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Degradation of ^{13}C-labeled pyrene in soil-compost mixtures and fertilized soil

Iris K. U. Adam[1] · **Anja Miltner**[1] · **Matthias Kästner**[1]

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Abstract Polycyclic aromatic hydrocarbons (PAH) are toxic pollutants widely distributed in the environment due to natural and anthropogenic processes. In order to mitigate tar oil contaminations with PAH, research on improving bioremediation approaches, which are sometimes inefficient, is needed. However, the knowledge on the fate of PAH-derived carbon and the microbial degraders in particular in compost-supplemented soils is still limited. Here we show the PAH carbon turnover mass balance in microcosms with soil-compost mixtures or in farmyard fertilized soil using [$^{13}C_6$]-pyrene as a model PAH. Complete pyrene degradation of 100 mg/kg of soil was observed in all supplemented microcosms within 3 to 5 months, and the residual ^{13}C was mainly found as carbon converted to microbial biomass. Long-term fertilization of soil with farmyard manure resulted in pyrene removal efficiency similar to compost addition, although with a much longer lag phase, higher mineralization, and lower carbon incorporation into the biomass. Organic amendments either as long-term manure fertilization or as compost amendment thus play a key role in increasing the PAH-degrading potential of the soil microbial community. Phospholipid fatty acid stable isotope probing (PLFA-SIP) was used to trace the carbon within the microbial population and the amount of biomass formed from pyrene degradation. The results demonstrate that complex microbial degrader consortia rather than the expected single key players are responsible for PAH degradation in organic-amended soil.

Keywords Biodegradation · Pyrene · Stable isotope probing · PLFA · Carbon mass balance

Introduction

Polycyclic aromatic hydrocarbons (PAH) are hazardous contaminants mainly formed by incomplete combustion processes of organic material and are widely distributed in the environment (Srogi 2007). Consisting of two or more aromatic rings, PAH are hydrophobic and persistent in soils and sediments. They can be metabolized to reactive intermediates, which are cytotoxic, mutagenic, and carcinogenic (ATSDR 1995). Therefore, PAH are of environmental concern, and remediation of PAH- and tar oil-contaminated sites is of high relevance.

Although PAH are harmful to most organisms, many bacteria, cyanobacteria, algae, and fungi are capable of metabolizing these compounds (Bamforth and Singleton 2005; Cerniglia 1993; Kästner 2000; Vila et al. 2015). Furthermore, certain soil fauna is known to accumulate PAH or enrich PAH-degrading microorganisms (Contreras-Ramos et al. 2009; Haimi 2000). In order to exploit this natural potential, intensive research has focused on the bioremediation of PAH-contaminated sites as an efficient approach for decontamination (Megharaj et al. 2011; Romantschuk et al. 2000). One historically developed and established remediation method is the addition of compost to contaminated soil or sediment. This method has been found to be cost-efficient and highly effective (Loick et al. 2009; Semple et al. 2001). Compost provides a wide variety of microorganisms, nutrients, extra carbon

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✉ Matthias Kästner
matthias.kaestner@ufz.de

[1] Department of Environmental Biotechnology, Helmholtz-Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany

sources, enhanced aeration, as well as water and pH buffer capacities and thus is a valuable biostimulant (Kästner and Mahro 1996; Plaza et al. 2009). Although much research has been done to identify specific PAH-degrading bacteria and their metabolism, the details of the biostimulation mechanisms and processes in real habitats have not yet been identified (Vila et al. 2015). Therefore, improving techniques with compost application for most efficient PAH bioremediation is still a field of research, since the mechanisms underlying the observed effect of compost addition, e.g., the introduction of microorganisms, nutrients, primary substrates, or all of them, are not yet clear. Supplementing contaminated soils with various types of compost material or other organic supplements, e.g., yard waste compost (Carlstrom and Tuovinen 2003; Kästner and Mahro 1996; Kästner et al. 1995; Winquist et al. 2014; Wu et al. 2013), spent mushroom substrate (Cajthaml et al. 2002; Lau et al. 2003; Reid et al. 2002; Šašek et al. 2003), animal manure (Atagana 2004; Wong et al. 2002; Yuan et al. 2009), and sludges (Hamdi et al. 2006, 2007; Petersen et al. 2003), has been successfully applied in order to remove PAH; a detailed summary is given elsewhere (Loick et al. 2009). For optimal PAH removal, fully rotted compost has often been found to be much more effective than fresh compost (Haderlein et al. 2006; Plaza et al. 2009), and mature compost is considered to provide available nutrients with low sorption potential for high-molecular-weight PAH (Antizar-Ladislao et al. 2005; Hafidi et al. 2008).

In addition, the positive effect of organic matter input by compost addition to PAH-contaminated soil raises the question of how soil management can contribute to building up or maintaining the PAH degradation potential of a soil. For example, long-term fertilization of agricultural soil with farmyard manure (FYM) may also contribute to enhancing PAH degradation, as compost addition does. The regular treatment of agricultural soil with manure increases the soil organic matter content and alters soil microbial communities. Therefore, the influence of soil fertilization on the PAH removal potential may be significant, but knowledge in this field is lacking.

Investigation of PAH degradation processes in soil systems require sophisticated analytical techniques. The application of stable or radioisotopes has provided valuable process and source information in environmental systems (Gutierrez-Zamora and Manefield 2010). Research studies have been successfully conducted using ^{14}C-labeled PAH (Carlstrom and Tuovinen 2003; Cheng and Wong 2008; Haderlein et al. 2001, 2006; Johnsen et al. 2002; Kästner et al. 1995, 1999; Reid et al. 2002) for investigating the fate of the PAH-derived carbon in pure soil, soil-compost mixtures, or composting systems. These studies, however, mainly focused on PAH mineralization. Therefore, information on the fate and identity, i.e. the chemical composition, of residual PAH-derived labeled carbon in the solid matrix is still limited. On the other hand, studies using ^{13}C-labeled PAH were mainly focused on the analysis of PAH degraders (Bamforth and Singleton 2005; Johnsen et al. 2002; Peng et al. 2013; Vila et al. 2015; Zhang et al. 2011a). ^{13}C-analyses including turnover mass balances are scarce (Richnow 2000), and thus a comprehensive investigation to elucidate the fate of PAH-derived carbon in soil and soil-compost environments is missing.

Therefore, the present study aimed at (1) testing the effect of two different soil management strategies on PAH bioremediation: compost addition or long-term fertilization of soil with FYM, (2) balancing the carbon turnover from PAH e.g. conversion to CO_2 as well as extractable and non-extractable residue in the soil matrix, and (3) investigating the molecular structure of the remaining carbon. The carbon turnover was analyzed using ^{13}C-labeled pyrene as a high-molecular-weight model PAH in a microcosm approach and phospholipid fatty acid stable isotope probing (PLFA-SIP) to evaluate the pyrene-derived carbon within the microbial food web.

Materials and methods

Chemicals

Unlabeled pyrene (>96 % chemical purity) was purchased from Merck Schuchardt (Hohenbrunn, Germany), [$^{13}C_6$]-pyrene (>99 % chemical purity, 99.5 % ^{13}C isotopic enrichment) was custom-synthesized by Alsachim (Illkirch, France), and anthracene (99 % chemical purity) was obtained from Sigma-Aldrich (Steinheim, Germany). All solvents used for extractions were HPLC grade and purchased from Carl Roth GmbH (Karlsruhe, Germany). Stock solutions of unlabeled and [$^{13}C_6$]-pyrene (4 g l^{-1}) were prepared in ethyl acetate.

Soil and compost

The experiments were performed with agricultural soil (Ap horizon of a Haplic Chernozem) of the Static Fertilization Experiment Bad Lauchstädt, Germany (Altermann et al. 2005). For investigating the influence of the soil management on the PAH degradation, unfertilized soil and soil fertilized with 30 tons FYM per hectare every second year (long-term fertilization) were used. Commercially available, fully rotted mature compost (Bio-Komp SAS GmbH, Weißenfels, Germany), made from a mixture of vegetable and animal organic waste derived from private households and green waste, was applied as soil supplement. Pyrene background concentration was <0.1 mg kg^{-1} in all materials. A detailed characterization of the soil mixtures is given in Table 1.

Microcosm setup and conditions

The soil and compost material was sieved to a particle size of ≤4 mm. For the experiments with compost

Table 1 Characterization profile of the soil and compost used in the experiments

	Unfertilized soil	FYM-fertilized soil	Compost
pH ($CaCl_2$)	5.6	6.2	7.8
Water content (%)	11.6	12.3	46.5
Maximum water-holding capacity (%)	36.1	39.3	56.8
Total N content (mg g^{-1} dw)	n.d.	n.d.	14
organic Matter (mg g^{-1} dw)	n.d.	n.d.	365
Organic carbon content (mg g^{-1} dw)	16	22	n.d.
Salt (g kg^{-1} fw)	n.d.	n.d.	5.8
Pyrene concentration (mg kg^{-1})	<0.1	<0.1	<0.1
PLFA (μg PLFA g^{-1} dw)	5.0 ± 0.3	9.9 ± 0.1	82.4 ± 1.4

n.d. no data

addition (unfertilized and fertilized soil), the soil and the compost were mixed at a ratio of 3:1 (fw/fw; representing ≈ 4:1 dw/dw). The water content of the soils or soil-compost mixtures was then adjusted to 60 % of their maximum water-holding capacity (Kästner and Mahro 1996). Except for the control, the soil materials were spiked with [$^{13}C_6$]-pyrene or, for the analysis of the ^{13}C background, with unlabeled pyrene, to a final concentration of about 100 mg kg^{-1}. The spiking was conducted following a procedure described previously (Kästner and Mahro 1996). Briefly, for optimal homogenization without affecting microbial activity, the spiking solution in ethyl acetate was added to about one third of the material before thoroughly mixing this part with the residual material. The spiking solution was added drop-wise to the soil or soil-compost material via a glass syringe while continuously mixing the material with a commercial stand mixer. The procedure was conducted until the solvent was completely evaporated. Finally, 250-g (fresh weight) samples of the soils or soil-compost mixtures were transferred to 1 l glass bottles. For analyzing the mineralization background of soil and compost organic matter, similar microcosms were set up without pyrene addition. The microcosms were sealed with Teflon-coated screw caps and were incubated at 20 °C; soil samples were taken after 0, 35, 48, 64, 80, 96, and 160 days from the unfertilized soil-compost microcosms; after 0, 16, 31, 46, 71, 95, and 161 days from the unfertilized soil and fertilized soil-compost microcosms; and after 0, 16, 31, 46, 71, 95, 161, and 203 days from the fertilized soil microcosms. For mineralization analysis, a test tube containing 10 ml of 2 M NaOH was placed in each microcosm for trapping the CO_2 released into the microcosm atmosphere. In order to aerate the microcosms and to prevent CO_2 saturation, the NaOH was replaced once a week in the beginning of the experiments and every 2 weeks later. The soil, soil-compost, and NaOH samples were stored at −20 °C until further analysis.

CO_2 recovery and analysis

For determining the mineralization, 1 ml of each NaOH sample was measured using the Total Organic Carbon Analyzer TOC-5000 with the ASI 5000 auto sampler (Shimadzu, Duisburg, Germany). For analysis of the ^{13}C-pyrene-derived carbon mineralization ($^{13}CO_2$), 2 ml of the NaOH was transferred to 15-ml crimp-capped glass vials. The vials were sealed and acidified with 400 μl of phosphoric acid (85 %). After gas production seized, headspace samples were taken and the ratio of ^{13}C to ^{12}C was analyzed using a gas chromatograph (7890A, Agilent Technologies, Waldbronn, Germany) equipped with a CP-PoraBOND Q column (50 m × 0.32 mm id, 5-μm film, Agilent Technologies) coupled to an isotope ratio mass spectrometer (MAT 253, Thermo Fisher Scientific, Bremen, Germany) with a ConFlo IV interface (Thermo Fisher Scientific). Samples were analyzed isothermally at 40 °C. In order to maintain optimal analytical conditions, the column was heated to 120 °C for 10 min after 120 min of analyses. The injector was set to 250 °C. The isotopic composition of [$^{13}C_6$]-pyrene-treated microcosms was corrected against the natural ^{13}C abundance based on microcosms amended with unlabeled pyrene. Mineralized ^{13}C was calculated from the inorganic carbon content in NaOH and the isotopic composition of the headspace samples in relation to initial ^{13}C introduced by [$^{13}C_6$]-pyrene.

Analysis of residual ^{13}C in soil and soil-compost mixtures

Soil and soil-compost samples were dried at 105 °C overnight and ground to fine powder. The isotopic composition was measured using an elemental analyzer (EuroVector, Milan, Italy) coupled to an isotope ratio mass spectrometer (MAT 253, Thermo Fisher Scientific) with a ConFlo IV interface (Thermo Fisher Scientific). The combustion unit was operated at 1020 °C, the reduction unit at 650 °C.

Pyrene extraction and analysis

Pyrene was extracted from the soil and soil-compost samples by a two-step procedure (Eschenbach et al. 1994). In the first step, samples were extracted with ethyl acetate and ultrasonication treatment, and in the second step, with methanolic KOH. After the methanolic hydrolysis, 1 ml distilled water was added to the supernatant followed by three extraction steps, each with 1 ml of hexane, prior to the gas chromatographic analysis, in order to prevent damage of the GC column due to oxidation. Anthracene was chosen as internal standard to check the extraction efficiency by anthracene recovery, which was in the range of 84 to 98 %. [$^{13}C_6$]-pyrene and unlabeled pyrene were quantified with the aid of a gas chromatograph (7890A, Agilent Technologies) equipped with a BPX5 column (30 m × 0.25 mm id, 0.25 μm film, SGE Analytical Science, Melbourne, Australia) coupled to a quadrupole mass spectrometer (5975C, Agilent Technologies). The GC oven temperature was programmed as follows: initial 40 °C, hold for 2 min, then heat to 180 °C at 40 °C min^{-1}, 2-min hold, heat to 240 °C at 5 °C min^{-1}, 2-min hold and heat to a final temperature of 300 °C at 15 °C min^{-1}, 5-min hold, with a helium flow of 1.5 ml min^{-1} (based on David and Klee 2009). The injector was set to 280 °C. The MS was operated in the electron impact ionization mode at 70 eV. The pyrene concentration was determined by means of the internal standard method.

PLFA extraction and analysis

The PLFA were extracted from the soil and soil-compost samples according to the method described previously (Bligh and Dyer 1959) modified by Miltner et al. (2004). Briefly, lipids were extracted with phosphate buffer, methanol, and chloroform from the soil and soil-compost samples. The chloroform phase was applied to columns of silica gel for separation of the lipid classes (neutral lipids, glycolipids, and phospholipids) by solvent washing. The phospholipid fraction was collected and methylated with trimethylsilyl chloride in methanol. Heneicosanoate methyl ester (21:0) was added to each sample as an internal standard for quantification. The extracts of methylated PLFA were analyzed on a gas chromatograph coupled to a mass spectrometer as described above. The GC oven temperature was programmed as follows: initial 50 °C, hold for 1 min, then heat to 250 °C at 4 °C min^{-1}, 0-min hold, and heat to a final temperature of 300 °C at 20 °C min^{-1}, 5-min hold, with a helium flow of 1.2 ml min^{-1}. The injector was set to 280 °C. The isotopic composition was determined with the aid of a gas chromatograph (TRACE 1310, Thermo Fisher Scientific) equipped with a BPX5 column (50 m × 0.32 mm id, 0.5-μm film, SGE Analytical Science) coupled to an isotope ratio mass spectrometer (MAT 253, Thermo Fisher Scientific) with a ConFlo IV interface (Thermo Fisher Scientific). The GC oven temperature was programmed as follows: initial 70 °C, hold for 1 min, then heat to 130 °C at 20 °C min^{-1}, 0-min hold, heat to 150 °C at 2 °C min^{-1}, 5-min hold, heat to 165 °C at 2 °C min^{-1}, 5-min hold, heat to 230 °C at 2 °C min^{-1}, 0-min hold, and heat to a final temperature of 300 °C at 20 °C min^{-1}, 5-min hold, with a helium flow of 2 ml min^{-1}. The injector was set to 250 °C. All PLFA peaks were identified on the basis of retention time compared to a standard mixture of bacterial acid methyl esters (47080-U, Sigma-Aldrich) and by inspection of the mass spectra. The PLFA nomenclature is based upon counting the carbon atoms from the carboxyl end of the fatty acid chain (IUPAC rule for numbering fatty acids).

PLFA were used for taxonomic investigations because some PLFA groups are regarded characteristic for certain groups of microorganisms. PLFA with a chain length of ≤20 carbon atoms are typically found in prokaryotes and microbial eukaryotes. Saturated straight-chain PLFA are unspecific and are found in both prokaryotes and eukaryotes (Zelles 1999). They are therefore inappropriate for taxonomic assignments. The iso and anteiso isomers of PLFA are considered as indicators for Gram-positive (Gram+) bacteria in general, and methyl branching at the 10th carbon atom as indicators for actinomycetes (Amelung et al. 2008; Zelles 1999) in particular. Monounsaturated PLFA and cyclopropyl PLFA are considered as markers for Gram-negative (Gram−) bacteria (Zelles 1999). 18:1(9)cis and 18:2(9,12) are indicators for fungi (Zelles 1999). As there is some controversy about these indicator assignments, PLFA should be interpreted with caution in terms of microbial analysis (Frostegård et al. 2011). For example, 18:1(9)cis and 18:2(9,12) are good fungal biomarkers, but only if both PLFA co-occur (Frostegård et al. 2011). Furthermore, in particular, the PLFA 18:1(9)cis and 18:1(11)cis are not exclusively found in Gram− bacteria but also in Gram+ bacteria (Schoug et al. 2008), particularly in mycobacteria (Wick et al. 2003) which are well known for PAH degradation. Therefore, we will not attribute the PLFA 18:1(9)cis and 18:1(11)cis to a certain group of microorganisms except where the PLFA 18:1(9)cis and 18:2(9,12) co-occur; they are then considered indicators for fungi. Absolute abundances of PLFA and distribution of ^{13}C-enriched PLFA given as means are compiled in Supplemental Table S1a-d.

Statistics

All microcosm experiments were performed in replicates as indicated. Changes in the PLFA concentrations with time and their ^{13}C enrichment based on the PLFA ^{13}C amounts were tested for significance by performing repeated measurement ANOVA with post hoc Bonferroni paired *t* tests for pairwise multiple comparison analyses. The detailed results of the PLFA test statistics are compiled in Supplemental Tables S2a-d (PLFA concentrations) and Supplemental

Tables S3a-c (^{13}C enrichment). All tests were computed using SigmaPlot 12.5 (Systat Software, Erkrath, Germany) test statistics, based on a 0.05 significance level. For better visualization of the relationship of ^{13}C-enriched PLFA and the three different types of organic-amended soils, multidimensional scaling was performed using a principal coordinates analysis (PCoA) based on a Bray-Curtis distance matrix of ^{13}C PLFA data at the incubation time when most intense pyrene degradation started. The results are presented in an ordination biplot. PCoA was computed using the "vegan package" (Oksanen et al. 2013) of the "R Project" for Statistical Computing (R Core Team 2013).

Results

Carbon mass balances

The mass balances of the unfertilized soil-compost, fertilized soil-compost, and fertilized soil microcosms were compared in order to investigate the fate of pyrene-derived carbon. For control, we also analyzed the pyrene degradation in unfertilized soil without amendment. The concentration of pyrene decreased from the initial 119.2 ± 13.7 to 49.1 ± 11.9 mg kg^{-1} dw after 161 days of incubation in the microcosms with unfertilized soil (Fig. 1a), indicating that only around half of the initially added pyrene was degraded in the unfertilized soil during the 203-day incubation. Therefore, no additional fate analysis was performed in the unfertilized soil.

The effect of long-term fertilization of soil with FYM on pyrene degradation was analyzed by the carbon mass balance of the fertilized soil (Fig. 1b). Pyrene degradation showed a logistic progression and only 4.5 ± 0.8 mg kg^{-1} dw (4 ± 1 %) of the initial 107.5 ± 5.3 mg kg^{-1} dw pyrene was left after 203 days. Pyrene degradation started only after a 31-day lag phase and was most intense between days 46 and 71. The $^{13}CO_2$ production from the [$^{13}C_6$]-pyrene amendment increased over time to an overall 57 ± 1 % of the initially added label at the end of the experiment; 42 ± 2 % residual label remained in the soil at the end of the experiment, resulting in a 99 ± 4 % overall label recovery. Based on the PLFA concentrations and isotopic enrichments (Supplemental Table S1b) and assuming a PLFA content in bacterial biomass of 100 μmol PLFA per gram dry biomass (Green and Scow 2000), we estimated that 6 ± 1 % of the initially added pyrene-derived ^{13}C was found to be incorporated into biomass after 203 days. However, the highest incorporation of the label into microbial biomass was found earlier, on day 161 (10 ± 0 %), indicating a decay of the pyrene-degrading biomass.

In contrast to the findings for the fertilized soil, the pyrene degradation in the unfertilized soil-compost mixture described first-order kinetics without a pronounced lag phase, rather than a logistic progression. The pyrene concentration decreased from the initial 130.5 ± 2.5 to 0.1 ± 0.1 mg kg^{-1} dw (0.1 ± 0.1 %) after 160 days (Fig. 1c). The most intense degradation was already detected between day 35 and 48 of the incubation with only 47 ± 0.3 % of the ^{13}C label being mineralized after 160 days; 36 ± 1 % remained as residual ^{13}C in the soil-compost mixture with a maximum incorporation into the biomass of about 14 ± 1 % on day 48 and a final value of 8 ± 1 % on day 160. The overall label recovery in the unfertilized soil-compost microcosms was 83 ± 2 %.

Similar to the unfertilized soil-compost mixture, the pyrene degradation in the fertilized soil-compost mixture started without any pronounced lag phase (Fig. 1d). The pyrene concentration decreased rapidly from the initial 115.1 ± 5.8 to 1.3 ± 0.1 mg kg^{-1} dw (1 ± 0.1 %) after 161 days. The most intense pyrene degradation was found between 31 and 46 days. After 161 days, 49 ± 1 % of the pyrene-derived ^{13}C label was mineralized and 39 ± 6 % residual label was detected in the soil-compost mixture, resulting in an overall label recovery of 88 ± 7 %. Finally, 9 ± 1 % of the label was incorporated into biomass with a transient maximum (14 ± 0 %) on day 71. The compost-supplemented soils showed lower mineralization (47–49 % compared to 57 %) and higher maximum incorporation of the label into the biomass (14 % compared to 10 %) than the FYM-supplemented soil.

PLFA-SIP

In order to describe the size and activity of the microbial community within the fertilized soil and soil-compost mixtures, we investigated the PLFA patterns as a proxy for the living biomass. An overview on the absolute PLFA abundances during incubation is shown in Fig. 2a–d. For the sake of clarity, PLFA concentrations are shown only for four time points (start of the experiment, start and end of the period of most intense pyrene degradation, and end of the experiment; see Supplemental Material). The addition of compost to soil strongly increased the PLFA concentrations, as shown for the unfertilized soil-compost mixture (Fig. 2a) and the fertilized soil-compost mixture (Fig. 2b) in comparison to the lower PLFA concentrations in the fertilized soil (Fig. 2c); the lowest concentrations were found in the unfertilized soil (Fig. 2d). Prior to the experiments, the compost contained 82.4 ± 1.4 μg PLFA g^{-1} dw, the fertilized soil 9.9 ± 0.1 μg PLFA g^{-1} dw, and the unfertilized soil 5.0 ± 0.3 μg PLFA g^{-1} dw (Table 1), indicating that long-term fertilization doubled the amount of microbial biomass compared to unfertilized soil, whereas the biomass in the compost was one order of magnitude higher than in the soils.

In the unfertilized soil-compost mixture microcosms, a significant decrease of certain PLFA markers for Gram+ bacteria

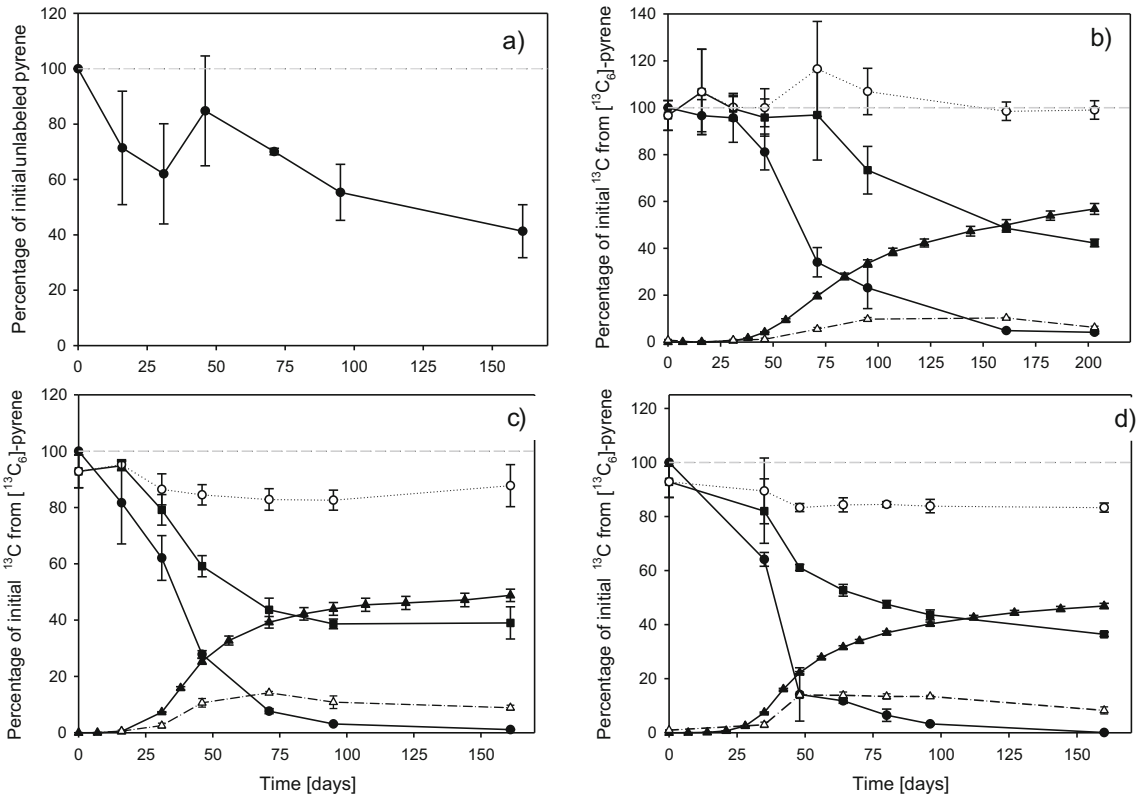

Fig. 1 **a**) Pyrene removal in pure unfertilized soil and percentage of the initially added unlabeled pyrene over time; **b**) carbon balance of ^{13}C-labeled pyrene in pure fertilized soil; **c**) carbon balance of ^{13}C-labeled pyrene in unfertilized soil-compost mixtures; and **d**) carbon balance of ^{13}C-labeled pyrene in fertilized soil-compost mixtures. Percentage of the initial ^{13}C label derived from the [$^{13}C_6$]-pyrene in soil or soil-compost microcosms over time: [$^{13}C_6$]-pyrene (*black circles*), $^{13}CO_2$ (*black triangles*), residual ^{13}C (*black squares*), and total label recovery (*open circles*) and estimated ^{13}C conversion to biomass based on PLFA data (*open triangles*). The calculations were based on triplicate microcosms; the *error bars* indicate standard deviations

(i-16:0, a-17:0), actinomycetes (10Me17:0), Gram− bacteria (17:1cis), and of 15:0 was observed after the start of the incubation (Fig. 2a). On the other hand, the concentrations of the PLFA 10Me16:0 and 10Me18:0 (actinomycetes), 16:1(11)cis (Gram− bacteria), and of 18:1(9)cis and 20:0 did not change initially but increased significantly between day 35 and day 48. However, a significant increase of the Gram− biomarker 16:1(y) from day 0 to day 35 was detected.

Certain biomass indicators decreased even more distinctly after incubation started in the fertilized soil-compost mixture microcosms (Fig. 2b) than in unfertilized soil-compost mixture. Markers for Gram+ bacteria (i-16:0, i-17:0, a-17:0); actinomycetes (10Me17:0); Gram− bacteria (16:1(9)cis, 17:1cis); and 15:0, 17:0, 18:2(9,12), 18:1(9)cis, 18:0, and 20:0 decreased significantly from day 0 to day 31. Among these PLFA, only 16:1(9)cis (indicator for Gram− bacteria), i-17:0 (Gram + bacteria), and 10Me17:0 (actinomycetes) recovered and reached the starting concentrations by day 161. In contrast, other markers for Gram− bacteria (16:1(x), 16:1(y)) and actinomycetes (10Me18:0) already increased significantly between day 31 and day 46. The concentrations of 16:1(11)cis, 17:0cyclo, and 19:0cyclo (Gram− bacteria); i-15:0 (Gram+ bacteria); and 10Me16:0 (actinomycetes) and 18:1(11)cis increased significantly between day 46 and day 161.

All PLFA detected in the fertilized soil microcosms decreased significantly from the beginning of incubation (Fig. 2c) with the exception of some saturated PLFA. Only i-15:0 and a-15:0 (Gram+ bacteria) and 16:1(y) (Gram− bacteria) recovered by day 71, and 16:0 and 10Me18:0 (actinomycetes) by day 203. In contrast to the other microcosms, very few significant variations in the PLFA concentrations over time were detected in the unfertilized soil microcosms (Fig. 2d). Only the saturated PLFA 20:0 increased significantly from day 31 to day 46, and 10Me18:0 (marker for actinomycetes) from day 31 to day 161.

For identifying the active pyrene-degrader organisms, the pyrene-derived label distribution in the detected PLFA was calculated for the treatments with [$^{13}C_6$]-pyrene amendment. For better comparison, the label incorporation is standardized by setting the sum of the quantified ^{13}C in all detected PLFA of each microcosm and sampling time to 100 % (Fig. 2e–g). At the end of the experiments, the incorporation of pyrene-derived ^{13}C into PLFA was detected for almost all PLFA and

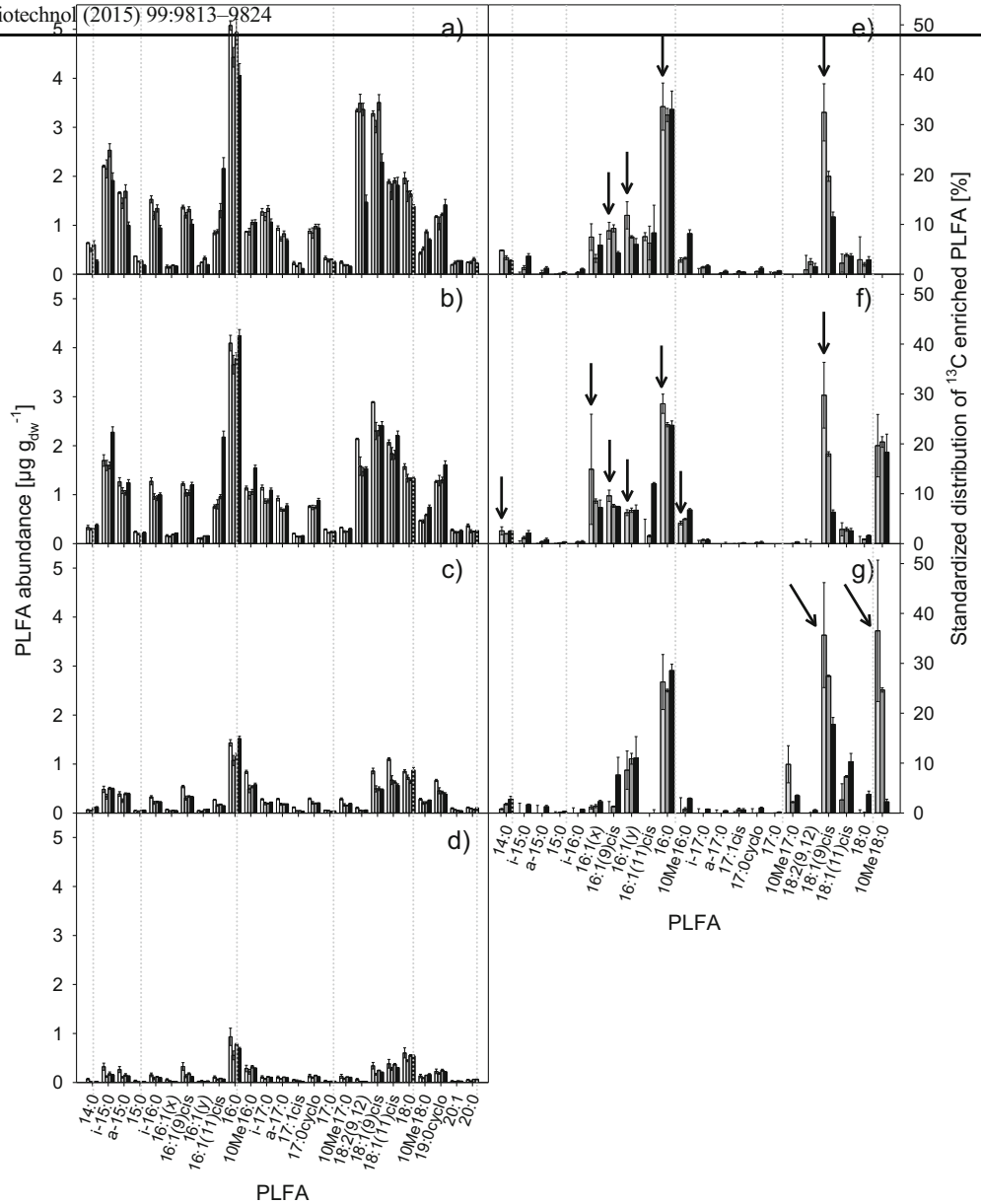

Fig. 2 Absolute abundances of PLFA (**a**, **b**, **c**, **d**) with **a** unfertilized soil-compost mixture after 0 (*white*), 35 (*light grey*), 48 (*dark grey*), and 160 days (*black*) of incubation; **b** fertilized soil-compost mixture after 0, 31, 46, and 161 days; **c** fertilized soil after 0, 46, 71, and 203 days; and **d** unfertilized soil after 0, 31, 46, and 161 days, all given as µg g_{dw}^{-1}. Standardized distribution of ^{13}C-enriched PLFA (**e**, **f**, **g**) with **e** unfertilized soil-compost mixture after 35 (*light grey*), 48 (*dark grey*) and 160 days (*black*); (**f**) fertilized soil-compost mixture after 31, 46, and 161 days; and **g** fertilized soil after 46, 71, and 203 days, all given in percent *Arrows* indicate statistically significant enriched PLFA when most intense pyrene degradation occurred. *Dashed lines* indicate the position of the saturated straight-chain fatty acids 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0 (panels **a**–**d** only) for easier orientation in the graphs. All calculations were based on triplicate microcosms; the *error bars* indicate standard deviations. (Due to the high background noise, the ^{13}C incorporation into the PLFA 19:0cyclo, 20:1, and 20:0 could not be calculated. Since no labeling experiment with solely unfertilized soil was conducted, Fig. 2d shows the data of the microcosms incubated with unlabeled pyrene; all other panels show data of incubations with [$^{13}C_6$]-pyrene.)

in all microcosms indicating a complete spread of the carbon within the microbial food web. Multidimensional scaling using a PCoA for the times when most intense pyrene degradation in unfertilized soil-compost mixture (day 35), fertilized soil-compost mixture (day 31), and fertilized soil (day 46) showed distinct changes in specific PLFA according to the treatments (Fig. 3), which are also indicated by the arrows in Fig. 2e–g.

In the unfertilized soil-compost microcosms, a significant ^{13}C enrichment was detected in the PLFA markers for Gram− bacteria (16:1(9)cis, 16:1(y)) and for 16:0 and 18:1(9)cis by day 35 (Fig. 2e). Among these PLFA, a concomitant increase in absolute abundance between day 0 and day 35 was only observed for 16:1(y), and it should be noted that 16:1(y) was one of the less abundant PLFA. By day 48, nearly all markers PLFA for Gram− bacteria (16:1(x), 16:1(11)cis, 17:1cis);

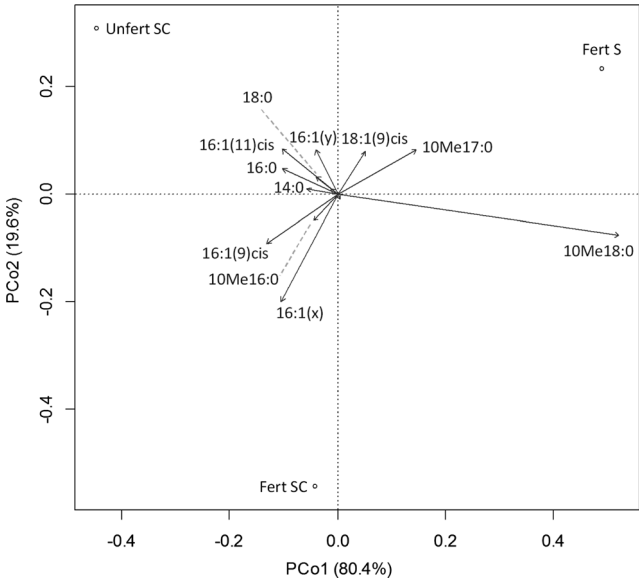

Fig. 3 PCoA of ^{13}C-enriched PLFA when most intense pyrene degradation in unfertilized soil-compost mixture (*Unfert SC*, day 35), fertilized soil-compost mixture (*Fert SC*, day 31), and fertilized soil (*Fert S*, day 46) occurred. Microcosm triplicates are presented as means. PCoA is based on a Bray-Curtis distance matrix. PLFA clustering too close at the origin of coordinates are not designated by their names; for lack of space, the PLFA 18:0 and 10Me16:0 are designated by means of *dashed lines*

Gram+ bacteria (i-17:0); actinomycetes (10Me16:0); and the PLFA 14:0, 17:0, 18:1(11)cis, and 18:0 were enriched in ^{13}C.

In the fertilized soil-compost microcosms, PLFA markers for Gram− bacteria (16:1(x), 16:1(9)cis, 16:1(y)); actinomycetes (10Me16:0); and 14:0, 16:0, and 18:1(9)cis were significantly enriched by day 31 (Fig. 2f). This is partly in accordance with the increase in concentrations of 16:1(x) and 16:1(y) from day 31 to day 46. Indicator PLFA for Gram+ bacteria (i15:0, i17:0), actinomycetes (10Me18:0), and 18:1(11)cis and 18:0 were significantly enriched by day 46.

In the fertilized soil microcosms, only the non-specific PLFA 18:1(9)cis and 10Me18:0 were significantly enriched by day 46 (Fig. 2g). 18:1(9)cis may be indicative for mycobacteria (Wick et al. 2003). These PLFA had only low abundances in these samples, with almost no change in concentration time. No significant ^{13}C enrichment before day 71 could be detected in the PLFA markers for Gram− bacteria (16:1(x), 16:1(y)) and in the PLFA 14:0, 16:0, and 18:1(11)cis.

Discussion

In the present study, the experiments were set up for combining the information on the fate of the PAH-derived carbon (carbon turnover mass balance) with biomarker analysis for microbial food web investigation. This approach was used for evaluating the effect of compost amendment or soil fertilization with FYM on the PAH degradation, and thus, we did not enable aging of the pyrene or introduction of the compound within a tar oil matrix. The compost addition to soil facilitated complete pyrene degradation, and the results confirm the previous observation that compost addition enhances complete microbial degradation of PAH (Kästner and Mahro 1996; Loick et al. 2009). Also, long-term organic fertilization resulted in almost complete pyrene degradation. In contrast, only about half of the pyrene was metabolized in unfertilized soil without compost amendment. The agricultural unfertilized soil used in the present study did not originate from a contaminated site, and thus, the pyrene background concentration in the soils used was very low (<0.1 mg kg^{-1}). However, the ambient PAH input may be sufficient to maintain the low pyrene degradation potential found in the unfertilized soil microcosms.

Interestingly, the soil management by long-term fertilization with FYM resulted in a pyrene degradation capacity similar to that found for soil with compost amendment. Long-term organic fertilization contributes to the maintenance of microbial biomass and activity in soil and apparently provides the conditions for sustaining microbial consortia able to degrade organic contaminants like PAH. However, one striking difference was observed between the microcosms with compost treatment and the FYM-fertilized soil. Pyrene degradation started almost immediately in the soil-compost mixtures and followed first-order-like kinetics, whereas in the fertilized microcosms the degradation required a 31-day lag phase resulting in a logistic degradation curve. This kind of kinetics is typically found for low initial degrader populations characterized by an initial phase of acclimation and exponential growth (Ottow and Bidlingmaier 1997). In addition, the presence of the long lag phase was accompanied by higher mineralization and lower incorporation of the label into the biomass. In contrast, the active pyrene-degrader populations in the soil-compost mixtures were sufficiently large and active compared to the relatively low initial substrate concentrations to allow for first-order-like degradation kinetics. During composting, the microorganisms are permanently in contact with a high amount of aromatic compounds, e.g., lignin-containing plant material. Apparently, this leads to a highly active compost microflora in comparison to the unfertilized agricultural soil. Except for the control, the initial concentration of pyrene finally decreased to values below the detection limit in all microcosms, although more than 35 % of the label remained in the soil materials. In contrast to the present results, a previous study found a high mineralization of ^{14}C-pyrene of about 57 % in a spiked contaminated soil-compost mixture but did not analyze the carbon flux (Haderlein et al. 2001).

In terms of risk assessment, the chemical nature of the residual label in the soil and soil-compost mixtures needs to be investigated. Therefore, we evaluated the sorption and

sequestration of pyrene or primary metabolites in comparison to the conversion of ^{13}C to non-toxic biomass components by a two-step pyrene and metabolite extraction including methanolic hydrolysis. The additional alkaline hydrolysis was used to cleave ester bonds of the soil organic matrix and to release sorbed and sequestered compounds (Eschenbach et al. 1994). Most metabolites should have been recovered with the two-step extraction (Eschenbach et al. 1994), but we did not find any pyrene-derived primary metabolite and, thus, we consider that metabolites accounted for only <1 ‰ of the initial pyrene concentration. Therefore, the decrease of the pyrene concentration to values below the detection limit is an indicator for real pyrene degradation and not for sequestration and decreased bioavailability as found in other previous studies (Berns et al. 2005; Ghanem et al. 2013). This result is in accordance with findings that compost humic acids were suggested to improve bioremediation efforts based on their solubilization properties (Haderlein et al. 2001; Montoneri et al. 2009; Smith et al. 2009). Other studies (Kästner and Mahro 1996) did not find any indication for reduced bioavailability of PAH due to sorption to the compost matrix resulting in decreased biodegradation. Some studies could even show degradation of PAH sorbed to the solid compost matrix (Marchal et al. 2013). According to the literature about the compost process, PAH sorption with limited degradation was mostly detected during the stabilization phase of composting (Amir et al. 2005; Wu et al. 2013), whereas the highest pyrene degradation was detected with mature compost (Haderlein et al. 2006; Kästner and Mahro 1996; Marchal et al. 2013) as used in the present study. The difference is presumably caused by changes in compost composition during maturation.

The productive pyrene degradation in the present study was also evidenced by carbon conversion to PLFA, indicating the utilization of pyrene-derived carbon for microbial growth. The estimated label incorporation into the biomass reached a maximum of 10–14 % during a period from 50 to 100 days after onset of pyrene degradation. Finally, the values decreased until the end of the experiment to 6–9 %. The intermediate maximum and subsequent decrease of the PLFA concentrations over time reflect the transient nature of microbial PLFA. This indicates a fast turnover of these biomolecules, similar to what was found in experiments on the microbial turnover of 2, 4-dichlorophenoxyacetic acid (Nowak et al. 2011) and ibuprofen (Nowak et al. 2013). In these studies, residual ^{13}C in the soil was found to be mainly of biogenic origin. However, transfer of the label turnover into proteins (analyzed as amino acids after acid hydrolysis) was two to three times higher than into PLFA. PLFA are major components of the microbial cell wall and undergo fast turnover in soil after cell death (Amelung et al. 2008). Accordingly, the extrapolation of biomass from PLFA underestimates the actual pyrene-derived carbon incorporation into the microbial biomass, and amino acids may thus be the more reliable biomarker for quantification of microbial residues. Considering the biomass conversion factor found for label incorporation into amino acids, presumably all residues from pyrene degradation in the present fertilized soil or soil-compost mixtures can be considered to be of biogenic origin without any toxic potential.

The transient nature of PLFA is an advantage for the characterization of the microbial communities responsible for the pyrene degradation and food web analysis. As shown by the PLFA concentrations, compost addition and long-term fertilization with FYM both provide high amounts of active biomass. Compost showed biomass values one order of magnitude higher than the unfertilized soil whereas the fertilized soil only showed a doubled amount. This accounts for microbial biomass increase, combined with the improved conditions of enhanced aeration plus the input of additional carbon sources and nutrients, and thus the positive effect of the supplementations on the pyrene degradation. In all four microcosms, an initial decrease of the concentrations of many of the PLFA was observed (Fig. 2a–d), which is considered to be due to the physical stress during extensive mixing of the materials and the altered water content resulting in the turnover burst after mixing. However, the PLFA patterns did not change significantly and the recovery of the PLFA concentrations was observed in most cases.

Some PLFA are considered to be biomarkers for a simple assessment of the microbial communities (Amelung et al. 2008; Zelles 1999). ^{13}C incorporation into PLFA requires productive pyrene degradation, and therefore, the labeled microbial groups at the time of most intense degradation are presumed to be responsible for the pyrene degradation. With proceeding incubation time, cross-feeding of bioprocessed ^{13}C within the microbial network will occur. In the unfertilized and fertilized soil-compost mixtures, Gram− bacteria appeared to dominate the pyrene degradation. However, the label simultaneously increased in Gram+ bacteria, including actinomycetes, indicating a highly complex community utilizing the pyrene-derived carbon during the time of most intense pyrene degradation. This evidenced a sufficient number of degrader bacteria to be present initially allowing pyrene degradation to start immediately. The ^{13}C enrichment of the PLFA in the unfertilized and fertilized soil-compost mixture does not fully parallel the changes in the PLFA concentrations, indicating that the most active pyrene-degrading organisms were not among the most abundant ones. With proceeding incubation time, ^{13}C was distributed within the microbial network over Gram− bacteria, Gram+ bacteria, and actinomycetes. The observed degrader pattern was less diverse in the fertilized soil microcosms, where only two PLFA showed a statistically significant ^{13}C enrichment. This indicates that only a small group of organisms metabolized pyrene in these microcosms, which is presumably the reason for the lag phase prior to pyrene degradation started in these microcosms. With proceeding incubation time, further ^{13}C incorporation, mainly into biomass

of Gram− bacteria, was observed, indicating an increase in abundance and diversity of bacteria using pyrene-derived carbon.

In the past, manifold evidence for tracking enhanced PAH degradation by single microbial strains (see "Introduction") has been presented, but complex consortia have not been in the focus in the past. Here, we provide evidence for complex consortia rather than specific key players being responsible for PAH degradation in compost supplemented soil. The ^{13}C incorporation into various marker PLFA and the pattern of enriched PLFA differed between the unfertilized and fertilized soil-compost mixtures and in the pure fertilized soil indicating different degrader populations. In contrast to compost amendment, long-term fertilization with FYM provided less diverse and less abundant degrader populations, with more pronounced participation of Gram+ bacteria with apparently lower carbon use efficiency and yield. However, after initial adaptation and growth, the degrader population was as efficient as the populations in the compost-amended treatments. A dominance of Gram− bacteria and fungi in contaminant removal accompanied by an increase of Gram+ bacteria was also reported for PAH composting experiments (Zhang et al. 2011b) and for microcosm studies with hydrocarbon contamination simulating land farming (Chaineau et al. 1995). None of these studies, however, specifically tracked the carbon flux.

The concentrations of the fungal PLFA marker were generally high in the compost-supplemented microcosms indicating some level of fungal activity, but the participation of fungi in pyrene degradation can be considered to be low. In contrast to bacteria, fungi oxidize PAH co-metabolically with no incorporation of the carbon from pyrene into their biomass, and thus enrichment of fungal PLFA indicators cannot be expected. Label incorporation into fungal biomass can only occur by cross-feeding towards the end of the experiment. However, no enrichment of the fungal indicator PLFA (18:1(9)cis and 18:2) at all was observed in the present experiments. Also no accumulation of dead-end metabolites, typically found for fungal co-metabolic substrate turnover (Ottow and Bidlingmaier 1997), was observed here, which is in line with previous studies (Haderlein et al. 2006; Johnsen et al. 2002).

In summary, we could show that both compost amendment and long-term fertilization with FYM enhance pyrene mineralization by complex microbial communities. The degradation kinetics differed between the treatments. Compost addition facilitates degradation without pronounced lag phase, whereas the degraders present in FYM-fertilized soil require time for initial growth prior to efficient pyrene mineralization. Complex low-abundance microbial communities rather than specific key players were responsible for pyrene degradation induced by compost addition. In contrast, the complexity of the degrading community after long-term fertilization with FYM was lower. A much more detailed molecular analysis of the microbial communities was performed for the present microcosm experiments but the details will be shown elsewhere soon.

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Conflict of interest The authors declare that they have no competing interests.

Compliance with ethical standards No humans or animals were involved in the experiments.

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