Environ Sci Pollut Res (2014) 21:3670–3679
DOI 10.1007/s11356-013-2330-5

RESEARCH ARTICLE

Biostimulation as an attractive technique to reduce phenanthrene toxicity for meiofauna and bacteria in lagoon sediment

Hela Louati · Olfa Ben Said · Amel Soltani · Patrice Got · Cristiana Cravo-Laureau · Robert Duran · Patricia Aissa · Olivier Pringault · Ezzeddine Mahmoudi

Received: 18 August 2013 / Accepted: 4 November 2013 / Published online: 26 November 2013

Abstract A microcosm experiment was setup to examine (1) the effect of phenanthrene contamination on meiofauna and bacteria communities and (2) the effects of different bioremediation strategies on phenanthrene degradation and on the community structure of free-living marine nematodes. Sediments from Bizerte lagoon were contaminated with (100 mg kg^{-1}) phenanthrene and effects were examined after 20 days. Biostimulation (addition of nitrogen and phosphorus fertilizer or mineral salt medium) and bioaugmentation (inoculation of a hydrocarbonoclastic bacterium) were used as bioremediation treatments. Bacterial biomass was estimated using flow cytometry. Meiofauna was counted and identified at the higher taxon level using a stereomicroscope. Nematodes, comprising approximately two thirds of total meiofauna abundance, were identified to genus or species. Phenanthrene contamination had a severe impact on bacteria and meiofauna abundances with a strong decrease of nematodes with a complete disappearance of polychaetes and copepods. Bioremediation counter balanced the toxic effects of phenanthrene since meiofauna and bacteria abundances were significantly higher ($p<0.01$) than those observed in phenanthrene contamination. Up to 98 % of phenanthrene removal was observed. In response to phenanthrene contamination, the nematode species had different behavior: *Daptonema fallax* was eliminated in contaminated microcosms, suggesting that it is an intolerant species to phenanthrene; *Neochromadora peocilosoma*, *Spirinia parasitifera*, and *Odontophora* n. sp., which significantly ($p<0.05$) increased in contaminated microcosms, could be considered as "opportunistic" species to phenanthrene whereas *Anticoma acuminata* and *Calomicrolaimus honestus* increased in the treatment combining biostimulation and bioaugmentation. Phenanthrene had a significant effect on meiofaunal and bacterial abundances ($p<0.05$), with a strong reduction of density and change in the nematode communities. Biostimulation using mineral salt medium strongly enhanced phenanthrene removal, leading to a decrease of its toxicity. This finding opens exciting axes for the future use of biostimulation to reduce toxic effects of PAHs for meiofauna and bacteria in lagoon sediment.

Keywords Phenanthrene · Biostimulation · Bacteria · Meiofauna · Free-living nematodes · Community structure · Bizerte lagoon

Responsibility editor: Philippe Garrigues

H. Louati (✉) · O. B. Said · A. Soltani · P. Aissa · E. Mahmoudi
Laboratoire de Biosurveillance de l'Environnement, Faculté des Sciences de Bizerte, Bizerte, Tunisia
e-mail: hela.louati@yahoo.fr

O. B. Said · A. Soltani · C. Cravo-Laureau · R. Duran
Equipe Environnement et Microbiologie–UMR CNRS IPREM 5254- IBEAS, Université de Pau et des Pays de l'Adour, Pau, France

H. Louati · P. Got · O. Pringault
Laboratoire Ecosystèmes Marins Côtiers, UMR 5119 CNRS-UM2-IFREMER-IRD-ECOSYM, Station Méditerranéenne de l'Environnement Littoral, 2, rue des chantiers, 34200 Sète, France

Introduction

Coastal marine ecosystems are often contaminated by PAHs (Louati et al. 2001; Soclo et al. 2000), and the biota is affected by this pollution. The toxicity and lethality of PAHs have been assessed for a variety of marine organisms such as fish, copepods, and amphipods (Engraff et al. 2011; Lotufo 1997;

Shailaja and D'Silva 2003). Some of these compounds (e.g., pyrene, anthracene, phenanthrene) are of major public concern due to their toxicity to organisms in carcinogenic and mutagenic potential. Phenanthrene, one of the most abundant PAHs in the environment (Cerniglia 1993), is included in the U.S. Environmental Protection Agency list of priority pollutants (Keith and Telliard 1979). Since phenanthrene is the smallest tricyclic aromatic hydrocarbon to have a "bay-region" and a "K-region" (Ouyang 2006), i.e., highly reactive regions of PAH molecules where the main carcinogenic species can be formed, it is commonly used as a model substrate for studies on metabolism of carcinogenic PAHs (IARC 2010). Phenanthrene was chosen as the model compound since it exhibits intermediate toxicity, hydrophobicity, and environmental persistence (Stringer et al. 2012).

Once in aquatic systems, PAHs tend to adsorb on particles and accumulate in sediments, and undergo various degradation, transformations, and sequestration (Haritash and Kaushik 2009; Yang et al. 2010). Biodegradation under aerobic or anaerobic condition is a major process for PAH removal (Hale et al. 2010; Ulanowicz et al. 2009). Nevertheless, natural attenuation cannot appreciably remove pollutants mostly because nutrient limitation is one of the major factors limiting biodegradation of PAHs in sediments (Smith et al. 1998). For this reason, increasing attention has been directed toward the research of new strategies and environmental-friendly technologies to be applied for the remediation of sediments contaminated by hydrocarbons. Among these, biotechnological strategies based on the biostimulation of autochthonous microbial communities to speed up biodegradation processes of organic pollutants are of particular relevance (Beolchini et al. 2010). Generally, mineralization of organic matter is enhanced and bacterial production stimulated in the presence of meiofauna (Gerlach 1978). Meiofaunal assemblages are ideal for microcosm experiments. They have a short generation time, a high density, and continuous reproduction (Suderman and Thistle 2003). These small animals are also easily maintained and sensitive to many toxicants (Boufahja et al. 2011; Mahmoudi et al. 2007). Free-living nematodes, the most abundant taxa among the meiofauna (defined here as microscopic metazoan invertebrates passing through 1 mm mesh size and retained on 40 μm mesh size sieves), are relevant indicators of environmental perturbation (e.g., Beyrem and Aissa 2000; Burton et al. 2001). Field and laboratory studies have documented that the meiofaunal component of the benthos is sensitive to petroleum contaminants (Louati et al. 2013b; Mahmoudi et al. 2005) and that meiobenthic nematode are relatively more susceptible to petroleum hydrocarbons than other meiofaunal taxa, such as copepods (Beyrem and Aissa 2000; Lindgren et al. 2012; Mahmoudi et al. 2005).

Biological effects of phenanthrene have been examined on many taxa such as diatoms, gastropods, mussels, and fish (Albers 2003; Ana et al. 2007; Einsporn and Koehler 2008), but the influence of this contaminant on benthic communities is poorly understood, and no experimental study has assessed the impacts of phenanthrene contamination on Mediterranean benthic organism assemblages. In the present study, we present the results of a microcosm experiment designed to compare the response of Mediterranean benthic nematodes and bacteria facing contamination of phenanthrene as a function of the bioremediation treatments used for PAH biodegradation. The investigation focused on the comparison of densities, diversity, and species composition of nematode assemblages from control microcosm and phenanthrene bioremediation treatments. Bioremediation schemes included biostimulation and the combination of biostimulation with bioaugmentation by the inoculation of a marine PAH degrading bacterium, *Bacillus megaterium*, previously isolated from Bizerte lagoon contaminated sediment (Ben Said et al. 2008).

Materials and methods

Field site

Natural meiobenthic communities were collected from Bizerte lagoon (Tunisia) on Mars 2010 (Fig. 1). Hand-cores of 10 cm^2 were used to a depth of 15 cm to transfer sediment into a bucket. The physical–chemical characteristics of the field site on the sampling day were: water depth 1.3±0.2 m; salinity 36.2±1.2 PSU; temperature 15.3±0.2 °C; water pH 8.1±0.3; dissolved oxygen content 8.3±1.5 mg l^{-1}. The sediment had a median particle diameter of 0.43±0.12 mm and an organic content of carbon 0.79±0.02 %. Sediment total nitrogen content was 1.03±0.01 %.

On return to the laboratory, sediments were homogenized by gentle hand stirring with a large spatula before they were

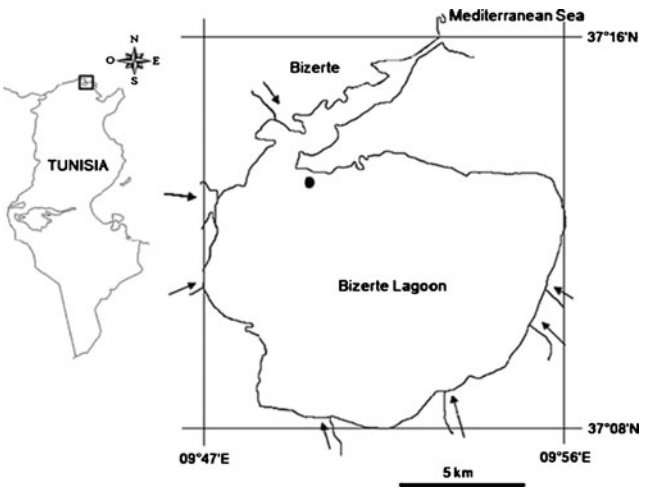

Fig. 1 Map of Bizerte lagoon showing the site from which sediment was collected (Echaraà)

used for phenanthrene contamination and bioremediation or microcosm filling.

Phenanthrene contamination of sediments

Stock solution of phenanthrene (Sigma–Aldrich Chemical A8, 920–0) was prepared in acetone (5 mg mL^{-1}). Next, phenanthrene solution was added into the sediment slurry and shaken overnight to let the PAH adsorbed onto the sediments. Final concentration of phenanthrene in sediment was 100 mg kg^{-1} of dry sediment. Sediment used for phenanthrene contamination was first alternately frozen and thawed three times to defaunate it (Gyedu-Ababio and Baird 2006), and then it was wet-sieved to remove the larger particles (>63 μm).

Microcosm experiment

Microcosms consisted of 1,600 ml glass bottles. One control and three treatments (Table 1) with three replicates each were set up. Contaminated microcosms were gently filled with 200 g (wt) of homogenized sediment (100 g of natural sediment and 100 g contaminated sediment) topped up with 1 L of filtered (1 μm) natural lagoon water at 30 PSU. In control microcosm, the contaminated sediment was replaced by 100 g of the defaunated sediment. Each bottle was stoppered 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 phenanthrene contamination. Biostimulation was achieved by amending two types of compositions: (1) slow-release particle fertilizers (70 mg kg^{-1} of nitrogen fertilizer ($NaNO_3$) and 35 mg kg^{-1} of phosphorus fertilizer (KH_2PO_4) and (2) mineral salt medium (MSM) using the protocols of Yu et al. (2005) and Jacques et al. (2008). The MSM has the following composition (milligrams per liter): $(NH_4)_2SO_4$, 1,000; K_2HPO_4, 10,000; KH_2PO_4, 5,000; $MgSO_4$ $(H_2O)_7$, 200; $CaCl_2$ $(H_2O)_2$, 100; and trace elements made up of $FeSO_4$ $(H_2O)_7$, 5; $MnSO_4$ (H_2O), 3; $CuSO_4$ $(H_2O)_5$, 0.015; $(NH_4)_6Mo_7O_{24}$ $(H_2O)_7$, 1; Na_2MoO_4 $(2H_2O)_2$, 0.01. Bioaugmentation was achieved by inoculating with a marine PAH-degrading bacterium, *B. megaterium* strain isolated from Bizerte-polluted sediments (Ben Said et al. 2008).

Table 1 Experimental treatments

Experimental treatment	Name
Control sediment	T
Contaminated sediment with phenanthrene (100 ppm)	C
Contaminated sediment with phenanthrene+ N+P	CBS1
Contaminated sediment with phenanthrene +MSM	CBS2
Contaminated sediment with phenanthrene + N+P+ bacteria	CBS1+BA
Contaminated sediment with phenanthrene + MSM+ bacteria	CBS2+BA

The strain was previously grown in 50 ml of LB broth. After 1 week cultivation, cells were harvested by centrifugation at 10,000×*g* for 15 min at 4 °C. The initial inoculum was 6.34×10^8 cell ml^{-1}. For the combination of biostimulation and bioaugmentation, cells of bacterial strain were suspended in the two types of biostimulation treatments (1: N+P; 2: MSM), and 2 ml of the culture was introduced into the microcosms previously amended with nutrients. After bacterium inoculation and nutrient addition, sediment was agitated for half an hour for homogenization. All microcosms were incubated in laboratory at room temperature (22–24 °C). After 20 days of incubation, microcosm sediments were fixed in 4 % formalin.

Phenanthrene analysis

Phenanthrene analysis in the sediments was conducted by gas chromatography (GC). At the end of incubation, sediment were homogenized, and samples of 1 g (dry weight) of each microcosm was extracted with 40 ml of acetone/hexane (*v*/*v*) and with 2,2,4,4,6,8,8-heptamethylnonane as internal standard, in an ultrasonic bath (15 min). The GC (GC Agilent Technologies) was equipped with a flame-ionization detector (290 °C) and a capillary column HP 5 (30 m×320 μm×0.25 μm, Hewlett-Packard, Palo Alto, CA, USA). The injector temperature was maintained at 280 °C. The carrier gas (He) was maintained at 1.7 ml/min. The oven temperature was programmed from 60 °C (1 min) to 200 °C (1 min) with a ramp of 15 °C/min, and then to 300 °C (2 min) with a ramp of 5 °C/min.

Flow cytometry measurements

Bacteria were extracted from sediment 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 a 2.6 % (final concentration of work solution) and incubated in the dark 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 FACS Calibur 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.

Sample processing

Meiofauna samples were rinsed with a gentle jet of freshwater over a 1 mm sieve to exclude macrofauna, decanted over a 40 μm sieve, and stained with Rose Bengal (0.2 g l^{-1}). Meiofauna was counted and identified at the higher taxon level using a stereomicroscope. Nematodes, comprising approximately two thirds of total meiofauna abundance, were identified to genus or species using the pictorial keys of Platt and Warwick (1983, 1988) and Warwick et al. (1998).

Data processing

The majority of data analysis followed standard community analysis methods described by Clarke (1993) and Clarke and Warwick (2001) using the Plymouth Routines in Multivariate Ecological Research (PRIMER) software package.

Univariate indices were computed: total nematode abundance (I, ind. microcosm^{-1}), number of species (S), diversity (Shannon-Wiener index H=loge), species richness (Margalef's d) and evenness (Pielou's J) were calculated for each microcosm. The one-way ANOVA was used to test for overall differences between these indices, and the Tukey HSD multiple comparisons test were used in pairwise comparisons of treatments and control. A significant difference was assumed when $P<0.05$. For statistical analysis of nematode community structure, relative abundances of nematodes were transformed with arcsin (x^0.5) to get a normal distribution of data. Principal component analysis (PCA) was performed with MVSP v3.12d software (Kovach Computing Service, Anglesey Wales). Pairwise analysis of similarities (ANOSIM) was carried out to determine if there were any significant differences between nematode assemblages in different treatments. SIMPER (Bray-Curtis similarity index) was used to determine the contribution of individual species towards similarity between treatments and control.

Results

Phenanthrene removal

During 20 days biodegradation experiment, the percentage of phenanthrene (Phe) removal in the contaminated microcosms C was very small (20±4 %) (Fig. 2). In contrast, phenanthrene was significantly removed when bioremediation treatments were used, although the efficiency varied between treatments of biostimulation. Biostimulation with mineral salt medium (CBS2) strongly enhanced phenanthrene removal, with up to

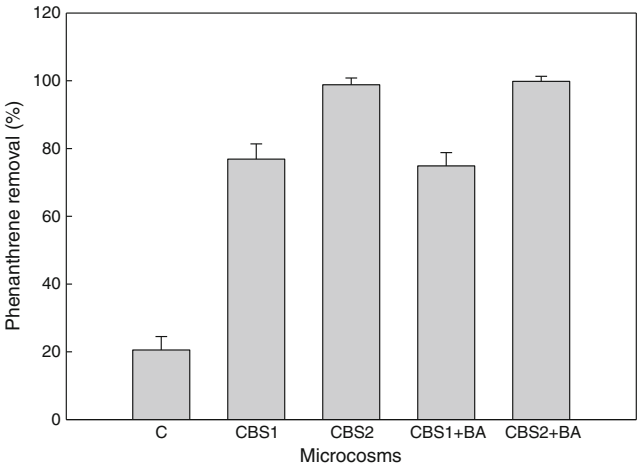

Fig. 2 Removal of phenanthrene (Phe) in the sediment according to different treatments (*T* uncontaminated, *C* contaminated, *CBS* biostimulation; *CBS+BA*: biostimulation and bioaugmentation) after 20 days of incubation (average±SD, $n=3$)

98±0.2 % whereas Phe removal was lower in CBS1 when nitrogen and phosphorus fertilizer were used (76±0.4 %) (Fig. 2). Combination of biostimulation and bioaugmentation did not significantly enhance PAH removal in comparison to the biostimulation protocols.

Bacterial and meiofaunal abundance

After 20 days of incubation, phenanthrene contamination resulted in significant differences in the benthic bacterial abundances relative to the T microcosms (Fig. 3, upper panel). In T microcosms, bacterial abundance averaged $3.84\pm0.29\times10^7$ cells cm^{-3}. Phenanthrene contamination had a significant effect on bacterial abundance which was reduced in C microcosms ($1.65\pm0.18\times10^7$ cells cm^{-3}) relative to T microcosms. Nevertheless, biostimulation CBS and biostimulation coupled with bioaugmentation (CBS+BA) resulted in a significant increase ($P<0.001$) of indigenous bacterial abundance relative to C microcosms irrespective of the type of nutrients added. Interestingly, bacterial abundance in CBS1 was lower relative to CBS2 microcosms ($4.6\pm0.46\times10^7$ and $7.66\pm0.62\times10^7$ cells cm^{-3}, respectively; Fig. 3, upper panel). The highest value of bacterial abundance was observed in combination of both treatments of bioremediation CBS2+BA ($8.81\pm0.57\times10^7$ cells cm^{-3}) corresponding to a twofold increase relative to the control microcosm.

In T microcosms, after 20 days of incubation, total meiofauna represented on average 513±4 Ind/microcosm with the following repartition, 91 % nematodes, 7 % copepods, and 2 % polychaetes (Fig. 3, lower panel). Contamination by the phenanthrene had a clear effect on meiofauna with a strong reduction of total density (72±2 vs 513±4 Ind./microcosm, for C and T microcosms, respectively) together with a complete disappearance of polychaetes and copepods. As a consequence, nematodes represented the

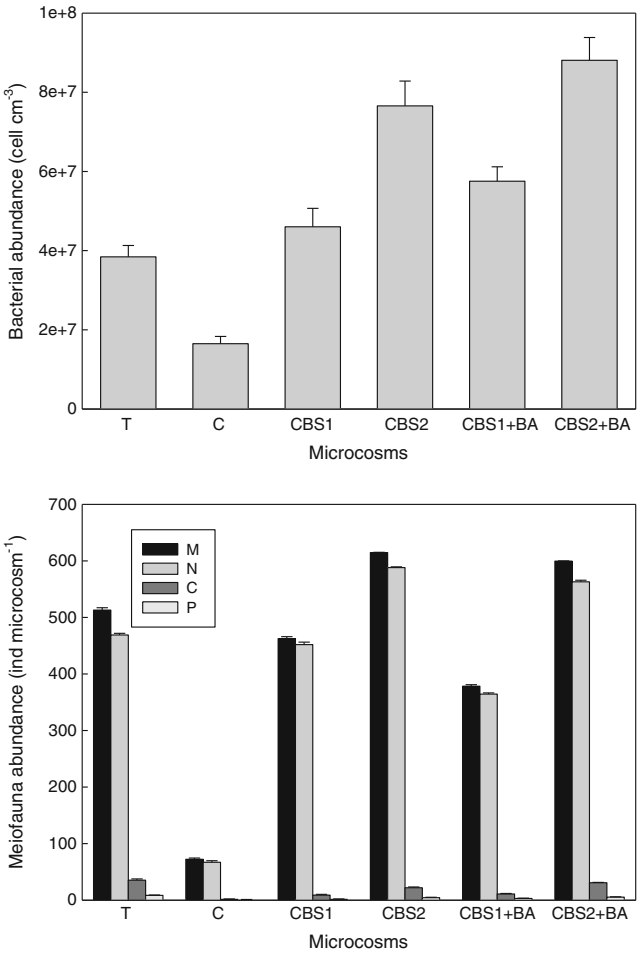

Fig. 3 Bacterial and meiofaunal abundances in the sediment of different microcosms (*T* uncontaminated, *C* contaminated, *CBS* biostimulation; *CBS+BA*: biostimulation and bioaugmentation) at the end of the 20-day incubation. U*pper panel*: Bacterial abundance determined by flow cytometry (average±standard deviation). *Lower panel*: Absolute abundance (individuals per microcosm) and standard deviation of total meiofauna (M) and major groups: nematodes (N), copepods (C), and polychaetes (P)

single main taxon identified in C microcosm. In contrast, both biostimulation treatments strongly enhanced meiofaunal density compared with C microcosms. The biostimulation when mineral salt medium were used (CBS2) resulted in higher densities relative to the CBS1 treatment with nitrogen and phosphorus fertilizer (614±0.7 vs 462±3 Ind/microcosm, respectively). Nevertheless, this increase was observed for nematodes; the density of copepods and polychaetes remained similar to that of the T microcosms.

Nematofauna diversity

Diversity of the nematode community and univariate indices

A total of 20 nematode species were recorded in all the microcosms (Table 2). All microcosms except C replicates were dominated by *Oncholaimus campylocercoides*. Further in the control microcosm T, *Daptonema fallax* (13 %) and *Neochromadora peocilosoma* (9 %) were the two next most frequent species besides *O. campylocercoides* (40 %). Significant differences between control (T) and contaminated microcosms (C) mainly resulted from changes in the abundances of the most dominant species (Table 3). Elimination of *D. fallax*, decreasing abundance of *O. campylocercoides* and increasing numbers of *N. peocilosoma, Spirinia parasitifera*, and *Odontophora* n. sp. were responsible for the significant difference between T and C microcosms. Use of bioremediation treatment resulted in a decrease of the effects of phenanthrene contamination on free-living nematodes. Only biostimulation (CBS2) treatments resulted to a similar community structure observed in T microcosms. Differences were observed for the other treatments. Combination of both treatments resulted in different community structures, especially for CBS1+BA treatment with dominance of *Anticoma acuminata* which showed a very strong increase from 1 % (T) to 30 % (CBS1+BA). Increase in *Calomicrolaimus honestus* (3±0.5 % to 12±1 %) was responsible for significant differences between (T) and bioremediation microcosms (CBS2+BA).

Mean values of univariate indices for nematodes as a function of treatments are given in Table 4. Phenanthrene contamination resulted in significant changes of univariate measures for all indices except for the eveness. Total nematode abundance (*I*), species richness (*d*), diversity (H'), and number of species (*S*) decreased significantly with phenanthrene contamination (Table 5). In contrast, these univariate indices were not affected in all bioremediation microcosms except the abundance that was significantly higher in biostimulation and combination treatments (CBS2 and CBS2+BA) than in the control and contaminated microcosms. Biostimulation treatments strongly enhanced the density of nematodes compared with the T microcosms (588±1.4 vs 469±2.8 Ind/microcosm, for CBS2 and T microcosms, respectively).

Distributional plots

The *k*-dominance curves (Fig. 4) illustrate a clear effect of phenanthrene on nematode community. An increase of dominance concomitant with a decrease of diversity was obvious at this phenanthrene contamination level. Strong changes in *K* dominance were also observed in CBS1 and CBS1+BA where only two species accounted for more 75 % of the total community. In T microcosms, the nine most dominant species represented 75 % of the total community whereas for C microcosms only five dominant species accounted for 75 % of the total community. Biostimulation treatment using a

Table 2 Relative abundance (±SD) for nematode species identified in microcosms

	T	C	CBS1	CBS2	CBS1+BA	CBS2+BA
Anticoma acuminata	1.1±0.1	0.6±0.0	0.8±0.1	0.6±0.1	**30±1.5**	6.0±1.1
C. honestus	3.3±0.5	6.9±1.0	1.0±0.0	2.3±0.5	0.6±0.1	**11±1.5**
Chromadora nudicapitata	3.0±0.8	0.3±0.0	0.6±0.0	1.1±0.1	0.3±0.0	1.6±0.4
D. fallax	**13±2.0**	0	1.0±0.0	**20±1.5**	2.0±0.4	**11±1.4**
Desmodora n. sp.	1.6±0.3	0.3±0.0	0.6±0.1	1.1±0.0	0.6±0.1	1.0±0.0
Enoplolaimus longispiculosus	2.5±0.2	0.3±0.1	2.5±0.4	0	1.3±0.4	1.6±0.1
Marylynia stekoveni	0.9±0.0	0.3±0.0	1.6±0.2	0.6±0.1	0.6±0.0	1.0±0.0
Metachromadora macroutera	3.0±0.5	0.3±0.0	4.0±0.3	3.9±0.8	0	0
Mesacanthion diplechma	2.0±0.3	0	2.8±0.2	1.9±0.2	1.3±0.5	0
Odontophora n. sp.	4.8±1.1	**14±1.5**	4.0±0.1	2.7±0.4	1.0±0.0	5.6±0.1
N. peocilosoma	9.3±1.5	**40±2.5**	2.0±0.1	7.1±1.1	3.6±0.5	8.8±1.0
O. campylocercoides	**39±2.0**	9.0±1.5	**67±2.5**	**45±2.5**	**53±1.1**	**38±3.0**
Paracomesoma dubium	0.7±0.0	0	0.8±0.1	0.5±0.0	0.5±0.0	0
Paramonystera pilosa	2.4±0.1	2.5±0.4	1.6±0.2	2.1±0.3	1.3±0.1	1.7±0.1
Prochromadorella neapolitana	0.8±0.0	1.6±0.5	0.6±0.0	1.1±0.0	0.8±0.2	0.4±0.0
S. parasitifera	5.3±1.5	**18±1.0**	3.6±0.9	2.0±0.4	0	5.7±0.6
Synonchiella edax	0.9±0.1	0.3±0.1	0.9±0.1	1.2±0.1	0.6±0.1	0.1±0.0
Thalassironus britannicus	1.3±0.1	0.8±0.1	0.6±0.1	0.4±0.0	0.3±0.0	0.5±0.0
Terschellingia longicaudata	2.0±0.4	1.0±0.0	1.8±0.2	1.3±0.1	0.6±0.1	1.1±0.0
Viscosia sp.	1.5±0.1	2.3±0.1	1.0±0.0	3.5±0.9	0.6±0.0	0.6±0.0

Values of relative abundance are means of three replicates per treatment. Abundances of dominant species (>10 %) are indicated in bold

T uncontaminated, *C* contaminated, *BS* biostimulation, *BS+BA* biostimulation and bioaugmentation

combination of different nutrient (CBS2) changed moderately, relative to the control; the number of dominant species, on average eight species, accounted for 75 % of the total community.

Multivariate analysis

The PCA analysis, based on relative abundance of species (Fig. 5), illustrates a strong impact of phenanthrene contamination on nematode assemblages. Contaminated microcosms (C) are distinct from other microcosms with dominance and disappearance of specific species. The replicates of biostimulation treatments (CBS2) are grouped with the control T.

Additionally, the ANOSIM results showed a significant impact of phenanthrene contamination on nematode assemblages. All microcosms were significantly different from the control and from each other (*R* statistic=1, significance level=2.9 %) except for T and CBS2 microcosms. Bray-Curtis similarity index reveals the lowest average similarity was recorded between C and T microcosms (Table 6). In contrast, the highest value of average similarity was observed between control and CBS2 microcosms (Table 6).

Discussion

This study has shown that phenanthrene is toxic for both meiofauna and bacteria in sediment from Bizerte lagoon. Field and laboratory studies have documented that the meiofaunal and bacterial components of the benthos is sensitive to petroleum contaminants (Mahmoudi et al. 2005; Sundback et al. 2010; Zhou et al. 2009). Bacterial abundance

Table 3 Species responsible for differences between control and treated microcosms based on similarity percentages (SIMPER) analysis of square-root transformed data

C	CBS1	CBS2	CBS1+BA	CBS2+BA
N. peocilosoma (+)	*O. campylocercoides* (+)	*D. fallax* (+)	*A. acuminata* (+)	*C. honestus* (+)
O. campylocercoides (−)	*D. fallax* (−)	*O. campylocercoides* (+)	*O. campylocercoides* (+)	*A. acuminata* (+)
D. fallax (elim)	*N. peocilosoma* (−)	*S. parasitifera* (−)	*D. fallax* (−)	*D. fallax* (−)

Species accounting for ~70 % of overall dissimilarity between treatment groups are ranked in order of importance of their contribution to this dissimilarity

Plus sign more abundant, *minus sign* less abundant, *elim* elimination

Table 4 Univariate indices for nematode assemblages from each microcosm

Microcosm	*I*	*H′*	*d*	*J′*	*S*
T	469±2.8	2.17±0.1	3.6±0.3	0.75±0.0	20±1.5
C	67±2.8	1.80±0.1	2.3±0.2	0.73±0.0	11±1.1
CBS1	452±4.2	1.47±0.0	3.4±0.2	0.52±0.0	17±1.0
CBS2	588±1.4	1.88±0.0	3.4±0.0	0.66±0.1	19±0.0
CBS1+BA	364±2.1	1.39±0.0	3.5±0.4	0.52±0.1	14±0.5
CBS2+BA	563±0.5	2.01±0.1	3.3±0.4	0.74±0.0	15±1.0

I abundance, *H′* Shannon-Weaver index, *d* species richness, *J′* evenness, *S* number of species

and community structure changes as response to PAH addition have been often observed using a single molecule, e.g., anthracene (Louati et al. 2013a), phenanthrene (Muckian et al. 2009), or a complex mixture of PAH (phenanthrene, fluoranthene, and benzo(K)fluoranthene) (Verrhiest et al. 2002). Similarly, in our study, we observed a strong decrease of meiofauna total density in contaminated microcosms concomitant with a high reduction in the abundance of nematodes and a complete disappearance of polychaetes and copepods. This variable response of meiobenthos to the phenanthrene contamination suggests that resistance mechanisms have been developed in nematodes to face PAH contamination. In this context, molecular studies are needed to isolate the resistance genes of marine nematodes to face pollutants, as it has been done with soils nematodes (Broeks et al. 1996; Cui et al. 2007). However, for the other groups of meiofauna (polychaetes and copepods), their complete disappearance suggests a non-tolerance to the high dose of phenanthrene used in this study. As phenanthrene contamination severely affected the repartition of major meiofauna taxa, competition for space and resources might have favored phenanthrene-tolerant species due to the disappearance of non-tolerant species. Similarly, Lotufo (1997) also found that relatively high-level phenanthrene contamination may cause many ecologically important impacts on copepod community with modification of

Table 5 Multiple comparison tests for significant differences between nematode assemblages as a function of the treatment

Microcosm	Effects of treatment on univariate indices for nematode assemblages				
T vs C	I: −	H': −	d: −	J': ns	S: −
T vs CBS1	I: −	H': −	d: ns	J': −	S: ns
T vs CBS2	I: +	H': −	d : ns	J': −	S: ns
T vs CBS1+BA	I: −	H': −	d: −	J': −	S: −
T vs CBS2+BA	I: +	H': −	d: ns	J': ns	S: ns

The T microcosms were considered as reference

Plus sign increase in univariate measure, *minus sign* decrease in univariate measure, *ns* no significant difference at $p<0.05$

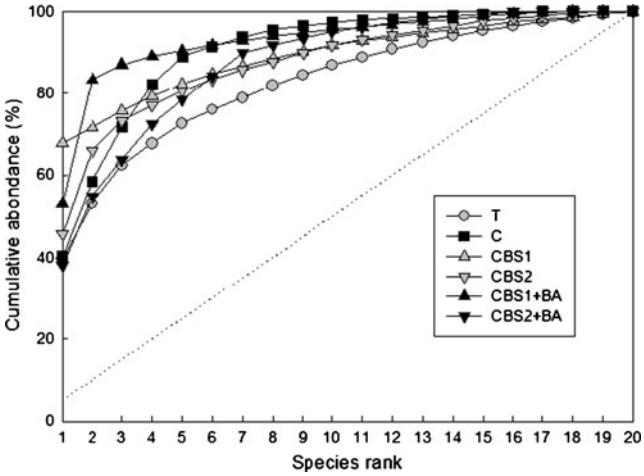

Fig. 4 *k*-dominance curves of the nematode communities as a function of bioremediation treatment after 20 days of incubation. (*T*: uncontaminated, *C*: contaminated, *CBS*: biostimulation, *CBS+BA*: biostimulation and bioaugmentation). The *dotted line* represents an equal distribution of each species

distribution and abundance of *Schizopera knabeni* in heavily contaminated sites.

Despite the nematode group was still observed in contaminated microcosms, the negative effect of phenanthrene contamination is obvious. The univariate descriptors of diversity in the contaminated microcosms as well as the *k* dominance were significantly reduced in comparison with controls (Tukey's HSD test, $p<0.05$). The toxic effects of phenanthrene observed on abundance were also accompanied by strong changes in nematode community structure (Table 2). We selected phenanthrene as a reference PAH due to its relatively high solubility and high toxicity to benthic organisms. Our results confirm the observations made by Mahmoudi et al. (2005) who showed that exposure to a mixture of PAH (diesel) altered the response of nematode communities. Changes in nematode abundance and diversity were accompanied by modification of the structure. Control microcosms were mainly dominated by three main species, *O. campylocercoides*, *D. fallax*, and *N. peocilosoma* whereas in contaminated microcosms the dominant species were *N. peocilosoma, S. parasitifera*, and *Odontophora* n. sp. Such changes in the dominance repartition were followed by significant modifications of the nematode structure as revealed by the PCA analysis (Fig. 5). The differences observed between C and T microcosms were partly explained by the elimination of *D. fallax* suggesting that this species might be sensitive to phenanthrene. This species has been reported as an opportunistic species upon a low diesel contamination (5 ppm) in Ghar El Melh lagoon (Tunisia) (Mahmoudi et al. 2005) but was eliminated for diesel concentrations above 5 ppm. Similarly, *Oncholaimus campylocercoïdes* was significantly affected by phenanthrene; nevertheless, it was not eliminated; therefore this species can be categorized as

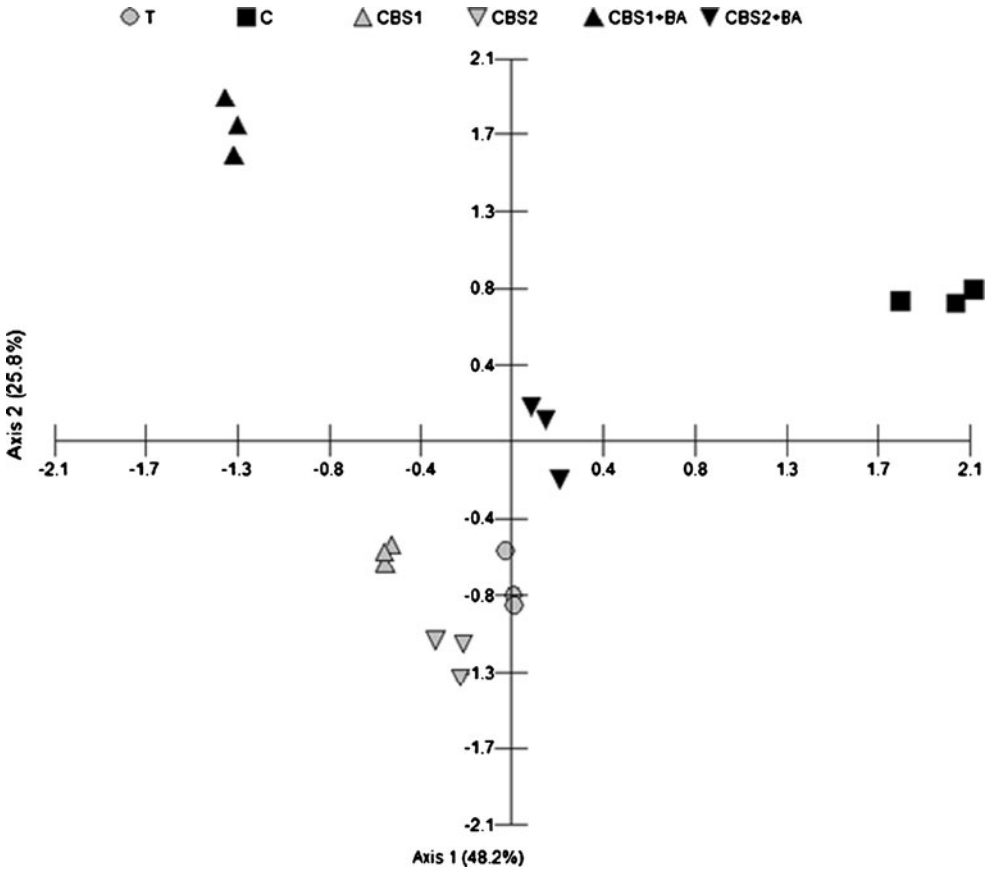

Fig. 5 Correspondence analysis (CA) of nematode species absolute abundance data from uncontaminated sediment control microcosms (*T*), contaminated microcosms (*C*), and bioremediation treatments (CBS1; CBS2; CBS1+BA; CBS2+BA)

"phenanthrene-sensitive." In contrast, *N. peocilosoma*, *S. parasitifera* and *Odontophora* n. sp., characterized by increased abundances in contaminated microcosms, appeared to be "opportunistic" species to phenanthrene. This variable response of different nematode species to phenanthrene suggests that the development of free-living nematodes in polluted environments is subject to very precise control mechanisms to face contamination. Due to their sensitivity to contaminants at environmentally relevant concentrations, the use of free-living nematodes as bioindicator organisms in ecological risk assessments is often proposed to evaluate the impacts of hydrocarbons, lubricants, metals, and other xenobiotic contamination and their bioavailability in aquatic systems (Beyrem et al. 2010; Hedfi et al. 2007; Mahmoudi et al. 2007).

The present study showed that phenanthrene removal was minimal in C microcosms suggesting the low capacity of indigenous microorganisms to degrade phenanthrene in sediment in the experimental conditions imposed. However, biostimulation with addition of mineral salt medium (combination of different nutrient) and the combination of biostimulation and bioaugmentation (CBS2+BA) can efficiently remove phenanthrene (98 %). This nutrient composition was more efficient to remove phenanthrene than the addition of nitrate and phosphate (CBS1 and CBS+BA). In biostimulation treatments, the toxic effects of phenanthrene on meiofauna and bacteria observed in C microcosms were removed. Indeed, bacterial and meiofauna abundances strongly increased in both bioremediation treatments, with significantly higher densities than those observed in T and C microcosms. The

Table 6 Average (standard deviation, $n=3$ replicates) similarity between microcosms

Average similarity (%)	T	C	CBS1	CBS2	CBS1+BA	CBS2+BA
T	84.7 (3.3)					
C	40.8 (3.8)	90.2 (2.2)				
CBS1	66.2 (1.9)	27.3 (3.3)	89.8 (2.3)			
CBS2	80.2 (3.2)	32.2 (2.9)	67.0 (2.6)	88.1 (1.0)		
CBS1+BA	56.0 (1.7)	20.8 (2.4)	66.0 (1.8)	60.3 (2.4)	93.2 (0.3)	
CBS2+BA	77.4 (3.2)	43.4 (2.0)	57.5 (1.6)	68.1 (1.0)	57.1 (3.3)	88.6 (1.2)

effectiveness of these strategies of bioremediation varies from sediments to sediments and from contaminants to contaminants (Balba et al. 1998). Biostimulation has long been used as a strategy to enhance the biodegradation rate of contaminants in nutrient limited environments (Yu et al. 2005), especially in environments such as Bizerte sediments where nutrients are often limiting (Hlaili et al. 2006). Nevertheless, the changes observed in the *K* dominance indicate that biostimulation can affect nematode diversity even so the structure was relatively similar in T and biostimulation treatment (Fig. 4 and Table 4).

The addition of nutrients favored growth of hydrocarbon-degrading bacteria that were probably nutrient-limited in C microcosms, since little natural phenanthrene biodegradation was observed. Therefore, nematodes in bioremediation microcosms benefit from both the decrease in phenanthrene toxicity and the nutrient addition that alleviated nutrient limitation. In addition, significant correlation ($P<0.05$) between phenanthrene removal and nematode abundance could suggest an effective direct or indirect participation of nematodes in phenanthrene degradation by the selective pressure exerted on bacteria involved the degradation of complex molecules (Louati et al. 2013b). Nevertheless, addition of nitrate and phosphate (CBS1 and CBS1+BA) modified the structure of free-living nematodes. In contrast, the biostimulation treatment with addition of mineral salt medium (CBS2) resulted to a similar abundance and community structure observed in T microcosms together the highest efficiency (up to 98 % removal).

The efficiency of the inoculation of a marine PAH-degrading bacterium is not observed in phenanthrene biodegradation. It is likely that combination of both treatments has caused a competition between indigenous and introduced microorganisms. Similar results have been observed in mangrove sediments, where biodegradation of the mixed PAHs (fluorene, phenanthrene, and pyrene) were low after bioaugmentation, suggesting some negative interaction occurred between inoculum and indigenous microbial community such as competition for resources (Yu et al. 2005).

Conclusion

The results from our study demonstrate that phenanthrene had a significant effect on meiofaunal and bacterial community with the selection of nematode species that could be proposed as bioindicators of PAH pollution such as *S. parasitifera* or *N. peocilosoma*. Altered species composition could significantly influence interactions between nematodes and interactions among major benthic taxa. Response of free-living nematodes to phenanthrene contamination could lead to food limitation for their predators, which ultimately could alter entire communities and ecosystems. This finding opens exciting axes for the future use of the biostimulation with a complex mixture of nutrients to reduce toxic effects of PAHs for meiofauna and bacteria in polluted sediment. This bioremediation strategy has shown the highest efficiency in phenanthrene degradation but also for other PAH compounds.

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).

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