Environ Sci Pollut Res
DOI 10.1007/s11356-015-4105-7

DECAPAGE PROJECT: HYDROCARBON DEGRADATION IN COASTAL SEDIMENTS*

Changes of benthic bacteria and meiofauna assemblages during bio-treatments of anthracene-contaminated sediments from Bizerta lagoon (Tunisia)

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Received: 31 May 2014 / Accepted: 11 January 2015

Abstract Sediments from Bizerta lagoon were used in an experimental microcosm setup involving three scenarios for the bioremediation of anthracene-polluted sediments, namely bioaugmentation, biostimulation, and a combination of both bioaugmentation and biostimulation. In order to investigate the effect of the biotreatments on the benthic biosphere, 16S rRNA gene-based T-RFLP bacterial community structure and the abundance and diversity of the meiofauna were determined throughout the experiment period. Addition of fresh anthracene drastically reduced the benthic bacterial and meiofaunal abundances. The treatment combining biostimulation and bioaugmentation was most efficient in eliminating anthracene, resulting in a less toxic sedimentary environment, which restored meiofaunal abundance and diversity. Furthermore, canonical correspondence analysis showed that the biostimulation treatment promoted a bacterial community favorable to the development of nematodes while the treatment combining biostimulation and bioaugmentation resulted in a bacterial community that advantaged the development of the other meiofauna taxa (copepods, oligochaetes, polychaetes, and other) restoring thus the meiofaunal structure. The results highlight the importance to take into account the bacteria/meiofauna interactions during the implementation of bioremediation treatment.

Keywords Anthracene · Bacteria diversity · Bioremediation · PAH degraders · Microcosms · Bizerta lagoon · Meiofauna · Community structure · Degradation

Responsible editor: Philippe Garrigues

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Introduction

Petroleum hydrocarbons are frequently detected in marine sediments of industrial areas as results of accidental spills, industrial and urban runoffs, and shipping activities (Bossert and Compeau 1995; Onwurah et al. 2007). Among petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) are of primary environmental concern due to their toxicity and persistence in the environment (Angerer et al. 1997). They accumulate in bottom sediments affecting benthic organisms directly and indirectly pelagic organisms via re-suspension (Boonyatumanond et al. 2006) as well as organisms at higher trophic levels (Meador et al. 1995). In general, PAHs do not degrade easily under natural conditions and their persistence increases with increasing molecular weight (Bamforth and Singleton 2005).

Published online: 25 January 2015

As other PAHs, anthracene, a tricyclic PAH derived from coal tar or through incomplete combustion of organic material or existing in petroleum, enters aquatic environments through run-off or atmospheric deposition. It tends to bind fairly strong to sediment and can bioaccumulate in biota (Cornelissen et al. 1998). It is highly toxic to wildlife (Nkansah et al. 2011); its toxicity has been reported to amphipods (Gomes et al. 2009), animals (Mekenyan et al. 1994), oligochaetes (Erickson et al. 1999), and nematodes (Sese et al. 2009). It has been listed as one of the priority environmental pollutants by the United States Environmental Protection Agency (Zhang et al. 2011).

Microbial degradation of PAHs has been demonstrated several times in marine sediments (Goni-Urriza and Duran 2010). The catabolic pathways for PAHs under aerobic conditions, especially those for PAHs with two and three rings, have been widely reported and the mechanisms involved clearly illustrated (Cerniglia 1992; Bamforth and Singleton 2005; Cao et al. 2009). Microbial degradation has been recognized as an attractive non-invasive remediation strategy for removing hydrocarbon compounds in PAH-contaminated sediments (Hughes et al. 1997; Juhasz and Naidu 2000; Miyasaka et al. 2006; Atlas and Bragg 2009). It presents advantages over physical and chemical treatments due to its relatively low cost and little disturbance on the environment (Morgan and Watkinson 1989; Swannell et al. 1996; Head and Swannell 1999). In order to enhance the biodegradation efficiency, three strategies, namely natural attenuation, bioaugmentation, and biostimulation, have been proposed (Goni-Urriza et al. 2013). Natural attenuation utilizes the intrinsic degradation capabilities of autochthonous microorganisms (Mills et al. 2003). However, it often takes a long time to complete because the population size of the autochthonous degrading microorganisms is low (Forsyth et al. 1995). Bioaugmentation, i.e., inoculation of microorganisms with desired degradation capability, is a potential approach to enhance the biodegradation (Vogel 1996; Abbondanzi et al. 2006). Biostimulation, i.e., supplying additional nutrients or substrates to stimulate degradation of native microorganisms, is another strategy to promote the biodegradation (Riser-Roberts 1998; Roling et al. 2004). However, the effectiveness of these strategies varies from sediments to sediments and from contaminants to contaminants (Balba et al. 1998).

In this perspective, sediments of coastal lagoons deserve special attention because of their natural inclination in accumulating contaminants. Lagoons are considered environments with high ecological values because they are highly productive ecosystems and contribute to the overall productivity of coastal waters (Anthony et al. 2009). Sediments of coastal lagoons, owning high biodiversity, are complex ecosystems where important biological processes take place. They are thus important nursery and breeding areas and constitute habitats for diverse aquatic organisms. They are particularly vulnerable due to their exploitation by humans, which includes aquaculture and recreational activities for example. They are also indirectly threatened by human activities because they constitute the receptacle of terrestrial hydrosystems carrying diverse pollutants such as hydrocarbon and pesticide compounds.

The activities of benthic organisms (meiofauna and macrofauna) and the fluctuations of environmental parameters are important drivers for microbial organization and activities. The meiofauna/microorganisms interactions, including trophic and non-trophic interactions (De Mesel et al. 2003, 2004; Moens et al. 2005), play a major role in the overall carbon fluxes (Meysman et al. 2005; Van Oevelen et al. 2006) and organic matter mineralization (Aller and Aller 1992; Alkemade et al. 1992). We recently demonstrated that the benthic meiofauna influenced the prokaryotic hydrocarbon-degrading community structure and composition without affecting the overall hydrocarbon-degradation capacity (Stauffert et al. 2013; 2014; Cravo-Laureau and Duran 2014). The presence of pollutants such as hydrocarbon compounds may disrupt the balance of sediments ecosystem, affecting particularly microbial and meiofaunal communities (Louati et al. 2013a, b). Similarly, bioremediation treatments, by adding nutrients and/or bacterial strains, may result in a novel ecosystem balance with a concomitant pollutant degradation (Louati et al. 2013b) and toxicity reduction (Louati et al. 2014a, b).

The main aim of the present study was to determine the meiofaunal-bacterial relationships in anthracene-contaminated sediments subjected to different bioremediation treatments in order to develop new strategies for the restoration of contaminated sediments. For that purpose, we performed microcosm incubations with sediments sampled in the Bizerta lagoon (Southern Mediterranean). Anthracene was used as model compound because it is ubiquitous and persistent in aquatic environments (Müncnerová and Augustin 1994; Krivobok et al. 1998; Cheung et al. 2008) with demonstrated toxicity against benthic organisms (Erickson et al. 1999; Gomes et al. 2009; Sese et al. 2009).

Materials and methods

Site description

Bizerta lagoon is a canalized lagoon system located in an economically important area in northern Tunisia. This area extends over 150 km^2 and has a mean depth of 7 m, and is connected to the Mediterranean Sea through a 6-km-long inlet and to the Ichkeul Lake through the Tinja channel. Bizerta lagoon constitutes a receptor of several industrial sewages, aquaculture wastes, fertilizers, and pesticides through runoff and soil erosion, and wastewaters from towns implanted around (Yoshida et al. 2002; Derouiche et al. 2004; Ben

Said et al. 2010). Consequently, Bizerta sediments are contaminated by PAH compounds especially in the canal where most of the industrial activities are concentrated (Ben Said et al. 2010).

Sampling and field measurements

Sediments were collected by scuba diving at the Echaraà station (37.13° N, 9.49° E), a site with low contamination (Trabelsi and Driss 2005; Ben Said et al. 2010), in September 2009. The contamination of this station by anthracene was below 5 ppm (Louati et al. 2014a) and the concentration of fluoranthene, phenanthrene, and pyrene were under the detection limit (Louati et al. 2013b). Handcores of 10 cm^2 were used to a depth of 15 cm to transfer sediments into a bucket. Back in the laboratory, sediments were homogenized by gentle hand stirring with a large spatula before microcosm setup and anthracene contamination. Buckets and spatula were all acid-rinsed before use.

PAH choice and microcosm setup

Microcosms consisted of 1600-mL glass bottles as previously described (Louati et al. 2013a). One control and seven treatments (Table 1) were set up in triplicates. All triplicate tests were duplicated, one set was sampled after 20 days and the other set after 40 days of incubation, thus we started with 48 different microcosms. Spiked microcosms (S) were gently filled with 200 g (wet weight) of homogenized sediments (100 g of natural sediments and 100 g of anthracene-spiked sediments) topped up with 1 L of filtered (1 μm) natural lagoon water at 30 PSU. Sediments used for contamination was first alternately frozen (−80 °C) and thawed three times to defaunate it (Gyedu-Ababio and Baird 2006). Then the larger particles (>63 μm) were removed by sieving. Two milliliters of stock solution (100 mg L^{-1}) of high-purity anthracene (Sigma–Aldrich Chemical A8, 920-0) dissolved in acetone was added into the microcosm. After acetone evaporation, the sediments were added and shaken overnight to let the anthracene adsorb onto the sediments as previously described (Miyasaka et al. 2006). Final concentration of anthracene in sediments was 1 ppm. The un-spiked control (C) microcosms were constituted by 100 g of natural sediments and 100 g of defaunated sediments without anthracene addition. Each bottle was stopped with a rubber bung with two holes and aerated via an air stone diffuser. Air flux was filtered on 0.2 μm to prevent contamination.

Bioremediation treatments were started 1 day after anthracene contamination. Biostimulation (BS) was achieved by amending two types of slow-release particle fertilizers, 70 mg kg^{-1} of nitrogen fertilizer ($NaNO_3$) and 35 mg kg^{-1} of phosphorus fertilizer (KH_2PO_4), using the protocol of Jacques et al. (2008).

Bioaugmentation (BA) was achieved by adding the marine PAH-degrading bacterium, *Acinetobacter* sp., isolated from Bizerta lagoon sediments (Ben Said et al. 2008). The strain was previously grown in 50 mL of Luria-Bertani broth. After 1 week of cultivation, cells were harvested by centrifugation at 10,000×*g* for 15 min at 4 °C. Cells were suspended in seawater solution and introduced into the microcosms. Biostimulation-bioaugmentation (BS+BA) microcosms were amended with nutrients as described for BS before adding *Acinetobacter* sp. cells. The initial inoculum was 5.30×10^8 cells mL^{-1}. After nutrient addition and bacterium inoculation, sediments were agitated for half an hour for homogenization.

Anthracene and TOC analysis

Anthracene analyses in the sediments (initial, after 20 and 40 days of incubation) were conducted by gas chromatography coupled with atmospheric pressure ion source tandem mass spectrometry (APGC/MS–MS). Approximately 1 g (dry weight) of the samples was extracted with 40 mL of nonane (*v*/*v*) and with 2,2,4,4,6,8,8-heptamethylnonane and naphthalene as internal standards (1 ppm, ISTD) in an

Table 1 Microcosm treatments' codes, anthracene concentrations, and total organic carbon (TOC) contents in the microcosms

	Treatment	Code	$[anthracene]_{20d}$ (μg g^{-1})	$[anthracene]_{40d}$ (μg g^{-1})	TOC (%)
Un-spiked control	No treatment	C	5.58±0.1	4.31±0.1	0.66±0.04
	Biostimulation	CBS	5.25±0.02	3.44±0.08	0.64±0.02
	Bioaugmentation	CBA	5.13±0.02	2.41±0.02	0.69±0.06
	Biostimulation+bioaugmentation	CBS+BA	4.62±0.17	3.1±0.15	0.77±0.1
Spiked+1 ppm anthracene	No treatment	S	6.51±0.5	4.79±0.2	0.62±0.04
	Biostimulation	SBS	4.53±0.04	2.1±0.02	0.75±0.001
	Bioaugmentation	SBA	4.29±0.13	1.93±0.09	0.67±0.02
	Biostimulation + Bioaugmentation	SBS+BA	3.42±0.15	1.52±0.02	0.63±0.02

Anthracene concentrations were determined at 20 (*20d*) and 40 (*40d*) days. TOCs were determined at 40 days. Values are average±SD (n=3)

ultrasonic bath (15 min). The gas chromatograph (GC 7890, Agilent Technologies) was equipped with a capillary column DB5MS (30 m × 250 μm × 0.25 μm) and with a splitless injector (splitless time, 1 min; pulse, 0.15 min). The setting parameters of the APGC ion source (Waters) are source temperature, 150 °C; cone voltage, 25 V; and extractor voltage, 2.3 V. The injector temperature was maintained at 290 °C. The carrier gas (He) was maintained at 3 mL min^{-1} (constant flow mode). The oven temperature was programmed from 110 °C (1 min) to 290 °C (3 min) with a ramp of 35 °C min^{-1}.

Total organic carbon (TOC) analyses were performed by the Laboratoire des Pyrénées (Lagor, France). TOC contents were determined by dry combustion (LECO analyzer) after treatment with 6 N HCl to remove the inorganic carbon (Schumacher 2002).

Bacterial counts by flow cytometry

Bacteria were extracted from sediments (after 20 and 40 days of incubation) following the protocol of Duhamel and Jacquet (2006) as detailed in Louati et al. (2013a). For the enumeration of total bacteria, cells were stained with the nucleic acid stain SYBR Green I (Marie et al. 1997). Working stocks of SYBR Green I (10^{-3} of the commercial solution; Molecular Probes) were freshly prepared on the day of analysis. Bacterial samples were stained with 2.6 % (final concentration of work solution) and incubated in the dark at 4 °C for 15 min before analysis. The stained bacterial cells, excited at 488 nm, were enumerated using side scatter and green fluorescence at 530 nm. Fluorescent beads (1 and 2 μm; Polysciences, Inc., Warrington, PA, USA) were added to each sample as an external standard. True count beads (Becton Dickinson, San Jose, CA, USA) were added to determine the volume analyzed. Samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson) equipped with a 15-mW argon ion laser emitting at 488 nm for excitation. Data analyses were carried out with CellQuest Pro 5 software obtained from BD Biosciences.

T-RFLP analysis

Mixed-community DNA was extracted directly from sediments microcosms (after 20 and 40 days of incubation) using an Ultra Clean® Microbial DNA Isolation DNA Kit (MoBio Laboratories, Carlsbad, CA, USA) by following the manufacturer's protocol. Genes encoding 16S rRNA were PCR amplified from extracted samples using primers 8F (5′-AGAGTT TGATCCTGGCT-CAG-3′) and 1489R (5′-TACCTTGTTA CGACTTCA-3′) as previously described (Guyoneaud et al. 2002). Primer 8F was fluorescently labeled with 5-tetrachlorofluorescein E.SG.C. (Cybergene Group, France). PCR and terminal restriction fragment length polymorphism (T-RFLP) analysis were carried out as described previously (Ben Said et al. 2010) using the Taq DNA polymerase (Eurobio). The fluorescent PCR products were cleaned using GFX PCR DNA and Gel Band Purification kit (Amersham-Pharmacia), and 10 μL of purified product was digested separately with 3 U of enzyme HaeIII and HinfI for 3 h at 37 °C (New England Biolabs). One microliter of restriction digests was then mixed with 20 μL of deionized formamide and 0.5 μL of a TAMRA-labeled Gene scan 500 bp internal size standard (Applied Biosystems), denatured for 5 min at 95 °C, and immediately transferred to ice. Samples were loaded onto an ABI PRISM 310 automated genetic analyzer (Applied Biosystems). T-RFLP profiles were aligned by identifying and grouping homologous fragments and normalized by calculating relative abundances of each terminal restriction fragment (T-RF) from height fluorescence intensity of each T-RF as described (Duran et al. 2008; Pringault et al. 2008). For statistical analysis, T-RF heights of the three microcosm replicates were averaged.

Meiofauna extraction

After 20 and 40 days of the microcosm incubation, sediments were fixed in 4 % neutralized formalin (Mahmoudi et al. 2005). Sediments were washed through nested 1-mm-mesh sieves to separate macrofauna from meiofauna, which are retained on a 40-μm sieve, using the protocol described in Mahmoudi et al. (2007). Meiofauna (M) were counted and identified at the higher taxon level, free nematodes (N), copepods (C), and polychaetes (P), using a stereomicroscope (magnification ×40).

Statistical analysis

Statistical analysis of the effects of treatment on anthracene biodegradation and biological parameters was performed using a two-way (treatment×time) analysis of variance (ANOVA). Prior ANOVA test normality (Shapiro-Wilk Test) and homogeneity of variance were tested. If conditions to use ANOVA were not met, differences between treatments were tested using the non-parametric Kruskal-Wallis ANOVA test. A posteriori paired multiple comparisons were performed using Tukey HSD test. Statistically significant differences were assumed when $p < 0.05$. For statistical analysis of bacterial community structure, relative abundances of T-RFs were transformed with arcsin ($x^{0.5}$) to get a normal distribution of data (Legendre and Legendre 1998). Clustering (Bray-Curtis similarity with Ward linkage method) and correspondence analyses (CA) were performed to assess difference in the microbial community structure as a function of treatments and time (20 and 40 days of incubation). Canonical correspondence analysis (CCA) was performed with meiofauna data to estimate the role of biotic factors in the microbial community assemblages. Multivariate analyses (clustering, CA, and CCA) were performed with MVSP v3.12d software (Kovach Computing Service, Anglesey, Wales).

Results

Anthracene biodegradation

In order to investigate the bacteria/meiofauna relationship during the restoration of PAH-contaminated marine sediments, a microcosm experiment was set up with low-contaminated anthracene (5.5±0.1 μg g^{-1}) sediments from Bizerta lagoon. A low dose (1 ppm) of fresh anthracene, with limited effect on the meiofauna, was added to compare old and fresh anthracene contamination. The anthracene degradation was determined after 20 and 40 days of incubation. Before anthracene addition, the in situ anthracene concentration in sediments was 5.5±0.1 μg g^{-1} (dry weight sediments). Therefore, the initial concentration in un-spiked control (C) microcosms (C, CBS, CBA, CBS+BA) was 5.5±0.1 and 6.5±0.1 μg g^{-1} for anthracene-spiked microcosms (S, SBS, SBA, SBS+BA) considering the artificial spiking of 1 μg g^{-1} (the initial concentration in each microcosm was unfortunately not measured). The biodegradation (%) of anthracene was calculated from the difference between the initial concentration and concentration measured after 20 and 40 days of incubation. Treatments and incubation time had a significant effect ($p<0.05$, ANOVA test) on anthracene concentrations. The anthracene concentration was maximal in anthracene-spiked microcosms (S), 6.5±0.5 μg g^{-1} (dry weight sediments) at 20 days of incubation, and it was reduced to 4.79 ±0.5 μg g^{-1} (dry weight sediments) after 40 days of incubation (Table 1) indicating that anthracene degradation was effective in the microcosms. The bioremediation treatments enhanced the biodegradation, which was maximal when the combination of biostimulation (nutrients addition) and bioaugmentation (addition of a hydrocarbon-degrading bacterium) treatments (BS+BA) was applied (Fig. 1). Interestingly, significant ($p<0.05$, ANOVA test) anthracene degradation was observed in the un-spiked control microcosms, the bioaugmentation treatment (CBA) being the most efficient.

At the end of incubation, the total organic carbon (TOC) content was roughly similar in all microcosms ranging from 0.62±0.04 % (S) to 0.77±0.1 % (CBS+BA; Table 1) indicating that the microcosms had the same carbon limitation.

Effect of anthracene and bioremediation treatments on bacterial abundance and diversity

Flow cytometry analyses showed that the artificial addition of anthracene had a significant negative effect on bacterial abundance ($p<0.05$ Kruskal-Wallis ANOVA test) at both 20 and 40 days of incubation (Fig. 2). After 40 days of incubation, bacterial abundance ranged from 4.62±0.3×10^7 to 4.88±0.09×10^7 cells cm^{-3} in un-spiked control microcosms whereas in S bottle the bacterial abundance was five times lower, 0.86±0.1×10^7 cells cm^{-3} (Fig. 2). The bioaugmentation treatment (SBA) limited the impact of the added anthracene maintaining the bacterial abundance at 2.5±0.05×10^7 cells cm^{-3} at d40, the same level observed at d20 in the S microcosm (2.3±0.03×10^7 cells cm^{-3}), while it was three times lower in the S microcosm at d40 (0.86±0.1×10^7 cells cm^{-3}; Fig. 2). The stimulation treatment (SBS) and the combined treatment (SBS+SBA) restored the bacterial abundance reaching 5±0.2×10^7 cells cm^{-3}, the same level observed in the un-spiked control without addition of anthracene (Fig. 2). It is important to notice that the bioaugmentation combined with artificial anthracene spiking (SBA) had a significant ($p<0.05$, Tukey HSD test) negative impact on both bacterial abundance and OTUs richness even in un-spiked control microcosms (CBA) indicating that the addition of bacteria disorganized

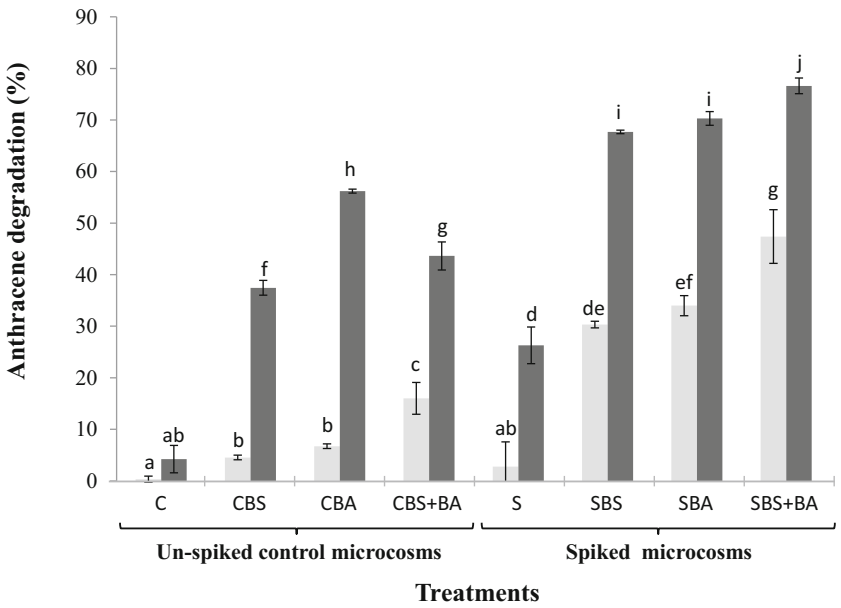

Fig. 1 Anthracene degradation (%, calculated as a function of the initial anthracene concentration) according to different treatments after 20 (*gray bars*) and 40 (*black bars*) days of incubation. *C* Un-spiked control, *S* anthracene-spiked, *BS* biostimulation, *BA* bioaugmentation, *BS+BA* biostimulation and bioaugmentation. Average±SD ($n=3$). *Letters* refer to homogenous groups according to post hoc Tukey test

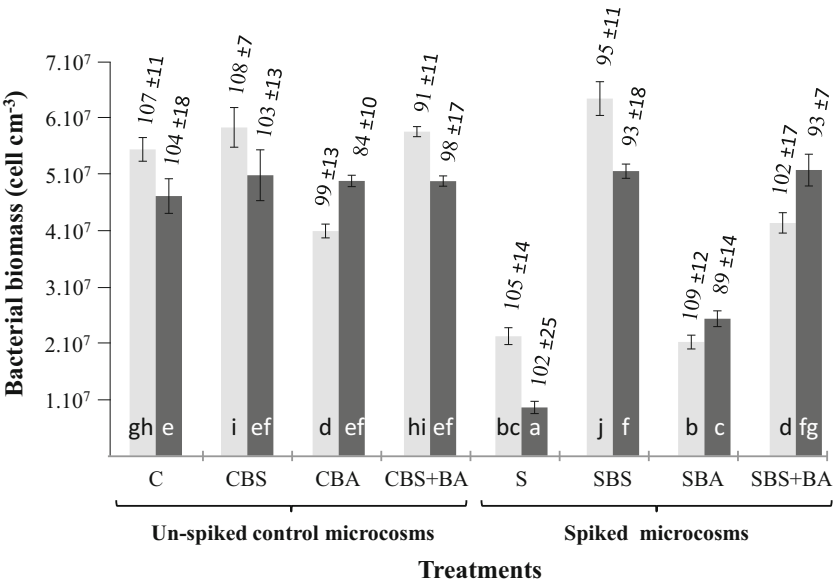

Fig. 2 Bacterial abundance (average±SD; n=3) counted by flow cytometry in sediments of different treatments (*C* un-spiked control, *S* anthracene-spiked, *BS* biostimulation, *BA* bioaugmentation, *BS+BA* biostimulation and bioaugmentation) after 20 (*gray bars*) and 40 (*black bars*) days of incubation. *Numbers* indicate the OTUs richness (average number of T-RFs in T-RFLP patterns± SD). *Letters* refer to homogenous groups according to post hoc Tukey test

the initial bacterial community structure. Indeed, T-RFLP patterns showed between 84±10 and 109±12 OTUs (T-RFs or peaks), the lowest OTUs number observed in both bioaugmentation treatments [84±10 and 89±14 OTUs in control (CBA) and contaminated (SBA) microcosms, respectively]. However, no clear relationship could be established between OTU numbers and treatments.

The comparison of bacterial community structure by correspondence analyses (CA) showed that bacterial community structure was modified according to treatments and incubation time (Fig. 3a, b). In un-spiked control microcosms (C), the bacterial community structures were more dispersed at the end of incubation (40 days, Fig. 3b) suggesting that each treatment resulted in a specific bacterial community. In contrast, in the anthracene-spiked microcosms (S), the bacterial community structures tended to be more similar at the end of incubation (40 days) rather than they were at 20 days (Fig. 3a).

Effect of anthracene and bioremediation treatments on meiofauna abundance and diversity

Meiofauna were counted and sorted into different taxonomic groups in order to evaluate the effect of anthracene and bioremediation treatments on meiofauna abundance and diversity. In the un-spiked control microcosms (C), the treatments had significant (p<0.05, ANOVA test) positive effect on total meiofauna abundance in the CBS+BA at 20 days and in the CBS at 40 days (Fig. 4). After 40 days, the total meiofauna abundance was similar in all treatments (around 2.41±0.05 ind/cm^3) except for the CBS where 3.37±0.1 ind/cm^3 were counted.

The meiofauna abundance was reduced drastically (p<0.05, ANOVA test) to 0.66±0.07 and 0.55±0.09 ind/cm^3 at 20 and 40 days, respectively, in anthracene-spiked microcosms (Fig. 4). This impact might be due to a direct effect of anthracene or lack of food since bacteria were affected by the addition of anthracene as shown in Fig. 2. Bioremediation treatments seemed to reduce anthracene impact with BS+BA (2.56±0.08 ind/cm^3) and BS (2.77±0.11 ind/cm^3) as the most effective treatments for restoring meiofauna at 20 and 40 days, respectively (Fig. 5).

The relative abundance of main meiofauna groups showed a large dominance of nematodes in all microcosms (Fig. 5a). The comparison of meiofauna community structures by clustering analysis showed a clear difference between spiked (S) and un-spiked control microcosms (C) (Fig. 5b). The addition of fresh anthracene increased by ca. 20 % the relative abundance of nematodes while a negative effect was observed for copepods, which decreased by ca. 4 %. The anthracene affected also the unclassified group, i.e., the meiofauna that could not be sorted in taxonomic groups, which decreased by ca. 20 %, respectively. These effects were more pronounced with the addition of hydrocarbon-degrading bacterium in the BA treatment where the nematodes' relative abundance was increased by ca. 30 %, and the decrease of copepods and unclassified groups was also maximal representing ca. 7 and 22 %, respectively. The BA treatment also negatively affected the polychaetes group, which decreased by 4 %. Similar effects were observed with the BS treatment where nematodes increased by ca. 25 % and the unclassified group decreasing by ca. 14 %, but the copepods group was less affected, decreasing by ca. 2 % (Fig. 5). It is noteworthy that the combination of both treatments BS+BA at 40 days had similar meiofauna community structure than that observed in un-spiked control microcosm (Fig. 5) indicating that this treatment was the most efficient in mitigating the anthracene effect.

In order to estimate whether the effect of anthracene and treatments on the meiofauna were associated with that observed on bacterial community structures, we performed canonical correspondence analyses (CCA) correlating T-RFLP community structure patterns with the abundances of the main

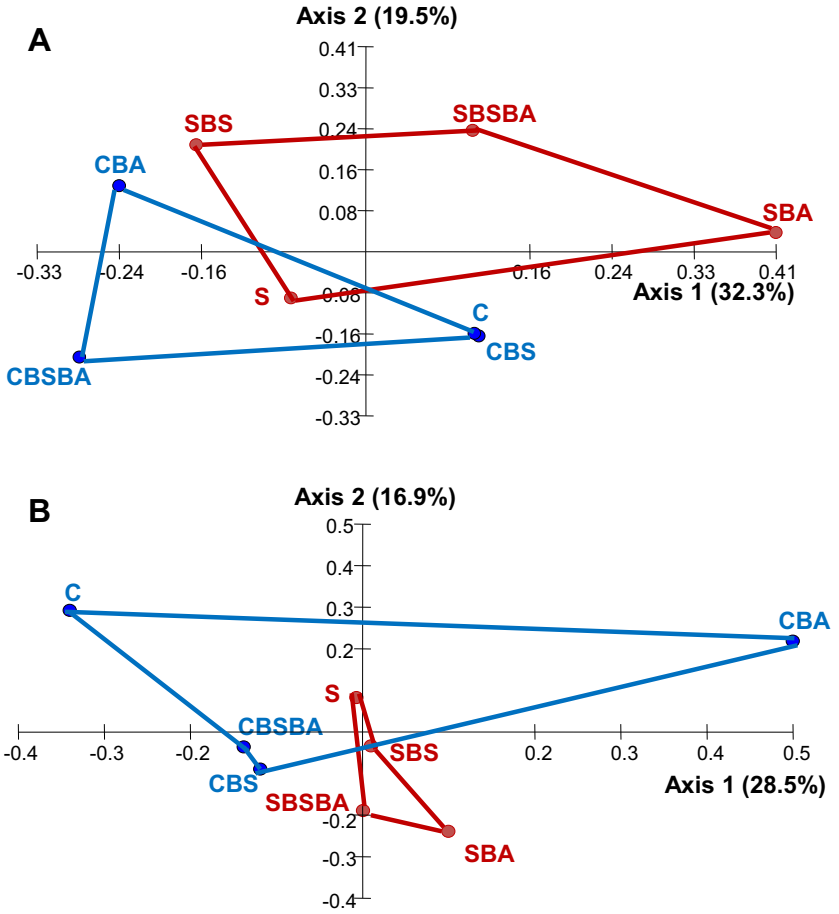

Fig. 3 Comparison of bacterial community structures of the different treatments at 20 days (**a**) and 40 days (**b**). The comparison was performed by correspondence analysis (CA) based on T-RFLP of 16S rRNA genes [mean profile of replicate samples (n=3) of HaeIII- and HinfI-digested 16S rRNA gene]. The letter *C* indicates the un-spiked control (*blue*), the letter *S* indicates the anthracene-spiked microcosms (*red*). *BS* Biostimulation, *BA* bioaugmentation, *CBS+BA* biostimulation and bioaugmentation

meiofauna groups. So as to observe the effect of anthracene in the modifications of the relationships linking meiofauna and bacterial communities according to time, CCA were performed at each incubation time for the control and anthracene conditions. The CCA showed differences according to the presence of anthracene at 20 days (Fig. 6a, b). For example, regarding the most abundant meiofauna group, nematodes and copepods were associated with the bacterial communities

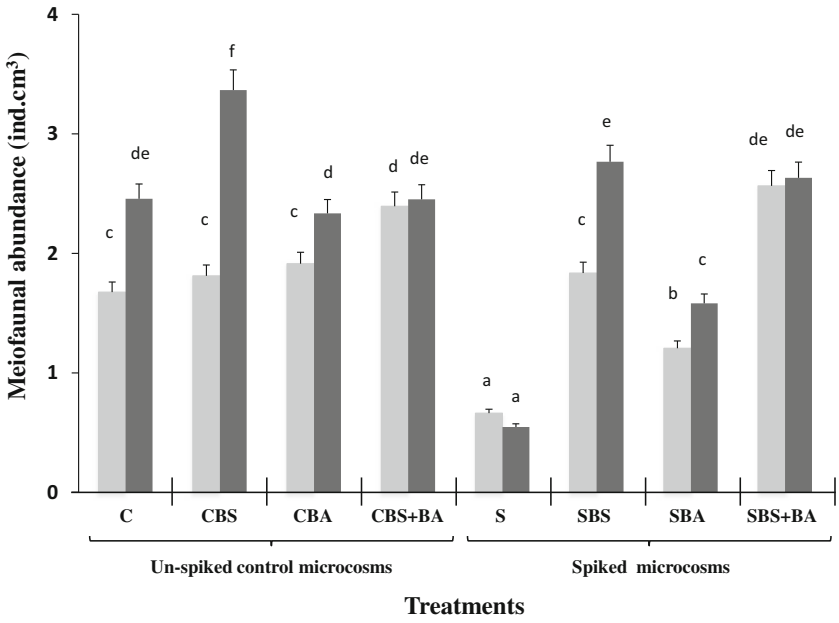

Fig. 4 Meiofauna abundances (average±SD; n=3) in sediments of different treatments (*C* un-spiked control, *S* anthracene-spiked, *BS* biostimulation, *BA* bioaugmentation, *BS+BA* biostimulation and bioaugmentation) after 20 (*gray bars*) and 40 (*black bars*) days of incubation. *C* un-spiked control, *S* anthracene-spiked, *BS* biostimulation, *BA* bioaugmentation, *BS+BA* biostimulation and bioaugmentation. *Letters* refer to homogenous groups according to post hoc Tukey test

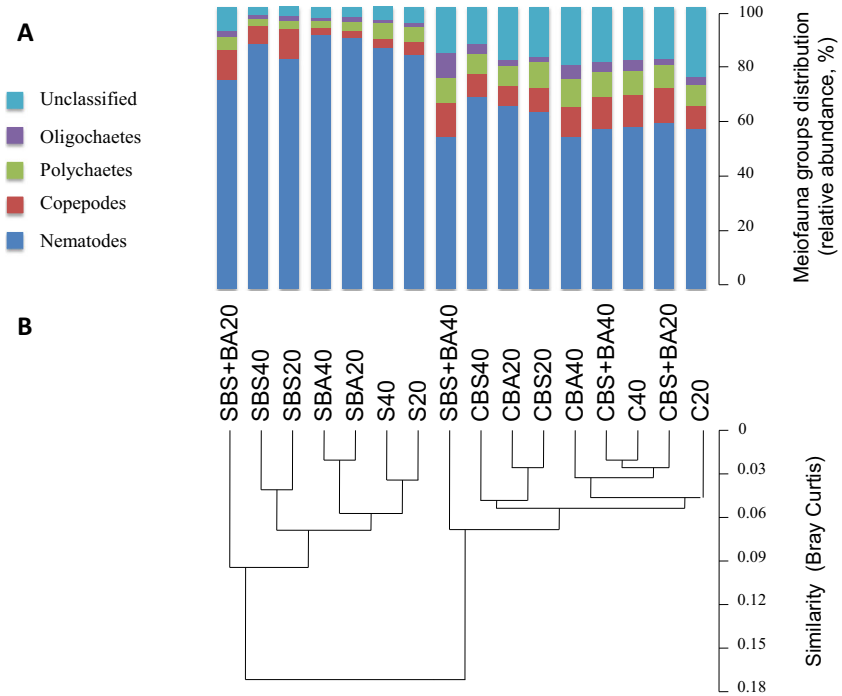

Fig. 5 Comparison of meiofauna community structures. **a** Relative abundance of the major meiofauna taxonomic groups in the different treatments after 20 and 40 days of incubation. **b** Clustering analysis based on Bray-Curtis similarity, Ward linkage method was applied. For (**a**) and (**b**), *C* un-spiked control, *S* anthracene-spiked, *BS* biostimulation, *BA* bioaugmentation, *BS+BA* biostimulation and bioaugmentation. Unclassified corresponds to the meiofauna that could not be sorted in the taxonomic groups

from BS+BA and BA treatments in un-spiked control microcosms (Fig. 6a) while nematodes were associated to the community structures of the BA treatment and copepods to those of BS treatments in anthracene-spiked microcosms (Fig. 6b). These trends were modified with the incubation time. Indeed at 40 days (Fig. 6c, d), nematodes were associated with the bacterial communities from BS and BS+BA treatments in un-spiked control microcosms (Fig. 6c). Interestingly, nematodes were also correlated with the bacterial community of the BS treatments in anthracene-spiked microcosms (Fig. 6d) showing the positive effect of nutrient addition for nematode survival that may probably be due to bacteria-feeding meiofauna. However, in the anthracene-spiked microcosm, the other meiofauna taxa (copepods, oligochaetes, polychaetes, and other) were correlated with the bacterial communities from the BS+BA treatment at 40 days (Fig. 6d) suggesting that this treatment was efficient in reducing anthracene toxicity.

Discussion

In order to evaluate the effect of bio-treatments, for the remediation of PAH contaminated marine sediments, on benthic bacterial and meiofaunal communities, we conducted a microcosm experiment with lightly anthracene-contaminated (5.5± 0.1 μg g^{-1}) sediments from Bizerta lagoon. In a previous attempt, the addition of 10 ppm anthracene in the sediments resulted in the disappearance of major meiofaunal groups including copepods, oligochaetes, and polychaetes. We thus added a lower dose of fresh anthracene (1 ppm) in order to limit the anthracene effect on the meiofauna. Nevertheless, even at low dose, the added anthracene affected both bacterial and meiofaunal communities suggesting that the anthracene initially present in situ permitted the development of bacteria and meiofauna populations sensitive to anthracene. This observation suggested that both bacterial and meiofaunal communities were not completely adapted to hydrocarbon compounds. It is known that anthracene preferentially binds on fine particles (Cornelissen et al. 1998) with slow desorption rates (Brion and Pelletier 2005). Because the studied sediments were dominated by the fine fraction (70 %<63 μm), we assume that the initial anthracene content in sediments was not sufficiently bioavailable to exert a selective pressure on both bacterial and meiofaunal communities as it is usually observed in highly polluted environments (Carman et al. 1995; Paisse et al. 2010). Freshly added contaminants are considered more bioavailable than aged materials (MacRae and Hall 1998; Alexander 2000). Adsorption of PAH molecules on sediments is a slow process, and freshly added PAH are still easily extractable for many days (Brion and Pelletier 2005), resulting in high bioavailability and therefore toxicity that may explain the detrimental effect of the added anthracene observed on both bacterial and meiofaunal communities in our microcosms.

As anthracene degradation was effective in the microcosms, different effects on bacterial and meiofaunal communities were observed according to the bioremediation treatments. Anthracene degradation was observed in all conditions including the un-spiked control microcosms, but a higher

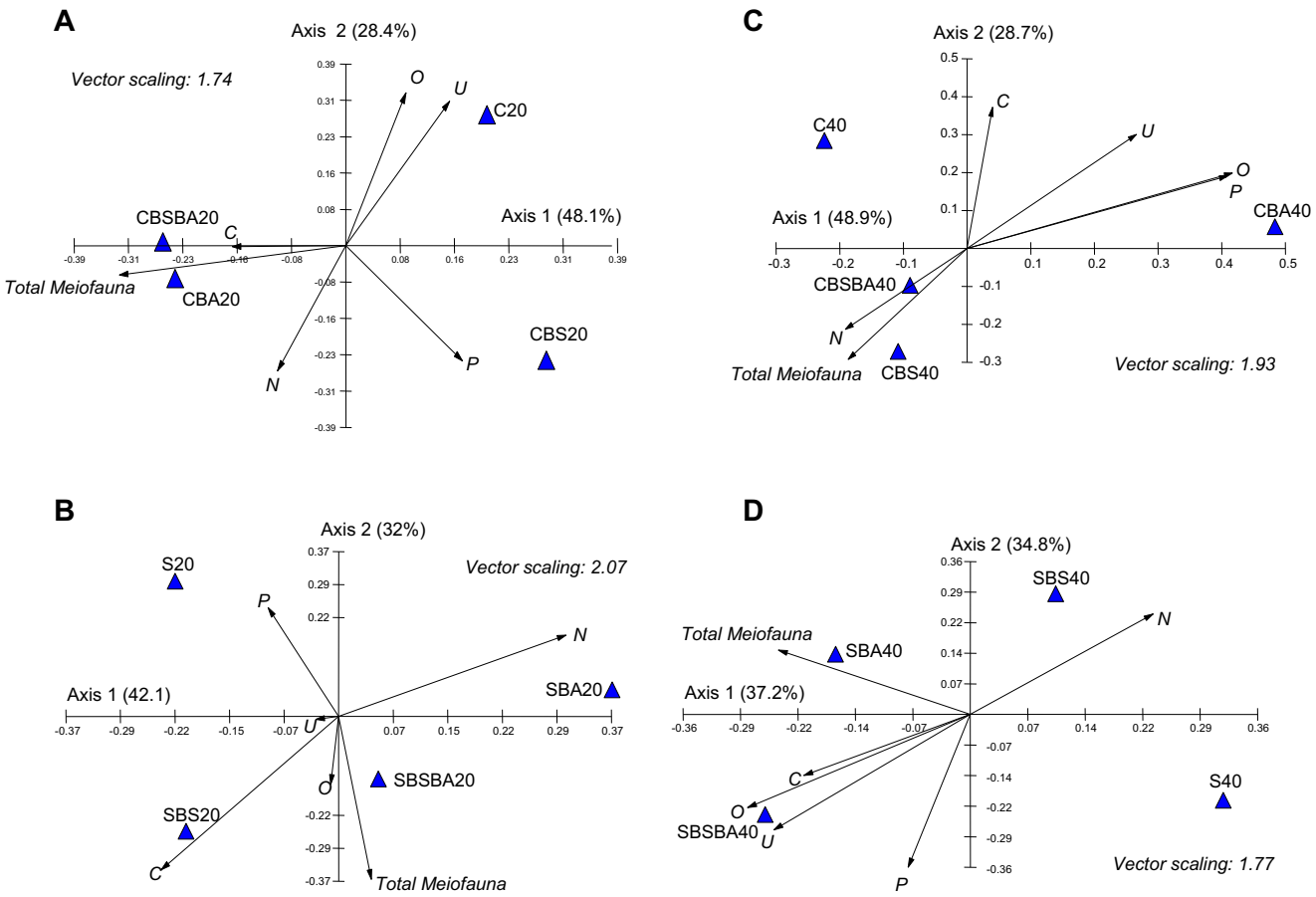

Fig. 6 Canonical correspondence analysis (CCA) correlating T-RFLP community structure patterns based on T-RFLP of 16S rRNA genes [mean profile of replicate samples (n=3) of HaeIII- and HinfI-digested 16S rRNA gene] with the abundances of the main meiofauna taxonomic groups after 20 (**a**, **c**) and 40 (**b**, **d**) days of incubation. *N* Nematodes, *C* copepods, *P* polychaetes, *O* oligochaetes, *U* unclassified. *C* Un-spiked control, *S* anthracene-spiked, *BS* biostimulation, *BA* bioaugmentation, *BS+BA* biostimulation and bioaugmentation

degradation was observed in all anthracene-spiked microcosms. It is likely that the spiking of fresh anthracene favored the degradation of "old" anthracene suggesting the presence of a priming effect as observed in soils for organic matter degradation (Castro-Silva et al. 2013). Overall, the bioremediation treatments obviously increased the anthracene degradation. The bioremediation treatments included biostimulation (nutrients addition), bioaugmentation by the inoculation of *Acinetobacter* sp., a marine PAH-degrading bacterium previously isolated from Bizerta lagoon sediments (Ben Said et al. 2008), and the combination of biostimulation and bioaugmentation. Many studies demonstrated that biodegradation rates were enhanced by the addition of soluble inorganic fertilizer to sediments (Lee et al. 1993; Yu et al. 2005b); by the addition of biostimulators such as Tween 80, silicone oil, pig dung, and NPK fertilizer, alone or in combination (Agarry and Owabor 2011); and the addition of nutrients and/or inoculation of bacterial consortia to PAH-contaminated sediments (Guo et al. 2005; Miyasaka et al. 2006; Louati et al. 2013a, b). In our study, the combination of biostimulation and bioaugmentation was significantly (p<0.05, ANOVA test) the most effective for anthracene biodegradation reaching 72± 2 %, which represents approximately 8 % more than the other treatments (Fig. 1). The anthracene degradation resulted in a reduction of the sediment toxicity restoring the meiofauna abundance, but not the group similarities in comparison with un-spiked control (Fig. 5).

In our study, the bacterial community structures of the bioremediation treatment microcosms were closer at the end (40 days) of incubation (Fig. 3b) than at 20 days (Fig. 3a) suggesting that anthracene affected the bacterial community organization. The presence of anthracene imposed a selection pressure although treatments were performed. Thus, it is likely that the presence of anthracene softens the influence of treatment on bacterial communities indicating that anthracene was a stronger driver of the bacterial community structure than treatments. Similar toxic effects of anthracene on bacterial community structure were reported in soils (MacNaughton et al. 1999; Vinas et al. 2005; Gandolfi et al. 2010; Castro-Silva et al. 2013) and sediments (Flores et al. 2010; Louati et al. 2013a). In our study, the bacterial abundance at 40 days was three times higher in the bioaugmentation treatment than

in the untreated contaminated control (S microcosm) indicating that the bioaugmentation limited the anthracene effect. In the stimulation (SBS) and in the combined (CBS+CBA) treatments, the bacterial abundance was 5.8 times higher than in the untreated contaminated control (S microcosm) restoring the same level observed in the un-spiked control C without addition of anthracene (ca. $5 \pm 0.2 \times 10^7$ cells cm^{-3}). Surprisingly, the bioaugmentation had a negative impact on both bacterial abundance and OTU richness even in un-spiked control microcosms. These results suggest that the addition of bacteria disorganizes the initial bacterial community structure as observed in bioaugmentation studies for the mitigation of creosote-contaminated soil (Simarro et al. 2013) due to the competition for space and resources. Nevertheless, this disorganization was less observed when the exogenous bacteria were added concomitantly with nutrients. This would suggest that the competition between indigenous and exogenous bacteria decreased once nutrients are in excess in the sediment resulting in a slight modification of the community structure. In principle, bioaugmentation is expected to enhance the metabolic potential by increasing the genetic diversity (Dejonghe et al. 2001). However, in our study, the bioaugmentation resulted in a decrease of indigenous bacterial abundance and diversity as observed in bioreactors for the remediation of PAH-contaminated sediments (Launen et al. 2002). It is likely that the inoculated strain was in competition with the autochthonous populations for an ecological niche as already reported (Yu et al. 2005a, b; Vinas et al. 2005; Simarro et al. 2013).

The toxic effects of anthracene on meiofauna community composition were characterized by a large dominance of nematodes in all microcosms (Fig. 5a) as reported for PAH (Lin et al. 2011) and phenanthrene contamination (Louati et al. 2014b). The observed toxic effects on the meiofauna may also be due to secondary metabolites produced during PAH biodegradation (Traczewska 2000). Nematodes are sensitive to the effects of oil and bioremediation treatments because they are in direct contact with contaminants in the interstitial water through their permeable cuticle throughout their life cycle (Schratzberger et al. 2003). Nevertheless, toxicity of PAH may vary according to nematode species. Louati et al. (2014a) observed that anthracene degradation resulted in significant changes of the nematode assemblages with the disappearance of some species (*Mesacanthion diplechma*) and the increase of *Spirinia parasitifera*. The high nematode abundance observed in bioremediation treatments suggests that the decrease of anthracene-sensitive species was counterbalanced by an increase of tolerant species. The biostimulation treatment exhibited the highest meiofauna abundance indicating that the addition of nutrients promoted the bacterial growth that in turn feeds the meiofauna growth. Our results confirm that bioremediation using both addition of fertilizers and addition of hydrocarbonoclastic bacteria can be an attractive solution for enhancing the degradation of anthracene, decreasing thus its potential toxic effects. Such treatments might promote the natural recovery of ecosystems. Additionally, our results confirm the potential use of nematodes for assessing the ecological conditions of coastal environments (Losi et al. 2013) since it was the sole meiofaunal group present in all treatments throughout the present experiment.

Interestingly, the results presented here showed that nematodes were correlated with bacterial community structure in the biostimulation treatment with spiked anthracene. However, in the combination of biostimulation and bioaugmentation treatment, the bacterial community structure was correlated with the other meiofauna taxa (copepods, oligochaetes, polychaetes, and other), confirming that this treatment was efficient in reducing anthracene toxicity. These observations suggested that the bio-treatments, by stimulating autochthonous bacteria, also affected the relationships between bacterial and meiofaunal communities, bacteria feeding the meiofauna. The bacterial community structure and activities are under the influence of meiofaunal groups such as nematodes (De Mesel et al. 2004; Moens et al. 2005), directly as main bacterial predators and indirectly modifying the environmental conditions. An increase of nematode abundance resulted in higher organic matter mineralization (Nascimento et al. 2012). It has been reported that the presence of polychaetes enhanced microbial pyrene mineralization, probably by increasing oxygen supply due to burrow ventilation (Timmermann et al. 2008). In contrast, it was found that the increase of nematode abundance resulted in an increase of hydrocarbonoclastic bacteria grazing and therefore a decrease of PAH degradation efficiency (Naslund et al. 2010). Bacterial community structure can also be influenced by the bioturbation activity without affecting the biodegradation capacities (Stauffert et al. 2013). In a previous study, we demonstrated that biological top-down control by meiofauna was more effective in shaping bacterial community structure than the selective pressure exerted by a PAH cocktail (Louati et al. 2013b). The benthic meiofauna strongly influence microbial structure (Stauffert et al. 2013; Cravo-Laureau and Duran 2014), particularly functional groups such as sulfate-reducing (Stauffert et al. 2014a) and denitrifying (Stauffert et al. 2014b) microorganisms.

Conclusion

In conclusion, addition of a low dose of anthracene had a severe impact on bacteria and meiofauna communities in terms of abundances and diversity. The treatment combining biostimulation and bioaugmentation was the most efficient for anthracene elimination on both freshly and old contaminated sediments, reducing drastically the sediment toxicity and restoring thus the meiofaunal structure. The different treatments

affected the meiofaunal community directly and indirectly by modifying the bacterial communities that in turn influenced the meiofauna. Such treatments provide the opportunity to manipulate the biological communities inhabiting sediments allowing to study the bacteria/meiofauna relationships. The recently developed metagenomic approaches are promising tools that will shed more light on the biological networks involved in hydrocarbon degradation processes in marine sediments.

Acknowledgments This work was supported by a funding of the CMCU program (PHC-UTIQUE, n° 09G 0189), Centre National de la Recherche Scientifique (CNRS), Institut de Recherche pour le Développement (IRD), and the Faculté des Sciences de Bizerte (FSB). We acknowledge the Regional Platform for Environmental Microbiology PREMICE supported by the Aquitaine Regional Government Council (France) and the urban community of Pau-Pyrénées (France). Thanks also to anonymous reviewers for their helpful comments and suggestions.

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