# Hydrocarbon degrading microbial communities in bench scale aerobic biobarriers for gasoline contaminated groundwater treatment

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## 1. Introduction

Petroleum hydrocarbons are extensively used both as fossil fuel and for chemical production. Due to spills, hydrocarbons contaminate soil, sediments and groundwater (Rosell et al., 2006; Höhener and Ponsin, 2014). Monoaromatic hydrocarbons, such as

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BTEX compounds (benzene, toluene, ethylbenzene and xylenes), and additives, such as methyl *tert*-butyl ether (MTBE), are some of the main constituents of gasoline and cause concern because of their recalcitrance, toxicity and mobility in groundwater (Poulsen et al., 1992; Levchuk et al., 2014). Biological permeable reactive barriers, also known as "biobarriers" (BBs), are an *in situ* bioremediation technology based on the placement of a permeable medium into the aquifer to intercept the contaminants and promote the biodegradation by stimulating the microbial activity (Careghini et al., 2013). BBs are currently a developing technology and little information is available about the microbial communities developing and operating on the supporting material. Microorganisms have been largely described to degrade hydrocarbons with a variety of electron acceptors in both aerobic and anaerobic conditions (Jindrová et al., 2002; Kleinsteuber et al., 2012; Hyman, 2013). Bioaugmentation is a bioremediation strategy that involves the addition of specialized microorganisms to the contaminated site; it is especially used when the indigenous communities do not have the necessary metabolic abilities. A successful application of this strategy is however highly site specific, and requires a deep characterization of the system (El Fantroussi and Agathos, 2005). Monitoring techniques, such as quantitative PCR (qPCR) for detection of catabolic genes, can be exploited to gain more insight into complex bioremediation processes by giving information about the abundance of bacteria with specific degradation potential (Atlas and Hazen, 2011). This approach has been recently used to quantify aromatic oxygenase genes in gasoline contaminated sites to evaluate the effect of different remediation strategies (Nebe et al., 2009; Baldwin et al., 2009). Furthermore, 16S rRNA gene sequencing has been broadly used to describe the taxonomic composition of the microbial communities in hydrocarbon contaminated sites (Chakraborty et al., 2012; Singleton et al., 2013).

In this study we characterized the microbial communities developed in two bench scale aerobic biobarriers for gasoline contaminated groundwater treatment. In one column an allochtonous bacterial consortium was added as microbial inoculum. At the end of the experiments, the communities enriched were described by Illumina sequencing of the 16S rRNA gene and catabolic genes were quantified by qPCR. The aim of our study was to describe the microbial communities developed during the treatment and to understand whether (i) the microbial inoculum persisted, and (ii) the bioaugmentation improved the biodegradation potential.

## 2. Materials and methods

### 2.1. Column experiments

The column tests were carried out as described in (Careghini et al., 2015). A Teflon-coated basin (250 cm long, 10 cm wide) filled with volcanic pumice was used to perform the experiments. Eight piezometers ('A' to 'H') were distributed along the column to collect water samples (a list of the abbreviations is reported in Table S1). In the inoculated test (IN), an allochthonous microbial consortium containing *Rhodococcus* sp. CE461, *Rhodococcus* sp. CT451 and *Methylibium petroleiphilum* LMG 22953 ($OD_{540}$ = 0.1 each, volume = 5 L each) was added into the column and was recirculated for 3 d before starting the test. The microbial inoculum was selected as previously reported for its ability of efficiently degrading hydrocarbons in batch experiments (Careghini et al., 2015). The cultures were incubated 24 h in LB rich medium at 30 °C under shaking, washed and resuspended in a phosphate buffer solution to $OD_{540}$ = 0.1 (Franzetti et al., 2011). The duration of the tests was 43 d for the uninoculated test (UN) and 85 d for IN. Tap water artificially contaminated with gasoline was used as the inflow solution at 3.0 L $d^{-1}$. To ensure aerobic conditions, a commercial oxygen-release compound (EHC-O, Adventus, 0.3% on dry weight basis of pumice) was uniformly distributed before starting the tests. Further EHC-O (250 mg twice a week) were added during the IN test. Nitrogen (27.7 g of $NH_4NO_3$) and phosphorus (2.1 g of $H_2KPO_4$ and 2.7 g of $HK_2PO_4$) were also added as nutrient source. Water samples were collected to measure BTEX, MTBE, ETBE and *tert*-Butyl Alcohol concentrations, temperature, pH (electronic pH-meter XS pH 6, Oakton), and dissolved oxygen (DO) (OXI 340i probe, WTW). Hydrocarbons were extracted by solid-phase microextraction and quantified by GC–MS (UNICHIM 1210:1997). At the end of the experiments pumice samples were collected and stored at − 20 °C for the microbial characterization.

### 2.2. Amplification of the 16S rRNA gene, sequencing and sequence analyses

Total bacterial DNA was extracted using the FastDNA Spin for Soil kit (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. The presence of environmental inhibitors was investigated by performing serial dilutions of the extracted DNA and inhibition effect was not observed. The V5-V6 hypervariable regions of the 16S rRNA gene were PCR-amplified and sequenced by MiSeq Illumina (Illumina, Inc., San Diego, CA, USA) using a 250 bp × 2 paired-end protocol. The multiplexed libraries were prepared using a dual PCR amplification protocol. The first PCR was performed in 3 × 75 μL volume reactions with GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) and 1 μM of each primer. 783F and 1046R primers were used (Huber et al., 2007; Wang and Qian, 2009) and the cycling conditions were: initial denaturation at 98 °C for 30 s; 20 cycles at 98 °C for 10 s, 47 °C for 30 s, and 72 °C for 5 s and a final extension at 72 °C for 2 min. The second PCR was performed in 3 × 50 μL vol-ume reactions by using 23 μL of the purified amplicons (Wizard® SV Gel and PCR Clean-up System, Promega Corporation, Madison, WI, USA) from the first step as template and 0.2 μM of each primer. Primers sequences contained the standard Nextera indexes and the cycling conditions were: initial denaturation at 98 °C for 30 s; 15 cycles at 98 °C for 10 s, 62 °C for 30 s, and 72 °C for 6 s and a final extension at 72 °C for 2 min. DNA quantity after the amplification was evaluated using Qubit® (Life Technologies, Carlsbad, CA, USA). The sequencing was carried out at the Parco Tecnologico Padano (Lodi, Italy). Each sequence was assigned to its original sample according to its barcode. Forward and reverse reads were merged with perfect overlapping and quality filtered with default para-meters using Uparse pipeline (Edgar, 2013). 6000 randomly select-ed sequences were retrieved from each library and taxonomically classified by RDP classifier (>80% confidence). The sequences were deposited in the European Nucleotide Archive with accession number PRJEB7329.

### 2.3. Catabolic genes and 16S rRNA gene quantification

The abundance of total bacteria was estimated by the quantification of the number of copies of the 16S rRNA gene. A 466-bp fragment of the bacterial 16S rRNA gene (331–797 according to *Escherichia coli* position) was PCR-amplified with a universal primer set (Table S2) (Nadkarni et al., 2002). The PCR was performed in a total volume of 10 μL using the FluoCycleII Sybr reaction mix (Euroclone, Pero, Italy) with 0.3 μM (final concentration) of forward and reverse primers. The amplification was carried out as previously described (Bertolini et al., 2013). The abundance of hydrocarbon-degrading bacteria was estimated by quantification of catabolic genes. Xylene monooxygenase (*xylM*) and toluene monooxygenase (*tmoA*) were chosen as target genes using respectively TOL and RDEG primers (Table S2) (Baldwin et al., 2003). The PCR mix was prepared as previously described for the 16S rRNA gene and the amplification was performed in both cases under

the following conditions: 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s, 57 °C for 30 s and 72 °C for 45 s, with acquisition of the fluorescence at the end of each 72 °C elongation step.

### 2.4. Statistical analysis

A Principal Component Analysis (PCA) of the genus relative abundance and the Welch's *t*-test on the most abundant genera (95% of confidence) were performed using the STAMP software (Parks et al., 2014).

## 3. Results

### 3.1. Column parameters

At the end of the experiments, DO showed generally lower values compared to the beginning. Moreover, it decreased throughout the column in both UN and IN test (Fig. S1). After a fast decreasing next to the inlet, however, UN showed a higher DO (ranging from 4.8 mg $L^{-1}$ to 5.4 mg $L^{-1}$) than IN (ranging from 2.1 mg $L^{-1}$ to 4.8 mg $L^{-1}$). Since DO drop observed in IN occurred after only 6 d of operation, to ensure aerobic conditions during the experiment, ECH-O was periodically added into the column. MTBE removal efficiency in UN was about 78%, slightly higher than that in IN (63%) (Fig. S2). Toluene was almost completely removed from the column during the treatment (Fig. S2).

### 3.2. Taxonomic composition and biodiversity

The classification of the sequences was performed at the genus level and a comparison of the communities developed throughout the column was performed. The Shannon Index was calculated for each sample as an estimation of the alpha diversity (Fig. S3). The diversity decreased in UN throughout the column and the Shannon Index reached the lowest values near the piezometers E and G. IN showed a constant biodiversity before the treatment and an increase of the alpha diversity was observed after the treatment.

PCA revealed that pumice before the treatment, samples collected from UN, samples collected from IN before the treatment and samples collected from IN after the treatment formed separate clusters (Fig. 1). A clear separation between the microbial communities developed in the first part of the column (piezometers A–C, Cluster 1) and the second part of the column (piezometers D–H, Cluster 2) in UN was also observed (Fig. 1).

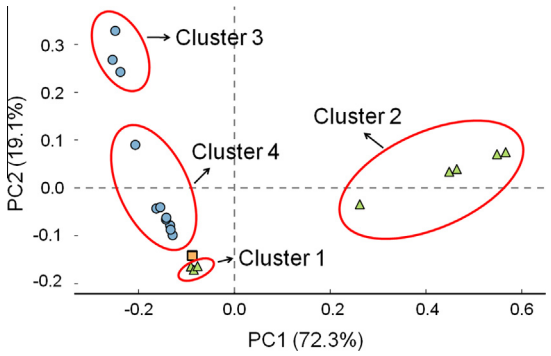


**Fig. 1.** Principal Component Analysis of the genus relative abundance. Uninoculated column (UN-green triangles); inoculated column (IN-blue circles); dry pumice (P-orange square); t0 = sample collected before the treatment; tf = sample collected after the treatment; letters (A–H) indicate the sampling point in the column. Cluster 1 = A-UN-tf, B-UN-tf, C-UN-tf; Cluster 2 = D-UN-tf, E-UN-tf, F-UN-tf, G-UN-tf, H-UN-tf; Cluster 3 = A-IN-t0, D-IN-t0, G-IN-t0; Cluster 4 = A-IN-tf, B-IN-tf, C-IN-tf, D-IN-tf, E-IN-tf, F-IN-tf, G-IN-tf, H-IN-tf. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The taxonomic composition in UN changed heavily throughout the column (Fig. 2). The most abundant genus in the first part of the column (samples A-UN-tf, B-UN-tf, and C-UN-tf) was *Acidovorax* (between 8.0% and 15.4%). In the same samples *Hydrogenophaga* genus was between 5.7% and 14.7% of the sequences. Genera *Pseudoxanthomonas*, *Bdellovibrio* and *Serpens* were particularly abundant respectively in samples A-UN-tf, B-UN-tf, and C-UN-tf. In the second part of UN, from piezometer D until the end of the column, the microbial communities were dominated by facultative anaerobes. *Thauera* was the most abundant genus (Fig. S4) with a relative abun-dance up to 82.0%. Samples collected from IN before the treatment showed that genera *Rhodococcus* and *Micrococcus* were the most abundant. No sequences belonging to *M. petroleiphilum* were detect-ed. After the treatment, *Rhodococcus* was the most abundant genus in IN but its abundance decreased with respect to the initial value (Fig. S4). Microorganisms belonging to *Serpens* were found through-out the whole column but they were more abundant near the piezometers from C to H. Similarly *Acidovorax* represented up to 14.6% of the sequences near the piezometer C, but in lesser extent it was detected in all the samples. *Mycobacterium* was enriched in the first part of the column and represented 16.2% of the sequences in the sample B-IN-tf. The facultative anaerobe *Azoarcus* colonized the second part of the column, from piezometer D to piezometer H and represented up to 19.4% of the sequences.

### 3.3. Gene quantification

The number of ribosomal operons per g of pumice in sample P was about $10^7$ while, in the samples collected from UN at the end of the treatment, it increased of an order of magnitude (Fig. 3). When the bacterial inoculum was added into the column, the number of ribosomal operons did not increase in samples A-IN-t0, D-IN-t0 and G-IN-t0 compared to P. After the treatment, the number of ribosomal copies in IN did not differ from that observed in UN and increased up to $10^8$ compared to the beginning of the experiment.

In both IN and UN *tmoA* was not detected. The presence of *xylM* was not observed before the treatment in both the conditions but it became relevant at the end of the experiment (Fig. 3), Moreover, in the IN experiment, the number of copies of *xylM* was generally one order of magnitude higher compared to the UN test.

## 4. Discussion

Gasoline contaminated water treatment was performed with a bench scale biobarrier. The removal efficiency was tested both with and without the addition of a selected microbial inoculum, however the addition selected microorganisms did not lead to an increasing of hydrocarbon removal. These data are in contrast to what previously observed in a similar system. In a permeable reactive barrier with cell-immobilized beads for gasoline contaminated groundwater treatment the bioaugmentation improved the removal efficiency (Xin et al., 2013). The different behavior could be due to the immobilization of the microbial inoculum on the support material in this last case, allowing an easier competition with the indigenous microorganisms in the column. The use of support materials could enhance the degradative activity and promote the persistence of external inocula (Rivelli et al., 2013). In the present study pumice did not show the ability to promote neither the degradative activity nor the persistence of the microbial inoculum. However, native bacteria on the pumice were already able to degrade the contaminants and thus the stimulation of the native communities, rather than the addition of an external inoculum, is an attractive strategy.

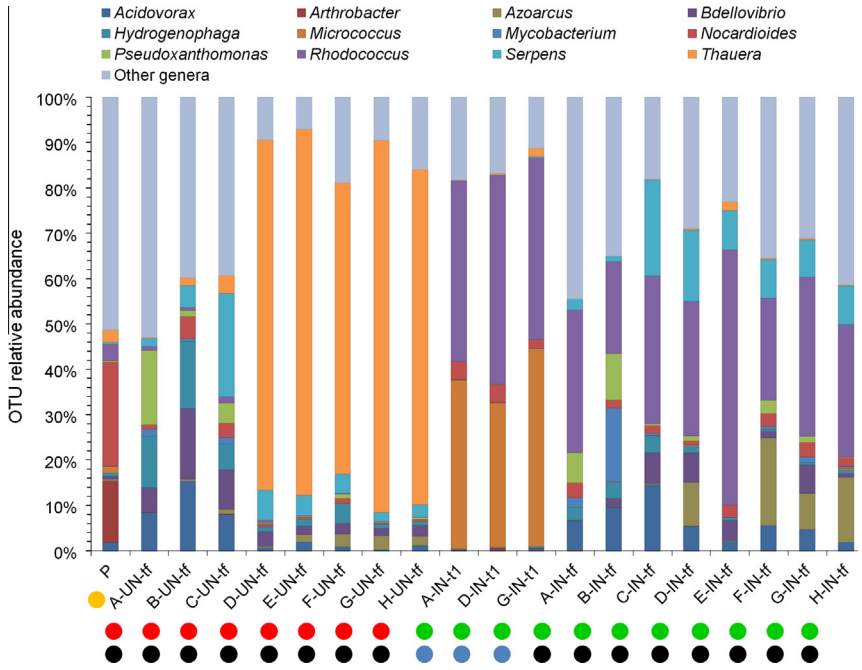


**Fig. 2.** Taxonomic composition at genus level of the microbial communities selected into UN (red circles) and IN (green circles), before (t0) (blue circles) and after (tf) (black circles) the treatment and on the dry pumice (P) (yellow circle). The letters (A–H) indicate near which piezometer the sample was collected. Only the OTUs with a relative abundance of 10.0% (or higher) in at least one sample were reported. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

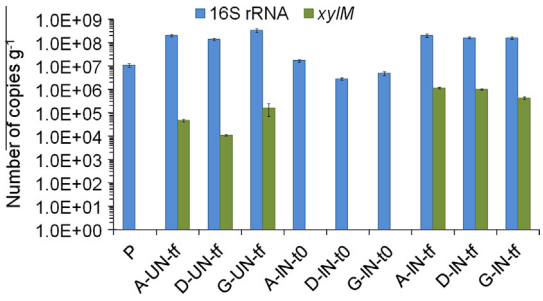


**Fig. 3.** Number of copies per g of pumice of the 16S rRNA gene and *xylM* detected by qPCR into UN and IN, before (t0) and after (tf) the treatment and on the dry pumice (P). The letters (A–H) indicate near which piezometer the sample was collected. *XylM* was under the detection limit in samples P, A-IN-t0, D-IN-t0, G-IN-t0.

The taxonomic composition of the microbial communities enriched during the treatment was performed at genus level. The Shannon Index showed an increasing of the diversity in IN after the treatment, which suggested that the microbial inoculum was outcompeted by the native microorganisms of the pumice. This observation was confirmed by the PCA because the samples collected from IN formed two different clusters. Furthermore, both the Shannon Index and the PCA showed that the microbial communities enriched in UN had a spatial separation throughout the column and changed from the first part of the column to the second part of the column. These findings were also consistent with the difference in the degradation rate constants measured in the two parts of the column (Table S3). This difference is clearly visible for m-, p-xylene and ethylbenzene (Careghini et al., 2015).

The most abundant genera in the first part of UN were *Acidovorax* and *Hydrogenophaga*. Microorganisms belonging to the genus *Acidovorax* were described as polycyclic aromatic hydrocarbon (PAH) degraders, and were found in column reactors designed to stimulate the biodegradation in contaminated soil (Singleton et al., 2013). The microbial characterization of the communities of a site contaminated with BTEX and MTBE showed that *Acidovorax* was abundant in oxic samples (Tischer et al., 2013). *Hydrogenophaga flava* ENV735 was able to degrade both TBA and MTBE in pure culture (Hatzinger et al., 2001). This genus was also found in MTBE degrading communities enriched both in bioreactors and in microcosms (Raynal and Pruden, 2008; Bastida et al., 2010). The presence of *Hydrogenophaga* was also described in a microcosm set up using groundwater from a BTEX contaminated site as inoculum and a mixture of toluene and benzene as carbon source (Aburto and Peimbert, 2011), and seemed to have a role in PAH degrading communities (Martin et al., 2012). Other abundant bacteria enriched in UN (piezometers A–C) were *Pseudoxanthomonas*, *Bdellovibrio* and *Serpens*. *Pseudoxanthomonas* genus was broadly described as able to degrade PAH and several strains were isolated from contaminated soil and sediment (Klankeo et al., 2009; Patel et al., 2012), while *Pseudoxanthomonas spadix* BD-a59 was reported to be able to degrade all the BTEXs (Kim et al., 2008). *Bdellovibrio* was previously enriched in a BTEX-fed reactor for water treatment (Li and Goel, 2012), while, to the best of our knowledge, this is the first time that *Serpens* genus is described in a hydrocarbon degrading community. *Thauera* was the dominant microorganism in UN from the piezometer D to the piezometer H. A member of this genus is *Thauera aromatica* K 172, a microorganism well known for its ability to degrade aromatic hydrocarbons both in aerobic and denitrifying conditions (Anders et al., 1995). *Thauera* was also found in a microcosm set up with toluene as carbon source in denitrifying conditions (An et al., 2004; Li et al., 2012).

The most abundant microorganisms in IN before the treatment were *Rhodococcus* and *Micrococcus*. While *Rhodococcus* was inoculated in the column with the artificial consortium used as inoculum, microorganisms belonging to the genus *Micrococcus* grew in the column during the colonization phase. *M. petroleiphilum* was probably outcompeted by other bacteria during the 3-d recirculation phase. The high similarity among microbial communities detected in different piezometers of IN before the treatment confirmed that the recirculation phase was effective in the homo-

geneous distribution of the inoculum. However, since less than half of the sequences belong to microorganisms added with the inoculum, it is reasonable to speculate that most of the inoculated bacteria could not survive during the initial recirculation.

After the treatment *Rhodococcus* was the most abundant microorganism in IN, which was expected since it was added in the column with the microbial inoculum. However, the abundance of this genus was significantly higher before the treatment than after the treatment, suggesting that the inoculum did not success-fully colonize the pumice. Other taxonomic groups that were abun-dant in IN after the treatment were *Serpens*, *Acidovorax*, *Mycobacterium* and *Azoarcus*. *Mycobacterium austroafricanum* IFP 2015 was reported as MTBE degrader and was isolated from an MTBE degrading microcosm (Lopes Ferreira et al., 2006). In a pre-vious work the microbial biodiversity in BTEX contaminated groundwater was studied and *Mycobacterium* sp. C3, able to degrade both toluene and m-xylene, was isolated (Cavalca et al., 2004). Several microorganisms belonging to the genus *Azoarcus* were reported to degrade hydrocarbons in nitrate reducing conditions. *Azoarcus evansii* KB 740 was able to use a variety of aromatic substrates as sole carbon source in the presence of nitrate (Anders et al., 1995), while *Azoarcus*-like strain EbN1, isolated from anoxic freshwater mud, was previously used as model microorganism to describe the oxidation mechanism of toluene and ethylbenzene in anaerobic conditions (Rabus et al., 2002). Microorganisms belonging to *Azoarcus* genus were also isolated from a BTEX contaminated groundwater (Cavalca et al., 2004).

The enrichment of microbial communities dominated by facultative anaerobic microorganisms in UN (piezometers D–H) could be explained referring to the DO concentrations measured during the experiment. DO measured in the area next to every piezometer was approximately 5 mg $L^{-1}$, but the heterogeneity of the system could have caused the presence of microenvironments with a vari-able DO concentration, or even without oxygen. However, the available information about DO concentration in the system con-cern only the environment external to the pores of the pumice and oxygen diffusion into the pores could be difficult. Thus, bacte-ria able to grow both with and without oxygen as electron acceptor (e.g. *Thauera*), could have taken advantage over the aerobic microorganisms. The presence of *Thauera* was significantly higher in the second part of the column. Furthermore, sequences belonging to the facultative anaerobe, hydrocarbon degrader, *Azoarcus* were observed to be more abundant from the piezometer E to the piezometer H, although it represented less than 3% of the microbial communities. These findings enforce the hypothesis that microenvironments with low DO characterized the second part of the column in UN leading to an increase in the hydrocarbon degradation using $NO_3^-$ as electron acceptor. Thus, the $NH_4NO_3$ added served not only as a nutrient source but also as an electron sink.

The higher abundance of aerobic microorganisms in IN compared to UN explained the faster oxygen consumption in this experiment. Furthermore, aerobic microorganisms could have been advantaged from the periodical spiking with EHC-O during the treatment, leading to an homogeneous DO concentration in the column. In contrast, since $NH_4NO_3$ was not further added in IN during the experiment, there was a lack of $NO_3^-$ that could have been used as electron acceptor, limiting the growth of $NO_3^-$ – reducers, with the only exception of a small amount of *Azoarcus*. The taxonomic composition analysis of IN confirmed the findings of the Shannon Index and PCA results. In fact, the microbial inocu-lum persisted into the column only partially and was outcompeted by the native microorganisms of the pumice.

The taxonomic analysis performed on both columns revealed that, although the gasoline contaminated water treatment led to microbial communities with a completely different composition in the two conditions, the measured degradation kinetic constants were similar for both toluene and MTBE (Table S3) (Careghini et al., 2015).

The number of copies of the 16S rRNA gene was quantified by qPCR in order to compare the bacterial abundance at the beginning and at the end of the column experiments. This parameter is not directly linked to cell number due to the presence of multiple ribosomal operons in the bacterial genomes; however, it can be used to look at relative shifts in microbial biomass as the bias in copy number is likely constant (Bertolini et al., 2013).

It was observed that the number of ribosomal operons per g of pumice did not increase after the inoculum addition. Therefore, the addition of the inoculum did not lead to an increasing of the biomass into the columns, confirming that it was, at least partially, washed away not only after the treatment, but also after the 3-d recirculation phase. The microbial inoculum used for IN test was composed by two Gram-positive strains (*Rhodococcus* sp. CE461 and *Rhodococcus* sp. CT451). Gram-positive microorganisms are reported to use mainly dioxygenases to degrade monoaromatic hydrocarbons. However, these enzymes are mainly represented by naphthalene dioxygenases that can also degrade monoaromatic hydrocarbons with more aspecific mechanisms (Maruyama et al., 2005). However, *xylM*-like and *tmoA*-like genes were also detected in Gram-positive bacteria, e.g. *Rhodococcus* and *Arthrobacter* strains, probably because of horizontal gene transfer (Hendrickx et al., 2006). For this reasons, in this study we focused our attention on monooxygenases specific for BTEX degradation. The presence of both *tmoA* and *xylM* genes was assessed, since these genes were sug-gested to be the most frequently retrieved BTEX catabolic genes in environmental samples (Hendrickx et al., 2006). Increases in oxyge-nase copy number were found to be related to the catabolic ability to degrade hydrocarbons during bioremediation processes (Baldwin et al., 2009). In our experiments, *tmoA* was not detected in any sam-ple, suggesting that this gene was not crucial during the BTEX degra-dation in this system. However, the increasing in the number of copies of *xylM* indicated that BTEX degrading microorganisms were selected. Furthermore, the higher number of copies of *xylM* observed in IN compared to the UN test may be due to the higher presence of aerobic bacteria, thus indicating that the degradation mechanisms occurred in the two conditions were different.

Both the 16S rRNA gene sequencing and the qPCR results clearly showed that (i) the inoculum was outcompeted by the native microorganisms of the pumice and persisted only partially into the column, and (ii) the bioaugmentation did not lead to an increase in the degradative potential. The overall results of this study showed that the use of an aerobic BB is an effective strategy to remediate gasoline contaminated groundwater and that the addition of a microbial inoculum may be not necessary to reach a suitable removal efficiency. However, results also showed that different DO concentrations along the simulated biobarrier affect-ed the microbial community composition and, subsequently, the biodegradation rate of pollutants.


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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version.

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