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RESEARCH ARTICLE

# Effect of oxic/anoxic switches on bacterial communities and PAH biodegradation in an oil-contaminated sludge

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**Abstract**

*Purpose* We studied the effect of alternations of aeration on both the autochthonous bacterial communities from an oily sludge to the endogenous polycyclic aromatic hydrocarbons (PAH) biodegradation compared to a permanent oxic condition.

*Methods* Genomic and transcriptional analyses associated with chemical measurements were used to assess the dynamics of bacteria coupled to PAH removal during an incubation of 26 days.

*Results and conclusions* The autochthonous bacterial communities of an oil sludge showed a strong potential to adapt and degrade PAH when they were subjected to alternating anoxic/oxic conditions, as well as under an oxic condition. In addition, changes in the bacterial communities were related to the different phases of hydrocarbon degradation, and the removal efficiency of PAH was similar in both switching and permanent oxic conditions. This methodology could be useful for an alternative solution of oil sludge treatment with a low-cost processing, as its efficiency is similar to that of a permanent oxic incubation which is more expensive in oxygen supply.

**Keywords** Anoxic/oxic alternations · Biodegradation · Polycyclic aromatic hydrocarbons · Bacterial communities · Oil sludge

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## 1 Introduction

Among the organic pollutants detected in contaminated environments, hydrocarbons represent the most widespread concern because of their persistence and toxicity to biological functions (Doyle et al. 2008). The toxic oil-recovery wastes and oil sludge can be treated by various physiochemical methods that are very expensive (Boopathy 2000; Vidali 2001), but biodegradation represents an alternative approach for the removal of organic chemicals from contaminated sites. In fact, microorganisms are known to have a strong potential for the degradation of a wide range of organic chemicals, either in oxic or in anoxic conditions (Heider et al. 1999; Head et al. 2006). Several techniques including bioaugmentation or biostimulation of such environments have been reported to be efficient (Juteau et al. 2003; Chaîneau et al. 2005). In the context of bioremediation, most studies were restricted to either oxic or anoxic conditions for the biodegradation processes (Eriksson et al. 1999; Boopathy 2004; Gafarov et al. 2006). Nevertheless, oxic/anoxic switches could be useful for a new strategy of bioremediation, by using the different biodegradation capacities of autochthonous bacteria. Indeed, two previous studies have focused on this alternative method with a specific interest on the fate of

hydrocarbons (Löser et al. 1998; Vieira et al. 2009) but not addressing the microbial communities. Even though, bacteria have demonstrated to play a major role in the transformation and biodegradation of organic pollutants (Lovley 2003). Thus, the understanding of their role and behavior in the biodegradation processes of contaminated environments and their responses to environmental parameter changes are necessary to improve bioremediation processes.

Molecular-based techniques are useful to assess the dynamics and the involvement of microbial communities in polluted environments. Most studies have focused on the characterization of bacterial communities at a genomic level during long treatment periods, without differentiating between living, dormant, and dead microbial cells (McNaughton et al. 1999; Iwamoto et al. 2000; Mills et al. 2003). However, the characterization of the metabolically active microflora is of particular importance in obtaining a global picture of the true bacterial community involved in the removal of contaminants. Recent studies have combined both genomic and transcriptional analyses, giving a more complete vision of the total microbial community and its active fraction (Nogales et al. 2001; Moeseneder et al. 2005; Mills et al. 2005; Gentile et al. 2006).

The aim of this work was to characterize the successive changes in the total and active autochthonous bacterial communities in relation to polycyclic aromatic hydrocarbons (PAH) depletion in an oil sludge during periodical anoxic/oxic switches, using genomic and transcriptional analyses. The impact of oxic/anoxic alternations has not so far been studied, simultaneously for both the dynamics of microbial communities and hydrocarbon degradation.

## 2 Materials and methods

### 2.1 Sludge sample and slurry preparation

The sample used in the experiments was collected from a sedimentation tank supplied with discharges from a petrochemical industry treatment plant to contaminated rainfall water from these industrial areas (Etang de Berre, south of France; 43°29′05″ N, 5°11′17″ E). This sample was a viscous sludge composed of aged organic matter and sediment subjected to long-term contamination by a wide range of petrochemical products.

A slurry was obtained by homogenization of 20% (*w*/*v*) oily sludge with a mineral medium (grams per liter of distilled water): $CaCl_2.2H_2O$, 0.04; $KH_2PO_4$, 0.1; NaCl, 6.56; $NH_4Cl$, 1; $MgSO_4$, 0.2; KCl, 0.1; vitamin V7 solution (Pfennig and Trüper 1992), 1 ml; trace element solution SL12 (Overmann et al. 1992), 1 ml; selenite–tungstate solution (Widdel and Bak 1992), 1 ml; pH 7.4.

### 2.2 Reactor systems and experimental conditions

The experimental system employed in this study consisted of two reactors with a working volume of two liters in batch conditions with stirring (350 rpm). The slurry (1.5 l) was distributed in each reactor.

One reactor was maintained in permanent aeration with an oxygen concentration over 2 mg/l. The second was exposed to two anoxic/oxic periods of 10 days in the anoxic condition and 3 days in the oxic condition. Aeration in the reactors was provided by filtered air using bubble diffusers, and the anoxic condition was obtained by stopping the aeration. The reactors were incubated at room temperature in the dark during 26 days. During the incubation, the temperature ranged from 22°C to 27°C, and the pH ranged from 6.5 to 8. The oxygen concentrations were over 2 mg/l during the oxic periods and 0 mg/l during the anoxic periods. Sampling for chemical and bacterial analyses was carried out in both reactors at the beginning of incubation (t0, no aeration in either reactors), after 10 days (t10), after 13 days (t13), after 23 days (t23), and finally, after 26 days (t26).

### 2.3 Total DNA and RNA isolation

At the time of each switch, 2 ml in duplicates from both reactors was collected to centrifuge at 4°C for 5 min at 10,000×*g*. Pellets were stored at −80°C until nucleic acid extraction. DNA and RNA of the total bacterial community were extracted using the Ultra Clean Soil DNA kit (MoBio Laboratories) and RNA Powersoil Total RNA Isolation Kit (MoBio Laboratories), respectively, according to the manufacturer's instructions. All extractions were performed in duplicates.

### 2.4 Reverse transcription of total RNA

Reverse transcriptions were performed on the RNA extracts with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with the modification of using the 1387R primer (5′-GGGCGGWGTGTACAAGGC-3′) (Marchesi et al. 1998) instead of random primers. Degradation of DNA was ensured by two DNAse treatments with Turbo DNA-free Kit (Ambion, Applied Biosystems) according to the manufacturer′s instructions. Complementary DNA (cDNA) was stored at −20°C prior to amplification. DNA contamination of RT samples was

controlled by amplifying nonreversed transcript samples prior to PCR amplification.

### 2.5 DNA and cDNA amplification, purification, and T-RFLP analysis

DNA and cDNA were amplified using bacteria primers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387R (Marchesi et al. 1998). The primer 63F was labeled at the 5′ end with the phosphoramidite carboxyfluorescein dye FAM. For each amplification reaction, 1 µl of diluted DNA extract (1/5) or 2 µl of cDNA sample was added to the PCR mix to a final volume of 50 µl. The mix consisted of 50 mM buffer, 0.4 mM dNTP, 0.2 µM of each primer, and 1.25 U of Taq polymerase (Ozyme). DNA was amplified in a thermocycler (Eppendorf) using the following amplification program: 95°C for 5 min (1 cycle), 45 s for 95°C, 58°C for 45 s, 72°C for 1 min (35 cycles), and 72°C for 10 min (1 cycle).

PCR products were purified with GFX PCR Purification Kit (Amersham Biosciences) and digested with 3 U of *Hae*III or 3 U of *Hinf*I in a total volume of 10 µl at 37°C for 3 h. These enzymatic restrictions (1 µl) were mixed with 20 µl of deionised formamide and 0.5 µl of internal size standard (TAMRA Size Standard Gene Scann-500, Applied Biosystems). The samples were denaturated for 5 min at 95°C and immediately chilled on ice then electrophoresed in a capillary electrophoresis (ABI Prism 310 Genetic Analyser, Applied Biosystems). The lengths of fluorescently labeled terminal restriction fragments (TRFs) were determined by comparison with internal standards, using Genescan software (Applied Biosystems). TRFs with a peak height lower than 30 fluorescence units were excluded from the analyses. Similarities between communities were analyzed by Principal Component Analysis and Cluster Analysis with Primer 6 version 6.1.6 (Primer-E) using S17 Bray Curtis similarity. Canonical Correspondence Analysis was performed with MVSP v3.13p.

### 2.6 cDNA library and sequencing

Pooled RNA extraction duplicates from each bioreactor sample were amplified as described above using 16S ribosomal RNA (rRNA) bacteria primers 63F and 1387R. PCR products were cloned into *Escherichia coli* using the TOPO Cloning Kit (Invitrogen). The ligated plasmids were transformed in *Escherichia coli* TOP10F′ (Invitrogen) following the manufacturer′s instructions. Cloned 16S rRNA gene fragments were amplified using primers M13 surrounding the cloning site. Inserts were sequenced by GATC Biotech SARL (Konstanz, Germany). Sequences (about 850 bp) were first analyzed using the CHECK CHIMERA program on the Ribosomal Database Project website (http://rdp8.cme.msu.edu/html/) (Cole et al. 2003). Then, the sequences were compared with the GenBank nucleotide database by basic local alignment search tool online searches (Altshul et al. 1997). Multiple sequence alignment of clones was performed using CLUSTALX (Thompson et al. 1997). Phylogenies were constructed with the Molecular Evolutionary Genetics Analysis version 3.0 (MEGA) program (Kumar et al. 2004) using the Kimura (1980) two-parameter model and the neighbor-joining algorithm (Saitou and Nei 1987). The significance of the branching order was determined using bootstrap analysis with 1,000 resampled data sets. Paleontological Statistics version 1.60 software (http://folk.uio.no/ohammer/past/) was used to perform rarefaction analysis and to calculate diversity indices for each clone library, with the clone phenotype similarity defined at 97% of the 16S rRNA gene sequence similarity. In order to determine the significance of differences between the clone libraries, the LIBSHUFF (LIBrary SHUFFling) method was applied (Singleton et al. 2001).

### 2.7 Nucleotide sequence accession numbers

The sequences determined in this study have been submitted to the EMBL database and assigned accession numbers FN429386–FN429651.

### 2.8 Hydrocarbons extraction and purification

Ten milliliters of slurry were filtered through glass microfiber filters (grade GF/F, Whatman). Filters were spiked with five aromatic perdeuterated hydrocarbons surrogate standard (LGC standard; Molshein, France) and then extracted twice with 40 ml of dichloromethane in an ultrasonic bath (15 min). Organic extracts were dried over $Na_2SO_4$ and concentrated by rotary evaporation. Samples were purified through low-pressure liquid chromatography on an open silica–alumina column (saturate and aromatic compounds were eluted simultaneously with a mixture of dichloromethane/pentane, 20/80). The solvent was then evaporated to 2 ml, and samples were analyzed by gas chromatography coupled with mass spectrometry.

### 2.9 Hydrocarbons analysis

Hydrocarbons were analyzed with an HP 6890 N (Hewlett–Packard; Palo Alto, CA, USA) gas chromatograph connected to an HP MSD 5973 mass spectrometer. The gas chromatograph was equipped with a capillary column

HP 5 MS, 60 m×0.25 mm ID×0.25 μm film thickness. The splitless injector temperature was maintained at 270°C. The carrier gas (He) was maintained at 1 ml/min. The oven temperature was programmed from 50°C (1 min) to 300°C with a ramp of 5°C/min. Hydrocarbons were quantified using the single ion monitoring mode with the molecular ion for each compound at 1.4 cycles/s. The Chemstation software was used for determining the concentration of individual PAH in sediment samples. The analysis of hydrocarbons was normalized with 17 αβ-hopane (m/z= 191) as a conserved internal standard according to Prince et al. (1994).

## 3 Results and discussion

### 3.1 Removal of PAH

The concentration of total PAH in the sludge was about 62 ±2 mg/g of dry sediment, with 61%, 30%, 8%, and 0.2% of hydrocarbon molecules with 2, 3, 4, and 5–6 aromatic rings, respectively. For each family of PAH, the abundance of alkylated compounds appeared to be more important than that of the parent compounds. This profile is typical of petrogenic oil (Wang and Stout 2007).

Total PAH removal was about 93% after 26 days in both the switching anoxic/oxic and permanent oxic conditions (data not shown). By using the same incubation methods with pristine sand contaminated with diesel, Löser et al. (1998) observed less than 50% of diesel biodegradation and noticed no difference of biodegradation efficiency between the two conditions over a period of 27 days. In contrast, Vieira et al. (2009) obtained similar total petroleum hydrocarbon depletion than in our study by using the same methodology, with addition of adapted bacteria. These authors also observed a better efficiency when switching between anoxic/oxic conditions.

Figure 1 shows the removal percentages of six families of PAH for the five sampling points (t0, t10, t13, t23, and t26) in the reactors. In the case of switching anoxic/oxic condition, PAH were not, or weakly, depleted during the first 10 days of the anoxic period. After the three subsequent days of aeration (t13), an important removal of PAH with two and three aromatic rings was observed (Fig. 1a). After 23 days of incubation, they were all depleted to about 95%. In contrast, fluoranthenes/pyrenes

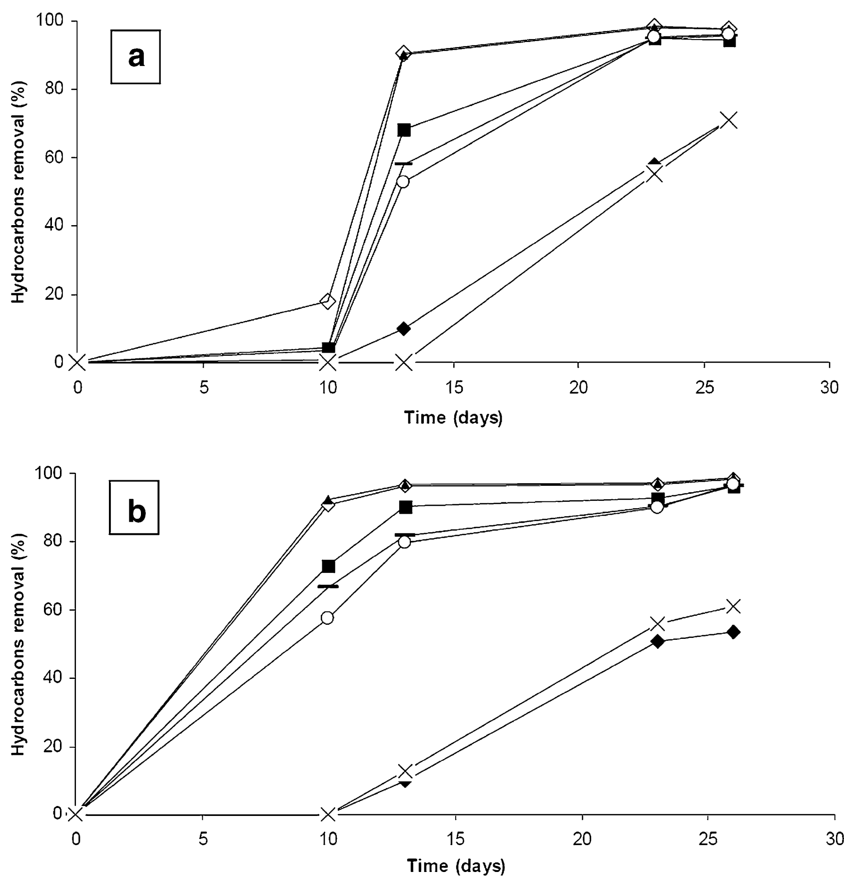


**Fig. 1** Removal percentages of PAH families (parent and alkylated forms) during 26 days of incubation for the switching anoxic/oxic (**a**) and the permanent oxic (**b**) reactors. *white diamond* benzo(b)thiophenes, *black triangle* naphthalenes, *black square* fluorenes, *straight line* phenanthrenes+anthracenes, *white circle* dibenzothiophenes, *black diamond* fluoranthenes +pyrenes, *multiplication symbol* chrysenes

(4 aromatic rings) showed only a weak depletion at t10, and chrysene (4 aromatic rings) removal started only after t13 (Fig. 1a). In the permanent oxic condition (Fig. 1b), the lighter PAH (i.e., with 2 and 3 aromatic rings) were almost completely removed after 10 days of incubation. In contrast, fluoranthenes, pyrenes, and chrysenes (heavier PAH) started to decrease in concentration only after 10 days of incubation. The removal of the lighter PAH (2 and 3 aromatic rings) in both incubation conditions was obtained either by the activity of aerobic autochthonous bacteria in the oil sludge or by volatilization (in particular, of 2 aromatic rings), after the air supply. Furthermore, in the switching anoxic/oxic condition, chrysenes and fluoranthenes showed a similar removal after only 3 days of aeration (following 10 days of anoxia) than in the oxic condition after 13 days. These hydrocarbons are known to be relatively recalcitrant to natural degradation processes, but their degradation has been reported both in anoxic (Foght 2008) and oxic conditions (Peng et al. 2008), sometimes in co-metabolism (Beckles et al. 1998; Baboshin et al. 2008). In our study, the anoxic period appears to have an influence on the degradation of these molecules, probably by stimulating anaerobic metabolisms, thus preparing the conditions for a better degradation by aerobic metabolisms after the oxygen supply. Similarly, Abril et al. (2010) and Bastviken et al. (2004) demonstrated that an anoxic period preceding an oxic period promotes the organic matter biodegradation. In addition, Hulthe et al. (1998) showed that re-exposure to oxygen of oxic coastal sediments buried to anoxia stimulates the biodegradation of organic matter. Thus, a step of anoxia by switching between anoxic/oxic conditions stimulates the biodegradation of hydrocarbons (see discussion below). Further studies are needed to understand the role of anaerobic metabolisms in this process.

Moreover, the removal of alkylated forms of the heavier PAH started in the same period as the parent compounds in both reactors. These molecules were also removed to similar proportions as the parent compounds by the end of incubation (data not shown). This result is surprising because alkylated hydrocarbons are more complex molecules and known to be more persistent than their parent compounds (Folsom et al. 1999). The presence of an alkyl branch may inhibit the orientation and accessibility of the PAH to dioxygenases (Seo et al. 2009). Again, in our study, a step of anoxia before an oxic period favored the degradation of alkylated compounds. In addition, oxic/anoxic switches stimulate both anaerobic and aerobic metabolisms, enhancing the degradation of refractory organic compounds (Aller 1994; Hulthe et al. 1998).

### 3.2 Dynamics of bacterial communities in permanent oxic and in switching anoxic/oxic conditions

The terminal restriction fragment length polymorphism (T-RFLP) fingerprints, based on 16S rRNA genes from bacterial communities, were analyzed from samples collected in the two reactors subjected to switching anoxic/oxic and permanent oxic conditions. Cluster analysis (data not shown) showed that duplicate T-RFLP patterns have more than 80% similarity; thus, the mean of the duplicates is presented in the results.

The principal component analysis (PCA) based on T-RFLP fingerprints accounted for 89% of the distribution (Fig. 2) and showed strong changes in the bacterial communities during the 26 days of incubation in both reactors. When considering an average similarity of 50%, the samples were distributed in three distinct groups. The cluster a contained all samples collected in all reactors at t0. All samples subjected to switching anoxic/oxic condition, at t10, t13, t23, and t26, clustered together (cluster b). The cluster c grouped all samples from permanent oxic

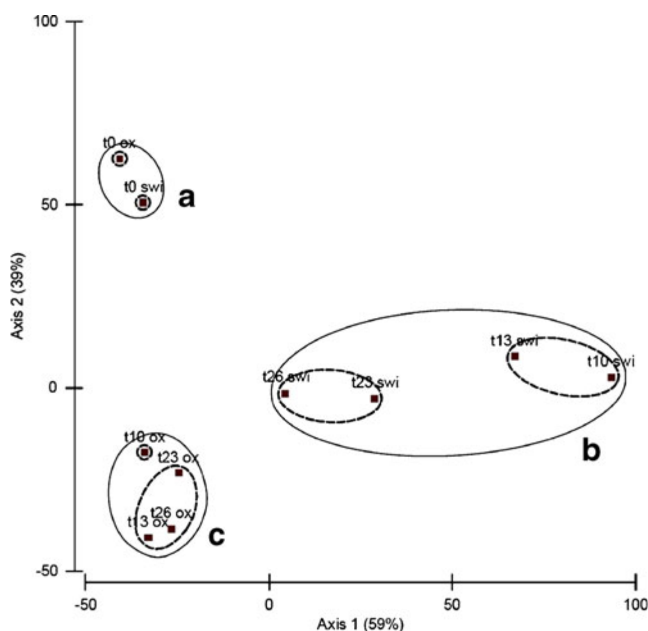


**Fig. 2** Bacterial community structure assemblages from switching anoxic/oxic (*swi*) to permanent oxic (*ox*) reactors. PCA based on the analysis of T-RFLP patterns of PCR-amplified 16S rRNA gene (mean of duplicates). Percent similarity represents the similarity between T-RFLP profiles, based on the presence of common TRFs within the profiles (Bray Curtis similarity). Each number corresponds to the day of sampling relative to the start of the experiment. *straight line* 50% of similarity, *broken line* 75% of similarity. Clusters *a*, *b*, and *c* correspond to *t0* of both conditions, *t10*, *t13*, *t23*, and *t26* of switching and permanent oxic conditions, respectively

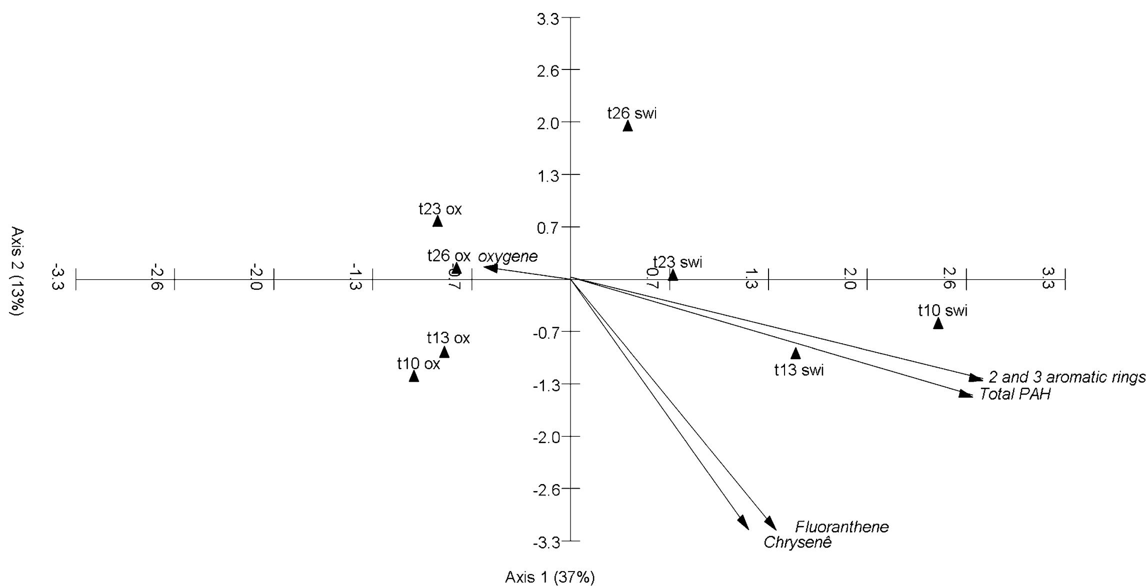


**Fig. 3** Canonical correspondence analysis between total PAHs, two and three aromatic rings, chrysene and fluoranthene contents, and oxygen and bacterial communities (mean of duplicates based on T-RFLP profiles) from each reactor. *OX* permanent oxic condition, *SWI* switching anoxic/oxic condition. Each number corresponds to the day of sampling relative to the start of the experiment

condition. A modification of bacterial community structure between the initial time and the end of incubation in switching anoxic/oxic and permanent oxic conditions revealed an adaptation of each community, specific to the incubation. The incubation mode during the first 10 days (i.e., oxic period in the case of permanent oxic condition and anoxic period in the case of switching anoxic/oxic condition) caused a disturbance on the bacterial community with a selection of the most adapted bacteria. These observations are in accordance with previous studies that showed a disturbance and a selection of adapted or resistant bacteria during a limited period after a stress (Zucchi et al. 2003; Noll et al. 2005; Konopka et al. 2007; Bressan et al. 2008).

When considering an average similarity of 75%, we observed a differentiation in the bacterial community

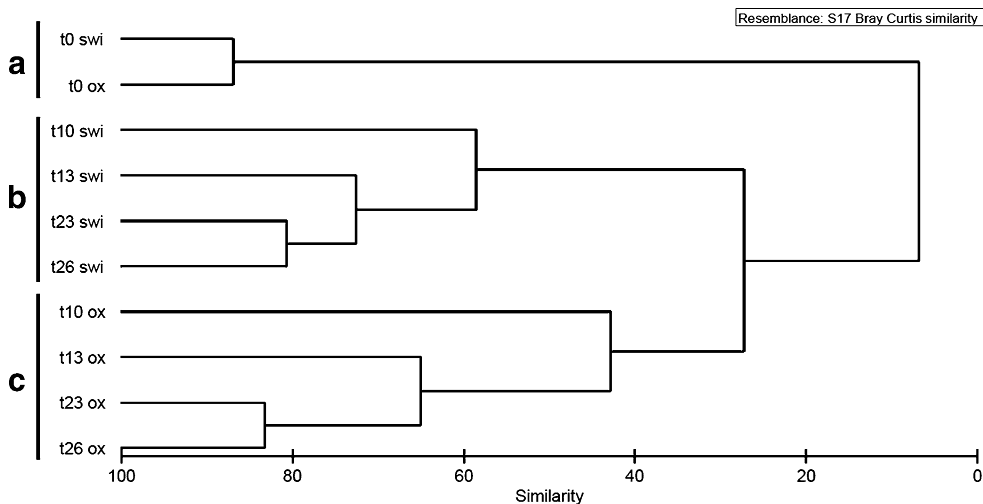


**Fig. 4** Cluster analysis of the metabolically active bacterial communities in switching anoxic/oxic (*swi*) and permanent oxic (*ox*) reactors, based on T-RFLP patterns of 16S rRNA (mean of duplicates). Each number corresponds to the day of sampling relative to the start of the experiment. Clusters *a*, *b*, and *c* correspond to *t0* of both conditions, *t10*, *t13*, *t23*, and *t26* of switching and permanent oxic conditions, respectively

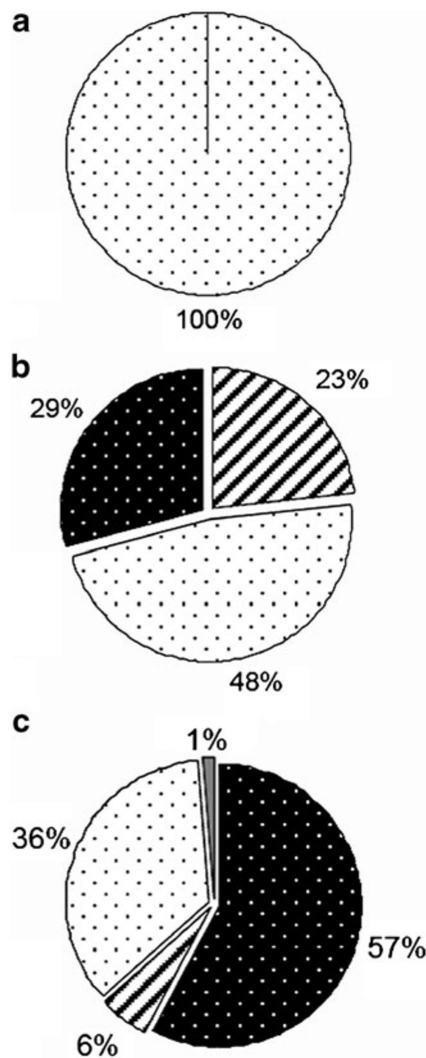


**Fig. 5** Relative abundance (%) of phylogenetic groups in 16S rRNA clone libraries from the beginning of the experiment for both reactors (**a**) and after 26 days of incubation for switching anoxic/oxic condition (**b**) and permanent oxic condition (**c**). *Gammaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*

structure between 13 and 23 days, i.e., after 3 days of aeration following the 10 days of anoxia, in the switching anoxic/oxic condition and between 10 and 13 days in the permanent oxic condition. The air supply could be the origin of the second disturbance in the case of the switching anoxic/oxic reactor, but the same disturbance was observed in the permanent oxic reactor. Thus, the air supply is not the only factor that can explain the changes in the bacterial community structure.

Figure 3 shows the influence of PAH concentrations on the bacterial community distribution. Total PAH, two- and three-aromatic ring hydrocarbons, and fluoranthene and chrysene contents were the main variables measured that

**Fig. 6** Phylogenetic tree based on the analysis of 16S rRNA cloned sequences (730-bp aligned) from 26 days of incubation in switching anoxic/oxic condition (*SWI*) to permanent oxic condition (*OX*). The name of the clone corresponds to the code of the reactor followed by the number of the clone. Percentages of 1,000 bootstrap resampling are shown above or near the relevant nodes. The *scale bar* corresponds to 0.02 substitutions per nucleotide position

accounted for 50% of the bacterial community distribution. The pattern of bacterial community structures from the switching condition followed the total PAH and two- to three-aromatic ring hydrocarbon contents until 23 days (axis 1). The pattern of community structures from the permanent aeration condition followed the oxygen variable. These results suggest a succession of bacterial populations within each community due to the loss of available substrates (depletion of a part of organic matter including two- and three-aromatic ring hydrocarbons) and the production of biosurfactants, facilitating the biodegradation, and intermediate metabolites that can serve as new sources of carbon, energy, or as co-metabolites. Similarly, Viñas et al. (2005) described changes in bacterial communities during the incubation in an aerated creosote-contaminated soil due to changes of substrates during the kinetics of total petroleum hydrocarbon depletion. Kaplan and Kitts (2004) observed a change in a petroleum land unit community after the fast-degradation phase. Thus, in our study, the air supply could cause a rapid aging of slurry in the reactors, in turn stimulating bacterial activity for the utilization and transformation of substrates. The combination of these two parameters (air supply and hydrocarbon utilization) explains the changes in diversity by selecting the bacteria most adapted to new environmental parameters, including oxygenation and available substrates, in the course of the incubation.

To confirm these observations and to have a true picture of the active fraction of bacteria involved in the communities, we focused on the dynamics of the metabolically active bacterial communities. Metabolically active bacterial communities from both reactors were analyzed for the five sampling points. As observed at the taxonomic level (16S rRNA gene), clustering analysis of T-RFLP profiles based on 16S rRNA transcripts (Fig. 4) showed three distinct clusters. Cluster a contained the samples at t0 (average similarity of 7%). Clusters b and c contained all samples of the switching anoxic/oxic condition and of the permanent oxic condition, respectively (average similarity of 27%), highlighting that each incubation condition involved a specific structure of the metabolically active bacterial communities. In addition, the increase of similarity over time, in both reactors (Fig. 4), showed constant changes in both bacterial community structures, according to the profiles of clusters b and c. These results confirm previous

SWI (16 clones)
Pseudomonas alcaligenes (Z76653)
Azotobacter beijerinckii strain G7 (AB429527)
Pseudomonas panipatensis strain Esp1 (EF424401)
SWI G07
SWI G09
OX E12
OX A04
Uncultured bacterium clone W9 (AY770949)
OX C04
SWI C01
SWI F10
Pseudomonas oleovorans (Z76665)
Pseudomonasmultiaromavorans (AB302402)
Pseudomonas putida strain F29 (EF204245)
Pseudomonas stutzeri strain CONC17 (EU275359) - SWI (2 clones) - OX (2 clones)
OXG08
OXF06
SWI H06
Pseudomonas putida (EU849665)
Pseudomonas stutzeri CCUG 11256 (U26262)
Pseudomonas stutzeri isolate st108 (AM905856)
SWI F04
OX E06
Pseudomonas stutzeri strain hyss62 (FJ613315)
SWI (9 clones) - OX (3 clones)
Uncultured bacterium clone G-39 (FJ900985)
SWI G10
Pseudomonas balearica strain SP1402 (NR 025972)
Pseudomonas stutzeri strain SP1402 (PSU26418)
OX F04
OX C03
OX F10
OX C08
OX E01
Rhodanobacter lindaniclasticus strain RP5557 (NR 024878)
OX H05
Rhodanobacter sp. CC-JY-1 (DQ239766)
Pseudomonadales
Uncultured pseudomonas sp. clone HKT140 (DQ309374)
Uncultured proteobacterium clone DR938CH110701SA (DQ230965)
Uncultured bacterium clone G3DCM-210 (EU037349)
Uncultured bacterium clone G1DCM-167 (EU037294)
SWI E02
SWI (5 clones)
Marine gamma proteobacterium BBFL3 (AF366048)
Gammaproteobacteria
SWI G12
Uncultured bacterium clone HM-51 (EU755081)
OX G09
OX D07
Uncultured bacterium clone EV818SWSAP61 (DQ337085)
OX G05
OX D08
Uncultured bacterium clone B4 (EF655621)
OX C12
Uncultured gamma proteobacterium clone CM37B4 (AM936379)
OX (5 clones)
Uncultured chromatiales bacterium clone Sc16 (EU375158)
Uncultured gamma proteobacterium clone 45 T9d-oil (FM242315) - OX (5 clones)
OX E07
Uncultured Gammaproteobacteria
OX A06
Uncultured proteobacterium clone MPCa6_H05 (EF414107)
OX B01
Uncultured bacterium clone LYC178 (DQ984610)
SWI H11
Burkholderiales
Sutterella morbirenis type strain CCUG 49335T (AJ832129)
Brachymonas petroleovorans strain CHX (AY275432) - SWI (5 clones)
Parasutterella excrementihominis (AB370250)
Ralstonia detusculanense (AF280433)
Limnobacter thiooxidans strain HLSB157 (FJ999570)
Burkholderia andropogonis strain 6177 (FJ595135)
Uncultured bacterium clone C46 (EU234261)
Ralstonia sp. P-4CB2 (AF229890)
Uncultured Burkholderiales bacterium clone REV_R1P (FJ933489)
Uncultured bacterium clone FS90 (EU593871) - SWI (4 clones) - OX (2 clones)
Betaproteobacteria
OX E02
Stella vacuolata strain DSM 5901 (NR 025583)
OX B09
OX D10
OX D06
OX A07
Azospirillum sp. 5C (AF413109)
Caenimicrobium bisanense strain K93 (EF100695)
SWI B01
SWI D01
OX E04
OX F05
Uncultured bacterium clone E91-35 (DQ001629)
OX H03
OX H10
Agrobacterium sanguineum ATCC 25661 (AB062106) - OX (2 clones)
Novosphingobium pentaromativorans strain 17-34 (EU167958)
OX H07
Sphingomonas bosoensis (AB275605)
OX A10
Sandaracinobacter sibiricus (Y10678)
OX H11
SWI G11
OX (4 clones)
Hyphomonas jannaschiana strain ATCC 33883 (NR 025326) - OX (1 clone) - SWI (2 clones)
SWI A05
SWI B11
OX D12
OX E11
SWI (6 clones) - OX (1 clone)
Parvibaculum lavamentivorans (AY387398)
SWI F01
OX G04
Rhodobacter aflagellatum strain JA194T (AM421024)
SWI B10
SWI E01
Uncultured bacterium clone CHUN-s-2 (EF632760)
Uncultured alpha proteobacterium clone AS_27_BAC (AB473971)
OX F01
OX C01
OX A08
OX C02
Paracoccus aminovorans isolate GP43 (AJ505785)
OX E08
OX B05
OX C05
OX A05
Rhodobacter sp. NO3B (DQ228136) - SWI (7 clones) - OX (1 clone)
OX (5 clones)
Rhodobacter sp. 13630K (EU741065)
SWI (2 clones) - OX (16 clones)
Alphaproteobacteria
Algoriphagus olei strain CC-Hsuan-617 (FJ231215)
OX E09
Bacteroidetes

observations, i.e., a selection of the bacteria most adapted to the incubation pressures and a succession of bacterial populations according to the sequential utilization of substrates over time.

### 3.3 Characterization of metabolically active bacterial communities by clone libraries

In order to further characterize the modifications observed in the metabolically active bacterial community structures in each reactor, cDNA libraries were constructed at t0 (pool of the two reactors) and t26 (t26ox and t26swi) (Fig. 5). A total of 266 sequences of 16S rRNA were analyzed. The comparison of the clone libraries with LIBSHUFF highlighted that 16S rRNA transcript sequences of the three libraries (t0, t26ox, t26swi) were significantly different ($XY_{t0\ t26swi}$, *P* value=0.003; $XY_{t0\ t26ox}$, *P* value=0.001; $XY_{t26swi\ t26ox}$, *P* value=0.001). A rarefaction analysis was performed in order to determine whether the number of clones analyzed was representative of the diversity. Although the curves did not reach a plateau (data not shown), the homologous curves obtained by comparison of t0, t26swi, and t26ox clone libraries with LIBSHUFF indicated that the libraries were representative of the most abundant populations in the original communities (high homologous coverage at an evolutionary distance of up to 0.05) (data not shown). The Simpson's index of the three libraries was 0.1297 for t0, 0.8445 for t26 in the switching anoxic/oxic condition, and 0.9317 for t26 in the permanent oxic condition, revealing an important increase in diversity over the 26 days of the experiment.

The three cDNA libraries showed that all sequences clustered with the phylum *Proteobacteria* (Fig. 5). All sequences obtained at t0 were related to the sequences belonging to the *Gammaproteobacteria*, affiliated to *Pseudomonadales* (Fig. 5a). According to several reports, it was not surprising to find an active bacterial community related to *Pseudomonas* at the beginning of the experiment: different substrates, including low-weight polyaromatic compounds, are bioavailable for bacteria and could be oxidized mainly by the pseudomonads (Singleton et al. 2005; Cappello et al. 2006; Popp et al. 2006). In addition, several strains of the *Pseudomonas* genus have been reported to produce biosurfactants (Ron and Rosenberg 2002). They can be considered as pioneer bacteria in the degradation of PAH.

At the end of the experiment, in the switching anoxic/oxic condition, *Gammaproteobacteria* (clone sequences close to *Pseudomonas* genus; Fig. 6) were dominant. *Alphaproteobacteria* (clone sequences close to *Stella*, *Caenimicrobium*, *Sphingomonas*, *Sandaracinobacter*, *Hyphomonas*, *Parvibaculum*, *Paracoccus*, and *Rhodobacter* genera; Fig. 6) and *Betaproteobacteria* (clone sequences close to *Ralstonia* and *Brachymonas* genera and to *Burkholderiales*; Fig. 6) were found at lower percentages (Fig. 5b). In contrast, *Alphaproteobacteria* were dominant in permanent oxic condition (Fig. 5c); *Betaproteobacteria* and *Gammaproteobacteria* were present in lower abundance. In addition, few members of the phylum *Bacteroidetes* were detected (Fig. 6) and were closely related to *Algoriphagus olei*, a new species isolated from an oil-contaminated soil (Young et al. 2009). Most of the sequences obtained were affiliated to strains isolated from oil-contaminated sites (Greene et al. 2000; Singleton et al. 2006; Shokrollahzadeh et al. 2008).

These results highlight an effective succession of bacterial populations, in both reactors, related to the incubation mode and to the changes in bioavailable substrates over time. This phenomenon of successive bacterial populations has been previously described by Kaplan and Kitts (2004) who observed a dominance of *Flavobacterium* and *Pseudomonas* during rapid hydrocarbon degradation in a petroleum land treatment unit, followed by a dominance of other bacterial phylotypes. They suggested specific bacterial phylotypes associated with different phases of petroleum degradation. A similar bacterial community succession was also observed by Greene et al. (2000) in an enrichment of a soil with a mixture of aromatic hydrocarbons.

This work highlights the link between the dynamics of autochthonous bacterial communities and the degradation of PAH in an oil-contaminated sludge subjected to anoxic/oxic switches compared with a permanent oxic condition. Genomic and transcriptional analyses of bacterial communities appeared useful to obtain a global picture of the dynamics of bacteria and for the characterization of the metabolically active microflora. The bioavailability of sources of carbon and energy and the production of intermediate metabolites, whether toxic or not, cause a rearrangement of the bacterial communities with specific phyla associated with the different steps of substrate biodegradation, i.e., a sequential utilization of substrates. In addition, similar PAH removal efficiencies in switching anoxic/oxic and permanent oxic conditions were observed. Thus, autochthonous bacteria show a strong ability to modify and adapt to changes of their environment parameters (aeration versus anoxia). The switching anoxic/oxic condition present the advantage to be less expensive. According to the literature, switching conditions provide less biomass during anoxic periods and, consequently, less sludge to be eliminated in posttreatment. This lower-cost method, without continuous air supply, could be a useful approach for bioremediation techniques especially for the treatment of PAH contaminated sludge or effluents.

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## References

Abril G, Commarieu MV, Etcheber H, Deborde J, Deflandre B, Zivadinovic MK, Chaillou G, Anschutz P (2010) *In vitro* simulation of oxic/suboxic diagenesis in an estuarine fluid mud subjected to redox oscillations. Estuar Coast Shelf Sci 88:279–291

Aller RC (1994) Bioturbation and remineralization of sedimentary organic matter: effects of redox oscillation: controls on carbon preservation. Chem Geol 114(3–4):331–345

Altshul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Baboshin M, Akimov V, Baskunov B, Born TL, Khan SU, Golovleva L (2008) Conversion of polycyclic aromatic hydrocarbons by *Sphingomonas* sp. VKM B-2434. Biodegradation 19:567–576

Bastviken D, Persson L, Odham G, Tranvik L (2004) Degradation of dissolved organic matter in oxic and anoxic lake water. Limnol Oceanogr 49(1):109–116

Beckles DM, Ward CH, Hughes JB (1998) Effect of mixtures of polycylic aromatic hydrocarbons and sediments on fluoranthene biodegradation patterns. Environ Toxicol Chem 17(7):1246–1251

Boopathy R (2000) Factors limiting bioremediation technologies. Bioresour Technol 74:63–67

Boopathy R (2004) Anaerobic biodegradation of no. 2 diesel fuel in soil: a soil column study. Bioresour Technol 94:143–151

Bressan M, Mougel C, Dequiedt S, Maron PA, Lemanceau P, Ranjard L (2008) Response of soil bacterial community structure to successive perturbations of different types and intensities. Environ Microbiol 10(8):2184–2187

Cappello S, Caruso G, Zampino D, Monticelli LS, Maimone G, Denaro R, Tripodo B, Trousselier M, Yakimov M, Giuliano L (2006) Microbial community dynamics during assays of harbour oil spill bioremediation: a microscale simulation study. J Appl Microbiol 102:184–194

Chaîneau CH, Rougeux G, Yéprémian C, Oudot J (2005) Effects of nutrient concentration on the biodegradation of a crude oil and associated microbial populations in the soil. Soil Biol Biochem 37:1490–1497

Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA, Chandra S, McGarrell DM, Schmidt TM, Garrity GM, Tiedje JM (2003) The ribosomal database project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31:442–443

Doyle E, Muckian L, Mickey AM, Clipson N (2008) Microbial PAH degradation. Adv Appl Microbiol 65:27–66

Eriksson M, Dalhammar G, Borg-Karlson AK (1999) Aerobic degradation of a hydrocarbon mixture in natural uncontaminated potting soil by indigenous microorganisms at 20°C and 6°C. Appl Microbiol Biotechnol 51:532–535

Foght J (2008) Anaerobic biodegradation of aromatic hydrocarbons: pathways and prospects. J Mol Microbiol Biotechnol 15:93–120

Folsom BR, Schieche DR, DiGrazia PM, Werner J, Palmer S (1999) Microbial desulfurization of alkylated dibenzothiophenes from a hydrodesulfurized middle distillate by *Rhodococcus erythropolis* I-19. Appl Environ Microbiol 65(11):4967–4972

Gafarov AB, Panov AV, Filonov AE, Boronin AM (2006) Change in the composition of a bacterial association degrading aromatic compounds during oil sludge detoxification in a continuous-flow microbial reactor. Appl Biochem Microbiol 42(2):160–165

Gentile G, Giuliano L, D'Auria G, Smedile F, Azzaro M, De Domenico M, Yakimov MM (2006) Study of bacterial communities in Antarctic coastal waters by a combination of 16S rRNA and 16S rDNA sequencing. Environ Microbiol 8(12):2150–2161

Greene EA, Kay JG, Jaber K, Stehmeier LG, Voordouw G (2000) Composition of soil microbial communities enriched on a mixture of aromatic compounds. Appl Environ Microbiol 66 (12):5282–5289

Head IM, Jones DM, Röling WFM (2006) Marine microorganisms make a meal of oil. Nat Rev Microbiol 4:173–182

Heider J, Spormann AM, Beller HR, Widdel F (1999) Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol Rev 22:459–473

Hulthe G, Hulth S, Hall POJ (1998) Effect of oxygen on degradation rate of refractory and labile organic matter in continental margin sediments. Geochim Cosmochim Acta 62:1319–1328

Iwamoto T, Tani K, Nakamura K, Suzuki Y, Kitagawa M, Eguchi M, Nasu M (2000) Monitoring impact of *in situ* biostimulation treatment on groundwater bacterial community by DGGE. FEMS Microbiol Ecol 32:129–141

Juteau P, Bisaillon JG, Lépine F, Ratheau V, Beaudet R, Villemur R (2003) Improving the biotreatment of hydrocarbons-contaminated soils by addition of activated sludge taken from the wastewater treatment facilities of an oil refinery. Biodegradation 14:31–40

Kaplan CW, Kitts CL (2004) Bacterial succession in a petroleum land treatment unit. Appl Environ Microbiol 70(3):1777–1786

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Konopka A, Carrero-Colon M, Nakatsu CH (2007) Community dynamics and heterogeneities in mixed bacterial communities subjected to nutrient periodicities. Environ Microbiol 9(6):1584–1590

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Löser C, Seidel H, Zehnsdorf A, Stottmeister U (1998) Microbial degradation of hydrocarbons in soil during aerobic/anaerobic changes and under purely aerobic conditions. Appl Microbiol Biotechnol 49:631–636

Lovley DR (2003) Cleaning up with genomics: applying molecular biology to bioremediation. Nat Rev Microbiol 1:35–44

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64(2):795–799

McNaughton SJ, Stephen JR, Venosa AD, Davis GA, Chang YJ, White DC (1999) Microbial population changes during bioremediation of an experimental oil spill. Appl Environ Microbiol 65 (8):3566–3574

Mills DK, Fitzgerald K, Litchfield CD, Gillevet PM (2003) A comparison of DNA profiling techniques for monitoring nutrient impact on microbial community composition during bioremediation of petroleum-contaminated soils. J Microbiol Meth 54:57–74

Mills HJ, Martinez RJ, Story S, Sobecky PA (2005) Characterization of microbial community structure in Gulf of Mexico gas hydrates: comparative analysis of DNA- and RNA-derived clone libraries. Appl Environ Microbiol 71(6):3235–3247

Moeseneder MM, Arrieta JM, Herndl GJ (2005) A comparison of DNA- and RNA-based clone libraries from the same marine bacterioplankton community. FEMS Microbiol Ecol 51:341–352

Nogales B, Moore ERB, Llobet-Brossa E, Rosselo-Mora R, Amann R, Timmis KN (2001) Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl Environ Microbiol 67(4):1874–1884

Noll M, Matthies D, Frenzel P, Derakshani M, Liesak W (2005) Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. Environ Microbiol 7(3):382–395

Overmann J, Fischer U, Pfennig N (1992) A new purple sulfur bacterium from saline littoral sediments, *Thiorhodovibrio winogradskyi* gen. nov. and sp. nov. Arch Microbiol 157:329–335

Peng RH, Xiong AS, Xue Y, Fu XY, Gao F, Zhao W, Tian YS, Yao QH (2008) Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol Rev 32:927–955

Pfennig N, Trüper HG (1992) The family *Chromatiaceae*. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) The Prokaryotes. Springer, New-York, pp 3200–3221

Popp N, Schlömann M, Mau M (2006) Bacterial diversity in the active stage of a bioremediation system for mineral oil hydrocarbon-contaminated soils. Microbiology 152:3291–3304

Prince RC, Elmendorf DL, Lute JR, Hsu CS, Haith CE, Senius JD, Dechert GJ, Douglas GS, Butler EL (1994) 17α (H), 21β (H)-hopane as a conserved internal marker for estimating the biodegradation of crude oil. Environ Sci Technol 28:142–145

Ron EZ, Rosenberg E (2002) Biosurfactants and oil bioremediation. Curr Opin Biotechnol 13:249–252

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Seo JS, Keum YS, Li QX (2009) Bacterial degradation of aromatic compounds. Int J Environ Res Public Health 6:278–309

Shokrollahzadeh S, Azizmohseni F, Golmohammad F, Shokouhi H, Khademhaghighat F (2008) Biodegradation potential and bacterial diversity of petrochemical wastewater treatment plant in Iran. Bioresour Technol 99:6127–6133

Singleton DR, Furlong MA, Rathbun SL, Whitman WB (2001) Quantitative comparison of 16S rRNA gene sequence libraries from environmental samples. Appl Environ Microbiol 67:4374–4376

Singleton DR, Powell SN, Sangaiah R, Gold A, Ball LM, Aitken MD (2005) Stable-isotope probing of bacteria capable of degrading salicylate, naphtalene, or phenanthrene in a bioreactor treating contaminated soil. Appl Environ Microbiol 71(3):1202–1209

Singleton DR, Sangaiah R, Gold A, Ball LM, Aitken MD (2006) Identification and quantification of uncultivated Proteobacteria associated with pyrene degradation in a bioreactor treating PAH-contaminated soil. Environ Microbiol 8(10):1736–1745

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Vidali M (2001) Bioremediation. An overview. Pure Appl Chem 73 (7):1163–1172

Vieira PA, Vieira RB, Faria S, Ribeiro EJ, Cardoso VL (2009) Biodegradation of diesel oil and gasoline contaminated effluent employing intermittent aeration. J Hazard Mater 168:1366–1372

Viñas M, Sabaté J, Espuny MJ, Solanas AM (2005) Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. Appl Environ Microbiol 71(11):7008–7018

Wang Z, Stout SA (2007) Oil spill environmental forensics. Fingerprinting and source identification. Academic, London, p 554

Widdel F, Bak F (1992) Gram-negative mesophilic sulfate-reducing bacteria. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) The prokaryotes. Springer, New-York, pp 3200–3221

Young CC, Lin SY, Arun AB, Shen FT, Chen WM, Rekha PD, Langer S, Busse HJ, Wu YH, Kämpfer P (2009) *Algoriphagus olei* sp. nov., isolated from oil-contaminated soil. Int J Syst Evol Microbiol. doi:0.1099/ijs.0.009415-0, In press

Zucchi M, Angiolini L, Borin S, Brusetti L, Dietrich N, Gigliotti C, Barbieri P, Sorlini C, Daffonchio C (2003) Response of bacterial community during bioremediation of an oil-polluted soil. J Appl Microbiol 94:248–257