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Bacterial communities and their hydrocarbon bioremediation potential in the Bohai Sea, China

Fenglong Yang[1], Jingshui Yang[1], Chunping Deng[1], Nan Chen[1], Shuangqing Wang[2], Entao Wang[3], HongLi Yuan[1,*]

[1]State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing 100193, PR China

[2]National Research Center for Geoanalysis, Beijing 100037, PR China

[3]Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico D. F. 11340, Mexico

[4]*Present address:* Center of Life Science, China Agriculture University, No.2 Yuanmingyuan West Road, Haidian District, Beijing 100193, PR China

ABSTRACT: Petroleum exploitation causes serious pollution to the semi-enclosed Bohai Sea (China) ecosystem. However, little is known about the influence of such pollution on the α- and β-diversity of the bacterial communities or the *in situ* hydrocarbon biodegradation potential present in the surface sediments. This was explored using 16S rRNA gene-based terminal restriction fragment length polymorphism, along with alkane hydroxylase (AlkB) and soluble di-iron monooxygenase (SDIMO) gene-based clone libraries. α-diversity (Shannon-Weaver index) was negatively correlated with the BTEX (i.e. benzene, toluene, ethylbenzene, and xylene) and total nitrogen contents. β-diversity at the phylum/class level (class level for *Proteobacteria*) and terminal restriction fragment (T-RF) level responded differently to the abiotic factors. At a small to intermediate scale (21.4 to 142.2 km), β-diversity at the T-RF level was closely correlated with geographic distances, while at the phylum/class level, it was significantly influenced by environment heterogeneity. In total, 72.61% of the AlkB sequences were related to *Gammaproteobacteria*, including *Alcanivorax* and *Marinobacter*, while the SDIMO genes were similar to *Phenol-2*, *Mmo*, *ThmA*, *PmoC* and *PrmA* genes, with 72% of the sequences found being novel. The high SDIMO gene diversity might be related to the complexity of the hydrocarbon pollution (a mixture of phenol, methane, tetrahydrofuran, propene and propane) and demonstrates the existence of *in situ* hydrocarbon biodegradation potential. Information about the diversity of bacteria and the hydroxylases is helpful to guide the *in situ* bioremediation of petroleum pollution and protect ecosystem function in the Bohai Sea.

KEY WORDS: Marine surface sediments · Petroleum pollution · Bacterial communities · Diversity · AlkB · SDIMOs

INTRODUCTION

During petroleum exploitation and transportation, spills occasionally occur, causing serious pollution to marine ecosystems throughout the world (Gong et al. 2014). The spilled oil is ultimately deposited on the ocean floor in sediments (Farwell et al. 2009), which are reservoirs of adsorbed nutrients and hazardous materials. Marine sediments harbor a large amount of microorganisms, which form the basis of the food web. These microorganisms play important roles in global biogeochemical cycles and the bioremediation of pollutants in marine ecosystems (Parkes et al. 2000, Spring et al. 2000, Köster & Meyer-Reil 2001,

*Corresponding author: hlyuan@cau.edu.cn

McGenity et al. 2012). Therefore, it is crucial to analyze the diversity of indigenous bacteria, especially the key oil-degraders and their potential to eliminate petroleum-derived pollutants. In addition, there is a clear need to accurately determine the influence of petroleum pollutants on bacterial communities, for the purpose of environmental protection and *in situ* petroleum pollution bioremediation (Yergeau et al. 2012).

Numerous field studies have spatially compared petroleum contaminated and uncontaminated sites. Various oil-degrading bacteria, mainly *Proteobacteria* (Gram-negative) and *Firmicutes* (Gram-positive), were significantly enriched by petroleum contamination (Head et al. 2006, Paissé et al. 2008, Kostka et al. 2011, Andrade et al. 2012, Rosano-Hernández et al. 2012, Acosta-González et al. 2013). However, due to the sensitivity and great plasticity of bacterial communities (Acosta-González et al. 2013, Cravo-Laureau & Duran 2014), it was suggested that the complexity of the whole sediment should be analyzed, to obtain a detailed record of the environmental conditions (i.e. physicochemical factors, nutrients and pollutants other than petroleum) and allow the identification of suitable taxa as microbial indicators of pollution in marine sediments (Martiny et al. 2006). Such studies could also reveal the mechanism driving variations in community structure, instead of mere phenomenological descriptions (Martiny et al. 2006).

Many studies have shown that microorganisms exhibit biogeographic patterns (Martiny et al. 2006), even in strongly connected marine surface sediment ecosystems (Xiong et al. 2014). A complex interplay of historical contingencies (geographic distance) and local environmental conditions influences the bacterial diversity and distribution (Schauer et al. 2010). However, it is still unknown whether this influence is the same at different taxonomic levels, for example the phylum/class level, or the terminal restriction fragment (T-RF) level.

Community functions associated with the *in situ* bioremediation of petroleum-polluted environments have received extensive attention. The hydroxylases responsible for the first step of aerobic alkane degradation in prokaryotes belong to different families: short-chain alkane-oxidizing soluble non-heme diiron monooxygenases (SDIMO); heme (cytochrome P450) and non-heme (AlkB) medium-chain alkane (C5–C16) monooxygenases; and other long-chain alkane hydroxylases (Van Beilen et al. 2006, Cappelletti 2009, Rojo 2009, Wang et al. 2010, Nie et al. 2014). AlkB and SDIMOs have been widely used to assess the diversity of oil-degraders and their oil-degrading potential (Cappelletti 2009). AlkB, as one of the key enzymes produced by aerobic alkane-degrading bacteria, was widely detected in α-, β-, and γ-*Proteobacteria*, as well as in *Actinobacteria* (Kuhn et al. 2009, Nie et al. 2014). Novel *alkB* gene sequences were obtained in hydrocarbon-seep-associated sediments in the Timor Sea, off Australia (Wasmund et al. 2009), and intertidal and shallow subantarctic coastal sediments (Guibert et al. 2012); this indicates that sediments are important sources of alkane-degrading bacteria.

SDIMOs could initiate the degradation of various petroleum-derived pollutants such as chlorinated solvents, aromatic hydrocarbons, short-chain alkanes and alkenes. SDIMOs can be divided into 5 groups, including soluble methane monooxygenases (sMMO), toluene monooxygenases, phenol hydroxylases, alkene monooxygenases, and tetrahydrofuran monooxygenase (ThmABCD) (Leahy et al. 2003, Cappelletti 2009, Li et al. 2013). Recently, a widespread distribution of SDIMO genes was found in various environments polluted with petroleum (Coleman et al. 2006, Miqueletto et al. 2011, Li et al. 2013, 2014); this suggests that SDIMOs could be used as a catabolic biomarker for the rapid assessment of bioremediation.

The Bohai Sea, in the northwestern Pacific Ocean, is an economically important marine ecosystem for fish, shrimp and crab in China (Sun et al. 2010). It is also rich in crude oil and surrounded by a string of industrial zones known as the Bohai Sea Economic Rim (BER). Along with the exploitation of crude oil and accelerated economic development of the BER, the Bohai Sea has been facing serious threats from petroleum pollution (Gao & Chen 2012, Wang et al. 2012, Gao et al. 2014). The semi-enclosed structure and relatively slow water exchange rates further aggravate the situation. As a result, the ecosystem is in poor health (Gao et al. 2014). The Chinese government launched a 15 yr program, known as the 'Bohai Blue Sea Action Plan', with a budget of about 55.5 billion yuan (9.1 billion USD) in 2001, and a further 40 billion yuan (6.6 billion USD) in 2008, to protect the Bohai Sea ecosystem. However, nothing definite has been accomplished and the species diversity of the macroorganisms has continued to decline (Gao et al. 2014). Although indigenous microbial populations adapt to the presence of toxic pollutants and play important roles in the bioremediation of contaminated environments (Pieper & Reineke 2000), little is known about the bacterial communities in the Bohai Sea. In addition, the driving force that shapes the

community structure at different taxonomic levels in this shallow marine surface sediment ecosystem is unknown.

The present study, with the main purpose of improving petroleum bioremediation techniques, focused on: (1) characterizing the α- and β-diversity of the bacterial communities in the Bohai Sea sediments using 16S rRNA gene terminal restriction fragment length polymorphism (t-RFLP); (2) identifying correlations among the diversity indicators and petroleum pollution; (3) identifying some microbial taxa as potential indicators of pollution; (4) determining the driving force (geographic distance, environmental heterogeneity, or both) that shaped the bacterial community structure at the T-RF and phylum/class levels; and (5) evaluating the *in situ* hydrocarbon biodegrading potential of the microbial communities, by constructing clone libraries using SDIMOs and AlkB monooxygenases as biomarkers.

MATERIALS AND METHODS

Sample collection and chemical analysis

Surface sediment samples (0–15 cm depth) were collected with a clamshell sampler at depths of 10–30 m in the Bohai Sea in October 2011 (Fig. 1). In total, 14 samples were collected from 3 different areas: Suizhong (SZ) oilfield, Jinzhou (JZ) oilfield and a nearshore non-oilfield (NOF) area, in Liaodong Bay (Fig. 1). Samples were kept in sterile polythene bags and transported to the laboratory (in Beijing) on ice. The sediment samples were stored at –20°C for subsequent DNA analysis. The contents of 6 BTEX compounds (i.e. benzene, toluene, ethylbenzene, *o*-, *m*-, and *p*-xylene) were determined in each sample as described previously (Qin et al. 2013), to provide an indication of petroleum hydrocarbon contamination.

Total organic carbon (TOC), total nitrogen (TN), available phosphorus (AP), available potassium (AK) and pH of the 14 samples were determined in triplicate using routine methods (Bao 2000). The contents of 24 heavy metals (HM) in the samples were detected using a polarized energy dispersive X-ray fluorescence spectrometer (P-EDXRF) X-lab 2000 (Chu & Luo 2010). The environmental heterogeneity among the sediment samples was analyzed using principal component analysis (PCA), based on the sediment physical and chemical properties (BTEX, HM, TOC, TN, AP, AK and pH), using the R package 'vegan' (Oksanen 2010).

Metagenomic DNA extraction and t-RFLP analysis

Metagenomic DNA was extracted and purified from 0.5 g (dry weight) of each sample with the

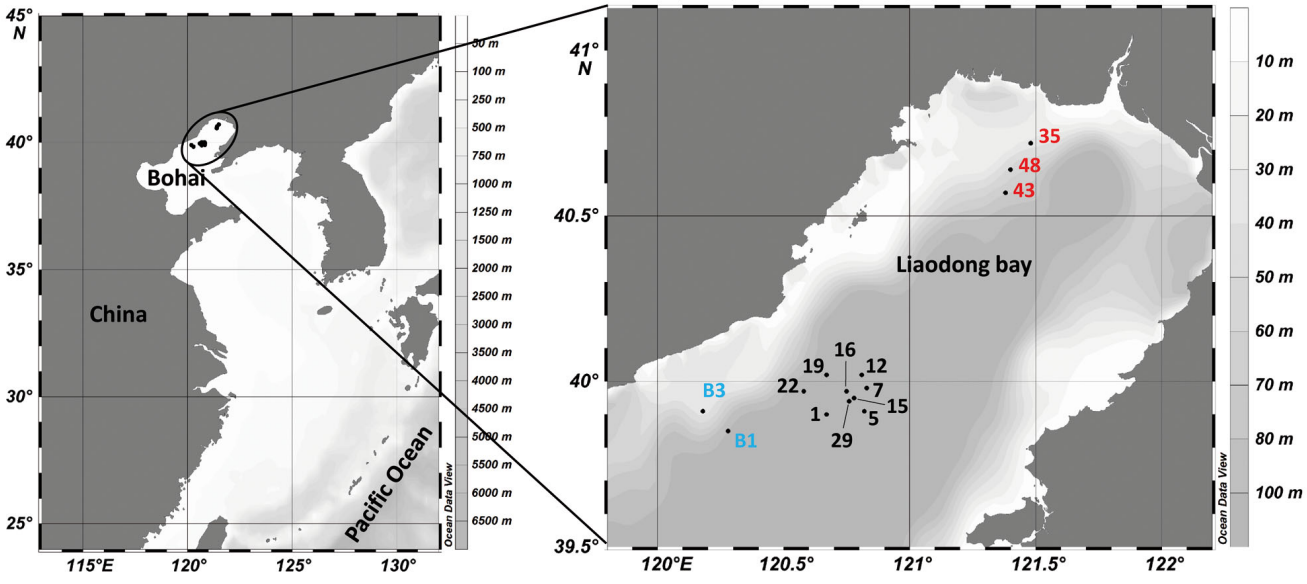

Fig. 1. Locations of the surface sediment samples collected from 3 different areas in the Bohai Sea, China, including a nearshore non-oilfield area (sample numbers in light blue), Suizhong oilfield (black numbers) and Jinzhou oilfield (red numbers). Water depths at the sampling sites are represented by the gray scale gradients. The map was generated using Ocean Data View software (Schlitzer 2015)

E.Z.N.A.™ Soil DNA Kit (Omega), according to the manufacturer's instructions, and was stored at –20°C. The fragments of 16S rRNA genes were amplified from the metagenomic DNA extracts by PCR using the primers 27F, fluorescently labeled with carboxyfluorescein (6-FAM), and 1495R (Barberio et al. 2001), as described by Sun et al. (2009). PCR products were visualized using electrophoresis in an agarose gel (1%, w/v), with 1 × TAE (Tris-acetate-EDTA) as the electrode buffer. They were pooled and then purified using the QIAquick PCR purification kit (Tiangen), according to the manufacturer's instructions. Triplicate 100 ng aliquots of purified fluorescent-labeled PCR products from each sample were digested separately with 10 U of *MspI, HhaI* and *HaeIII* in a 20 µl reaction volume for 3 h at 37°C, following the instructions of the manufacturer (Thermo Scientific). The digested products were purified and electrophoresed using an ABI3730 genetic analyzer (Applied Biosystems) as reported previously (Sun et al. 2009). The chromatograms were analyzed with GeneMarker software (version 2.2.0, SoftGenetics). The size of T-RF was estimated by referring to the size of the internal standard (GS500, Applied Biosystems), and only T-RFs with lengths of 50–500 bp and peak heights ≥ 25 fluorescence units were used for further analysis (Dunbar et al. 2001, Sun et al. 2009).

Statistical analysis of the t-RFLP patterns and phylogenetic assignment of T-RFs

T-Align (Smith et al. 2005) (http://inismor.ucd.ie/~talign/) was used to generate a matrix that showed whether a T-RF was present in a particular sample and its relative peak area. Operational taxonomic units (OTUs) were defined as unique fragment lengths. The abundance of each OTU was estimated from the relative areas of the T-RFs. Traditional α-diversity indices were calculated as described previously (Dunbar et al. 2000), including Shannon-Weaver index (H'), reciprocal of Simpson's index ($1/D$). Simpson Evenness index (E) was calculated using the formula $E = H'/\ln$ Richness, where Richness is the number of total T-RFs detected (Dunbar et al. 2000).

Only the T-RFs that had relative abundances >1% in at least one of the 14 samples were used to set up a new data matrix. The data were then square-root transformed, to downweight the contribution of highly dominant T-RFs, prior to hierarchical clustering and non-metric multidimensional scaling (NMDS) ordination (Lauber et al. 2008), done (based on Bray Curtis distance) with the R vegan package (Oksanen 2010). The robustness of the NMDS analysis was evaluated by calculating the Kruskal Stress.

The Phylogenetic Assignment Tool (PAT) (Kent et al. 2003) (http://mica.ibest.uidaho.edu/pat.php) was used to assign phylogeny to the formatted t-RFLP data, according to the PAT requirements and based on a database of fragments produced by known 16S rRNA gene sequences from the National Center for Biotechnology Information Database (NCBI) (Gil et al. 2006). As a conservative measure, high taxon levels (class level for *Proteobacteria* and phylum level for other bacteria) were used (Gil et al. 2006, Zhang et al. 2008). Only the phyla/classes that had relative abundances >1% in at least one of the 14 samples were used to set up a matrix. Redundancy analysis (RDA) was performed, using the R vegan package (Oksanen 2010), to analyze the correlations between β-diversity at the phylum/class level and the environmental factors. Significant tests of Monte Carlo permutations (999 permutations) were used to select the environmental variables (normalized by z-score transformation) and build the optimal RDA model of the bacteria–environment relationship (Oksanen 2010).

Simple and partial Mantel tests were used to determine the significance of the correlation coefficients between the community dissimilarities (at the T-RF and phylum/class level, based on Bray-Curtis distance), geographic distances and environmental dissimilarity matrices, using the R vegan package (Mantel 1967). Spatial dissimilarities, based on the geographic distance (latitude and longitude) between the sites and environmental dissimilarities (TOC, TN, AP, AK, pH, HM, BTEX and depth) were used to explain the community dissimilarity. Pearson correlation analysis was used to assess whether pollution and/or environmental heterogeneity had a significant effect on community diversity and, furthermore, to identify which individual bacterial phyla/classes could be used as bioindicators of environmental pollution.

Diversity of *alkB* and SDIMO genes

Partial *alkB* genes (approximately 550 bp) were amplified with primers and PCR protocols previously described (Kloos et al. 2006, Wasmund et al. 2009). A nested PCR strategy was used to amplify a region of the SDIMO alpha subunit gene (approximately

420 bp) using the degenerate primers NVC57, NVC58, NVC65 and NVC66, as described by Coleman et al. (2006).

For each sample, duplicate PCR reactions were performed, pooled and gel-purified using a gel extraction kit (TaKaRa) according to the manufacturer's instructions. The gene fragments were cloned using pGEM-T easy vector (Promega) and then transformed into competent cells of *Escherichia coli* DH5α following the manufacturer's guidelines. Transformed cells were detected on an LB/Amp/X-gal/IPTG plate, and the DNA inserts were verified by PCR using M13 primers. Clone libraries were constructed, and positive clones were selected randomly for sequencing. All nucleotide sequences were edited (vector sequences removed) and identified by comparison with closely related sequences via BLAST searches (Altschul et al. 1997). The program Bellerophon (Huber et al. 2004) was used to detect chimeric sequences. The remaining trimmed sequences were submitted to GenBank under the accession numbers KJ803283 through KJ803439 for *alkB*, and KJ803440 through KJ803564 for the SDIMO genes.

Bioinformatic and statistical analyses of the *alkB* and SDIMO data

All the translated AlkB or SDIMO protein sequences and the closely related sequences obtained from GenBank/NCBI were aligned with ClustalW. MEGA 6.0 (Tamura et al. 2013) was used to construct neighbor-joining (NJ) phylogenetic trees, with 1000 bootstrap replicates, and a pairwise distance matrix of the sequences was prepared as an input for the mothur platform (Schloss et al. 2009).

The deduced protein sequences were grouped into operational protein families (OPFs) using a distance cutoff value of 0.20 (Wasmund et al. 2009, Guibert et al. 2012). This cutoff value was selected based on the examination of previously constructed phylogenetic trees that included all the protein sequences. The generated OPFs were 100% identical to the phylogenetic clusters. Chao1 richness estimates (Chao 1987) and Shannon-Weaver diversity index (H') (Shannon & Weaver 1949) for each site were generated using the mothur program (Schloss et al. 2009). Coverage (C) was also calculated using the formula $C = [1 - (n/N)]$, where n is the number of OPFs that contained only 1 sequence, and N is the total number of sequences (Chelius & Triplett 2001).

RESULTS

Sample description and general sediment properties

PCA of the physicochemical properties (BTEX, HM, TOC, TN, AP, AK and pH) indicated that the environmental factors varied substantially among the sediment samples; PCA axis 1 explained 59.3% of the variability, and PCA axis 2 explained 17.7% (Fig. 2). Generally, samples from the same sampling area had similar environmental conditions and thus were grouped together in the PCA ordination plot, with the exception of Sample 48 from the JZ oilfield; this sample contained high levels of TOC (mean ± SD: 15.84 ± 0.20 g kg^{-1}) and AP (44.62 ± 3.43 mg kg^{-1}), making it distinct from all of the other samples tested. The total BTEX contents of the sediments in the NOF (0.477 ± 0.019 µg g^{-1}) were significantly higher than those in the SZ (0.225 ± 0.033 µg g^{-1}, paired t-test, $p < 0.001$) and JZ (0.273 ± 0.029 µg g^{-1}, paired t-test, $p < 0.001$) oilfields (Table S1 in the Supplement at www.int-res.com/articles/suppl/m538p117_supp.pdf). The results of our physicochemical analyses show that the sediments were markedly polluted by petroleum (100% BTEX detection rate) and heavy metals (Tables S2 & S3).

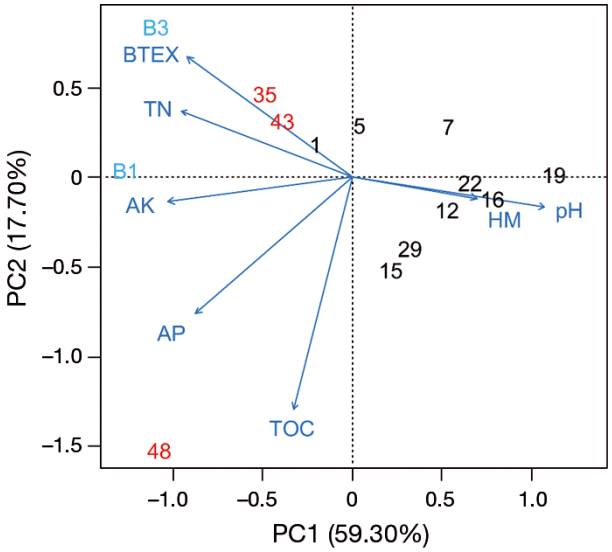

Fig. 2. A PCA ordination plot of the sediment samples and physicochemical properties at each site. Samples from the nearshore non-oilfield area are light blue; Jinzhou oilfield samples are red, and Suizhong oilfield samples are black. Arrows represent the influences of the physicochemical properties (BTEX: benzene, toluene, ethylbenzene, and xylene; HM: heavy metals; TOC: total organic carbon; TN: total nitrogen; AP: available phosphorus; AK: available potassium; and pH)

Community α-diversity at the T-RF level

The T-RFLP fingerprints generated from the *MspI, HhaI*, and *HaeIII* digestions showed consistent patterns, so the results of the *HaeIII* digestion were used to calculate the α-diversity indices. Bacterial communities from the 3 sample sites displayed a high species diversity (mean ± SD: H', 4.117 ± 0.177; $1/D$, 42.25 ± 6.193), Richness (Chao1, 115.8 ± 21.969) and evenness (E, 0.87 ± 0.020) (Table 1). In total, 13 dominant phyla/classes (with relative abundances > 1%) were detected in the Bohai Sea sediments (Fig. 3), among which *Firmicutes*, *Gammaproteobacteria* and *Betaproteobacteria* were the most abundant groups.

Community β-diversity at the T-RF and phylum/class levels

T-RF and phylum/class abundance metrics were used to document bacterial community structure at the T-RF and phylum/class levels. In the clustering results (Figs. 3 & 4), the communities were not separated based on spatial sampling regions; Sample B3 from the NOF was clustered with Sample 43 from the JZ oilfield, while Sample B1 from the NOF was clustered with 2 SZ oilfield samples (Samples 5 and 15) and 2 JZ oilfield samples (Samples 35 and 48).

Table 1. Indexes of α-diversity calculated for each sample, based on the terminal restriction fragment length polymorphism (t-RFLP) analysis. H': Shannon-Weaver index, where a higher number represents a higher diversity; $1/D$: reciprocal of the Simpson's index, where a higher number represents a higher diversity; Richness: number of terminal restriction fragments (T-RFs) detected; E: evenness index ($E = H'/\ln$ Richness)

Sample ID	H'	$1/D$	Richness	E
B1	4.123	45.891	132	0.844
B3	3.634	30.118	68	0.861
1	4.206	40.975	133	0.860
5	4.272	45.973	143	0.861
7	4.129	43.073	100	0.897
12	3.950	33.504	99	0.860
15	4.199	42.842	125	0.870
16	4.165	44.827	104	0.897
19	4.186	48.739	102	0.905
22	4.380	51.901	137	0.890
29	4.102	40.603	109	0.874
35	4.155	45.026	137	0.845
43	4.180	45.024	136	0.851
48	3.961	33.023	96	0.868
Average	4.117 ± 0.177	42.25 ± 6.19	115.8 ± 22.0	0.87 ± 0.02

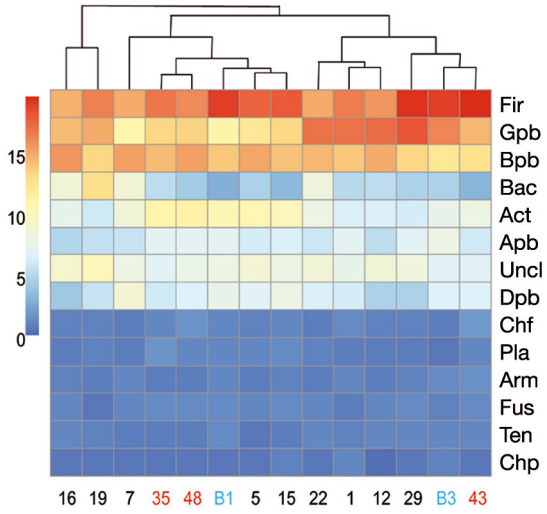

Fig. 3. β-diversity of the microbial communities at the phylum/class level. A color-scale heat map demonstrating the distribution of the 13 most dominant phyla/classes (>1%; estimated based on the t-RFLP data) across all 14 samples; red cells indicate higher proportional abundance (%), and blue cells indicate lower proportional abundance. Fir: *Firmicutes*; Gpb: *Gammaproteobacteria*; Bpb: *Betaproteobacteria*; Bac:, *Bacteroidetes*; Act: *Actinobacteria*; Apb: *Alphaproteobacteria*; Uncl: unclassified bacteria; Dpb: *Deltaproteobacteria*; Chf: *Chloroflexi*; Pla: *Planctomycetes*; Arm: *Armatimonadetes*; Fus: *Fusobacteria*; Ten: *Tenericutes*; and Chp: *Chlorophyta*

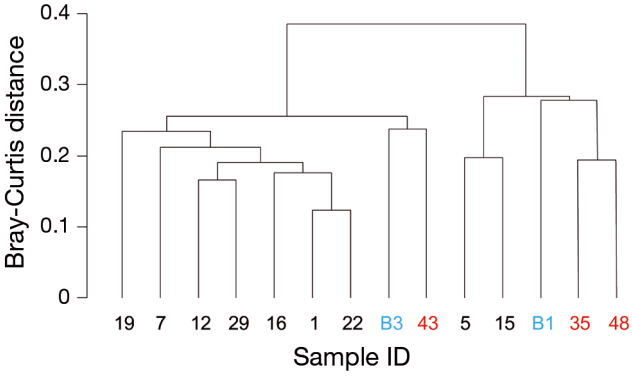

Fig. 4. β-diversity of the microbial communities at the T-RF level. Microbial communities, based on their between-sample similarities according to a Bray-Curtis distance matrix. Sample IDs are colored based on sampling area: Jinzhou oilfield, red; nearshore non-oilfield, light blue; Suizhong oilfield, black

The 2D stress (a measure of distortion) in the NMDS configuration is relatively low (0.06); thus, the 2D distance between points in the ordination plot provides a good representation of the degree of similarity between each sample's bacterial community (stress < 0.05, excellent representation; stress < 0.1, good) (Fig. 5 and Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m583p117_supp.pdf).

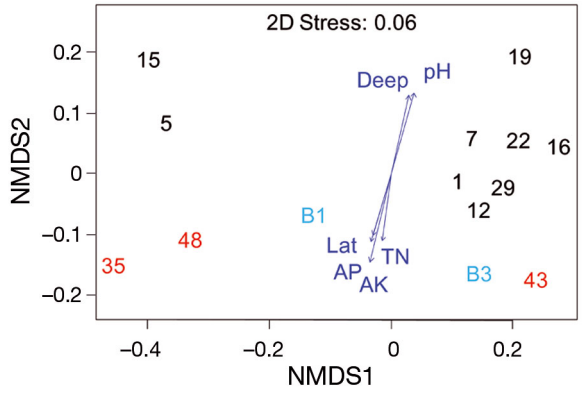

Fig. 5. β-diversity of the microbial communities at the T-RF level. NMDS ordination of the t-RFLP data, with bacterial community differences represented as Bray-Curtis distances. Sample IDs are colored based on sampling area: Jinzhou oilfield, red; nearshore non-oilfield, light blue; Suizhong oilfield, black. Arrows indicate the environmental factors (TN: total nitrogen; AP: available phosphorus; AK: available potassium; Deep: water depths at the sampling sites; Lat: latitude)

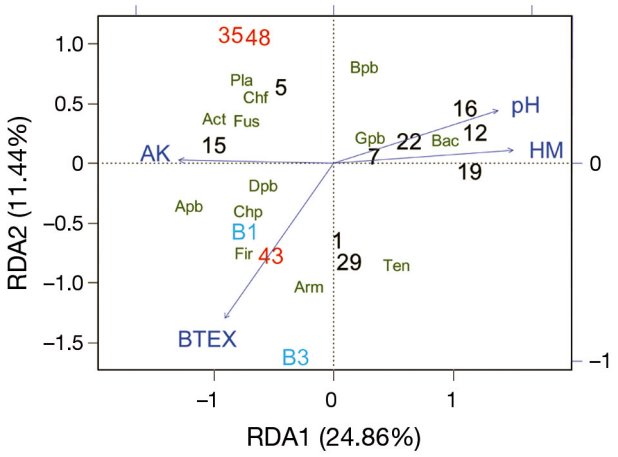

Fig. 6. Redundancy analysis (RDA) plot showing β-diversity of the microbial communities at the phylum/class level. Phyla/class names are abbreviations in green (see Fig. 3). Sample IDs are colored based on sampling (see Fig. 5). Arrows indicate the environmental factors (see Fig. 2)

The RDA model with 4 environmental variables (HM, BTEX, AK and pH), significantly correlated with the ordination axes ($p < 0.05$), is relatively strong (Monte Carlo permutation test, 999 permutation, $p = 0.019$). These 4 environmental variables explained 46.51% of the variance in the bacterial communities (RDA1 explained 24.86% and RDA2 explained 11.44%; Fig. 6). Both spatial parameters (water depth and latitude) and environmental factors (TN, AP, AK and pH) were significantly related to the dissimilarities of the microbial communities at the T-RF level (Monte Carlo permutation test, 999 permutation, $p < 0.05$) (Fig. 5 and Fig. S1). The HM, BTEX, and AK contents and the pH values were the best predictors of community variation at the phylum/class level (Fig. 6).

In Liaodong Bay, at a distance between sampling sites from 1.8 to 142.2 km, β-diversity at the T-RF level was significantly correlated with geographic distance, even when the local physicochemical factors were excluded (partial Mantel, $r_M = 0.309$, $p = 0.03$); while at the phylum/class level, environmental heterogeneity significantly influenced bacterial community structure, even when the spatial factor was excluded (partial Mantel, $r_M = 0.318$, $p = 0.013$) (Table 2). However, within the SZ oilfield, at a distance between sampling sites from 1.8 to 21.4 km, β-diversity at the T-RF level was significantly correlated with environment heterogeneity, even when the spatial factor was excluded (partial Mantel, $r_M = 0.462$, $p = 0.018$). At the phylum/class level, variation of the community structure was not significantly correlated with either geographic distance or with environmental heterogeneity (Table 2).

Table 2. Pearson correlations between the β-diversity (T-RF level and phylum/class level) and both the geographic distance and environmental heterogeneity. β-diversity was calculated based on Bray-Curtis distance matrices derived from the T-RF data set (square-root transformed) and the phylum/class abundance dataset. Geo: geographic distance; Env: environmental heterogeneity, based on the dissimilarities of TOC, TN, AP, AK, pH, HM, BTEX and depth; p-values, determined by the Mantel test and based on 999 permutations, are shown in parentheses after the Mantel r statistics and an asterisk (*) indicates a significant correlation

β-diversity	Simple Mantel tests		Partial Mantel tests	
	Geo	Env	Geo	Env
Liaodong Bay (n = 14)[a]				
1.8–142.2 km				
T-RF level	0.294 (0.036)*	0.151 (0.143)	0.309 (0.030)*	0.180 (0.088)
Phylum/class level	−0.111 (0.824)	0.323 (0.009)*	−0.093 (0.763)	0.318 (0.013)*
Suizhong oilfield (n = 9)[a]				
1.8–21.4 km				
T-RF level	−0.159 (0.746)	0.426 (0.042)*	−0.253 (0.890)	0.462 (0.018)*
Phylum/class level	−0.166 (0.800)	0.162 (0.188)	−0.196 (0.845)	0.194 (0.127)

[a]Geographic distances between the 14 samples in Liaodong Bay and 9 samples within the Suizhong oilfield

Correlations between community diversity, dominant phyla/classes and environmental factors

Pearson correlation analysis revealed that the Shannon-Weaver index correlated negatively with the BTEX and TN contents in the sediments ($p < 0.05$), while the Simpson Evenness index correlated negatively with the BTEX, TN and AK contents, but positively with the pH ($p < 0.05$) (Fig. 7A).

The phylotype richness patterns varied significantly among the samples, and 6 dominant phyla/classes (>1%) showed sensitivity to the environmental factors ($p < 0.05$); their abundances changed linearly along the environmental gradients (Fig. 7B).

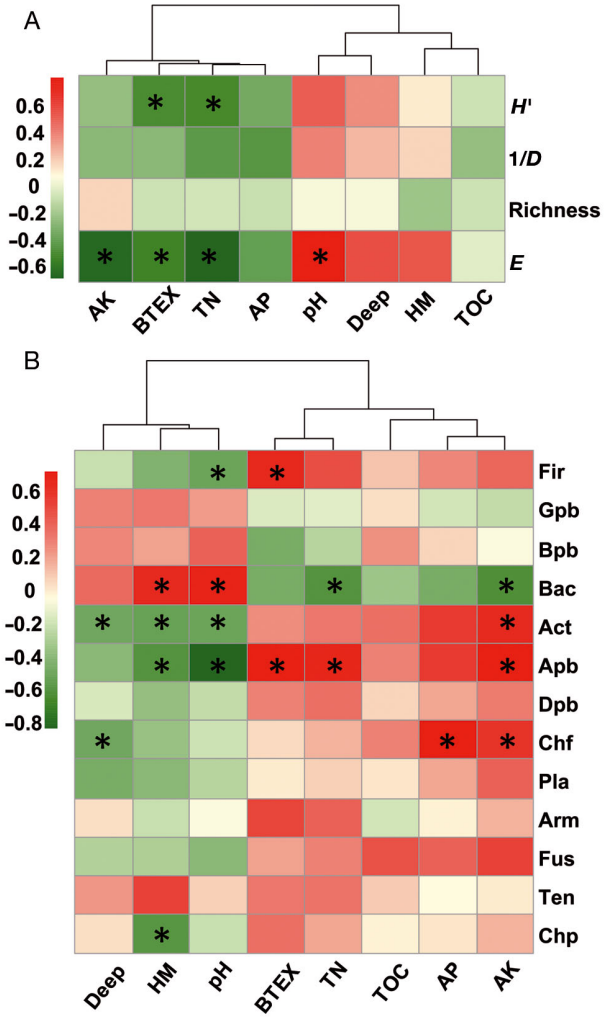

Fig. 7. Pearson correlations between (A) within-community (α) diversity (see Table 1 for definitions) and environmental factors (see Fig. 2); and (B) the abundance of dominant phyla/classes (see Fig. 3) and environmental factors. Measurements for the color scales represent Pearson correlation coefficients—Red: strong positive correlation; yellow: weak correlation; green:, strong negative correlation; *: significant correlation at $p < 0.05$

The relative abundance of *Firmicutes* was positively correlated with the concentration of BTEX and negatively correlated with pH. The relative abundance of *Bacteroidetes* was positively correlated with HM content and pH and negatively correlated with TN and AK levels. The relative abundance of *Actinobacteria* was positively correlated with AK and negatively correlated with HM, pH and the water depth of the sampling sites. The relative abundance of *Alphaproteobacteria* was positively correlated with AK, TN, and BTEX contents and negatively correlated with HM content and pH. *Chlorophyta* relative abundance was also negatively correlated with HM content. The relative abundance of *Chloroflexi* was reduced as the water depth increased and was positively correlated with AP and AK levels.

α-diversity indices of the *alkB* and SDIMO genes

To help uncover the key players in the biodegradation of alkanes and evaluate their *in situ* bioremediation potential in the petroleum-contaminated sediments, 3 representative samples from non-oilfield (Sample B3) and the SZ oilfield (Samplese 5 and 29) were chosen, according to the clustering dendrogram of the bacterial communities (Fig. 4). A total of 6 clone libraries of partial SDIMO and *alkB* genes were constructed to study the functional diversity involved in the biodegradation of the diverse petroleum pollutants. The α-diversity (*H'*), Richness (Chao1 and observed OPFs) and coverage (*C*) of each individual (and the pooled) clone library were calculated (Table 3). Generally, the library coverages were approaching the plateau stage (87.76 to 97.78%), indicating a satisfactory sequencing effort. Rarefaction analysis also indicated a saturated OPF 'discovery' for each of the libraries (data not shown). A total of 125 SDIMO gene sequences and 157 *alkB* sequences were retrieved from the genomic DNA of the 3 samples. These were clustered into 9 and 17 separate OPFs, based on the deduced protein sequences using a distance cutoff of 0.20.

Phylogenetic diversity of AlkB and SDIMO protein sequences

Among the 17 OPFs of AlkB, OPF1 (comprising 46 sequences) and OPF4 (16 sequences) matched the deduced AlkB protein sequences from *Marinobacter*, with high identity values (96 to 100%); OPF5 (8 sequences) was clustered with *Alcanivorax* sp. S9-

Table 3. Diversity and richness estimators of the *alkB* and SDIMOs gene clone libraries (cutoff = 0.20). *H'*: Shannon-Weaver index of diversity; Richness: number of operational protein families (OPF) observed (at the 80% level); numbers in parentheses are the number of clones used in the analysis. CHAO1: Chao1 richness estimate; C: library coverage

Sample ID	*H'*	Richness	CHAO1	C (%)
SDIMOs				
5	0.53	3 (45)	3.00	97.78
B3	0.64	4 (49)	5.00	95.92
29	1.50	7 (31)	8.50	90.32
Pooled	0.96	9 (125)	11.00	96.80
AlkB				
5	2.10	13 (49)	16.75	87.76
B3	2.23	13 (55)	16.33	90.91
29	0.93	7 (53)	17.00	90.57
Pooled	2.16	17 (157)	18.50	97.45

11 (96% identity); OPF13 (2 sequences) was grouped with *Dietzia* sp. UCD-THP (99% identity); OPF6 (7 sequences) and OPF7 (6 sequences) were grouped with sequences related to the uncultured bacteria from a hydrocarbon seep sediment, with a similarity above 91%; and OPF8 (4 sequences) was clustered with an uncultured bacterium from an Antarctic marine sediment, with a similarity above 80%. The other 10 OPFs (68 sequences) were single lineages distantly related to the reported genes. The affiliation of 26.11% sequences with uncultured bacteria, and the definition of 10 OPFs as being derived from novel genes, indicate that the sediments in the Bohai Sea served as a great source for novel genes and novel alkane-degrading species.

In the phylogenetic analysis of the SDIMO deduced protein sequences, 5 OPFs (22.4% relative abundance) matched *Phenol-2*-like, *Mmo*-like, *ThmA*-like, *PmoC*-like and *PrmA*-like genes. OPF5, with a 90% amino acid sequence identity to phenol-2 monooxygenase (*Phenol-2*-like) from *Marinobacter adhaerens* and OPF9 with a 90% identity to methane monooxygenase (*Mmo*-like) from *Catellibacterium nectariphilum* were only found in oilfield sediment (Sample 29). OPF7, with an average of 62% protein sequence identity to tetrahydrofuran monooxygenase (*ThmA*) from *Patulibacter* sp. I11, was only retrieved in the non-oilfield sediment (Sample B3). OPF2 had an average of 70% protein sequence identity to the propane monooxygenase hydroxylase (*PrmA*-like) large subunit from *Mycobacterium* sp. ENV421 and OPF4 had an average of 63% protein sequence identity to the epoxidase (*PmoC*-like) subunit from *Gordonia rubripertincta*; these were found in both oilfield and non-oilfield sediments (Samples B3 and 5, respectively). The other 4 OPFs (OPF1, 3, 6, 8; 77.6% relative abundance) were related to uncultured bacteria (Fig. 8B). 72% of the newly discovered SDIMO gene sequences (clustered in OPF1) that were retrieved in this study were distantly related (38% sequence identities) to previously reported sequences from *Mycobacterium rhodesiae* NBB3 (Table S6).

DISCUSSION

Pollution status of the Bohai Sea sediments

The Bohai Sea served as a good shallow marine ecosystem model for the study of the influences of petroleum pollution on bacterial communities. Petroleum and heavy metal pollutions existed in the sediment samples. Sediments collected from the NOF showed higher BTEX contents than those in the SZ and JZ oilfields (Table S1). The coastal region known as the BER is now one of the most densely industrialized and populated zones in China (Gao et al. 2014), displaying high levels of BTEX pollution from the petrochemical works along the coast. The average contents of Zn, Cu, and Pb (Table S4) in the sediments were higher than those in historical monitoring data (1980–2003) (Qin et al. 2007) and the 'national coastal background standard values' (Yan et al. 1986). This suggests that heavy metal pollution of the Bohai Sea ecosystem has increased since 1980. The mean TOC contents measured at all the 3 sites (mean ± SD: 15.21 ± 0.29 g kg^{-1}) were higher than the levels previously found along the BER (4.71 ± 3.65 g kg^{-1}) (Wang et al. 2012), indicating higher levels of hydrocarbon pollution in the sea sediments than in the intertidal sediments.

Influence of BTEX pollution on bacterial communities and the bioremediation hints

This is the first study to reveal the bacterial diversity in different surface sediments across Liaodong Bay. Both α- and β-diversity of the bacterial communities were significantly affected by BTEX (comprising the major pollutants of petroleum) contents. Shannon-Weaver index and Simpson Evenness of bacteria communities correlated negatively with the BTEX contents in the Bohai Sea sediments (Fig. 7A). Previous studies have also shown that bacterial diversity in a BTEX-contaminated aquifer was lower

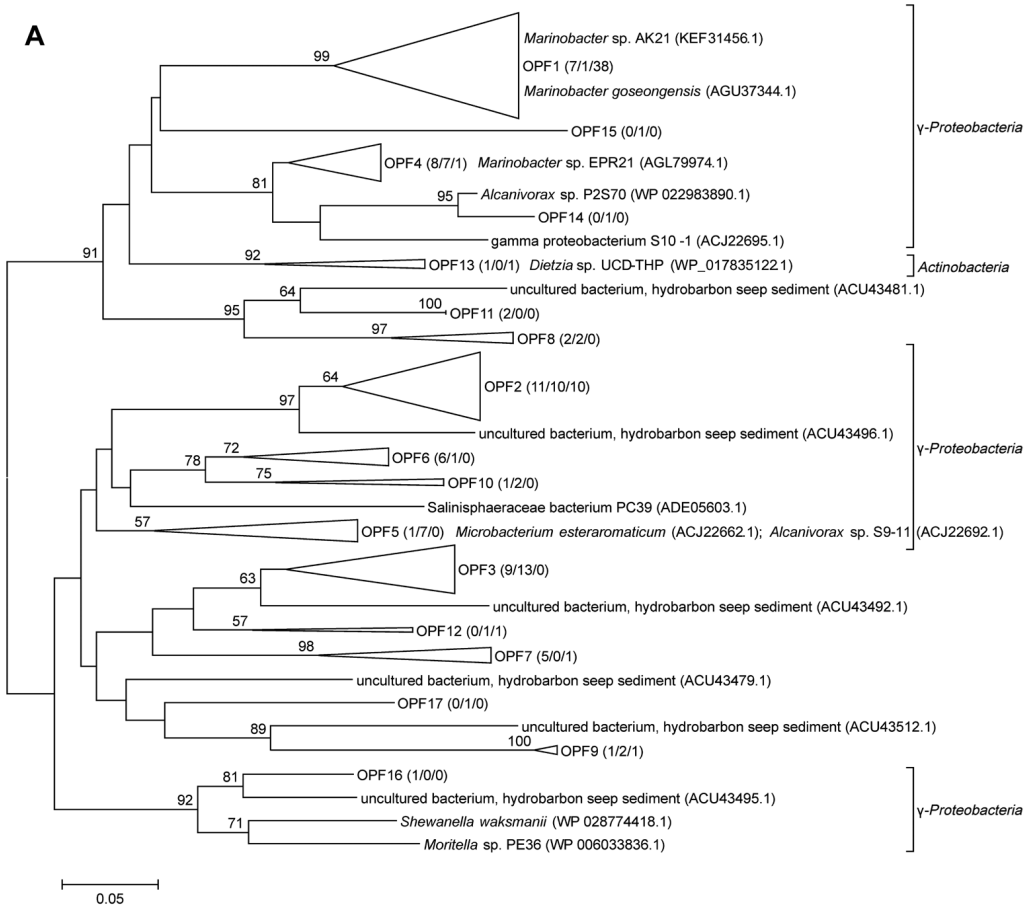

Fig. 8. A phylogenetic tree of the partial (A) AlkB and (B) SDIMO amino acid sequences retrieved from the metagenome of the Bohai Sea sediments and reference sequences. Numbers in parentheses after the operational protein family (OPF) indicate the number of sequences retrieved from the 3 sediment samples (B3, 5 and 29). Numbers in parentheses after the reference sequences are accession numbers. The scale represents 5% estimated sequence divergence

than in uncontaminated areas (Alfreider & Vogt 2007), indicating a toxic effect of BTEX on bacteria.

Firmicutes and *Alphaproteobacteria* were predicted to be dominant BTEX degraders, as their relative abundance was positively correlated with the concentration of BTEX. *Firmicutes* and *Bacteroidetes* were previously reported as dominant bacteria in high salinity and polluted aquatic environments (Kirchman 2002), indicating a high potential for adaptation by species of the 2 phyla. Previous studies have also shown that BTEX degraders are mainly from *Actinobacteria* and *Proteobacteria* (α-, β-, and γ-*Proteobacteria*) (El-Naas et al. 2014). In samples with high levels of salinity, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* were found to be dominant BTEX degraders (Li et al. 2012).

Degradation of oil is generally limited by the supply of inorganic nutrients. Röling et al. (2002) treated oil-contaminated beach sediment microcosms with different levels of inorganic nutrients and found that the nutrient amendments significantly improved oil degradation by strongly selecting bacteria that belonged to the alkane-degrading *Alcanivorax*/*Fundibacter* group. N and P were proven to be the limiting factors that influenced the oil degradation in their study. In the present study, *Firmicutes* and *Alphaproteobacteria* were enriched in a response to the BTEX pollution. *Alphaproteobacteria* was also positively correlated with TN and AK contents (Fig. 7B). Therefore, in the Bohai Sea sediments, *Alphaproteobacteria* and *Firmicutes* were inferred to be associated with BTEX biodegradation, and introducing a nutrition source (TN and AK) *in situ* might be an effective biostimulation strategy for the bioremediation of the BTEX-polluted ecosystem. At the same time, *Bacteroidetes*, *Actinobacteria*, *Alphaproteobacteria* and *Chlorophyta* might serve as good bioindicators of HM pollution.

Driving forces influencing the biogeographic patterns of microbial communities

Biogeographic patterns in microbial communities have been traditionally explained by environmental heterogeneity and geographic distance (Martiny et al. 2006, Ramette & Tiedje 2007, O'Malley 2008). Bacterial dispersal limitation existed in the surface sediments of Bohai Sea (Table 2). In the East China Sea, at small to intermediate scales (7.8 to 89.3 km), spatial distance between sampling sites was also found to contribute more to sediment bacterial community variation than any of the environmental factors tested (Xiong et al. 2014). Studies based on 16S rRNA gene sequences and t-RFLP fingerprints (Schauer et al. 2010) in deep-sea surface sediments (South Atlantic Ocean) at intermediate (10 to 3000 km) to large scales (43 000 km) indicated a complex interplay of local contemporary environmental factors and a dispersal limitation on the influence of bacterial diversity. The t-RFLP fingerprinting method screens all species above the detection threshold (Bent & Forney 2008). The data in the present study provided direct evidence (Table 2) that bacterial dispersal limitations existed in the surface sediment of the strongly connected shallow-sea ecosystem (Bohai Sea) at similar scales (21.5 to 142.2 km); this supports the hypothesis (Martiny et al. 2006) that microbial biogeographic patterns could reflect the influences of both geographic distance and contemporary environmental conditions. Local environmental heterogeneity might favor specific species, which would lead to distinctive microbial assemblages at the phylum/class level. This was shown in the present study, where pollution factors (BTEX and HM), nutrient factor (AK) and pH were significantly correlated with the dissimilarities of the microbial communities at the phylum/class level in the Bohai Sea ecosystem (Fig. 6). Potassium (K^+), as the most abundant intracellular cation, plays an essential role in the maintenance of both membrane potential and a nearly neutral intracellular pH, and enables the survival of bacterial cells under stressful conditions (Ochrombel et al. 2011). Thus, higher or lower ambient AK and pH might limit bacterial niche viability and may have led to the significantly different bacterial community structures observed in the present study.

Diversity of AlkB and SDIMO protein sequences and the hydrocarbon bioremediation potential

The Bohai Sea ecosystem had *in situ* hydrocarbon bioremediation potential, which was indicated by the diversity of the *alkB* and SDIMO genes. For *alkB* gene sequences, at the same cutoff value (0.20), 22 OPFs were defined from 17 water samples in the northern Gulf of Mexico, which experienced numerous hydrocarbon inputs (Smith et al. 2013); 30 OPFs were clustered from 202 clones (pooled from 5 libraries) obtained from chronically polluted coastal subantarctic sediments (Guibert et al. 2012); a greater richness (53 OPFs, pooled from 5 libraries) was found from 246 clones in Timor Sea sediments, where hydrocarbon seeps existed (Wasmund et al. 2009). In the present study, a lower *alkB* richness (17 OPFs) was retrieved, probably due to a smaller sequencing

effort of the *alkB* genes or because there are lower levels of alkanes in the Bohai Sea sediments.

For SDIMO, 7 OPFs were retrieved from a terrestrial petroliferous field (Potiguar Basin, Brazil) by Miqueletto et al. (2011), and 6 groups of SDIMOs were retrieved from various soil and sediment samples by Coleman et al. (2006). In the present study, a similar diversity was retrieved from the Bohai Sea sediments (9 OPFs), indicating that diverse SDIMO genes exist in the petroleum-polluted environments.

Phylogenetic analysis of the AlkB-deduced protein sequences (Fig. 8A) revealed that in the Bohai Sea, the AlkB protein sequences were closely related to previously identified AlkB sequences retrieved from databases (Wasmund et al. 2009, Guibert et al. 2012) with an average amino acid sequence identity of 86.82% (ranging from 75 to 100%; see Table S5). *Gammaproteobacteria* provided the greatest potential for alkane biodegradation in the Bohai Sea sediments, where it was the dominant class; 72.61% of the AlkB amino acid sequences obtained in the present study belonged to this class (Fig. 3). Previous studies showed that cultured bacteria (such as *Alcanivorax* and *Marinobacter*) belonging to *Gammaproteobacteria* played an important role in the degradation of alkanes in marine environments (Head et al. 2006). *Dietzia* is an aerobic, Gram-positive actinomycete (Koerner et al. 2009); strains of *Dietzia* have previously been isolated from hydrocarbon-contaminated ecosystems and shown able to degrade *n*-alkanes (Radwan et al. 2007, Sette et al. 2007). However, only 1.27% of the AlkB sequences retrieved from *Dietzia* (*Actinobacteria*) were retrieved in the petroleum-polluted Bohai Sea sediments, which might be due to their lack of adaptability to the anaerobic environment of the marine sediments.

Phenol-2, *Mmo*-, *ThmA*-, *PmoC*- and *PrmA*-like genes were retrieved in the Bohai Sea sediments, with 72% of the sequences being novel. This suggests that the Bohai Sea sediment might be a rich reservoir for novel SDIMO genes and new bacterial populations with undiscovered biodegradation functions. The catabolic substrates of the new SDIMO gene sequences were still unclear. However, the sequences obtained were useful as catabolic biomarkers for the assessment of *in situ* biodegradation processes in the Bohai Sea sediments.

Limitations of this study and future directions

Methodology is an important limiting factor in microbial ecology studies (Fuhrman 2009). Here, we conducted *in situ* sampling to study the entire bacterial communities in their actual complex biotic and abiotic contexts and evaluated both their hydrocarbon bioremediation potential and their response to environmental factors. However, it would be impossible to survey all the environmental factors that influence the communities via this approach, and ecophysiological data on the activity and degradation pathways of the oil-degrading microorganisms remain limited. Experimental ecology using simplified microbial systems that mimic (as close as possible) the real ecosystems, and allow experimental controls, is a promising approach to address these ecological questions (Cravo-Laureau & Duran 2014). Thus, further work using laboratory-controlled systems to test the biostimulation strategies proposed in this study are recommended before the application of *in situ* BTEX bioremediation.

Both medium-length alkane monooxygenase (P450) and long-length alkane hydroxylases still need to be taken into consideration, to provide a complete picture of the community functions in terms of alkane monooxygenation. Functional screening of the metagenomic libraries for enzymes with high petroleum-degrading efficiency and the development of a hydrocarbon-utilizing microbial consortium from the sediment samples are also needed for bioaugmentation techniques, to improve the elimination efficiency of petroleum-derived pollutants.

The effective prevention and control of pollution is more important for environmental protection. Further exploration of the available heavy metal and petroleum pollution monitoring methodologies, using sensitive microbial indicators to support the enforcement of environmental regulations, is very important for the protection of the Bohai Sea ecosystem.

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