tropical bacteria and via co-
540 metabolism with phenanthrene. Res. J. Environ. Sci. 4, 317-326.

541

542 Janbandhu, A., Fulekar, M., 2011. Biodegradation of phenanthrene using adapter microbial consortium
543 isolated from petrochemical contaminated environment. J. Hazard. Mater. 187, 333-340.

544

545 Jonsson, S., Persson, Y., Frankki, S., Van Bavel, B., Lundstedt, S., Haglund, P., Tysklind, M., 2007.
546 Degradation of polycyclic aromatic hydrocarbons (PAHs) in contaminated soils by Fenton's reagent: a
547 multivariate evaluation of the importance of soil characteristics and PAH properties. J. Hazard. Mater.
548 149, 86-96.

549

550 Kabata-Pendias, A., 2010. Trace Elements in Soils and Plants, forth ed. CRC Press, Boca Raton.

551

552 Kästner, M., Breuer-Jammali, M., Mahro, B., 1994. Enumeration and characterization of the soil
553 microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic
554 hydrocarbons (PAH). Appl. Microbiol. Biot. 41, 267-273.

555

556 Ke, L., Luo, L., Wang, P., Luan, T., Tam, N.F.Y., 2010. Effects of metals on biosorption and
557 biodegradation of mixed polycyclic aromatic hydrocarbons by a freshwater green alga *Selenastrum*
558 *capricornutum*. Bioresource Technol. 101, 6950-6961.

559

560 Khan, S., El-Latif Hesham, A., Qing, G., Shuang, L., He, J., 2009. Biodegradation of pyrene and
561 catabolic genes in contaminated soils cultivated with *Lolium multiflorum*. J. Soil. Sediments 9, 482-
562 491.

563

564 Kobayashi, T., Murai, Y., Tatsumi, K., Iimura, Y., 2009. Biodegradation of polycyclic aromatic
565 hydrocarbons by *Sphingomonas* sp. enhanced by water-extractable organic matter from manure
566 compost. Sci. Total Environ. 407, 5805-5810.

567

568 Kuppusamy, S., Thavamani, P., Megharaj, M., Lee, Y.B., Naidu, R., 2016. Kinetics of PAH
569 degradation by a new acid-metal-tolerant *Trabulsiella* isolated from the MGP site soil and
570 identification of its potential to fix nitrogen and solubilize phosphorous. J. Hazard. Mater. 307, 99-107,
571 a.

572

573 Kuppusamy, S., Thavamani, P., Megharaj, M., Naidu, R., 2016. Biodegradation of polycyclic aromatic
574 hydrocarbons (PAHs) by novel bacterial consortia tolerant to diverse physical settings - Assessments
575 in liquid- and slurry-phase systems. Int. Biodeter. Biodegr. 108, 149-157, b.

576

577 Landi, L., Renella, G., Moreno, J.L., Falchini, L., Nannipieri, P., 2000. Influence of cadmium on the
578 metabolic quotient, L-:D-glutamic acid respiration ratio and enzyme activity: microbial biomass ratio
579 under laboratory conditions. Biol. Fertil. Soils 32, 8-16.

580

581 Moreau, C.J., Klerks, P.L., Haas, C.N., 1999. Interaction between phenanthrene and zinc in their
582 toxicity to the sheepshead minnow (*Cyprinodon variegatus*). Arch. Environ. Contam. Toxicol. 37,
583 251-257.

584

585 Niepceron, M., Martin-Laurent, F., Crampon, M., Portet-Koltalo, F., Akpa-Vinceslas, M., Legras, M.,
586 Bru, D., Bureau, F., Bodilis, J., 2013. GammaProteobacteria as a potential bioindicator of a multiple
587 contamination by polycyclic aromatic hydrocarbons (PAHs) in agricultural soils. Environ. Pollut. 180,
588 199-205.

589

590 Obuekwe, I.S., Semple, K.T., 2013. Impact of Zn and Cu on the development of phenanthrene
591 catabolism in soil. Environ. Monit. Assess. 185, 10039-10047.

592

593 Samanta, K.S., Singh, O.V., Jain, R.K., 2002. Polycyclic aromatic hydrocarbons: environmental
594 pollution and bioremediation. Trends Biotechnol. 20, 243-248.

595

596 Shen, G., Lu, Y., Hong, J., 2006. Combined effect of heavy metals and polycyclic aromatic
597 hydrocarbons on urease activity in soil. Ecotoxicol. Environ. Saf. 63, 474-480.

598

599 Shen, G., Lu, Y., Zhou, Q., Hong, J., 2005. Interaction of polycyclic aromatic hydrocarbons and heavy
600 metals on soil enzyme. Chemosphere 61, 1175-1182.

601

602 Siddiqi, M.A., Yuan, Z.X., Honey, S.A., Kumar, S., Sikka, H.C., 2002. Metabolism of PAHs and
603 methyl-substituted PAHs by *Sphingomonas paucimobilis* strain EPA 505. Polycycl. Aromat. Compd.
604 22, 621-630.

605

606 Sokhn, J., De Leij, F.A.A.M., Hart, T.D., Lynch, J. M., 2001. Effect of copper on the degradation of
607 phenanthrene by soil microorganisms. *Lett. Appl. Microbiol.* 33, 164-168.

608

609 Spanish Government, Real Decreto 1310/1990. [http://www.boe.es/boe/dias/1990/11/01/pdfs/A32339-](http://www.boe.es/boe/dias/1990/11/01/pdfs/A32339-32340.pdf)
610 [32340.pdf](http://www.boe.es/boe/dias/1990/11/01/pdfs/A32339-32340.pdf), (accessed 5 March 2013).

611

612 Stroud, J.L., Paton, G.I., Semple, K.T., 2007. Importance of chemical structure on the development of
613 hydrocarbon catabolism in soil. *FEMS Microbiol. Lett.* 272, 120-126.

614

615 Subashchandrabose, S.R., Megharaj, M., Venkateswarlu, K., Naidu, R., 2015. Interaction effects of
616 polycyclic aromatic hydrocarbons and heavy metals on a soil microalga, *Chlorococcum* sp. MM11.
617 *Environ. Sci. Pollut. Res. Int.* 22, 8876–8889.

618

619 Tam, Y.W.F., Takeda, K., Sakugawa, H., 2008. Exploring the correlation of particulate PAHs, sulfur
620 dioxide, nitrogen dioxide and ozone, A preliminary study. *Water Air Soil Pollut.* 194, 5-12.

621

622 Thavamani, P., Malik, S., Beer, M., Megharaj, M., Naidu, R., 2012. Microbial activity and diversity in
623 long-term mixed contaminated soils with respect to polyaromatic hydrocarbons and heavy metals. *J.*
624 *Environ. Manage.* 99, 10-17.

625

626 Thiele-Bruhn, S., Brümmer, G., 2005. Kinetics of polycyclic aromatic hydrocarbon (PAH) degradation
627 in long-term polluted soils during bioremediation. *Plant Soil* 275, 31-42.

628

629 Tobiszewski, M., Namieśnik, J., 2012. PAH diagnostic ratios for the identification of pollution
630 emission sources. *Environ. Pollut.* 162, 110-119.

631

632 Wang, C., Sun, H., Li, J., Li, Y., Zhang, Q., 2009. Enzyme activities during degradation of polycyclic
633 aromatic hydrocarbons by white rot fungus *Phanerochaete chrysosporium* in soils. *Chemosphere* 77,
634 733-738.

635

636 Watanabe, K., 2001. Microorganisms relevant to bioremediation. *Curr. Opin. Biotechnol.* 12, 237-256.

637

638 Wentworth, C.K., 1922. A scale of grade and class terms for clastic sediments. *J. Geol.* 30, 377-392.

639

640 Wen, J., Gao, D., Zhang, B., Liang, H., 2011. Co-metabolic degradation of pyrene by indigenous
641 white-rot fungus *Pseudotrametes gibbosa* from the northeast China. *Int. Biodeter. Biodegr.* 65, 600-
642 604.

643

644 Wilson, S.C., Jones, K., 1993. Bioremediation of soil contaminated with polynuclear aromatic
645 hydrocarbons (PAHs): a review. *Environ. Pollut.* 81, 229-249.

646

647 Wuana, R.A., Okieimen, F.E., 2011. Heavy metals in contaminated soils: A review of sources,
648 chemistry, risks and best available strategies for remediation. *ISRN Ecology*, 1-20.

649

650 Yuan, S.Y., Chang, J.S., Yen, J.H., Chang, B.V., 2001. Biodegradation of phenanthrene in river
651 sediment. *Chemosphere* 43, 273-278.

652

653 Zehetner, F., Rosenfellner, U., Mentler, A., Gerzabek, M.H., 2009. Distribution of road salt residues,
654 heavy metals and polycyclic aromatic hydrocarbons across a highway-forest interface. *Water Air Soil*
655 *Pollut.* 198, 125-132.

656

657 Zhang, X.X., Cheng, S.P., Zhu, J.C., Sun, S.L., 2006. Microbial PAH-degradation in soil: degradation
658 pathways and contributing factors. *Pedosphere* 16, 555-565.

659

660 Zhou, L., Li, H., Zhang, Y., Han, S., Xu, H., 2016. *Sphingomonas* from petroleum-contaminated soils
661 in Shenfu, China and their PAHs degradation abilities. *Braz. J. Microbiol.* 47, 271-278.