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Activated carbon stimulates microbial diversity and PAH biodegradation under anaerobic conditions in oil-polluted sediments

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HIGHLIGHTS
- Activated carbon (AC) boosts PAH biodegradation under anaerobic conditions.
- Under aerobic conditions AC does not promote biodegradation.
- AC stimulates diversity of PAH degrading microbes both without and with oxygen.
- Bioremediation of oil-polluted sediments may be facilitated by AC treatments.

ABSTRACT
Biodegradation by microorganisms is a useful tool that helps alleviating hydrocarbon pollution in nature. Microbes are more efficient in degradation under aerobic than anaerobic conditions, but the majority of sediment by volume is generally anoxic. Incubation experiments were conducted to study the biodegradation potential of naphthalene—a common polycyclic aromatic hydrocarbon (PAH)—and the diversity of microbial communities in presence/absence of activated carbon (AC) under aerobic/anaerobic conditions. Radio-respirometry experiments with endogenous microorganisms indicated that degradation of naphthalene was strongly stimulated (96%) by the AC addition under anaerobic conditions. In aerobic conditions, however, AC had no effects on naphthalene biodegradation. Bioaugmentation tests with cultured microbial populations grown on naphthalene showed that AC further stimulated (92%) naphthalene degradation in anoxia. Analysis of the 16S rRNA gene sequences implied that sediment amendment with AC increased microbial community diversity and changed community structure. Moreover, the relative abundance of Geobacter, Thiothrix, Sulfuricurvum, and methanogenic archaea increased sharply after amendment with AC under anaerobic conditions. These results may be explained by the fact that AC particles promoted direct interspecies electron transfer (DIET) between microorganisms involved in PAH degradation pathways. We suggest that important ecosystem functions mediated by microbes—such as hydrocarbon degradation—can be induced and that AC enrichment strategies can be exploited for facilitating bioremediation of anoxic oil-contaminated sediments and soils.

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1. Introduction
Crude oil is a very complex matrix containing several hundreds of aliphatic and aromatic hydrocarbons (HC) (Atlas, 1981; Wang et al., 1994). Within the aromatic fraction, polycyclic aromatic
hydrocarbons (PAHs) are of high environmental concern in coastal oil-polluted sediments resulting from human activities and from the petrochemical industry (Blumer and Sass, 1972; Atlas, 1981; Das and Chandran, 2011; Gong et al., 2014). Low molecular weight (LMW) PAHs, such as naphthalene, are more water-soluble than high molecular weight (HMW) ones (Hylland, 2006), while HMW PAHs are of particular concern since they are potentially carcinogenic or teratogenic for marine organisms and humans. Due to their hydrophobicity, PAHs have a high affinity for clay particles and sedimentary organic matter and tend to accumulate in sediments (Yang et al., 2016). Large parts of PAHs in marine sediments are lost due to the breakdown by microbial communities, which represents the quantitatively most important degradation pathway for HC in natural ecosystems (Atlas, 1981, 1991; Leahy and Colwell, 1990; Lu et al., 2011).

There are many natural HC-degrading microorganisms in marine ecosystems, such as Pseudomonas, Mycobacterium, Klebsiella, Acinetobacter, Micrococcus and Nocardia (Roy et al., 2002). These organisms can utilize PAHs as primary carbon source, which may lead to their complete mineralization from HMW to LMW PAHs and ultimately to carbon dioxide and inorganic nutrients (Atlas, 1981). In nature, aerobic degradation is the most efficient HC mineralization pathway due to the high energy yield obtained using O₂ as a terminal electron acceptor (TEA) (Lu et al., 2011). Since the largest fraction of aquatic sediments is anoxic, most organic compounds, including HC, are therefore expected to be degraded or buried in anaerobic conditions (Canfield, 1994). Nitrate and sulfate, for example, can serve as alternate TEAs for hydrocarbon respiration (Atlas, 1981). However, to date there are only a few reports on anaerobic biodegradation of complex hydrocarbons, and in particular literature on anaerobic degradation of PAHs such as naphthalene is scarce (Rockne et al., 2000; Christensen et al., 2004). Moreover, HMW PAHs have half-lives of years in marine sediments because biodegradation happens very slowly for these refractory compounds (Wilcock et al., 1996). Interestingly, activated carbon (AC) has also been shown to act as conductive material mediating direct interspecies electron transfer (DIET) between bacteria attached to the HC particles (Lovley, 2017; Yee et al., 2019). For example, this has been shown for Geobacter oxidizing ethanol coupled to the reduction of CO₂ to methane (CH₄) by methanogenic archaea (Liu et al., 2012; Yee et al., 2019). DIET can also be conducted via extracellular electron transfer directly by bacteria attached to each other via pili (instead of AC substituting pili) (Rotaru et al., 2014). It is therefore possible that microbial degrada-
tion of complex hydrocarbons in AC amended sediment may be facilitated by DIET.

Traditional remediation techniques following oil spills or in oil refinery ponds generally consist of sediment dredging plus treatment ex situ or through isolation capping by thick layers of inert materials such as clay (Forstner and Apitz, 2007). Bioremediation, i.e., the stimulation of the degrading capacity of HC-degrading organisms, has also been proposed as an alternative or complementary technique for remediation of HC-polluted sediments (Leafy and Colwell, 1990; Atlas, 1991). Recently, another promising in situ remediation of polluted sediments through amendment with reactive carbonaceous sorbents, and especially with AC, has been proposed as a low-cost remediation alternative to dredging (Luthy et al., 1997; Cornelissen et al., 2011; Ghosh et al., 2011; Abel et al., 2017). Studies have shown that sorption to AC can decrease PAH availability to benthic organisms by over 80% in contaminated sediments, and that use of finer AC particles as well as enhanced contact time can greatly increase sorption capacity (Zimmerman et al., 2004, 2005). However, to date it is not clear whether AC could facilitate biodegradation of sediment-associated PAHs carried out by indigenous HC degraders. Earlier works on the effects of AC and biochar on PAH degradation show contrasting results leading either to enhanced bioremediation (Chen et al., 2012) or acting as an impediment to microbial degradation (Ogbonnya et al., 2016). A recent in situ study showed that AC amended with bacteria decreased polychlorinated biphenyls (PCBs) concentrations in the sediment by up to 52% (Payne et al., 2019). To our knowledge, however, no study has so far investigated whether microorganisms cultivated with AC under aerobic or anaerobic conditions may stimulate PAH degradation.

It is thus clear that the effects of AC treatment on microbial communities and on biodegradation of contaminants are still largely unknown. In this study we collected oil-polluted sediment with its native microorganisms to test whether: (1) AC amendment affects microbial diversity and community structure; (2) AC increases biodegradation of both native PAHs and added naphthalene; (3) bio-augmentation by addition of microbial communities cultured in the presence of AC stimulates further the degradation of naphthalene in contaminant sediments. These hypotheses were investigated with laboratory experiments using amplicon sequencing technology (microbial community analysis) and respirometry essays (potential for PAH degradation), both in aerobic and anaerobic conditions.

2. Materials and methods

2.1. Sediment sampling and experimental design

Oil polluted sediment was collected from the retention lagoon (58°55′45″N; 17°58′12″E) of the oil refinery Nynas AB (Nynäshamn, Sweden) in September 2017. Five sediment cores were collected at 2 m depth using a piston core (5.4 cm inner diameter) (Fig. S1). The upper 50 cm of sediment from each core was pooled into one composite sample in a 10-L container and transported to the lab at Stockholm University, where it was kept in a thermo-constant room at 5 ± 1 °C. The sediment was very soft with a strong smell of oil. Physico-chemical characteristics (pH, salinity and O₂ concentrations) of the overlying water were determined with a multi-meter (HQ40D, Hach®, USA) and 3 × 50 mL sediment samples were collected for analyses of water content, organic matter and initial concentration of PAHs (see section 2.5). This sediment was also sampled (3 × 1 mL samples) and immediately frozen at −80 °C for later DNA extraction and sequencing. Additionally, initial oil-polluted sediment samples from the 10-L container were collected in order to: (1) setup microbial cultures and microbial community analyses (metabarcoding), and (2) conduct PAH and naphthalene biodegradation assays. For the first experiment 200 mL sediment were collected and split into two 250-mL Erlenmeyer flasks (Fig. S2), while for the second experiment 1 L sediment was sampled and split into two 1-L glass bottles (Fig. S3).

2.2. Culture experiments to assess microbial diversity and community structure

Cultures and amplification of endogenous naphthalene degraders present in the oil polluted sediment were prepared in two 250 mL Erlenmeyer flasks, by adding 100-mL oil-contaminated sediment and 100-mL Bushnell-Haas mineral medium (Bushnell and Haas, 1941) to each flask, as recommended for growing microbial hydrocarbon degraders (Wang et al., 1998) (Fig. S2). One flask (OX) was used to culture microorganisms under aerobic conditions, by aeration with sterile filtered (pore size, 0.2 μm) ambient air. The other flask (AN) was purged with N₂ gas in order to get a culture under anaerobic conditions. The anaerobic culture was
also provided with NaNO₃ (6.2 mM) to initially serve as electron acceptor for the oxidation of organic compounds (Eriksson et al., 2003).

After an initial acclimatization period (13 d), sediment cultures were divided into four separate treatments (OX, OX + AC, AN, AN + AC), each consisting of five replicates (Fig. S2). Each culture consisted of 20 mL of either aerobic (OX) or anaerobic (AN) culture from the previous step (Fig. S2) and the addition of 40 μL (0.78 mM) naphthalene dissolved in ethanol, in 50 mL Falcon tubes. OX + AC and AN + AC cultures were amended with additionally 220 μg—corresponding to 10% (dw) addition—of powdered AC (PAC) made from anthracite (AquaSorb® BP2 PAC-S, Jacobi Carbons AB, Sweden) (Fig. S2). An aliquot of the PAC material used in these experiments was analyzed for elemental composition (see section 2.5).

Cultures were incubated at room temperature (20 ± 1 °C) and mixed twice a day by hand to prevent sedimentation (Fig. S2). After an incubation time of 5 weeks, 1 mL was taken from each sample for DNA sequencing (n = 20) and the volume of the cultures was restored by adding 1 mL of Bushnell-Haas medium, PAC and naphthalene in the same proportions as described above. Final microbial samples (n = 20) were collected also after 10 weeks as described above.

### 2.3. DNA extraction, sequencing and bioinformatics

Microbial composition of the sediment was determined by extracting DNA from the initial sediment (Zero time-points: n = 3), from the four treatments after 5 weeks (Mid; n = 20) and after 10 weeks (End; n = 20) of incubation, using the DNeasy PowerSoil® DNA Isolation Kit (QIAGEN Inc, Germantown, MD, USA). Extracted DNA was purified using Agencourt AMPure® XP beads according to the standard protocol (Beckman Coulter, USA). The concentration of extracted DNA was evaluated using Quant-iT™ PicoGreen® dsDNA Assay kit (ThermoFisher, USA). Library preparation followed the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, 2013). The 16S rRNA gene V3–V4 region was amplified using the universal primers 341F and 805R (Herlemann et al., 2011). Prior to sequencing DNA samples were amplified by a two-step PCR with Phusion High-Fidelity DNA Polymerase (New England Biolabs, USA). Quality control was performed on an Agilent 2100 BioAnalyser (Agilent Technologies, USA). The pooled libraries were denatured with NaOH and diluted to 6pM. The denatured amplicon library was combined with denatured Phix control (5%). Samples were sequenced on the Illumina MiSeq platform using the v3 reagent kit (600 cycles), resulting in paired ends with read-length of 2 × 300 bases. The raw sequence data was uploaded to NCBI GenBank and is available at the BioProject number PRJNA560138.

Initial demultiplexing and trimming (removal of primer sequences and indices) were performed with the default Illumina MiSeq Control Software (2.6.2.1) installed on the sequencer. Downstream quality control, trimming, filtering, merging of forward and reverse reads, chimera removal and identification of amplicon sequence variants was done with the R software version 3.4.4 (R Core Team, 2013), using the DADA2 R package version 1.6 (Callahan et al., 2016). Forward and reverse reads were trimmed by 25 and 10 bases respectively and truncated to 275 and 250 bases of sequence-length to account for sequence quality drop. Taxonomical classification of ASVs (Amplonc Sequence Variants) was performed using the Silva 16S database version 128 (Quast et al., 2013). Graphical presentation and statistical analysis of ASV sequence data was done with the software Explicet 2.10.5 (Robertson et al., 2013). A full list of ASVs with sequence counts is available in Supplementary Information.

### 2.4. Batch incubation experiments to assess PAH and naphthalene degradation

Biodegradation essays were performed by taking two subsamples (500 mL each) of the oil-polluted sediment, which were transferred to glass bottles and stored at 5 ± 1 °C in dark conditions for 60 d (Fig. S3). One bottle was left unamended, while the other bottle was amended with 5.5 g—corresponding to 10% (dw) addition—of PAC. Before and 60 d after amendment, water parameters and sediment samples were taken from both amended and unamended sediment batches and analyzed for concentration of PAHs with the methods described in section 2.5. The PAH concentrations before and after amendment were determined in only one bottle per treatment (Fig. S3) by taking three subsamples from each bottle and pooling them together into one sample. This was because of the low heterogeneity of the sediment slurries and because this was not the main focus of the study from the start.

To determine the combined effects of AC and oxygen availability on PAH biodegradation, radio-respirometry experiments were carried out. Briefly, 2-mL sediment from the glass bottles and 5-mL 0.2 μm filtered water from the lagoon were placed in a series of 12-mL gas-tight vials (Exetainer®, Labco, UK) and split into four treatments (Fig. S3a): (I) OX was aerated sediment slurry without AC; (II) AN was anoxic, N₂-purged sediment slurry without AC; (III) OX + AC was aerated sediment slurry inoculated with AC; (IV) AN + AC was anoxic, N₂-purged sediment slurry inoculated with AC (Fig. S3a). In addition, four additional blank controls (twooxic and two anoxic) with 0.2 μm filtered lagoon water (7 mL) were prepared (Fig. S3a).

The radio-respirometry experiment was conducted directly in the 24 Exetainers (Fig. S3a). In each Exetainer two hypodermic needles (0.8 × 100 mm and 0.4 × 40 mm) were inserted through the lid to let the gas in and out (Fig. S3b). The longest needle was used to let filtered (pore size, 0.2 μm) ambient air or N₂ gas inside the solution and the shortest needle to let gas out from the headspace (Fig. S3b). A valve was added to the inlet to be able to adjust the gas flow (air for the aerobic treatment, N₂ gas for the anaerobic treatment) for each replicate. The outgoing gas was directed into a second set of 12-mL Exetainers containing 7 mL of a high capacity ¹⁴CO₂ absorbing liquid (Carbo-Sorb® E, PerkinElmer, USA), in order to trap the produced carbon dioxide (CO₂). The second Exetainer also had two needles (0.8 × 100 mm and 0.4 × 40 mm) with the longest used as an inlet and gently bubbling inside the Carbo-Sorb, while the second was allowing gas to overflow (Fig. S3b).

The radio-respirometry was started by adding 20 μL of a [1-¹⁴C]-naphthalene stock solution (specific activity of 57 mCi/mmol; 99% purity, American Radiolabelled Chemicals, USA), containing 4.7 ± 0.2 kBq (average ± st.dev.; n = 3), and then turning on ambient air or N₂ gas flow. Repeated sampling of the Carbo-Sorb solution (1 mL) was done after 1, 2, 4, 6, 8, 24 and 28 h, and 1 mL of new Carbo-Sorb was replaced after each sampling time, in order to keep a constant volume of Carbo-Sorb in the second set of Exetainers (Fig. S3b).

After 28 h of incubation, each tube was supplied with cultured microbial communities from the experiment described in section 2.2, in order to test whether a bio-augmentation effect could be achieved, i.e., whether it was possible to increase the degradation rate of naphthalene into CO₂ by adding cultured microbial communities that had been selected for naphthalene degradation. Each of the respective microbial culture (0.5 mL) was added to the first Exetainers containing sediment slurries; the biodegradation measurements started approximately 1 h after addition of microbial cultures. Subsampling of 1 mL Carbo-Sorb was repeated after 1, 3, 5, 19, 24 and 43 h from the addition of the microbial cultures, and 1 mL of new Carbo-Sorb was replaced after each sampling.
The entire biodegradation experiment was conducted over a time-span of 71 h from the naphthalene addition and the $^{14}$CO$_2$ radioactivity was assessed as described by Nascimento et al. (2012). In short, the Carbo-Sorb liquid samples were added to 10 mL scintillation liquid (Permafluor® E+, PerkinElmer, USA) contained in 20 mL glass vials and measured on a Tri-Carb® liquid scintillation counter (PerkinElmer, USA). All samples were counted for 10 min and corrected for quenching, counting efficiency and background level (Nascimento et al., 2012). The vials were detached from the bubbling system and 80 h from the tracer addition the experiment was terminated by adding 500 µL of a 7 M ZnCl$_2$ solution in each Exetainer to stop microbial activity. Nitrate and methane analyses were carried out in each of the anoxic Exetainer.

2.5. Additional chemical analyses

Nitrate concentrations were determined spectrophotometrically following the method described in Caffrey et al. (2018). Methane was quantified by headspace analysis on a gas chromatograph (GC Trace 1300, Thermo) equipped with a flame ionization detector and using dinitrogen (N$_2$) as carrier gas. The CH$_4$ concentrations (µM) were measured based on the distribution coefficient of methane in gas-water (Wang et al., 2001) and temperature (Yamamoto et al., 1976). Precision was ± 1 nM for CH$_4$ concentrations.

Triplicate sediment samples were analyzed for water content by measuring the wet and dry weight of 5 mL sediment after drying at 105 °C. Organic content as loss of ignition (LOI) was estimated after exposing the sediment to 550 °C for 5 h. Total C, total organic C and total N contents were measured using an elemental analyzer (FLASH 2000 HT, Thermo) at the accredited laboratory at DEEP, Stockholm University.

Concentrations of PAHs in the oil-polluted sediments were determined by gas-chromatography mass-spectrometry (GC-MS) at the accredited laboratory at ALS Scandinavia AB, Sweden according to the International Standard ISO 18287 (ISO:18287:2016, 2006). The method is detailed in the Supplementary material. The AC material used in the experiment was analyzed for its elemental composition using a high-resolution scanning electron microscopy (SEM, Jeol JSM 7000F) equipped with an energy dispersive X-ray spectrometer (EDS, Oxford instruments) at Stockholm University according to previous protocols (Bonaglia et al., 2019).

2.6. Statistics and calculations

Microbial alpha diversity (Shannon’s H index) was calculated based on all ASVs in the software Explicet and was based on the distribution of all ASVs in the software Explicet was based sub-sampled sequence counts to the lowest sample size (19 583 counts) and the mean of bootstrap × 100. Tests for normal distribution, differences in alpha diversity, relative abundance of phyla, and lower taxonomic groups of ASVs between samples were conducted in SPSS 25 (IBM, USA). Data was checked for normal distribution using the Shapiro-Wilk test. Differences in alpha diversity was tested with One-Way ANOVA (when testing the zero time point against the treatment groups), and Three-Way ANOVA repeated measures to test the effect of AC on treatments. Non-metric multidimensional scaling (NMDS) multivariate analysis based on the Bray-Curtis distance matrix of all ASVs was conducted to find differences in beta diversity with the software past 3.25 (Hammer et al., 2001). Three-Way adonis PERMANOVA tests (9999 permutations with time, AC treatment, and oxygen condition as factors) were done with the R script adonis in the vegan package (Oksanen et al., 2018) to find differences in beta diversity between groups. Differences among taxonomic groups in relative abundance were tested with non-parametric Mann-Whitney U and Kruskal-Wallis tests because they were not normally distributed.

Naphthalene mineralization was expressed as cumulative % of $^{14}$C-naphthalene converted into $^{14}$CO$_2$, assuming a 1:1 M ratio. Differences in % of degraded naphthalene among treatments were compared using the Friedman Repeated Measures Analysis of Variance (ANOVA) on Ranks test, as the data were not normally distributed even after data transformation (log (x+1)). Pairwise post hoc comparisons among treatments were performed using the Tukey HSD test. Results from the biodegradation experiment with $^{14}$C-naphthalene were further analyzed with a two-parameter exponential regression using a best fit to first-order kinetics:

$$Y = a(1 - e^{-bx})$$

where $a$ is the % of degraded naphthalene when the plateau is reached, and $b$ is the initial degradation rate of naphthalene. Statistical analyses of chemistry data were performed with SigmaPlot 13.0 (Systat Software, USA). If not stated otherwise in the text, measurements are reported in the results as average ± st.dev.

3. Results and discussion

3.1. Sediment chemical properties and AC composition

In the initial sediment right after sampling, the pH of the overlying water ranged between 7.3 and 7.9 (Table 1). Salinity was 3.8–4.7 ppt and O$_2$ concentrations were 1.5–2.9 mg L$^{-1}$ (Table 1). The sediment had an average water content of 89% and organic carbon content of 39% (loss on ignition) (Table 1). Total carbon (TC) in the sediment ranged between 13 and 20% and was almost entirely organic carbon (TOC), indicating that carbonates were not present (Table 1). Total nitrogen (TN) ranged 0.5–0.7% (Table 1). The AC material used in this experiment was constituted by fine particles in the size range ($D_{50}$) of 15–35 μm. The AC was mainly composed of C (mean weight 87.1%), O (9.6%), Si (0.6%), Al (0.7%), S (0.6%), and Ca and Fe (0.5%) (Table S1). For the complete elemental spectrum of the AC and for atomic percentages of each element, refer to Table S1.

3.2. Microbial diversity and community structure

The DADA2 analysis resulted in 44 669 ASVs with sequence counts ranging from 19 583 to 52 716 (average 36 068) per sample. A full list of all ASVs, sequences, taxonomic classifications, and sequence counts per sample is available in Table S2. The alpha diversity (Shannon’s H index) was significantly higher in the initial samples (zero time-points) collected in the field ($n = 3$) compared to the OX, OX + AC, AN, and AN + AC treatments ($n = 5$ for each group) and was 10 ± 0.1 compared to a range of 4.0–7.9 in the treatment groups (One-Way ANOVA, $F_{(8, 34)} = 78.08, \ p < 0.0000001$, post hoc Tukey tests, average ± standard error reported; Fig. 1a). Interestingly, the treatments amended with AC had higher alpha diversity in both the oxic (OX + AC 8 ± 0.00 compared to OX 7 ± 0.1), and anoxic treatments (AN + AC 7 ± 0.1 compared to AN 5 ± 0.2) (Three-Way ANOVA repeated measures, $p < 0.05$; Fig. 1a). Multivariate analysis (NMDS) based on the Bray-Curtis beta diversity showed a difference in microbial community structure after AC treatment (OX, OX + AC, AN, and AN + AC) (adonis, 9999 permutations, $R^2 = 0.2439, p = 0.0001$; Fig. 1b). These differences in alpha and beta diversity in the treatments compared to the field samples are likely because our culture treatments selected for specific populations enhanced by the added medium and naphthalene (Pinhasi and Berman, 2003).

The literature on microbial community structure responses to AC
amendments has reported contradictory findings. An experimental study using contaminated sediment (upper 2 mm layer) with up to 20% AC addition showed no obvious effects on microbial community structure detectable by T-RFLP (Jonker et al., 2009). This was later supported by Meynet et al. (2012), who suggested that community structure of contaminated soils was more influenced by time rather than AC amendment. These studies, however, have only tested the effects of AC on microbial organisms under aerobic conditions. Conversely, recent experiments using intact sediment, where redox conditions and anoxic niches were preserved, showed an increase in bacterial production and alteration of the community structure with thin-layer capping with AC (Näslund et al., 2012). AC increases the available niches for HC-degrading bacteria due to its complex and large surface area (Liang et al., 2009), but to our knowledge this is the first study showing that AC increases bacterial alpha diversity in oil-polluted sediments.

Looking at major taxonomic phyla and proteobacterial classes at the end of the experiment, it is evident how presence of AC significantly stimulated Deltaproteobacteria, which reached higher relative abundances in treatments OX + AC and AN + AC compared to OX and AN, respectively (Mann-Whitney U tests, \( U = 0.00 \), \( p = 0.008 \) for both tests; Fig. 2). Among the members of these taxa there is for example Geobacter, a well-known PAH degrader (Zhang et al., 2010; Yu et al., 2017) (see section 3.3). The Archaea phylum Euryarchaeota had a higher relative abundance in the zero time-points and AN + AC compared to the other groups (Kruskal-Wallis test, \( df = 8, H = 38.45, p = 0.000006 \)). This might be due to the fact that members of Euryarchaeota, such as methanogens (methane producing), have been found to be associated with the degradation of PAH (Chang et al., 2006). Furthermore, the relative abundance of Epsilonproteobacteria was higher in the OX + AC treatment compared to the OX treatment (\( U = 0.00, p = 0.008 \) ). Betaproteobacteria had instead significantly higher relative abundance in the AN + AC treatment compared to AN (\( U = 0.00, p = 0.008; \) Fig. 2). Thus, there might have been a stimulation by AC, and microbial members belonging to Betaproteobacteria (Martin et al., 2012; Singleton et al., 2013) and Epsilonproteobacteria (Zhao et al., 2019) have both been shown to degrade PAHs.

Firmicutes and Gammaproteobacteria had significantly higher relative abundance in the AN treatment (without AC) compared to all other treatments (Kruskal-Wallis, \( df = 2, H = 12.5, p = 0.002, H = 10.1, p = 0.006 \), respectively; Fig. 2). Gammaproteobacteria in the AN treatment was dominated by the genus Pseudomonas (Table S2), which is known to be able to utilize naphthalene and other PAHs as sole sources of carbon and energy (See et al., 2009). However, Pseudomonas was not stimulated by AC in this study (see ASV table in Supplementary Information). The stark increase of opportunistic fast-growing Gammaproteobacteria (Pseudomonas) may have outcompeted other phyla such as Bacteriodetes, which had an overall lower relative abundance in the anerobic treatments compared to the aerobic treatments (Kruskal-Wallis, \( df = 2, H = 9.4, p = 0.009 \), with no effect of AC; Fig. 2).

### 3.3. Biodegradation of PAHs and naphthalene

Initial concentration of total low molecular weight (LMW) PAHs (sum of naphthalene, acenaphthene and acenaphthylene) in the sediment was 8.2 mg kg\(^{-1}\) dw. Total medium molecular weight (MMW) PAHs (sum of fluorene, phenanthrene, anthracene, fluoranthene and pyrene) was 88 mg kg\(^{-1}\) dw. Total high molecular weight (HMW) PAHs (sum of benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-c,d]pyrene, dibenzo[a,h]anthracene and benzo[g,h,i]perylene) was 13 mg kg\(^{-1}\) dw (Fig. S4). The oil-polluted sediment exposed for 60 days to AC had LMW, MMW and HMW PAH concentrations decreasing by 46%, 50% and 70%, respectively (Fig. S4), while the batch without AC had LMW, MMW and HMW PAH concentrations decreasing by 88%, 51% and 30%, respectively (Fig. S4). Thus, two months exposure of anoxic oil-polluted sediment to AC

Table 1

<table>
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<th>Sample conditions</th>
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Zero time-points data are derived from in situ microbial communities (\( n = 3 \)) while labels named “Mid” denote after 5 weeks of incubation with naphthalene, and “End” denotes after 10 weeks with naphthalene (end of the experiment). Abbreviations from the different treatments (\( n = 5 \) each) denote OX = oxic; OX + AC = oxic + activated carbon; AN = anoxic; AN + AC = anoxic + activated carbon.

![Fig. 1. Panel a) Shannon’s H alpha diversity index of the microbial cultures (mean of 100 × bootstrap is reported). The stars denote outliers three times the height the box. Panel b) NMDS showing the Bray-Curtis beta diversity of the different treatments. Zero time-points data are derived from in situ microbial communities (\( n = 3 \)) while labels named “Mid” denote after 5 weeks of incubation with naphthalene, and “End” denotes after 10 weeks with naphthalene (end of the experiment). Abbreviations from the different treatments (\( n = 5 \) each) denote OX = oxic; OX + AC = oxic + activated carbon; AN = anoxic; AN + AC = anoxic + activated carbon.](image-url)
increased degradation of HMW cancerogenic PAHs by 133% (~2.3-fold increase) compared to sediments without AC. However, LMW PAHs were higher in the sediment amended with AC compared to that without AC, possibly because microorganisms degrade HMW into intermediate compounds such as LMW PAHs (Lu et al., 2011). However, bacterial degradation of HMW PAHs generally involves the utilization of LMW PAHs as co-substrate and would thus reduce both compound groups (Lu et al., 2011). Thus, we cannot exclude that the higher concentrations of LMW PAHs in the AC-amended sediment were due to the increased retention of volatile compounds by sorption to AC particles.

In the radio-respirometry test with 14C-labeled naphthalene, all four treatments showed potential for naphthalene degradation (Fig. 3a), while the blank controls did not accumulate any 14CO2 with time, indicating that the measured 14CO2 associated with the four treatments was due to actual microbial degradation of naphthalene (Näsland et al., 2010). We did not test for volatility of naphthalene in our study but we are confident that, by choosing a short incubation time (3 d), naphthalene losses due to volatility were negligible and according to similar setups at maximum between 1 and 15% (Heitkamp et al., 1987; Tso and Taghon, 2006). There was a significant difference in biodegradation of naphthalene among treatments (repeated measures ANOVA, Friedman Repeated Measures Analysis of Variance on Ranks; $\chi^2 = 118.7; df = 3; p < 0.001$) (Fig. 3a). A Tukey HSD post hoc test for the treatment factor showed that: 1) the two OX treatments had significantly higher biodegradation than the two AN treatments ($p < 0.001$), and 2) AN + AC was significantly higher than AN ($p = 0.002$). There was no significant difference between OX and OX + AC ($p = 0.76$). These results confirm that aerobic degradation of naphthalene is significantly more effective than degradation under strict anaerobic conditions (Cerniglia, 1993). Oxygen may serve both as terminal electron acceptor (TEA) and in the oxidative opening of the aromatic ring (Cerniglia, 1993; Lu et al., 2011). The fact that rates of aerobic PAH degradation are faster than rates in anaerobic conditions is one of the reasons why most of the biodegradation studies have so far mainly focused on aerobic degradation of PAHs (Bregnard et al., 1996; Eriksson et al., 2003).

Because of high carbon mineralization and sharp redox gradients, oxygen diffusing from the water column is generally consumed after few mm both in pristine (Bonaglia et al., 2014) and polluted (Bonaglia et al., 2019) sediments, leaving the rest of the sediment strictly anoxic. This study clearly shows that under anaerobic conditions amendment with AC significantly increased the biodegradation of naphthalene (Fig. 3a) and HMW PAHs (Fig. 54). In the long run, AC addition to anoxic sediments (i.e., AN + AC vs. AN) would increase PAH degradation by 55%, while if the sediment was to be oxygenated for example by mechanical aeration (i.e., OX vs. AN) the degradation would increase by 433%. However, the second method would be costlier as mixing/aeration may be continuous. The AC enrichment, on the contrary, may be repeated after long time intervals (i.e., months or years). We suggest that AC may be added and mechanically mixed to the sediment and preferably not added by capping, as mixing via bioturbation is likely to be absent or inefficient in oil-polluted environments.

Bio-augmentation, the inoculation of cultured microbial consortia, has proven to promote in situ oil-product bioremediation (Bento et al., 2005). A response to the addition of the cultured increased degradation of HMW cancerogenic PAHs by 133% (~2.3-fold increase) compared to sediments without AC. However, LMW PAHs were higher in the sediment amended with AC compared to that without AC, possibly because microorganisms degrade HMW into intermediate compounds such as LMW PAHs (Lu et al., 2011). However, bacterial degradation of HMW PAHs generally involves the utilization of LMW PAHs as co-substrate and would thus reduce both compound groups (Lu et al., 2011). Thus, we cannot exclude that the higher concentrations of LMW PAHs in the AC-amended sediment were due to the increased retention of volatile compounds by sorption to AC particles.

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naphthalene degraders (i.e., bio-augmentation) on $^{14}$C-CO$_2$ production was studied by analyzing the slope of degradation rates before and after microbial culture addition (Fig. 3b). Under aerobic conditions, the added bacteria had no stimulatory effect on naphthalene degradation as rates decreased after bioaugmentation. Under anaerobic conditions, however, the rate of naphthalene biodegradation almost doubled in presence of AC (Fig. 3b). We exclude that nitrate initially added to the cultures played a role in the oxidation of naphthalene as the analyses conducted at the end of the bioaugmentation experiment showed nitrate depletion. These results combined strongly suggest an increase of biodegradation of native PAHs in the sediment treated with AC under anaerobic conditions, which can be further increased after microbial bioaugmentation.

A previous work using biochar—a carbonaceous material similar to AC produced from pyrolyzed biomass—showed opposing results on PAH degradation compared to ours. At increasing biochar concentrations, the mineralization of naphthalene by indigenous microorganisms in sandy soils decreased (Ogbonnaya et al., 2016). Those experiments, however, were conducted in aerobic conditions. Conversely, biochar enhanced the degradation of PAHs by inoculated microorganisms in a contaminated agricultural soil in China (Chen et al., 2012). It was suggested that activated carbon and biochar may enhance biodegradation by increasing the surface area available for microbial colonization (Liang et al., 2009), thus protecting degraders from predation (top-down control), and by facilitating the contact between degraders and contaminants (Chen et al., 2012; Rillig and Thies, 2012). It may be that in our study AC facilitated the contact between PAHs and degrading microbes. However, to explain why this was not observed in the aerobic treatments, a more likely hypothesis is that 1) AC mediated interspecies electron transfer between anaerobic bacteria, or 2) compounds leaching out of it such as Fe$^{3+}$ and Fe hydroxides (Table S1) may have served as terminal electron acceptors (TEAs) for PAH mineralization in absence of oxygen (Anderson and Lovley, 1999). However, we find the second hypothesis very unlikely, since with our AC addition (2.8 g L$^{-1}$) and with the low Fe content (Table S1), the maximum Fe content in slurries was only ca. 0.2 mM. Even if part of this Fe was released to the solution, the leaching Fe$^{3+}$ might not be sufficient for stimulating rates, as it can only accept one electron.

The microbial community data further indicated that AC promoted PAH-degrading populations under anaerobic conditions (Fig. 4). Anaerobic cultures containing sulfate-reducing bacteria, typical for anoxic sediments, have been observed to degrade naphthalene (Meckenstock et al., 2000). We found that the Deltaproteobacteria genus Geobacter had a significantly higher relative abundance in the AN + AC treatment compared to AN (Mann-Whitney U test, mid and end points tested together, $U = 4.0$, $p = 0.00013$; Fig. 4a). This is in accordance to previous studies showing that Geobacter adsorbs to AC (Liu et al., 2012), and is able to degrade PAHs (Zhang et al., 2010; Yu et al., 2017). Furthermore, the Beta- and Epsilonproteobacteria genera Thiobacillus and Sulfuricurvum both increased in relative abundance in the AN + AC treatment ($U = 0.0$, $p = 0.000011$, and $U = 21.0$, $p = 0.028806$, respectively; Fig. 4b). Kai et al. (1990) found in a laboratory experiment that up to 90% of free-living Thiobacillus adsorbed to activated carbon, and Thiobacillus has been shown to be stimulated in PAH-polluted soil (Martin et al., 2012; Singleton et al., 2013). Especially, Sulfuricurvum, a genus of sulfur-oxidizing bacteria, is the group that displayed the most pronounced (significant) increase with time under AN + AC conditions. This genus can be found in oil-polluted environments (Sun et al., 2017) and has been associated with the degradation of PAHs (Zhao et al., 2019). However, specific studies on the effects of AC on Sulfuricurvum are not currently available.

The relative abundance of methanogenic archaea genera Methanothermobacter and Methanosaeta was also higher in the AN + AC treatment compared to AN ($U = 1.0$, $p = 0.000022$, and $U = 0.0$, $p = 0.000011$, respectively; Fig. 4d). Methanogenic archaea have previously been observed to be stimulated by direct interspecies electron transfer (DIET) with Geobacter facilitated by AC in amended cultures (Liu et al., 2012). In addition, methanogens have been observed to be involved in the degradation of PAHs, including...
naphthalene (Chang et al., 2006). Furthermore, after AC addition we also saw an increase in the relative abundance of *Thiobacillus* previously shown to conduct interspecies electron transfer. For example, oxidation of acetate by *Geobacter* has been shown to transfer electrons over conductive material to *Thiobacillus* reducing nitrate (Kato et al., 2012). It is therefore possible that electrons were also transferred via AC between *Geobacter* and *Thiobacillus*.

Considering that mineralization of naphthalene increased in the ANþAC treatment compared to the AN control, it is possible that oxidation of PAH was a result of DIET with AC as a conduit. If this was, e.g., conducted by *Geobacter* and methanogens, such a process would have decreased the amount of naphthalene and produced methane. In fact, measured methane generated under anaerobic conditions was significantly higher (One-Way ANOVA; \( F_{(1,9)} = 6.3; p = 0.036 \)) in presence of AC (1.9 ± 0.9 \( \mu M \)) than without AC (0.8 ± 0.3 \( \mu M \)) (Fig. S5). All together these results indicate that AC addition promoted methanogenesis in oil-polluted sediments, possibly with AC mediating interspecies interactions or DIET between, e.g., *Geobacter* and methanogens.

4. Conclusions

Oil pollution is a serious threat to the environment, but microbial degradation of organic contaminants can facilitate in situ remediation strategies (Leahy and Colwell, 1990; Liang et al., 2009; Lu et al., 2011). As most of the volume of contaminated sediment is anoxic, our research focused on a potential, affordable method to boost bioremediation under anaerobic conditions. Our results indicate that commercially available AC in powder form may add electron acceptors for PAH degradation and likely

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**Fig. 4.** Microbial genera stimulated by AC under anaerobic conditions and their change over time. Panel **a)** shows the genus *Geobacter*, **b)** *Thiobacillus*, **c)** *Sulfuricurvum*, **d)** *Methanofollis*, **e)** *Methanosarcina*, and **f)** *Simplicispira*. The y-axis shows the relative abundance (%) of the whole microbial community. Labels denote “Zero” (zero time-points, in situ field data), “Mid” after 5 weeks of incubation with naphthalene, and “End” after 10 weeks of incubation with naphthalene. Labels denote OX = oxic; OX+AC = oxic + activated carbon; AN = anoxic; AN+AC = anoxic + activated carbon. Error bars represent average ± standard error of the mean (zero time-points \( n = 3 \), and each treatment \( n = 5 \)).
promote DIET between different microorganisms involved in the degradation pathways. Further studies are needed to elucidate that this is valid also for other organic pollutants. Nonetheless, amendment with carbonaceous materials such as AC might be an important tool not only for the sequestration of contaminants, but also for in situ bioremediation of anoxic sediments.

Credit author statement


Declaration of competing interest

The authors declare that they do not have financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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diversity shifts in the bacterial community of a PAH-contaminated soil exposed to phenanthrene. Environ. Pollut. 162, 345–353.


