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Acute effects of PAH contamination on microbial community of different forest soils☆

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are hazardous organic compounds with mutagenic, genotoxic and carcinogenic properties. Although PAHs in soil can cause toxicity to microorganisms, the microbial community is able to degrade these compounds. For this reason, it is important to study acute and short-term effects of PAH contamination on soil microbial community, also to shed light on its possible exploitation in soil restoration.

The effects of acute PAH contamination on the structure and metabolic activity of microbial communities in three forest (beech, holm oak, black pine) soils were studied. The soils were spiked with phenanthrene, pyrene or benzo[a]pyrene and incubated in experimental mesocosms, under controlled conditions. Enzymatic activities (laccase, total peroxidase and hydrolase), as well as microbial biomass and community structure (through phospholipid fatty acid and ergosterol analyses), were evaluated in the three soil systems 4 days after contamination and compared to no-spiked soils. In soil under holm oak, there was a stimulation of Gram+ bacteria after contamination with all the 3 PAHs, whereas in soil under pine, pyrene and phenanthrene additions mainly stimulated fungi and actinomycetes.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous semi-volatile organic pollutants, with toxic, mutagenic, genotoxic and carcinogenic properties (IARC, 2010). PAHs potentially cause adverse ecological and/or human health effects even at low levels. These pollutants are formed during incomplete combustion of organic materials. Forest fires are thus known as an important natural source of PAHs (Tsibart and Gennadiev, 2013), whereas combustion of fuel, coal and oil are known as the main anthropogenic sources (Gan et al., 2009). Forests play an important role in global distribution and fate of PAHs, increasing the deposition fluxes from air to soils by both the canopy scavenging and litter fall (Horstmann and McLachlan, 1998). The type of forest stand affects the PAH content in soils, with higher amounts under deciduous than coniferous stands (Komprdová et al., 2016). Both leaf (surfaces, hairs, stomata, lipids, waxes) as well as canopy (leaf density, production of new leaves, timing of bud burst) features affect scavenging and uptake rate of PAHs by different tree stands (Terzaghi et al., 2015; De Nicola et al., 2017), and in turn, the degree of PAH deposition to soil (Błońska et al., 2016). In soil, PAHs are subjected to a number of processes, following partition among solid, liquid and gaseous phases (Stokes et al., 2006). Loss of PAHs occurs by physico-chemical processes like volatilization and leaching, and by biological processes like microbial degradation.

Several soil microorganisms (for example fungi, actinomycetes, methanotrophic bacteria) have been identified as PAH degraders (Rockne et al., 1998; Wawra et al., 2018; Bellino et al., 2019). At the same time, however, microbes are susceptible to organic contaminants. In particular, high concentrations of PAHs in soil can negatively affect total bacterial and fungal populations, microbial metabolic processes (Zoppini et al., 2016), and enzyme activities (Pérez-Leblic et al., 2012). By causing pressure on sensitive soil microorganisms, PAHs could determine changes in microbial community composition (Su and Yang, 2009). The measurement of microbiological parameters, such as microbial biomass or enzyme activities, representing good indices of the impact of contaminants on soil health (Brohon et al., 2001; Eibes et al., 2006), provides information on intensity and duration of the effects of PAH pollution on soil community and *vice versa*. Indeed, several soil enzyme

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activities have been proposed for evaluating and monitoring remediation of hydrocarbon contaminated soils (Košnář et al., 2019; Polyak et al., 2018).

As microbial community responds to anthropogenic stresses with temporary dynamic changes (Ager et al., 2010) and successive recovers, it is possible that the effects of contamination on soil microbial community fade over the long-term. In addition, little is known on the response of indigenous microbial communities to acute PAH contamination following short-term exposure (Peng et al., 2013; Thomas and Cébron, 2016).

This study aimed to fill the gap in our knowledge on acute and recent soil contamination by PAHs. In particular, the goal of the study was to determine the short-term changes in soil microbial communities induced by PAH addition. The present research has its foundations on the hypothesis that several indigenous soil microbial communities show different short-term responses to acute PAH contamination events. To test this hypothesis, three different forest soils (sampled under holm oak, black pine and beech stands) were separately spiked in laboratory with three PAHs, one for each class of low, medium and high molecular weight: phenanthrene, pyrene and benzo[a]pyrene. The short-term impacts on microbial metabolic activity (enzyme activities), biomass and structure (phospholipid fatty acid and ergosterol) were analyzed by comparison with unspiked soils. A deep knowledge of microbial community involved in sustaining ecology of freshly contaminated soil could help to predict the natural attenuation processes and, in turn, aid in the identification of degrading-PAH consortia.

2. Materials and methods

2.1. Soil sampling

Three forests differing in dominant tree species, holm oak (H), black pine (P) and beech (B), were chosen for soil sampling. The three systems, widespread in the Matese Mountains (Apennines district, southern Italy), were on a calcareous substrate covered with pyroclastic materials (Di Gennaro, 2002). At each forest, about 20 Kg soil (0–10 cm depth) were randomly collected from 5 points after litter removal. The physico-chemical characterization of soils is reported in Table S1.

2.2. Mesocosms set-up

In the laboratory, H, P and B soils were separately treated with 3 PAHs, one for each class of molecular weight, obtaining the following treatments: 1) 125 mg/kg of phenanthrene (Phe); 2) 125 mg/kg of pyrene (Py); 3) 95 mg/kg of benzo[a]pyrene (Ba); 4) unspiked (US). PAH doses were chosen based on what reported in the literature in similar experiments in which effects on some enzymatic activities were observable (Scelza et al., 2007; Baldantoni et al., 2017). According to Brinch et al. (2002), a solution of each PAH (in acetone) was used to spike 25% of the soil. After acetone evaporation, the spiked soil was mixed with the remaining 75% of the soil sample to achieve homogeneity. The spiked and the relative unspiked soils were finally incubated in 20 × 30 × 10 cm mesocosms (2 mesocosms per each treatment per each soil type), at controlled temperature (22 °C) and relative humidity (88%) in the dark. After 4 days of incubation, 8 soil sub-samples were collected from the 2 mesocosms with the same treatment and pooled for the subsequent analyses. Hydrolytic, laccase and peroxidase activities, ergosterol and phospholipid fatty acid (PLFA) profiles were analyzed on fresh and sieved (2 mm) soils. All chemical and biological analyses were carried out in triplicates per each forest system and per each soil treatment.

2.3. Laboratory analyses

2.3.1. Enzyme activities

Fluorescein diacetate hydrolytic activity (FDAase) was determined by incubating soil samples at 25 °C for 30 min with phosphate buffer (60 mM, pH 7.6), adding FDA (2 mg/mL in acetone) as substrate and measuring the absorbance of the released fluorescein at 490 nm (Schnurer and Rosswall, 1982). The laccase activity was determined by incubating soil samples at 30 °C for 15 min with MUB buffer (0.1 M, pH 2.0), adding ABTS (0.1 M) as substrate and measuring the absorbance of the reaction product (ABTS+) at 420 nm (Floch et al., 2007). The peroxidase activity was determined by the method of Bach et al. (2013) modified. Soil suspensions were prepared by homogenizing 1 g of fresh soil in 125 mL of acetate buffer (50 mM, pH 5.3). Enzyme activities were measured by combining 800 μL of soil suspension with 200 μL of substrate solution (L-DOPA 25 mM) and by incubating soil samples at room temperature for 2 h. The absorbance of the reaction product was determined at 460 nm. The spectrophotometric measures were carried out by a 6715 Jenway (Cole-Parmer, UK) UV/Vis spectrophotometer.

2.3.2. Ergosterol

Ergosterol was determined using the method described by Grant and West (1986). Soil samples (1 g) were extracted in 4 mL 10% KOH in methanol by sonication for 15 min and incubated at 70 °C for 90 min. Samples were cooled, mixed with 1 mL of distilled water and 2 mL of cyclohexane and centrifuged (5 min at 3000 rpm). The upper polar phase was recovered and samples were washed again with 2 mL of cyclohexane and centrifuged (5 min at 3000 rpm). The upper polar phases from the same samples were combined and dried under a gentle nitrogen stream at 40 °C. The dried samples were dissolved in methanol, heated at 40 °C and filtered. The samples were analyzed by reverse-phase HPLC (Finnigan Surveyor, Thermo Scientific), using a LC Column 150 × 4.6 mm (Kinetex® 5 μm EVO C18 100 Å), a mobile phase constituted by 100% methanol at a flow rate of 1 mL/min, and a wavelength for the multi-wavelength detector equal to 282 nm.

2.3.3. PLFAs

PLFA analyses were carried out using the methods described by Bååth et al. (1992). Soil samples (1 g) were extracted in 10 mL of Bligh and Dyer reagent (chloroform:methanol:citrate buffer, 1:2:0.8, v/v/v). The lipid extract was fractionated into neutral, glycol- and polar lipids (phospholipids) on a silicic acid column. The phospholipids were subject to a mild alkaline methanolysis and the resulting fatty acid methyl esters were identified by a gas chromatograph with a flame ionization detector (GC-FID 6890 HP, equipped with a capillary column HP 5MS UI 60 m × 0.25 mm). The temperature program started at 80 °C for 1 min, raised at 20 °C per min up to 160 °C, followed by a further increase of 5 °C per min until the final temperature of 280 °C, and held until a total time of 50 min. Both injection temperature and detector temperature were 280 °C.

For PLFA nomenclature see Bååth et al. (1992). The sum of PLFAs i14:0, 14:00, i15:0, a15:0, 15:00, i16:0, 16:1ω9, 16:1ω7c, 16:1ω7t, 16:00, br17:0, 10Me16:0a, i17:0, a17:0, 17:1ω8, cy17:0, 17:00, 10Me17:0, 18:1ω9, 18:1ω7, 18:00, cy19:0, 20:00 was used as indicator of bacterial biomass (Bc), (Bååth and Anderson, 2003). PLFA 18:2ω6 was used as marker of fungi (F), (Bååth and Anderson, 2003), 16:1ω5 for mycorrhizae (M), (Pa, 1999), and 10Me18:0 for actinomycetes (A), (Vestal and White, 1989).

2.4. Statistical analysis

The differences in PLFA amounts (as specific group markers), ergosterol concentrations and enzymatic activities among treatments and soil types were evaluated through two-way multivariate analysis of variance (MANOVA). In order to evaluate the multivariate separation of the groups defined by soil treatments and forest systems, and to understand which variables mostly contributed to possible differences, the non-metric multidimensional scaling (NMDS), with the superimposition of the confidence ellipses (for $\alpha = 0.05$) for treatments and forest systems, was applied to the parameters analyzed.

Data of enzyme activities, PLFA (as specific group markers) and ergosterol amounts were tested for normality and homoscedasticity through Shapiro and Bartlett tests, respectively. The significance of the differences of these variables among Phe, Py, Ba and US mesocosms were evaluated by two-way analyses of variance (ANOVAs), with treatments and forest systems as fixed factors. The ANOVAs were followed by Tukey HSD *post hoc* tests.

The structure of soil microbial community in each forest system was analyzed by means of three separated Q-type principal component analyses (PCAs), based on the 26 PLFAs studied, expressed as mol%, calculated as the ratio (in percent) of the specific PLFA chromatogram area on the total area of the peaks.

The statistical analyses were performed using the “stats”, “vegan”, “agricolae”, “plotrix” and “plyr” packages within the R 3.4.3 programming environment (R Development Core Team, 2017).

3. Results

The MANOVA showed significant differences among the treatments ($F = 7.8$, $P < 0.001$), the forest systems ($F = 270.8$, $P < 0.001$), and for forest system × treatment interactions ($F = 5.4$, $P < 0.001$). The NMDS (Fig. 1) clearly separated unspiked soils (US) from the other treatments (Py, Phe, Ba), that overlapped. In addition, the biological variables determined a clear separation of the three forest systems, regardless of the treatment.

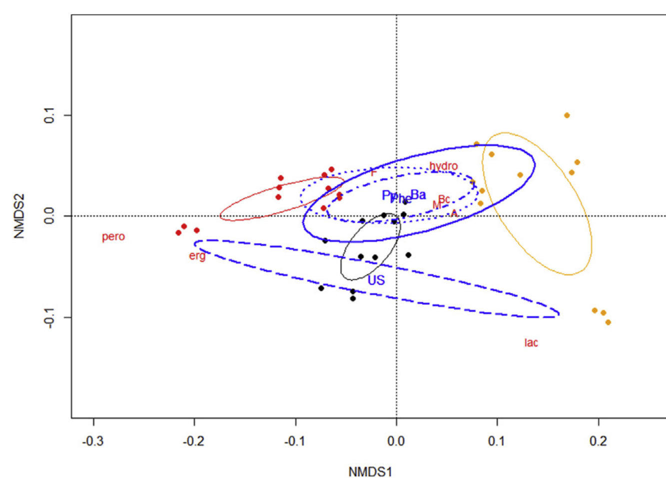

Fig. 1. Non-metric multidimensional scaling (NMDS) biplot, with superimposition of confidence ellipses (for $\alpha = 0.05$), based on biological parameters measured in soils of holm oak (black), black pine (red) and beech (orange) forests in unspiked (US, dashed), and in pyrene (Py, dotted), phenanthrene (Phe, dotdash) and benzo[a]pyrene (Ba, solid) treatments. The parameters included in the analysis are: hydrolytic activity (hydro), laccase activity (lac), peroxidase activity (pero), ergosterol (erg), fungi (F), bacteria (Bc), mycorrhizae (M) and actinomycetes (A). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.1. Enzyme activities

All the enzyme activities showed significant differences among the forest systems ($P < 0.001$) and the treatments ($P < 0.001$), as well as for forest system × treatment interactions (Table S2). In particular, FDAase (Fig. 2a), showed the highest values in US soil in P and B systems: significant differences were observed between US and spiked soils, both in P and B (except for Py treatment). In H system, FDAase (Fig. 2a) did not show differences between US and spiked soils. No significant differences among Phe, Py and Ba treatments were observed in any system (mean values of 0.64 mg FDA/g soil d.w. h for soil under holm oak, 1.2 mg FDA/g soil d.w. h under pine, 1.1 mg FDA/g soil d.w. h under beech). Laccase activity (Fig. 2b) showed significant differences between US and the spiked soils, with values higher in US than the spiked soils, both in B and H, whereas the activity was comparable among the treatments in P system, with a value around 0.65 μmol ABTS/g soil d.w. h. Peroxidase activity (Fig. 2c) showed values higher in US than spiked soils, both for H and P. On the contrary, in B system, the highest values were found in Py and Phe treatments.

3.2. Ergosterol and PLFAs

Ergosterol and PLFA abundances of specific microbial groups showed significant ($P < 0.001$) differences among US and Phe, Py and Ba treatments in the three forest soils, and for forest system × treatment interactions (Table S2). Ergosterol abundance (Fig. 2d) in B soil did not show significant differences among the treatments, in P soil it followed the order US > Py > Phe = Ba, in H soil only Py treatment showed a lower ergosterol abundance in respect to the US. Fungi abundance was higher (Fig. 2e) in all the spiked soils in respect to US both in B and P systems, while in H system fungal PLFA abundance in US was higher only in respect to Ba treatment.

Bacteria and mycorrhizae (Fig. 2f and g) showed similar PLFA abundances among treatments. Only in H soil, Ba treatment showed lower abundances of PLFA characterizing the two microbial groups, on average 35% less in respect to the unspiked soil. Actinomycetes abundance (Fig. 2h) increased in spiked Py, Phe and Ba, both in B and P soils. In H soil, only Py and Phe treatments showed actinomycetes 21% higher than US, whereas Ba was 17% lower in respect to US.

PCA analysis showed a separation between US and the other three treatments in all the forest soils (Fig. 3). The first two principal components accounted for more than 90% of the variation in the PLFA pattern. In all cases, PC1 differentiated US from spiked soils, whereas both PC1 and PC2 differentiated Ba from the other treatments (Fig. 3). In H soil (Fig. 3a, Table S3), PLFAs br17:0, 18:1ω7 (bacteria) and cy19:0 (a Gram- PLFA, Breulmann et al., 2014; Lange et al., 2014) were relatively more common in US treatment with values of 1.7, 14.9 and 7.5 mol%, respectively. PLFAs 18:1ω9 (bacteria) and i16:0 (Gram+ PLFA, Vestal and White, 1989), with values of about 9 and 5 mol%, respectively, mainly differentiated Py and Phe treatments, whereas a15:0 (Gram+ PLFA, Vestal and White, 1989) and 16:0 (bacteria), with about 5 and 17 mol%, respectively, mainly differentiated Ba treatment (Fig. 3a). In P soil (Fig. 3b, Table S3), PLFAs 16:1ω7c (a Gram- PLFA, Reinsch et al., 2014; Tavi et al., 2013) and 18:1ω7 (bacteria), with 8 and 16 mol%, respectively, were common in US soil; whereas a15:0 (a Gram+ PLFA, Vestal and White, 1989), with 6 mol%, in Ba treatment. PLFA 18:2ω6 (indicating fungi, Bååth and Anderson, 2003) was relatively more common in Py and Phe treatments, with a value of about 5 mol% (Fig. 3b, Table S3). In B soil (Fig. 3c, Table S3), PLFAs 18:1ω7 (bacteria) and cy19:0 (Gram- PLFA, Zelles et al., 1997), with values of 20 and 8 mol%, respectively, were common in US soil. 10Me:16a and

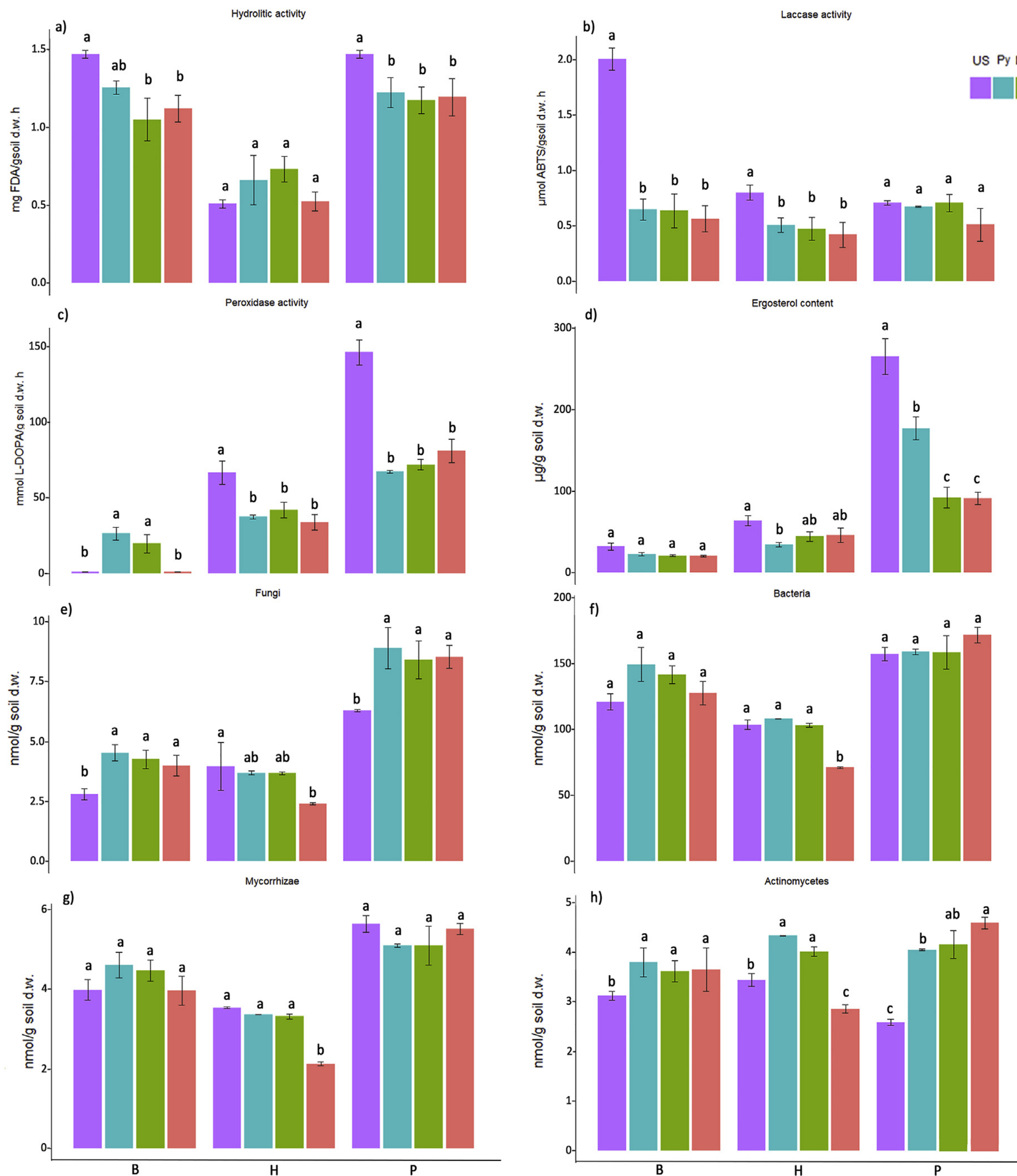

Fig. 2. Mean values ± s.e. of a) FDA hydrolase (FDAase), b) laccase, c) peroxidase activities, d) ergosterol abundance, e) fungi, f) bacteria, g) mycorrhizae, h) actinomycetes in beech (B), holm oak (H) and pine (P) forest soils. Different letters indicate significant differences ($\alpha = 0.05$) among unspiked (US), pyrene (Py), phenanthrene (Phe) and benzo[a]pyrene (Ba) treatments in each forest system.

18:0 (bacteria), with values of about 2.5 mol% for 10Me:16a and 3.6 mol% for 18:0, mainly differentiated US, Py and Phe treatments from Ba, whereas 14:0 (bacteria), i15:0, a15:0, i16:0 (Gram+ PLFAs, Vestal and White, 1989), 16:0 (bacteria) and 18:2ω6 (fungi) were common in Ba treatment (Fig. 3c, Table S3). PLFA 10Me18:0, indicating actinomycetes, was more common in the three soils spiked with Phe, Py and Ba (Fig. 3) in respect to US.

4. Discussion

The separation of the three forest soils, observed in the NMDS space based on their microbiological characteristics, highlighted that different tree canopies (beech, holm oak, black pine) influence the edaphic microbial communities, confirming what previously reported (Benizri and Amiaud, 2005; Iovieno et al., 2010; Grosso et al., 2018). This may be attributable to the effects of plant cover on soil physico-chemical properties, first of all the quality and quantity of organic matter (Table S1), that in turn affect microbial community characteristics. An effect of pedogenic substrate on soil microbial community is unlikely, since the investigated forests lay on the same calcareous substrate.

A clear separation between unspiked and spiked soils after 4

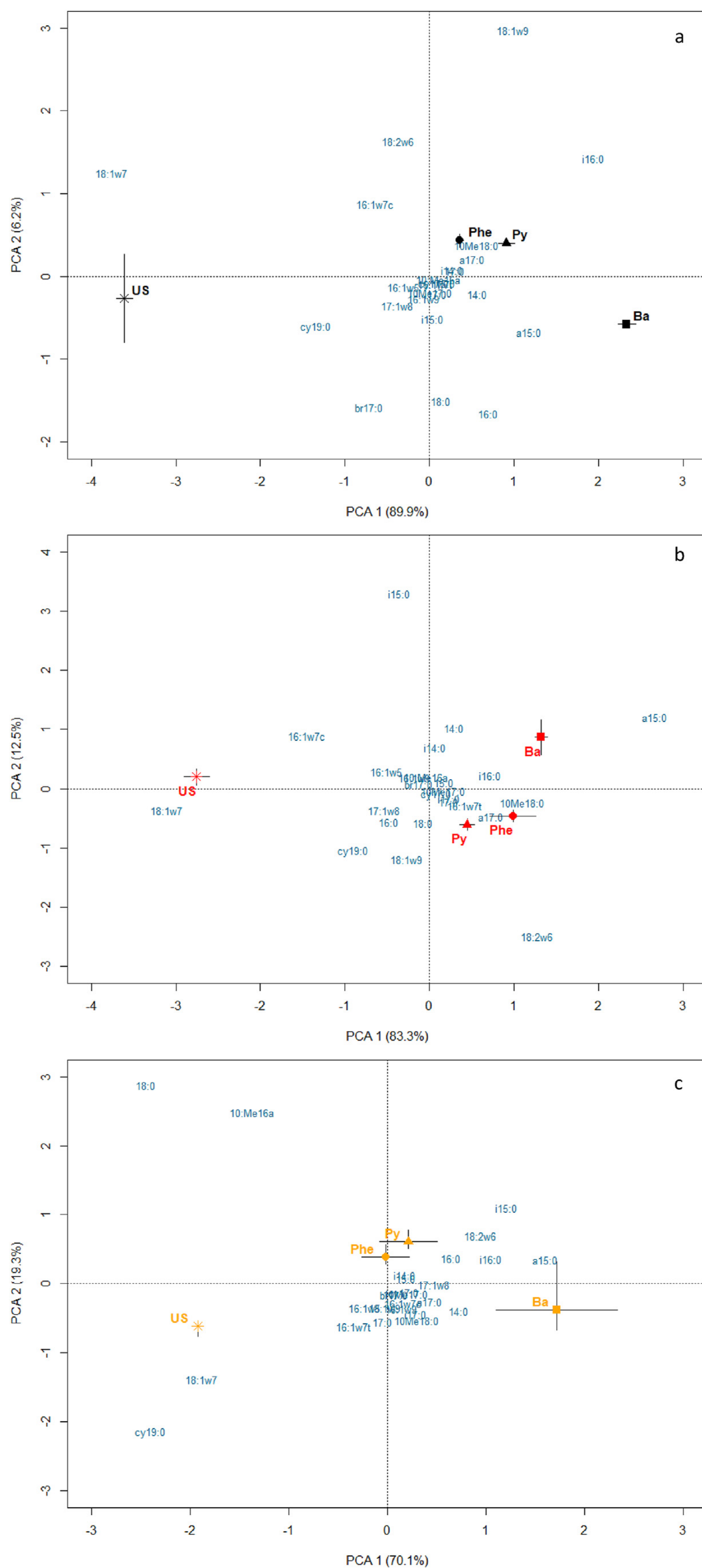

Fig. 3. Principal component analysis (PCA) of the relative abundance of phospholipids fatty acids (PLFAs) of soils under a) holm oak, b) black pine and c) beech. Unspiked (US, cross), pyrene (Py, triangle), phenanthrene (Phe, circle) and benzo[a]pyrene (Ba, square) treatments are indicated by mean ± s.e. Variance explained by principal component (PC) 1 and 2 are reported in parentheses. The 26 PLFAs detected are: i14:0, 14:0, i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ω7c, 16:1ω7t, 16:1ω5, 16:0, br17:0, 10:Me16a, i17:0, a17:0, 17:1ω8, cy17:0, 17:0, 10Me17:0, 18:2ω6, 18:1ω9, 18:1ω7, 18:0, 10Me18:0, cy19:0, 20:0.

days from pyrene, phenanthrene or benzo[a]pyrene addition was observed, shedding light on the response of different soil microbial communities to acute PAH contamination. The obtained results, in particular, suggest a short-term impact of PAH pollution on the edaphic community structure and on the activity of different microbial groups; differences were mainly observable in fungi and actinomycetes biomasses. The increase of actinomycetes biomass in all forest soils indicated a stimulation of this group after PAHs addition, already identified as phenanthrene degrader (Wawra et al., 2018). Accordingly, several studies showed a prevalence of Actinobacteriales species in hydrocarbon-contaminated soils, including some types of Actinomycetales (Kloos et al., 2006; Van Beilen and Funhoff, 2005). Moreover, it has been reported (Sawulski et al., 2014) that Actinobacteria dominate in fluoranthene spiked soils, after 2 days from contamination. Treatments with benzo[a]pyrene in soils under pine and beech, and with the three PAHs in soil under holm oak stimulated several Gram+ bacteria, ensuring soil resistance or resilience against hydrocarbon contamination (Bastida et al., 2010).

The involvement of fungi in the response to PAH contamination has been highlighted by the increase in both fungal biomass and enzyme activities. Fungi are well known PAH degraders (Bellino et al., 2019; Jove et al., 2016; Marco-Urrea et al., 2015; Mineki et al., 2015; Young et al., 2015). However, the contrasting pattern observed in fungal biomarkers employed (ergosterol and PLFA 18:2ω6), highlighted an involvement of different fungal species in relation to soil systems and PAH contamination. Although under certain environmental conditions fungi may not synthesize the PLFA 18:2ω6 (Klamer and Bååth, 1998), PLFA 18:1ω9, a fungal indicator (Frostegård et al., 2011), still indicates a shift of microbial community towards fungi in holm oak soils after phenanthrene and pyrene contamination.

Also the peroxidase activity response to the presence of phenanthrene and pyrene in soil under beech is likely related to the increase in biomass of fungi, that synthetize peroxidase enzymes (Kadri et al., 2017). Different effects of PAHs on enzymatic activities can be related to the different enzymes involved in PAH-oxidation. The investigated laccase and peroxidase activities are characteristic of ligninolytic fungi (Cerniglia and Sutherland, 2010; Hofrichter, 2002; Li et al., 2010), but biodegradation pathways of PAHs by non-ligninolytic fungi, that usually exhibited tolerance at high concentrations of pollutants, are also described (Marco-Urrea et al., 2015). The non-ligninolytic fungi synthetize Cytochrome P450 monooxygenase and lipases, enzymes not investigated in this study, able to degrade PAHs (Balaji et al., 2014; Bezalel et al., 1997). Laccase enzymes were resistant to PAH addition in soil under pine, whereas hydrolytic enzymes showed a resistance in soil under holm oak, being the activity nearly unchanged after 4 days from contamination. Even if the microbial degradation of PAHs depends on their molecular weight (Kanaly and Harayama, 2000), in our study acute PAH contamination affected the enzyme activity in the same way for the three PAHs, regardless of their molecular weight.

In the context of PAH degradation, it is worth mentioning that the degree to which PAHs are degraded or retained within the soil depends not only on biological activity, but also on the quantity and quality of soil organic matter, clay content, temperature, moisture, redox potential and nutrient availabilities (Duan et al., 2015; Wilson and Jones, 1993). The different soil properties of the three forest systems, mainly organic matter quantity (Table S1) and quality (unpublished data), may determine a different behavior in PAH resistance to degradation among them, since the sequestration of lipophilic contaminants by organic matter makes them less available for biodegradation (Cornelissen et al., 2005). According to the higher content and stability of organic matter in soils under pine and beech, PAHs should be more protected from microbial degradation in these systems and more susceptible to biodegradation in holm oak system.

5. Conclusions

Soil microbial community differently responds to acute PAH contamination in different forest systems. Not only the structure, but also the activity of soil microbial community changes after short-term PAH contamination. In soils under holm oak and beech, the microbial community composition shifts towards Gram+ bacteria after 4 days from contamination, whereas in soil under pine, pyrene and phenanthrene treatments mainly stimulate fungi and actinomycetes groups. In soil under beech, ligninolytic fungi, showing high peroxidase activity, are likely present. In addition, in soil under beech, benzo[a]pyrene treatment also stimulates fungi. Hydrolytic enzymes show a resistance in soil under holm oak, whereas laccase enzymes are resistant to PAH addition in soil under pine.

Since the use of indigenous microorganisms in bioremediation processes can reduce the risks associated with contaminated soils, a better understanding of the effects of hydrocarbon contaminants on soil microbial populations is critical for understanding microbial activity during bioremediation. Although the study focuses on soil responses to single PAHs, whereas PAHs are generally present as complex mixtures in soils, the findings may be of help in assessing the potential recovery of soils polluted by organic contaminants. Each mature soil is unique in terms of indigenous microorganisms and, for this reason, it is important to investigate the short-term effects of PAHs in different soil microbial communities, also to select suitable microbial groups to be employed in early soil restoration. Anyway, the study starts uncovering the importance of indigenous fungal populations in the treatment of soils recently contaminated by PAHs and suggests a high susceptibility of PAHs to biodegradation in holm oak forests, the climax community in the Mediterranean area.

Declaration of competing interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Enrica Picariello: Investigation, Writing - review & editing. **Daniela Baldantoni:** Conceptualization, Methodology, Resources, Writing - review & editing. **Flavia De Nicola:** Conceptualization, Methodology, Resources, Funding acquisition, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114378.

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