RESEARCH ARTICLE

Microbial community responses to bioremediation treatments for the mitigation of low-dose anthracene in marine coastal sediments of Bizerte lagoon (Tunisia)

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Abstract

Purpose The main goals of this study were to investigate (1) the behavior of microbial communities in response to low-dose bioavailable anthracene addition in lightly contaminated sediment from Bizerte Lagoon and (2) the effects of bioremediation treatments on microbial biomass, activity, and community structure.

Methods Sediment microcosms amended with 1 ppm anthracene were incubated in triplicate during 30 days. Biostimulation (addition of nitrogen and phosphorus fertilizer) and bioaugmentation (inoculation of a hydrocarbonoclastic bacterium) were used as bioremediation treatments. Bacterial biomass was estimated using flow cytometry. Sediment oxygen consumption was measured with oxygen microelectrodes. Bacterial community structure was assessed by molecular fingerprints (terminal restriction fragment length polymorphism; T-RFLP) analysis.

Results Anthracene contamination resulted in a significant reduction of bacterial abundance with an impact on cell integrity. Concomitantly, sediment oxygen consumption was strongly inhibited. Correspondence analysis on T-RFLP data indicated that bacterial community structures from anthracene-contaminated microcosms were different from that of the control. Interestingly, the changes observed in microbial biomass, structure, and activities as a result of anthracene contamination were not alleviated even with the use of biostimulation and combination of biostimulation and bioaugmentation strategy for anthracene bioremediation. Nevertheless, both treatment methods resulted in different community structures relative to the contaminated and control microcosms with the appearance of distinct populations.

Conclusion Anthracene spiking severely affected microbial communities, suggesting dominance of nontolerant populations in this lightly-contaminated sediment. Although biostimulation and/or bioaugmentation treatments did not alleviate the anthracene toxic effects, the changes observed in microbial population and structure suggest that the proposed treatments might be promising to promote bacterial growth. Further works are still required to propose a more efficient strategy to stimulate biodegradation that takes into account the complex interactions between species for resource access.

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1 Introduction

Pollution of coastal marine ecosystems by polycyclic aromatic hydrocarbons (PAHs) is a ubiquitous environmental problem (Tam et al. 2001). Polycyclic aromatic hydrocarbons are present in petroleum and formed during the incomplete combustion of almost any organic material (Atagana 2006). These compounds are considered hazardous because of their cytotoxic, mutagenic, and carcinogenic effects (Samanta et al. 2002). Petroleum hydrocarbons have been shown to affect life history parameters (Bridges et al. 1994) and immune systems (Tahir et al. 1993) of benthic organisms, as well as the structure of entire benthic communities (Nance 1991). Significant toxicological effects of PAHs on macrobenthic organisms have been observed at concentrations below 1 mg PAH/kg dry sediment (e.g., Long 1992), while in polluted sediments meio- and microbenthic communities were not significantly influenced by the addition of sublethal concentration of PAHs (0.3–3 mg kg^{-1}; Carman et al. 1995). Similarly, PAHs have been found to exhibit rather low toxicity toward sediment microbial activity (Myers et al. 1994) even at high concentrations (Bauer and Capone 1985).

Anthracene belongs to anthropogenic pollutants recommended for investigations by the US Environmental Protection Agency (Dabestani and Ivanov 1999) because of its high toxicity and wide occurrence in the environment. Anthracene is a three-fused-aromatic ring PAH, present in terrestrial and aquatic ecosystems primarily as a result of runoff from former wood-treating operations and coal gasification plants, and as a result of other types of petrochemical pollution (Brenner et al. 2002; Mueller et al. 1989). Anthracene was the first PAH for which phototoxicity was reported in aquatic systems (Bowling et al. 1983) and anthracene has been used as a representative phototoxic PAH (Kochevar et al. 1982). This molecule is known to be phototoxic to the luminescent bacterium *Vibrio fischeri* (El-Alawi et al. 2001; McConkey et al. 1997). Nevertheless, growth and activities of the bacterium were not affected by addition of photomodified or intact anthracene for concentrations up to 4 mg L^{-1} (El-Alawi et al. 2002).

Bioremediation has been recognized as an attractive decontamination strategy for a variety of polluted environments. The degradation of PAH by microorganisms depends to a large extent on their molecular weights among many other factors (Weissenfels et al. 1990). Two major bioremediation schemes have been proposed, biostimulation and bioaugmentation (Miyasaka et al. 2006). Biostimulation is defined as addition of various forms of rate-limiting nutrients and electron acceptors such as phosphorus, nitrogen, oxygen, or carbon in order to increase the population or activity of naturally occurring microorganisms available for bioremediation. Bioaugmentation is the addition, to the polluted environment, of microorganisms that can biotransform or biodegrade contaminants. The added microorganisms can be new for the environment or already observed on site. These techniques of bioremediation provided contrasting results in PAH biodegradation depending on the type of PAH and the sediment composition (Yu et al. 2005).

Polycyclic aromatic compounds are hydrophobic and readily adsorbed onto particulate matter, therefore, sediments represent the ultimate sinks and elevated concentrations have been recorded in coastal environments (Hughes et al. 1997; James 2002). As many Mediterranean coastal lagoons, the Bizerte lagoon (Tunisia) is a polluted ecosystem subject to both urbanization and industrialization (cement works, metallurgical industry, boatyard, and tire production factories), as well as naval and commercial shipping harbors. Runoff and discharges of urban and industrial wastes lead to significant pollution of sediments by anthracene and others PAHs (Louiz et al. 2008; Trabelsi and Driss 2005), pollution that promotes to the co-selection of strains able to cope with multiple contaminants (Ben Said et al. 2010). The aim of the present study was firstly to investigate the behavior of microbial communities in response to low-dose bioavailable anthracene addition (1 ppm) in lightly contaminated (4 ppm) sediment from Bizerte Lagoon. Secondly, the effect of bioremediation treatments on microbial biomass, activity, and community structure was investigated. Bioremediation schemes tested included biostimulation and the combination of biostimulation and bioaugmentation by the inoculation of a marine PAH-degrading bacterium, *Acinetobacter* sp., previously isolated from Bizerte lagoon sediment.

2 Materials and methods

2.1 Site description and sampling

Bizerte lagoon, the third important lagoon in Tunisia, is located in an economically important area in North Tunisia (Fig. 1). It is a 150 km² surface area depression known for its geostrategic position since it is connected to the Mediterranean Sea by a straight channel (Louiz et al. 2009). Sediment was collected by scuba diving from one site (Echaraà) located in the low-contaminated part of the lagoon (Ben Said et al. 2010; Trabelsi and Driss 2005). Handcores of 10 cm² were used to a depth of 15 cm to transfer sediment into a bucket. On return to the laboratory, sediments were homogenized by gentle hand stirring with a large spatula before microcosm setup and contamination. Buckets and spatula were all acid rinsed before use.

2.2 Microcosm set-up

Microcosms consisted of 1,600 ml glass bottles. One control and three treatments were setup. Contaminated microcosms
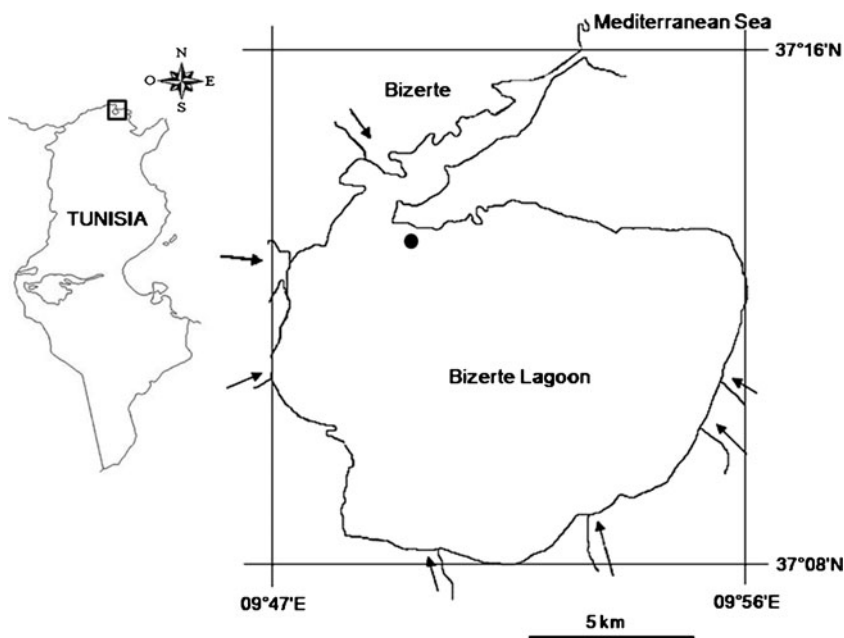
(C) were gently filled with 200 g (wet weight) of homogenized sediment (100 g of natural sediment and 100 g contaminated sediment) topped up with 1 L of filtered (1 μm) natural lagoon water at 30 PSU. Sediment used for contamination was first alternately frozen (−80°C) and thawed three times to defaunate it (Gyedu-Ababio and Baird 2006), and then it was wet sieved to remove the larger particles (>63 μm). Stock solution of high purity anthracene (100 mg L^{-1}; Sigma–Aldrich Chemical A8, 920–0), was dissolved in acetone. Next, 2 ml of the anthracene solution was added into the sediment slurry and shaken overnight to let the PAH adsorbed onto the sediments. Final concentration of anthracene in sediment was 1 ppm. Control consisted of uncontaminated and defaunated sediment (T). 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. All microcosms were performed in triplicates and incubated for 30 days in laboratory at room temperature (20–22°C).

Bioremediation treatments were started 1 day after anthracene contamination. Biostimulation (CBS) 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$_2$PO$_4$) using the protocol of Jacques et al. (2008). Biostimulation–bioaugmentation (CBS+BA): was achieved by adding the bacterium *Acinetobacter* sp. strain isolated from Bizerte-polluted sediments (Ben Said et al. 2008). 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. Cells were suspended in nutrient solution and introduced into the microcosms previously amended with nutrients (nitrogen and phosphorus fertilizer). The initial inoculum

was 5.30×10^8 cell ml^{-1}. After bacterium inoculation and nutrient addition, sediment was agitated for half an hour for homogenization.

2.3 Flow cytometry measurements

The method of bacteria extraction from sediment was derived from the protocol of Duhamel and Jacquet (2006). Sediment samples (1 g wet weight) were fixed with formalin (2 % final concentration) then 5 μl Tween 80 (10 %)+1 ml sodium pyrophosphate solution (10 mM)+4 ml MilliQ water were added. The samples were then sonicated three times for 1 min in a water bath and shaken manually after every minute. The samples were then centrifuged 1 min (800×g), filtrate supernatant on a 5-μm membrane and were diluted in TE-buffer 490 μl TE (prefiltered across 0.02 μm). For the enumeration of heterotrophic 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 and then 2.6 % at final concentration of work solution was used to stain bacteria sample incubated 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.

2.4 Oxygen microsensor measurements

2.4.1 Oxygen microsensor

Oxygen concentration was measured using a Clark-type oxygen microelectrode (Revsbech 1989) manufactured by Unisense (Denmark). These Clark-type oxygen microsensors are largely used in microbial ecology studies and contain a guard electrode to prevent internal oxygen consumption (Revsbech 2005). The electrode had a 90 % response time lower than 1 s and a stirring sensitivity of 1 %. Oxygen microprofiles were performed with a depth resolution of 100 μm using a motor-driven micromanipulator. The oxygen microprobe was manually positioned at the sediment surface, profiling and data acquisition were then controlled by computer. Linear calibration of the microprobe was determined from the electrode readings in air-saturated water above the sediment and in the anoxic part of the sediment. Oxygen concentration in air-saturated water was calculated from the solubility equation according to Garcia and Gordon (1992).

2.4.2 Diffusive oxygen flux calculation

Diffusive oxygen fluxes (J) were calculated from the steady-state oxygen profiles using Fick's first law of one-dimensional diffusion, written as (Eq. 1):

$$J = -\mathrm{Do} \times \frac{\delta C(z)}{\delta z}$$

where Do denotes the oxygen diffusion coefficient in water (Broecker and Peng 1974), C is the oxygen concentration, z is the depth and $\delta C(z)/\delta z$ the oxygen gradient in the diffusive boundary layer. Sediment oxygen consumption (SOC) was estimated from O_2 flux in the diffusive boundary layer according to Rasmussen and Jorgensen (1992). Depth profiles of SOC were estimated by using the software PROFILE (Berg et al. 1998). In this procedure, the rate of net consumption as a function of depth was calculated from steady-state oxygen microprofiles. The procedure is based on a series of least square fits of the second Fick's law to the measured steady-state oxygen profiles, followed by comparisons of these fits through statistical F testing. Thus, the simplest consumption profiles are objectively selected, which reproduce the measured concentration profiles within the chosen statistical accuracy. More details of the procedure can be found in Berg et al. (1998).

Porosity (ϕ) in the top millimeters was assumed to be constant and equal to 0.8. Sedimentary diffusion coefficient was estimated from Do according to the relation of Ullman and Aller (1982; Eq.2):

$$\mathrm{Ds} = \phi^2 \times \mathrm{Do}$$

For each microcosm, SOC vertical profiles were based on three oxygen microprofiles that were measured after 4 and 30 days of incubation.

2.5 T-RFLP analysis

Mixed community DNA was extracted directly from sediment microcosms 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′-AGAGTTTGATCCTGGCT-CAG-3′) and 1489R (5′-TACCTTGTTACGACTTCA-3′). Primer 8F was fluorescently labeled with 5-tetrachloro-fluorescein 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 were 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 analyser (Applied Biosystems). T-RFLP profiles were aligned by identifying and grouping homologous fragments and normalized by calculating relative abundances of
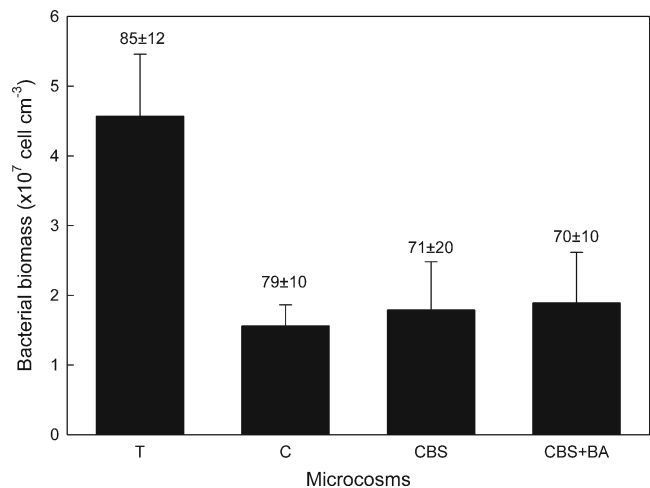

Fig. 2 Bacterial abundance (average±standard deviation) counted by flow cytometry in the sediment of different microcosms (*T* uncontaminated, *C* contaminated, *CBS* biostimulation, *CBS+BA* biostimulation and bioaugmentation). Numbers indicate the average number (±SD) of T-RFs observed in each microcosm

each terminal-restriction fragment (T-RF) from height fluorescence intensity of each T-RF as described in Pringault et al. (2008). For statistical analysis, T-RF heights of the three microcosm replicates were averaged.

2.6 Statistical analysis

One-way analysis of variance (Kruskal–Wallis test) was used to test for overall differences between controls and treatments and the Tukey HSD multiple comparisons test was used in pairwise comparisons of treatments and control. In all the above statistical significance, testing a significant difference was assumed when $p < 0.05$. For the statistical analysis of bacterial community structure, relative abundances of T-RFs have been transformed with arcsin ($\times^{0.5}$) according to Legendre and Legendre (1998) to get a normal distribution of the data. Correspondence analyses (CA) were then performed with MVSP v3.12d software (Kovach Computing Service, Anglesey, Wales).

3 Results

3.1 Bacterial abundance

Anthracene spiking resulted in significant differences in the benthic bacterial abundances relative to the control microcosms (Fig. 2). After 30 days of incubation, bacterial abundance in T microcosms averaged $4.57 \pm 0.89 \times 10^7$ cells cm^{-3}. Anthracene contamination had a strong impact on bacterial biomass which was significantly reduced in all contaminated microcosms. In C microcosms, bacterial abundance ($1.56 \pm 0.31 \times 10^7$ cells cm^{-3}.) was almost three times lower than in T microcosms. Similar strong reduction was observed for CBS and CBS+BA microcosms. Interestingly, CBS and biostimulation coupled with bioaugmentation (CBS+BA) resulted in similar ($p < 0.05$) bacterial biomass relative to the C microcosms ($1.79 \pm 0.69 \times 10^7$ and $1.89 \pm 0.73 \times 10^7$ cells cm^{-3}, respectively; Fig. 2).

The cytograms of the T microcosms clearly showed three distinct populations on the basis of nucleic acid content (NA) and the side scatter: NA1 (82 ± 4 %), NA2 (10 ± 3 %), and NA3(8 ± 3 %) with a positive growth (Fig. 3). Anthracene contamination resulted in the strong reduction of NA2 (3 ± 2 %), indicating inhibition of the bacterial growth for this population. Interestingly, biostimulation as well as combination of biostimulation and bioaugmentation resulted in similar cytograms as those observed in C microcosms with dominance of two distinct populations (NA1 and NA3). Nevertheless, in CBS and CBS+BA microcosms, two subpopulations were observed in NA1,

suggesting the presence of different populations as compared with the C microcosms. In addition, in all contaminated microcosms, the cytograms revealed high concentration of organic matter, indicating cell lysis induced by anthracene addition.

3.2 Sediment oxygen consumption

Oxygen consumption was measured 4 days after the microcosm settlement and after 30 days of incubation. At the beginning of the experiment, maximum oxygen penetration ranged from 0.22 to 0.38 cm, maximum penetration was observed in CBS+BA microcosms whereas minimum were observed in T microcosms (Fig. 4). Sediment oxygen consumption profiles were relatively similar in all microcosms, with a maximum value observed at the sediment surface and a second peak at the maximum oxygen penetration corresponding to the oxygen demand for the oxidation of reduced compounds. Areal sediment oxygen consumption rates (Fig. 5a) were similar in all microcosms with average rate of 1.25 nmol$\pm$0.28 O$_2$ cm^{-2} min^{-1}. After 30 days of incubation, oxygen penetrated deeper in the sediment irrespective of the treatment. In T microcosms, the maximum oxygen penetration averaged 0.6 cm, while for all contaminated microcosms oxygen penetration was significantly ($p < 0.05$) higher reaching 0.8 cm (Fig. 4). No significant difference was observed between contaminated microcosms. Interestingly, anthracene spiking resulted to a total inhibition of oxygen consumption at the sediment surface while for T microcosms surface activity was roughly similar as initially observed (0.14 vs. 0.12 nmol O$_2$ cm^{-3} s^{-1}, respectively). The strong inhibition of oxygen consumption by anthracene resulted to a significant decrease of sediment oxygen consumption ($p < 0.05$) relative both to the control (T) and to the rate measured at the beginning of the incubation (Fig. 5a). On average, rates measured at the end of incubation in anthracene microcosms represented only 25–30 % of the areal rates measured in T microcosms. As observed for bacterial biomass, both bioremediation treatments have no significant impact on oxygen consumption relative to the C microcosms; areal rates were not significantly different (Fig. 5a). Differences observed for sediment oxygen consumption between treatments were not explained by changes in total organic carbon since concentrations were roughly similar at the end of incubation in all microcosms (Fig. 5b).

3.3 Bacterial community structure

At the end of incubation, T-RFLP profiles showed an average species richness (S) between 70 ± 10 and 85 ± 12 T-RFs (Fig. 2), the lowest was observed in anthracene-contaminated microcosms (C) while control microcosms (T)
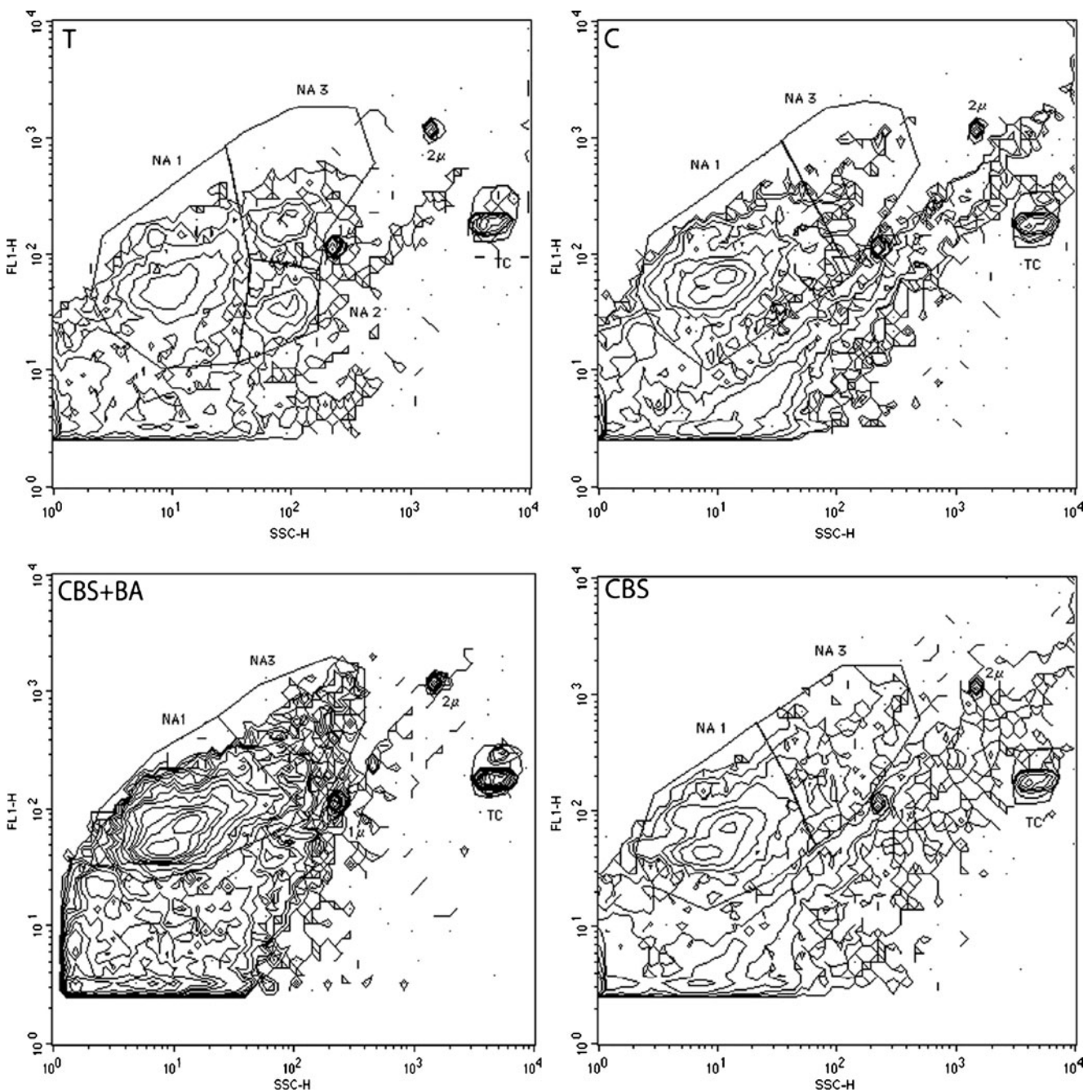
Fig. 3 Cytograms in contour plot of bacterial populations in the different treatments (*T* uncontaminated, *C* contaminated, *CBS* biostimulation, *CBS+BA* biostimulation and bioaugmentation). The *abscissa* indicates the fluorescence intensity (FL 1) of cells stained with SYBR Green I. The *ordinate* indicates the relative granularity (side scatter) of cells. *1 and 2 μm* calibration beads, *TC* Trucounts, *NA* nucleic acid

showed the highest (Fig. 2). The CA, based on T-RFLP fingerprints (Fig. 6), explaining more than 80 % of the distribution (53.3 and 27.5 % for axes 1 and 2, respectively) showed that bacterial communities from anthracene-contaminated microcosms were separated from the control according to axis 1 while the bacterial communities of the contaminated microcosms (C, CBS, CBS+BA) were distributed along axis 2. Among the different T-RFs observed, some T-RFs were detected in all microcosms with relative abundance >1 %, 33, 177, 192, 274 bp (HaeIII) and 116, 307, 331, 340 bp (HinfI). In contrast, relative abundance of specific T-RFs was subjected to changes as a function of the microcosm treatment. For example, T-RF 194 (HaeIII) showed a relative abundance of 5 % in anthracene microcosms while in T microcosms, its relative abundance was <1 %. In contrast, T-RF 269 (HaeIII) and 232 (HinfI) had a relative abundance >5 % in T microcosms and <1 % in anthracene microcosms, irrespective of the bioremediation
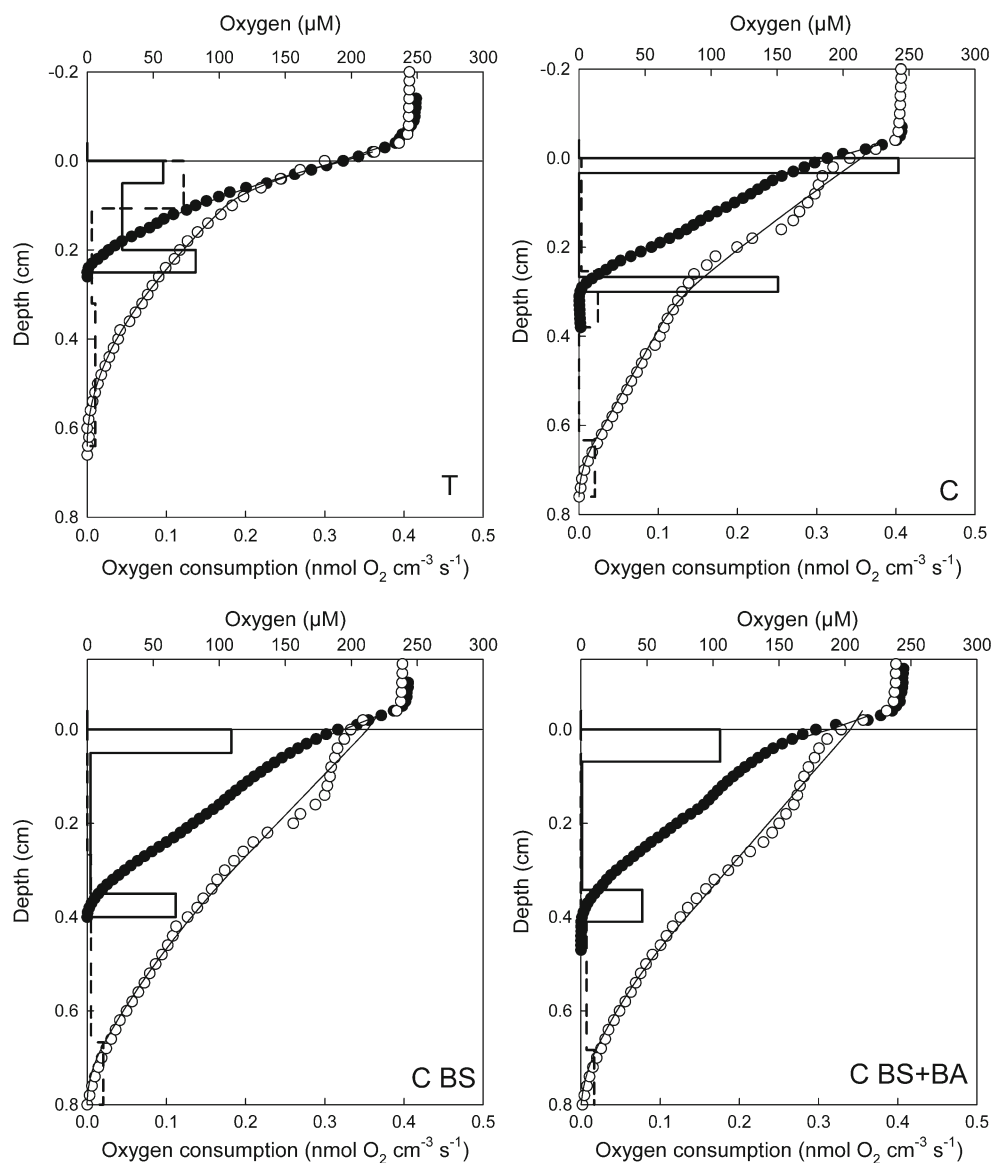
treatment. Interestingly the T-RFs 255 (HaeIII) which could be related to members of the *Acinetobacter* genera, genera from which the strain added in the bioaugmentation treatment belongs, was found the most abundant in CBS+BA microcosms with a relative abundance of 2.1 % while it represented 0.3, 0.2, and 0.1 % in the C, CBS, and T microcosms, respectively.

4 Discussion

Using a microcosm approach, we observed significant effects of anthracene on microbial biomass, activity and community structure despite the relative low concentration of anthracene added (1 ppm). Anthracene concentration in the studied sediment was around 4 ppm (unpublished results). As a consequence, the spiked concentration represented a small

enrichment of the in situ concentration. Nevertheless, effects of this low contamination were obvious for the microbial community suggesting that the in situ anthracene contamination of the studied sediment did not allow the dominance of tolerant anthracene populations as it can be observed in polluted environments (Carman et al. 1995; Paisse et al. 2010). Impact of pollutant on benthic organisms depends on its bioavailability. A pollutant can be in high concentration in a polluted system but moderately impact the microbial community because of strong adsorption onto particles decreasing its potential toxic effects (Talley et al. 2002). Anthracene is largely associated with organic particles in sediments decreasing its bioavailability (Talley et al. 2002). Nevertheless, our results suggest that the low anthracene concentration added was sufficient to induce significant changes in biomass and structure suggesting thus that the spiked anthracene was bioavailable for microorganisms in the experimental conditions imposed.
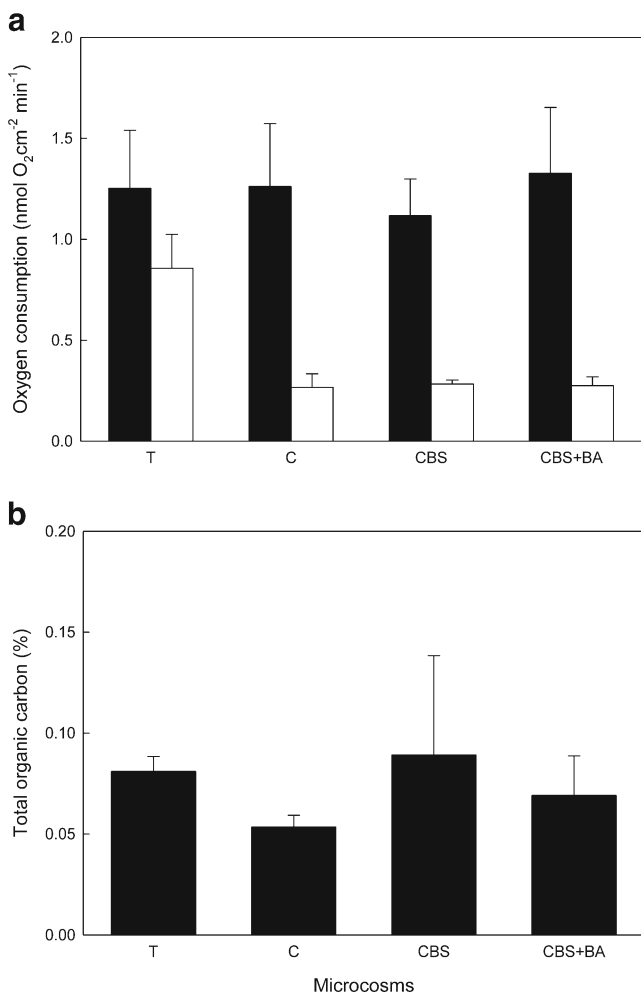
Fig. 5 Sediment oxygen consumption and total organic carbon as a function of the different treatments (*T* uncontaminated, *C* contaminated, *CBS* biostimulation, *CBS+BA* biostimulation and bioaugmentation). **a** Oxygen fluxes at the sediment/water interface after 4 days (*white bars*) and 30 days (*black bars*) of incubation (average±standard deviation). **b** Percentages of total organic carbon after 30 days of incubation (average±standard deviation)
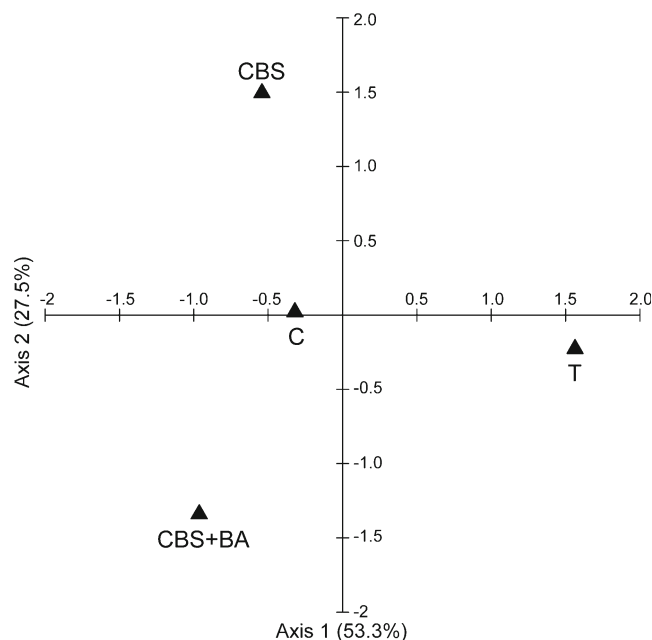

Fig. 6 Correspondence analysis (CA) of the microbial communities based on T-RFLP data (16S rRNA genes) as a function of the different treatments after 30 days of incubation (*T* uncontaminated, *C* contaminated, *CBS* biostimulation, *CBS+BA* biostimulation and bioaugmentation). The data analyzed correspond to the average for triplicate samples of HaeIII and HinfI-digested 16S rRNA genes yielding 5-end T-RFLP patterns

Analysis by flow cytometry showed that anthracene had a significant effect on bacterial abundance with inhibition of the population NA2 identified from acid nucleic content (Fig. 3). The decrease in bacterial abundance can be explained by the inhibition of bacterial growth of non-adapted populations. In addition, cytograms of contaminated microcosms showed that cell integrity was strongly impacted. Anthracene is considered toxic, but toxicology data specific to anthracene is uncommon, or somewhat conflicting. Dean-Ross et al. (2001) state that anthracene is neither genotoxic nor carcinogenic, but is a threat to the environment because of its toxicity to aquatic life, especially through photo-induced toxicity which was confirmed by El-Alawi et al. (2002) who showed that anthracene is very toxic in the presence of ultraviolet radiation for luminescent bacteria. In the present study, the photo-toxicity of anthracene can be excluded because microcosms were incubated in laboratory conditions, under free UV and solar radiations. Therefore, we measured the direct toxicity of anthracene on indigenous bacteria in marine coastal sediments. Flores et al. (2010) observed growth inhibition upon anthracene contamination (IC50 6 ppm) of microbial consortium isolated from polluted sediments. They suggested that the toxic effects of anthracene is due to the accumulation and mutagenic activity of anthracene, similar to the effects induced by polycyclic aromatic hydrocarbons on *Salmonella* strain YG1041 (Kummrow et al. 2006). Anthracene has a high octanol–water partition coefficient (log K_{ow}=4.1), and therefore can readily adsorb onto organic phases such as the phospholipids of bacterial membranes (Flores et al. 2010). This interaction provokes a hydrophobic region inside the bacterial membrane, which can act as a reservoir for anthracene accumulation (Bugg et al. 2000) enhancing its potential toxic effects.

Impact of anthracene on microbial biomass can also strongly change the sediment oxygen consumption since heterotrophic bacteria play a key role in the mineralization of the organic matter. The use of oxygen microelectrodes offers the possibility to determine with high-vertical resolution the sediment oxygen consumption, proxy of the organic matter mineralization under aerobic conditions (Viret et al. 2006). After 30 days of sediment contamination, anthracene had a strong impact on sediment oxygen consumption

(Fig. 5) with a complete inhibition of the oxygen consumption in the first 5 mm (Fig. 4), the oxygen consumption observed around 8 mm depth corresponding to the oxygen demand (biotic and abiotic) for the oxidation of reduced compounds. Inhibition of sediment oxygen consumption was demonstrated for various pollutants such as atrazine (Laursen and Carlton 1999), metals (Viret et al. 2006), and uranium (Lagauzere et al. 2009). Interestingly, in the present study, the specific activity (activity/biomass) after 30 days of incubation was similar in all treatments (control and contaminated microcosms) although the biomass strongly decreased in contaminated microcosms concomitant with high reduction in bacterial population NA2 (Fig. 3). This suggests that the remaining microbial populations observed at the end of the incubations in the contaminated microcosms were in the same physiological state than those observed in the control. In contrast, Hjorth et al. (2007) observed an increase of the specific activity for the bacterio- and phytoplankton upon pyrene few days of exposure. For both compartments, abundance strongly decreased while activity remained stable. They suggest a functional redundancy to explain this increase of the specific activity. Effect of pyrene was strong enough to eliminate sensitive groups, living space and resources to more tolerant and/or opportunistic groups, which responded with high specific activities (Hjorth et al. 2007). In our study, even so, anthracene had a strong selective pressure by decreasing abundance of nontolerant population, it did not apparently alleviate the competition for space and resources since specific activities were the same in all microcosms (contaminated and control).

The toxic effects of anthracene observed on biomass and cell integrity is accompanied by strong changes in bacterial community structure (Fig. 6) and in moderate decrease of species richness (Fig. 2). T-RFLP profiles revealed an important bacterial diversity in all microcosms as previously reported in marine sediments (Ben Said et al. 2010). The correspondence analysis, based on T-RFLP fingerprints (Fig. 6) showed that bacterial communities from anthracene-contaminated microcosms were separated from the control. Bacterial community structure changes as response to PAH addition have been often observed using a single molecule, e.g., anthracene (Zhang et al. 2011), phenanthrene (Muckian et al. 2009), or a complex mixture of PAH (Zhou et al. 2009). Changes in hydrocarbon content resulted in characteristic shifts of substrate utilization patterns which can correspond to similar changes in abundance of hydrocarbon-utilizing bacteria and the occurrence of specific bacterial groups (Wunsche et al. 1995). Interestingly, at the end of incubation, the T-RF 194 (HaeIII) was observed in abundance >5 % in the contaminated microcosms while its relative abundance was <1 % in the control. According to the Ribosomal Database Project, this T-RF may correspond

to an uncultured *Proteobacterium* frequently observed in PAH-contaminated environments (e.g., Paisse et al. 2010). It is likely that the dominance of this population could be attributed to the selective pressure exerted by anthracene and its degradation metabolites. The selective pressure can also result to the diminution of nontolerant populations as observed for the T-RFs 269 (HaeIII) and 232 (HinfI) for which a strong decrease of the relative abundance was observed in anthracene microcosms relative to the control.

Overall, the present study shows that addition of a low dose of anthracene strongly modified biomass, structure and function of the microbial community. Bioremediation using addition of fertilizers (CBS) and/or addition of hydrocarbonoclast bacteria (BA) can be attractive solutions to enhance degradation of organic pollutants, decreasing thus their potential toxic effects. Irrespective of the bioremediation treatments, the toxic effects of anthracene could not be diminished for microbial biomass, structure and function. Unfortunately, anthracene concentration was not measured at the end of incubation, nevertheless, the observed patterns clearly suggest that its toxic effects were still present after 30 days of incubation in both CBS and CBS+BA microcosms. Yu et al. (2005) observed that biostimulation and bioaugmentation are not always efficient in PAH biodegradation which can be explained by the bioavailability of PAHs and the competition between species for organic and inorganic substrates. In our study, the enrichment in nutrients (N and P) followed the protocols of Jacques et al. (2008) and represented an N/P ratio of 4. The cytograms of the CBS microcosms observed at the end of the incubations showed the presence of two subpopulations in the NA1 population (Fig. 3). These subpopulations were not observed in the C and T microcosms. This stimulation of these subpopulations was followed by a moderate change of the microbial community structure relative to the contaminated sediment (Fig. 6). The Bizerte lagoon exhibits N/P ratio around 2.5 in winter and around 20 in summer, suggesting different nutrient limitation for each season, N limitation in winter and P limitation in summer (Hlaili et al. 2006). According to the Redfield ratio, the N/P ratio used for the biostimulation should alleviate a P limitation. The presence of two subpopulations in NA1 population specific to CBS and CBS+BA microcosms suggests that the nutrient amendment partly alleviated P limitation resulting to different microbial community structures (Fig. 6) as also observed in other bioaugmentation studies (Di Gennaro et al. 2009; Miyasaka et al. 2006). However, this nutrient stimulation did not compensate the toxic effects of anthracene since total biomass and sediment oxygen consumption were similar in CBS and C microcosms. This noncompensation of the anthracene toxic effects was also observed when a hydrocarbonoclastic bacterium (*Acinetobacter* sp.) was inoculated together with the nutrients. The strain used for the

bioaugmentation was isolated from contaminated sediments of Bizerte lagoon and was chosen for its capacities to degrade hydrocarbons (Ben Said et al. 2008). The analysis of microbial community by T-RFLP fingerprints indicated that the T-RF255 (HaeIII) corresponding to this bacterium species had a relative abundance of >2 % in the CBS+BA microcosms while in other microcosms its relative abundance was <0.3 %. This suggests that the experimental conditions imposed favored the growth of *Acinetobacter* sp.; however, its potential hydrocarbon degradation capacities did not alleviate the toxic effects of anthracene. This might be due to the competition for resources as suggested by Yu et al. (2005) to explain the absence of effective biodegradation with bioaugmentation treatments as it is often reported (e.g., Perelo 2010). The fact that specific activities were similar in all treatments (see above) suggests that anthracene did not alleviate competition between microorganisms, which would support this hypothesis.

5 Conclusion

Using a microcosm approach, we showed that a low dose of anthracene (1 ppm) severely affect the microbial community, biomass, structure, and function in a sediment slightly contaminated by anthracene, suggesting the dominance of nontolerant microbial populations. The toxic effects of anthracene were not alleviated when bioremediation treatments, biostimulation, and/or bioaugmentation were used even so changes in microbial populations and structures were observed, suggesting that the proposed treatments might be promising to promote bacterial growth but further works are still required to propose a more efficient strategy to stimulate biodegradation that takes into account the complex interactions between species for resource access.

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