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

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Abstract

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

Keywords

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

1 Introduction

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

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

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

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

finding significant differences depending on the experimental conditions tested (Baldrian et al., 2000; Ke et al., 2010; Khan et al., 2009; Biswas et al., 2015; Deary et al., 2016). Other studies, show the biodegradation evaluation of one PAH in the presence of a mixture of heavy metal solutions at different pH (Kuppusamy et al. 2016b) and the comparison of the simultaneous biodegradation of two PAHs in the presence of individual metal solutions of Zn, Pb, Cu and Cd (Kuppusamy et al. 2016a).

The main objective of the present work is to study the influence of different concentrations of a mixture of metals on the biodegradation of some PAHs, under controlled conditions (i.e. temperature, light exposure, humidity, etc.). Contrary to evaluating specific single-metal/PAH interactions, focusing on mixtures allows to have a global picture of the mixed pollutants impact in the native soil. Hence, a group of soil based microcosms were prepared, spiked with a selected mixture of PAHs and different concentration levels of a mixture of metals. First, the selected soil was characterized in order to decide the concentration of PAHs to be added to the microcosms and to determine the original bioavailable metal content. Secondly, bacterial growth assays were performed in order to choose the range of metal concentration levels to be studied. Finally, microcosms were prepared and analyzed to carry out the biodegradation experiments to accomplish the aforementioned main objective.

2 Experimental

2.1 Chemicals

Toluene, acetone (HPLC grade) and nitric acid (69.5 %) were obtained from Sigma-Aldrich (Barcelona, Spain) and hydrochloric acid (37 %) from Panreac (Barcelona, Spain). Water was purified and deionized using an Elga Classic system from Veolia Water Solutions and Technology (Madrid, Spain). The internal standard, perdeuterated phenanthrene (Phe-D_{10}) and five PAHs used to spike microcosms (phenanthrene, fluoranthene, pyrene, benzo[a]pyrene, benzo[b]fluoranthene) were

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

2.2 Collection and characterization of soil samples

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

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

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

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

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

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

2.3 Selection of spiking concentrations for PAHs and metals

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

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

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

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

2.4 Preparation of microcosms

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

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

2.5 Microwave assisted extraction of PAHs from soil

Microwave assisted extraction (MAE) was performed using a MARS X equipment (CEM Corporation, Matthews, USA). Wet soils from microcosms were previously dried one night at 35°C and then crushed. Before the extraction, perdeuterated phenanthrene (Phe-D_{10}) was added as internal standard to reach a final concentration of 1.0 mg L^{-1}. A sample size of 1.5 g of crushed dry soil was extracted using 25 mL of acetone:toluene (1:1) during 30 minutes at 140 ºC and 1200 W. Then, extracts were filtered through 0.22 µm PVDF filters (Tianjin Heiaon Technology, Tianjin, China) in order to remove soil particles prior to the analysis. PAHs recoveries were found between 95-102 %.

2.6 GC/MS analysis of PAHs

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

temperature was set at 225 ºC (electron impact, 70 eV) and the transfer line at 300 ºC. The detection of the analytes was conducted in selected ion monitoring (SIM), with the following selected masses: Phe-D_{10} (188) as internal standard, Phe (178,179,176), Fluo (202, 201, 203), Pyr (202, 200, 203) and BbF and BaP (252, 253, 125). The detection and quantification limits of the complete method (MAE-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} for BbF and 94.2/314.0 ng g^{-1} for BaP. They were calculated as three and ten times the standard deviation of the blank sample noise area, respectively, after the extraction and analysis. The software used for data treatment was Xcalibur v.2.6.2.

2.7 Metal extraction from soil and ICP/MS analysis

The bioavailable fraction of metals in the native soil was determined using $MgCl_2$ as a single extracting solution. The extract, representing the exchangeable fraction, includes the metals adsorbed in the solid surfaces by weak electrostatic interactions and metals that can be released by ion exchange processes. It is accepted that the use of $MgCl_2$ in single extractions constitutes a quick method to estimate the bioavailable metal content in soil (Bakircioglu et al., 2011). Hence, a mixture of soil:$MgCl_2$ 0.1 mol L^{-1} (1:8) was agitated for 1 h, at room temperature, centrifuged for 15 min at 2,500 rpm and filtered through 0.22 µm PVDF filters. The supernatant was later analyzed by ICP/MS (Elemental X Series 2), with an autosampler (CETAC ASX520), from ThermoFisher Scientific (Barcelona, Spain). Monoelemental metal solutions were used to prepare the standards in 2 % nitric acid for the instrument calibration. Total dissolved solids (TDS) were fixed under 0.5 % to minimize depositions on skimmer and sampling cones. Sc, Ga, In and Tl were used as internal standards at a concentration of 5 µg L^{-1}. Determinations were done in triplicate and lines were rinsed during 1 min at 2.5 mL min^{-1} with 2 % nitric acid between samples. An auxiliary gas flow of He/H_2 (at 4.5 mL min^{-1}) was used in the collision cell to reduce interferences, principally formed as a result of species

recombination (*e.g.* $^{40}Ar^{12}C^{+}$ interferes with ^{52}Cr). The detection and quantification limits of the 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, 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 and ten times the standard deviation of the blank sample noise counts, respectively, after extraction and analysis. The software used for data treatment was PlasmaLab v.2.6.2.

2.8 Quality Assurance and Quality control (QA/QC)

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

3 Results and discussion

3.1 Determination of bioavailable fraction of metals in the soil

The analysis of the exchangeable fraction (accepted as the bioavailable fraction) in the native soil 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, 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. The total content of bioavailable metals was 1.61±0.05 µg g^{-1}. Therefore, these seven metals were

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

3.2 Microbiological growth assessment at different concentrations of metals

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

3.3 PAHs biodegradation at different concentrations of metals

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

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

Regarding PAH biodegradation kinetics, zero order, first order and second order were tested (Thiele-Bruhn and Brümmer, 2005). The best fit was generally found with equations of first order for all PAHs ($0.782\leq R^2\leq 0.956$) at all metals concentrations, with the exception of BbF and BaP which fit better with the zero-order kinetics model ($0.672\leq R^2\leq 0.960$), with a quite linear and very slow dissipation. The correlation coefficients with the logistic fitting and first order model, degradation rates and calculated and experimental DT50 values (time required for 50 % dissipation) are shown in Table 2. Figure 3 shows different degradation profiles which can be divided into two groups: Phe, Fluo and Pyr were together in the first group and BbF and BaP in the second one. The variation of the degradation at the distinct levels of metals is more important for the first three PAHs than for BbF and BaP.

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

x10 microcosms for Phe (k=0.1383 day^{-1}/0.1442 day^{-1}, respectively), Fluo (k=0.1211 day^{-1}/0.1174 day^{-1}, respectively) and Pyr (k=0.1021 day^{-1}/0.1051 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 $days^{-1}$; 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 $days^{-1}$/0.0146 $days^{-1}$ 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).

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

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

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

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

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

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

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

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

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

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