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Distribution of PAHs and the PAH-degrading bacteria in the deep-sea sediments of the high-latitude Arctic Ocean

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants, which can be transferred to a long distance and tend to accumulation in marine sediment. However, PAHs distribution and natural bioattenuation is less known in open sea, especially in the Arctic Ocean. In this report, sediment samples were collected at four sites from the Chukchi Plateau to Makarov Basin in the summer of 2010. PAH composition and total concentrations were examined with GC-MS, we found that the concentrations of 16 EPA-priority PAHs varied from 2.0 to 41.6 ng g^{-1} dry weight in total and decreased with sediment depths and as well as from the southern to northern sites. Among the targeted PAHs, phenanthrene was relatively abundant in all sediments. To learn the diversity of bacteria involved in PAHs degradation in situ, the 16S rRNA gene of the total environmental DNA was analyzed with Illumina high throughput sequencing (IHTS). In all the sediments, occurred the potential degraders including *Cycloclasticus*, *Pseudomonas*, *Halomonas*, *Pseudoalteromonas*, *Marinomonas*, *Bacillus*, *Dietzia*, *Colwellia*, *Acinetobacter*, *Alcanivorax*, *Salinisphaera* and *Shewanella,* with *Dietzia* as the most abundant. Meanwhile on board, enrichment with PAHs was initiated and repeated transfer in laboratory to obtain the degrading consortia. Most above mentioned bacteria in addition to *Hahella, Oleispira, Oceanobacter* and *Hyphomonas,* occurred alternately as a predominant member in enrichment cultures from different sediments, as revealed with IHTS and PCR-DGGE. To reconfirm their role in PAH degradation, 40 different bacteria were isolated and characterized, among which *Cycloclasticus* and *Pseudomonas* showed the best degradation capability under low temperature. Taken together, PAHs and PAH-degrading bacteria were widespread in the deep-sea sediments of the Arctic Ocean. We propose that bacteria of *Cycloclasticus*, *Pseudomonas*, *Pseudoalteromonas, Halomonas*, *Marinomonas* and *Dietzia* may play the most important role in PAHs mineralization in situ.

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1 Introduction

The Arctic Ocean is the smallest major ocean and almost completely surrounded by land. It has the most extensive shelves of any ocean basin. Terrigenous organic carbon load through rivers into the Arctic Ocean has drawn attentions (Opsahl et al., 1999; Lobbes et al., 2000; Benner et al., 2004), especially about its influences on bacterial diversity in water body (Ortega-Retuerta et al., 2012; Boeuf et al., 2014).

Polycyclic aromatic hydrocarbons (PAHs) are a kind of aromatic hydrocarbons with two or more fused benzene rings, which belong to persistent organic pollutants and tend to accumulate in marine sediments. They have gathered significant environmental concerns due to their toxicity, mutagenicity and carcinogenicity (Haritash and Kaushik, 2009). Being the main components of crude oil in addition to aliphatic hydrocarbons, PAHs in marine environments are mainly attributed to oil spills, discharge and natural seepage, river import, even air current transfer (Latimer and Zheng, 2003). With the increase of human activities globally, risks to marine environments increase.

The Arctic Ocean remains less exploited due to the remoteness and ice cover. Worries about the Arctic ecosystem increase in recent years. According to the assessment by the US Geological Survey, approximately 30 and 13 % of the world's undiscovered gas and oil reserves, respectively, may be found in the Arctic region (Gautier et al., 2009). Oil-drilling platforms have been set up in some offshore shelves (McClintock, 2011; Schmidt, 2012). With the oil exploitation and the future opening of the Northeast and Northwest passages, more input of PAHs into this area is unavoidable.

PAH contaminants are widespread in marine coastal sediments (Baumard et al., 1998; Witt, 1995). They have been also found in surface sediments of the Arctic Ocean, with varied concentrations from the shelf to basin (Yunker and Macdonald, 1995; Zaborska et al., 2011; Yunker et al., 2011). On the Beaufort Sea shelf, the total concentrations of PAHs with a molecular weight of 178–278 reached $850 \pm 230\,\mathrm{ng\,g^{-1}}$ dw, with phenanthrene, benzo(*ghi*)perylene and benzo[$b + k$]fluoranthene as the dominant constituents (Yunker and Macdonald, 1995). In the western Barents Sea, the values of

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ΣPAHs ranged from 35 to 132 ng g^{-1} dw, and Benzo[b + k]fluoranthene and phenanthrene dominated in the southern and northern areas, respectively (Zaborska et al., 2011). In contrast, the total concentration of PAHs is much lower in the high-latitude deep-sea basins, such as the Makarov Basin (35 ng g^{-1} dw) (Yunker et al., 2011).

As to the origin of PAHs in deep-sea sediments, both biotic and abiotic processes, both top-down processes (suspended particle absorption and sink) and upwelling (thermogenesis below the surface) are all believed to contribute to the accumulation of PAHs (Proskurowski et al., 2008). In the case of the Arctic Ocean, it receives large input of terrigenous and fossil particulate organic matters delivered by fluvial transport and coastal erosion, and combustion particulates contributed by atmospheric transport (Yunker et al., 2011). One example, industry in the former Soviet Union has provided a widespread source of atmospheric PAHs to the Canadian High Arctic, while substantially decreased in the 1990s (Halsall et al., 1997; Becker et al., 2006).

It is well known that bacterial degradation plays an important role in PAHs removal from the marine environment. Many PAH-degrading bacteria have been found in coastal sediments, including bacteria of *Cycloclasticus* (Dyksterhouse et al., 1995), *Marinobacter* (Hedlund et al., 2001), *Pseudoalteromonas*, *Marinomonas* and *Halomonas* (Melcher et al., 2002), *Sphingomonas* (Demaneche et al., 2004), and *Vibrio* (Hedlund and Staley, 2001). But knowledge about deep-sea environments is relatively less. In previous studies on the deep-sea sediments of Atlantic Ocean and Pacific Ocean, we found that *Cycloclasticus* was the most important bacterium, in addition to *Alteromonas* and *Novosphingobium* (Cui et al., 2008; Shao et al., 2010; Wang et al., 2008). In the case of the Arctic Ocean, *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter*, *Marinobacter* and *Shewanella* were frequently reported as the crude oil degraders in coastal seawater and sea ice (Deppe et al., 2005; Gerdes et al., 2005; Brakstad and Bonaunet, 2006; Giudice et al., 2010). *Pseudomonas* was found the predominant PAHs degrader in terrestrial soils (Whyte et al., 1997; Sorensen et al., 2010; Eriksson et al., 2003). Best to our knowledge, the diversity of PAH-degrading bacteria remains unknown in the deep-sea sediments of the high-latitude Arctic Ocean.

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In 2010, during the ecological survey of the "*Xuelong*" icebreaker, we sampled deep-sea sediments across the ocean and chose four sites, respectively at the Chukchi Plateau, Canada Basin, Alpha Ridge, and Makarov Basin to examine the distribution of PAHs and PAH-degrading bacteria therein. Bacterial diversity in both sediment and PAHs enrichment cultures were analyzed. The role of bacteria involved in PAH degradation was evaluated. Results will contribute to depict the distribution pattern of PAHs and PAH-utilizing bacteria in this extreme environment, and help to evaluate the fate of PAHs once contaminated in such like environments.

2 Material and methods

2.1 Sediment collection

A total of 19 sediment cores were collected from the Chukchi Plateau, Canada Basin, Alpha Ridge, and Makarov Basin during the fourth Arctic Research Expedition of the "*Xuelong*" icebreaker in the summer of 2010. Four sites, named BN03, BN06, BN09, and BN12, were chosen as the representatives of these four typical geographical regions to examine their PAHs and degrading bacteria (Fig. 1, Table S1). The sediment cores were first sampled using a box sampler (50 cm × 50 cm × 65 cm) then subsampled using a push core sampler (Φ10 cm × 60 cm) prior to releasing the box corers on deck. The length of BN03, BN06, BN09, and BN12 cores were 20, 30, 24 and 38 centimeters below surface, respectively. Subsequently, the cores were sliced into layers at depth intervals of 4 cm, except the surface layer that was sliced at a depth of 2 or 4 cm depending on the water content. Finally, three layers of each core, the surface, the bottom and the middle (Table S1), were selected for analyses in this report. Approximately 5 g of sediment of each selected layer was used for PAH enrichment on board. The remains of the sediments were frozen immediately at −20 °C on board, transported to the home laboratory on dry ice, and stored at −80 °C until further analyses of PAH content and microbial diversity.

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2.2 Chemicals and media

Naphthalene (> 99.8 %) was purchased from Sinopharm Chemical Reagent (Shanghai, China), and phenanthrene (> 97 %) and pyrene (> 98 %) were purchased from Sigma–Aldrich. The 16 EPA priority pollutant PAH standard and six types of surrogate standards (1,4-Dichlorobenzene-d4, Naphthane-d8, Acenaphthene-d10, Chrysene-d12, Phenanthrene-d10, and Perylene-d12) were purchased from AccuStandard Inc. Crude oil was from Iraq and imported by the SinoChem Quanzhou Petrochemical Corporation (Quanzhou, China). Mineral medium was used for enrichment of PAH-degrading bacteria, which contained 1 g of NH_4NO_3, 0.8 g of KH_2PO_4, 0.2 g of K_2HPO_4, 2.8×10^{-3} g of $FeSO_4$ and 1 L of in situ deep-sea water of the Arctic Ocean. ONR7a medium was used for the cultivation of bacteria from the enriched cultures (Dykstershouse et al., 1995).

2.3 PAHs quantification of deep-sea sediments

Extraction, purification, and gas chromatography-mass spectrometry (GC-MS) quantification of the PAHs in the deep-sea sediment samples were performed according to EPA method 8270D-2007 and previous reports (Zheng et al., 2002), with some modifications. In brief, six types of surrogate standards were spiked into 20 g of freeze-dried sediment before extraction. Then, the sediment was placed into Extraction System B-811 (Buchi) and extracted with 250 mL of solvent consisting of a mixture of *n*-hexane and dichloromethane (1 : 1 v/v) under the hot extraction mode for 4 h. The extract was concentrated using a vacuum rotary evaporator and cleaned using column chromatography. Each type of PAH was quantified using the internal standard method according to the concentrations of the six types of surrogate standard. The lowest detection limit of each type of the PAHs is from 0.13 to 0.97 ng g^{-1} dw.

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2.4 PAH-degrading bacteria enrichment

Approximately 5 g sediment of each selected layer was added to 250 mL of mineral medium in a 500 mL sterile polypropylene bottle and supplied with 1 mL of crude oil spiked with a PAHs mixture of naphthalene, phenanthrene, and pyrene at final concentrations of 0.02, 0.01, and 0.005 g L^{-1}, respectively. Enrichment was performed on board at 4 °C, and kept in the dark without agitation for two months. Only slight bacterial growth was observed after the first round of enrichment at 4 °C. Once back to the home laboratory, the enriched cultures were transferred with an inoculum of 5 % to 100 mL of fresh mineral medium in a 250 mL flask with the PAH mixture (without crude oil) as the sole carbon and energy source, and repeated twice in the rotary shakers (150 rpm) at 25 and 15 °C every one and two months, respectively. Finally, 36 PAH-degrading consortia were obtained, with three temperature treatments.

2.5 PAH-removal rate quantification by GC-MS

To determine the PAH-removal rates of each consortium, all of the consortia and uninoculated controls were incubated in a 250 mL flask that was loaded with 100 mL of fresh mineral medium containing 5 % inoculum and the above-mentioned PAH mixtures as the carbon source. After 45 day incubation at 15 and 25 °C, the residual PAHs were extracted with 100 mL dichloromethane separated into three parts. The purification and concentration of these combined extract was further processed according to the description for PAH quantification of the sediments prior to GC-MS quantification. PAHs recoveries during the incubation and extraction were determined by quantifying the residual PAHs in the uninoculated controls.

2.6 IHTS and data analysis

Community DNA of three selected layers of each core and enriched cultures was extracted using the PowerSoil DNA Isolation Kit (MoBio) according to the instruc-

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tion manual. Amplification of the 16S rRNA gene V6 region was performed using the universal bacterial primers 967F (5'-CNACGCGAAGAACCTTANC-3') and 1046R (5'-CGACAGCCATGCANCACCT-3') as described previously (Wu et al., 2010). A set of 10 nucleotide (nt) barcodes was designed and added to the 5' end of 967F for the multiplexing of samples in the Solexa paired-end (PE) sequencing runs. Each 25 µL PCR mixture consisted of approximately 10 ng of community DNA, 0.2 µM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 × TaKaRa *Ex Taq* Buffer (Mg^{2+} free), and 2.5 units of TaKaRa *Ex Taq* DNA polymerase. PCR amplification was conducted using the following thermocycles: initial denaturation at 94 °C for 2 min; 25 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. Equimolar amplicon suspensions were combined and subjected to paired-end sequencing using the PE75 strategy on the Illumina HiSeq2000 sequencing platform in Beijing Berrygenomics Company.

After sequencing, all of the raw reads were treated using the BIPES pipeline (Zhou et al., 2011), and the chimeric sequences were trimmed using the program Uchime (version 4.0.87) (Edgar et al., 2011). The clean reads were clustered into operational taxonomic units (OTUs) using UCLUST at 97 % sequence similarity (Edgar, 2010). Taxonomy assignment of the tags and OTUs was performed using the Global Alignment for Sequence Taxonomy (GAST) method (Sogin et al., 2006; Huse et al., 2008). Before analysis of the alpha (within-sample) and beta (among-sample comparisons) diversity, the clean reads of each sample were combined into a "*.fna" file using a custom-coded Perl script, and this file was used as the input file for the QIIME (v 1.7.0) pipeline (Caporaso et al., 2010). To compare the diversity indexes, the sequence number was normalized to 7047 reads (the fewest of the 48 samples). The observed OTU, Chao1, and Shannon were calculated, and a *t* test was used to test whether significant differences existed between the scores of the four types of sample (sediment, 4, 15, and 25 °C). The beta diversity indexes were compared using unweighted UniFrac distances in principal coordinate analysis (PCoA), and the R module Adonis was used to determine whether significant differences existed in the OTU diversity (with 999 permu-

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tations). In addition, a Mantel test was used to examine the correlations between the PAHs concentrations of in situ sediments and the abundant OTUs of the four types of sample (Smouse et al., 1986).

2.7 PCR-DGGE

PCR amplification and DGGE analysis of the 16S rRNA gene V3 fragments of the enriched consortia were performed as previously reported (Cui et al., 2008). The dominant bands in the DGGE profile of each consortium were excised, re-amplified, and then cloned into vectors. After sequencing, the bands (approximately 160–200 bp) were identified using the blastn program of NCBI.

2.8 Bacteria isolation, identification and phylogenetic analysis

Cultures enriched at 15 °C were chosen as representatives for degrading bacteria isolation, and approximately 10^{-4}, 10^{-5}, and 10^{-6} dilutions of theses cultures were spread onto ONR7a agar plates whose lids were supplemented with naphthalene crystals and incubated at 15 °C in dark. Colonies of different morphology were streaked onto fresh ONR7a plates twice to obtain pure cultures. PAH utilization of these isolates was tested in 100 mL ONR7a liquid medium supplemented with 0.2 g ultraviolet-sterilized naphthalene crystals at 15 °C, reflected by cultures color change and increase of cell optical density at 600 nm.

Genomic DNA of the bacteria was extracted using the TIANamp Bacteria DNA Kit (Tiangen) following the instruction manual. The isolates were screened for unique strains using Rep-PCR fingerprint analysis and primer BOX-A1R (5'-CTACGGCAAGGCGACGCTGACG-3') (Versalovic et al., 1991). Rep-PCR was carried out using the following cycle conditions: 5 min at 94 °C for denaturation; 35 cycles of 15 s at 94 °C, 30 s at 53 °C, and 8 min at 65 °C; and a final extension at 65 °C for 8 min. The 16S rRNA genes of the unique strains were amplified using the primer set 27F and 1502R (Lane, 1991), sequenced using the sequencing primer P300 (5'-

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CCAGACTCCTACGGGAGGCAGC-3'), and then submitted to the EzBioCloud website (http://www.ezbiocloud.net/) to identify their phylogenetic position (Kim et al., 2012). Finally, a neighbor-joining tree was constructed based on the 16S rRNA gene sequences of the strains and their closest type strains using the MEGA 5.0 software (Tamura et al., 2011).

2.9 Nucleotide sequence accession numbers

The Illumina high-throughput sequencing data that resulted from the present study were deposited into the NCBI Sequence Read Archive under run accession numbers SRR975490-SRR975525 and SRR768499-SRR768507. The partial sequences of the 16S rRNA gene that were obtained in this study were deposited into GenBank under accession numbers KF470969-KF471008 (strains) and KC581800-KC581881 (DGGE bands).

3 Results

3.1 PAHs in sediments

GC-MS quantification indicated that the total concentration of the 16 targeted PAHs (Σ_{16}PAH) ranged from 2.02 to 41.63 ng g^{-1} dw (Table S2) and decreased in the sediments from the southern to northern sites (Fig. 2). Among the sites, the southernmost site at Chukchi Plateau (BN03) ranked the highest, whereas the northernmost site at Makarov Basin (BN12) was the lowest. At each site, the Σ_{16}PAH decreased with sediment depth, except for the site at Chukchi Plateau (BN03). Among the detected PAHs, the concentration of phenanthrene ranked the highest and was followed by that of naphthalene, except in the sediments of the Chukchi Plateau (Fig. 2). PAHs with four to six rings, such as the four-ringed pyrene, five-ringed Benzo[*b*]fluoranthene, and six-ringed Benzo(*ghi*)perylene and Indenopyrene, were significantly higher at the Chukchi

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Plateau site (BN03) than in the other samples. At the Alpha Ridge site (BN09), phenanthrene ranked the highest, by 14.61 ng g^{-1} dw, among the PAHs of all samples.

3.2 Community structures of the in situ sediments revealed by IHTS

To obtain the bacterial composition and more insight into PAH degraders in the in situ sediments, all 12 samples were subjected to Illumina high-throughput sequencing. The sequencing efforts and bacterial diversity indexes were presented in the Table SS3. Finally, a total of 1 152 388 raw reads were obtained, and 1 051 978 clean reads were used for further analyses using QIIME (v 1.7.0).

At phylum level, Proteobacteria were the most abundant bacteria and occupied 44.7–57.3 % of the total tags of these sediments, which was followed by Acidobacteria, Actinobacteria, Gemmatimonadetes, and Planctomycetes (Fig. S1a). At order level, the abundant orders were presented in Supplement (Fig. S1b), and the top ten dominant bacteria belonged to *Oceanospirillales*, *Actinomycetales*, *Rhodospirillales*, *Planctomycetales*, *Gemmatimonadales*, *Acidobacteriales*, *Chromatiales*, *Alteromonadales*, *Pseudomonadales*, and *Bacillales*. Among them, *Oceanospirillales, Alteromonadales*, and *Pseudomonadales* actually contained most known oil and PAH-degrading bacteria by far, such as *Pseudomonas*, *Cycloclasticus*, *Alcanivorax*, *Pseudoalteromonas* and *Marinomonas*, which are documented in more details below.

The bacteria at the genus level are shown in Fig. 3a. The abundant bacteria that occupied more than 1 % of the total tags in at least one sample were presented, including 20 known genera and 3 uncultured bacterial groups (Table S4). Among them, *Dietzia, Salinisphaera, Pseudomonas, Acinetobacter, Pseudoalteromonas, Colwellia, Bacillus, Rhodovibrio, Marinomonas,* and *Halomonas* have been reported as hydrocarbon-degrading bacteria in marine environments. In addition, *Cycloclasticus* and *Alcanivorax* are noteworthy because they have been recognized as obligate marine hydrocarbon degraders (Yakimov et al., 2007), and they were widespread in all of the sediments of this report (Table S4). Specially, *Cycloclasticus* occupied 0.2–0.5 % of the 16S rRNA gene tags in each sample.

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3.3 PAH degradation of PAH enrichment consortia

To obtain the PAH-degrading bacteria from the sediments, PAH-degrading bacteria were enriched with the sediments supplied PAHs as sole energy and carbon source as described in materials and methods. After incubation under different temperatures (4, 15, and 25 °C), all treatments displayed obvious bacterial growth, reflected by color change and turbidity of the cultures compared to uninoculated controls. The PAH-removal rates of the consortia after 45 days of incubation at 15 and 25 °C were calculated according the PAH concentrations determined by GC-MS. In general, the removal rates of phenanthrene and pyrene at 25 °C were higher than those at 15 °C (Fig. 4). Notably, the consortia of the northernmost site BN12, generally displayed high rates at 15 and 25 °C. In contrast, the removal rates of the consortia from sites BN03, BN06, and BN09 were relatively low.

3.4 Community structures of the consortia enriched with PAHs

Corresponding to the in situ sediments, thirty-six enrichment cultures were also analyzed by using Illumina high-throughput sequencing (Table S3). In these consortia, nearly all of the dominant bacteria have been previously described as hydrocarbon degraders. For the 4 °C treatments that were enriched with crude oil-containing PAHs, the predominant bacteria included *Pseudomonas*, *Pseudoalteromonas*, *Marinomonas*, *Hahella*, *Marinobacter*, *Hyphomonas*, *Cycloclasticus*, *Colwellia*, *Halomonas*, *Oceanobacter*, *Salinisphaera*, *Oleispira*, *Alteromonas,* and *Alcanivorax* (Fig. 3b, Table S5). In the treatments that were enriched with PAHs at 15 and 25 °C, *Pseudoalteromonas*, *Cycloclasticus*, *Pseudomonas,* and *Halomonas* were selected as the most abundant. In the 15 °C consortia (Fig. 3c), *Pseudoalteromonas* was the most abundant in the consortia of site BN03 and from the upper layer of BN06. In contrast, *Cycloclasticus* dominated the three consortia of site BN12 (55.1–64.5 %), whereas *Pseudomonas* was clearly dominant in the consortia of the middle layer of site BN06 and upper layer of site BN09, and relatively dominant in all three consortia from site BN12 (Fig. 3c, Table S5).

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Halomonas was the most-dominant bacterium in the 25 °C consortia (Fig. 3d), occupying 33.4–71.0 % of the tags of the communities from sites BN03 and BN06, with the exception of the upper layer of BN03. *Pseudomonas* were dominant in the consortia of the middle layer of site BN09 (named 25BN09M, 54.1 % of all tags), the lower layer of BN03 (25BN03L, 32.3 %), the lower layer of BN06 (25BN06L, 31.6 %), and the upper layer of BN12 (25BN12U, 21.6 %). Similar to the 15 °C consortia, at 25 °C, *Cycloclasticus* dominated the northernmost consortia, e.g., the middle-layer consortium 25BN12M (46.0 %), the lower-layer consortium 25BN12L (30.5 %), and the upper-layer consortium 25BN12U (29.3 %) and were relatively abundant in the consortium of the upper layer of BN09 site, which was named 25BN09U (9.1 %).

3.5 Bacterial diversity comparison based on IHTS data statistics analysis and environment parameters

To compare the diversity indexes, the tags were normalized to 7047 (the fewest of the samples), and the observed OTUs, Chao1, and Shannon indexes were obtained using software package QIIME (Table S3). Overall, all of the diversity indexes indicated that the sediments had the highest bacterial richness and evenness (Fig. S2a–c). Among the enriched cultures, the bacterial diversity was increased with temperature rise (Fig. S2a and b). The Chao1 values of the 25 °C-enriched cultures were significantly higher than the others ($P < 0.01$); correspondingly, the observed OTUs number of the 25 °C-enriched cultures were also significantly higher than 4 °C treatments (P=0.029). Principal coordinate analysis (PCoA) showed that the communities of in situ sediment and all enriched cultures could be separated using the abundant OTU dataset (Fig. S2d), indicating that they had significantly different bacterial community structures, which were supported by the results of the nonparametric statistical Adonis method ($R^2 = 0.28$, $P = 0.001$). In addition, the Mantel test results showed no correlations between the community structures of four types of sample and the individual or total PAHs concentrations of the in situ sediments.

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3.6 Community composition revealed by PCR-DGGE

To reconfirm the bacterial composition, the PAH-degrading consortia were subjected to PCR-DGGE analysis in parallel. Figure 5 presents the DGGE profiles of 12 consortia that were enriched with PAHs at 25 °C. The bacteria, represented by bands, are listed in Table SS6, and some are noted on the pattern profiles. In general, the community structures corresponded well to the IHTS results, even though the two methods targeted different regions of the 16S gene. In these consortia, the three genera *Cycloclasticus, Pseudomonas,* and *Halomonas* alternatively dominated the communities. In detail, *Cycloclasticus* dominated all three consortia from site BN12 and the consortium (25BN09U) from the upper layer of sit BN09; interestingly, it was accompanied by *Alcanivorax* (Fig. 5, lanes 7 and 10–12). This finding was in agreement with the results of IHTS data (Fig. 3d). *Pseudomonas* dominated or shared dominance with *Cycloclasticus* or *Halomonas* in four consortia (Fig. 5, lanes 3, 6, 8 and 10) that were derived from each layer of the four sites, whereas they were relatively less abundant in three consortia (Fig. 5, lanes 2, 4 and 11) that were generated from three sites. *Halomonas* appeared as very stronger bands in five consortia (Fig. 5, lanes 2–6) from sites BN03 and BN06. *Pseudoalteromonas* mainly dominated in four consortia (Fig. 5, lanes 1, 3, 5 and 6) from sites BN03 and BN06. In addition, other bacteria, such as *Marinobacter, Alcanivorax*, *Marinobacterium*, *Colwellia*, *Thalassospira*, *Celeribacter,* and *Vibrio*, were occasionally strongly or weakly scattered in some of the consortia.

At low temperatures, the PAH-degrading communities varied to some extent in comparison to the 25 °C consortia, such as in the three consortia derived from site BN12, where such bacteria as *Cycloclasticus* and *Thalassolituus* became the dominant members at 4 °C (Fig. 6a, lanes 11–13) and such bacteria as *Pseudomonas*, *Maritalea*, and *Thalassospira* were only the dominant members in 4BN12U and 4BN12M (Fig. 6a, lanes 11–12). In contrast, at 15 °C, the most dominant member was the bacteria of *Cycloclasticus* (Fig. 6b, lane 11–13), which was consistent with the composition pattern revealed in Fig. 3c.

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3.7 Bacteria isolation and their potential in PAH degradation

On the ONR7a medium plates whose lids were supplied with naphthalene crystals as the sole carbon source, bacteria were isolated from all of the PAH-degrading consortia enriched at 15 °C. Forty isolates were obtained that were affiliated to 12 genera of γ- and α-proteobacteria and Actinobacteria (Fig. S3). Bacteria belonging to γ-proteobacteria were the predominant isolates, including *Pseudoalteromonas* (18 isolates), *Halomonas* (6 isolates), *Cycloclasticus* (3 isolates), *Pseudomonas* (3 isolates), *Marinobacter* (2 isolates), and *Shewanella* (2 isolates). PAHs utilization test showed that only the bacteria of *Cycloclasticus* and *Pseudomonas* showed obvious growth after two weeks in ONR7a liquid medium supplemented with naphthalene under 15 °C.

Both IHTS and PCR-DGGE results demonstrated that *Cycloclasticus* was predominant in the PAH-degrading consortia of site BN12 (Figs. 3, 5 and 6). From these consortia, three strains were isolated and named after the consortium as 15BN12U-14 (simplified as U-14), 15BN12L-10 (L-10), and 15BN12L-11 (L-11). These organisms have identical 16S rRNA gene sequence (1497 bp) and share 99.92 % similarity with the *C. pugetii* PS-1^T type strain. However, they varied in morphology (Fig. S4) and genome fingerprint patterning by Rep-PCR (Fig. S5). Strains L-10 and L-11 resemble each other in morphology and Rep-PCR profiles; therefore, only strain L-10 was chosen for further analyses. Growth tests were conducted at 15 °C with a single PAH as the sole carbon source in ONR7a liquid medium. The results showed that strains L-10 and U-14 could assimilate naphthalene and phenanthrene but neither could use pyrene (Fig. 7). In addition, strain U-14 exhibited better growth than strain L-10 when utilizing phenanthrene (Fig. 7a vs. b).

4 Discussion

This report examined PAHs and PAH-degrading bacteria in the deep-sea sediments across the Arctic Ocean. Based on the data available by far, the total PAH concen-

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trations decreased moving north toward the pole and generally decreased with sediment depth. Among the 16 EPA-priority PAHs targeted, phenanthrene was the most dominant one. Based on the bacterial diversity data of both culture-dependent and -independent methods, the general features of PAH-degrading bacteria were revealed in the sediment samples. According to our knowledge, this report is the first to consider the diversity and abundance of PAH-utilizing bacteria in the deep-sea sediments of the high-latitude Arctic Ocean.

Recently, to trace the origin of organic matter in the deep-sea sediments of the Arctic Ocean, Yunker et al. (2011) examine the distribution of PAHs, plant odd alkanes, hopanes, and steranes in the sediments based on large dataset (Yunker et al., 2011). They found that the central Arctic Ocean basins were compositionally distinct from rivers and shelves. And, in the case of PAHs, the concentrations decreased from 100–755 $ng\,g^{-1}$ dw in the coastal sea to 35 $ng\,g^{-1}$ dw in the central basin (Yunker et al., 2011). Our results in this report show that the PAH concentrations are generally in agreement with this tendency (Fig. 2). According to the theory of global distillation effects, it would be easier to transfer naphthalene than other PAHs of high molecular weight (HMW) over long distances (Goldberg, 1975; Friedman and Selin, 2012); however, the concentration of naphthalene was less than that of phenanthrene. This discrepancy might be partially due to its higher bioavailability and degradability. In contrast, HMW PAHs with four rings and above accumulated more in areas near the south of the continent, as mentioned above (Fig. 2). In addition, the establishment of PAHs composition is probably attributed to bacterial mineralization. To detect the relationship between PAHs composition and bacterial community, we analyzed the correlations between the community structures and the concentrations of individual or total PAHs using Mantel test method. However, no definite correlation was observed. Bacterial community in situ may be influenced by many other factors like nutrients and other carbons sources in addition to PAHs in the tested samples of the Arctic Ocean.

Various bacteria involved in PAH-degradation were found existing in all the sediments, including *Cycloclasticus*, *Pseudomonas*, *Halomonas*, *Pseudoalteromonas*,

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Marinomonas, *Bacillus*, *Dietzia*, *Colwellia*, *Acinetobacter*, *Alcanivorax*, *Salinisphaera* and *Shewanella*. But most of them only occupied less than 0.5 % of the total tags (Fig. S6). However, after PAH enrichment, *Pseudomonas*, *Pseudoalteromonas*, *Cycloclasticus*, *Halomonas*, and *Marinomonas* became the dominant members alternatively in the enriched cultures (Fig. S6). For example, when enriched at 4 °C with PAHs dissolved in crude oil, *Pseudomonas*, *Pseudoalteromonas*, *Marinomonas*, *Hyphomonas*, and *Cycloclasticus* turned to be the dominant members (Fig. 3b and S6). These bacteria have been previously detected as the dominant members in oil enriched consortia of the coastal seawater and sea ice from the Arctic Ocean (Deppe et al., 2005; Gerdes et al., 2005; Brakstad and Bonaunet, 2006; Giudice et al., 2010), but quite different with those from the deep-sea oil plume during the Deepwater Horizon oil spill, which were dominated by the bacteria of the order Oceanospirillales and the genus *Colwellia* (Hazen et al., 2010; Baelum et al., 2012). When enriched with PAHs as a sole carbon and energy source at 15 and 25 °C, the obtained PAH-degrading consortia were alternately dominated by *Pseudomonas*, *Pseudoalteromonas*, *Halomnonas*, and *Cycloclasticus*. A big difference in the community structures occurred between 4 and 15 °C treatments (Fig. 3 and S2); while the bacterial community structures under 25 °C also varied to some extent in comparison with those of 15 °C, but the dominant bacteria including *Cycloclasticus, Pseudomonas, Pseudoalteromonas* and *Halomnonas* remain predominant (Fig. 3).

Bacteria of the genus *Cycloclasticus* have been recognized as obligate marine PAH degraders (Dyksterhouse et al., 1995; Yakimov et al., 2007). They usually turn to be one of the most predominant in crude oil polluted sediments or seawater (Kasai et al., 2002; Maruyama et al., 2003; McKew et al., 2007a, b; Coulon et al., 2007; Kappell et al., 2014; Dubinsky et al., 2013). In addition to coastal environments, they were also found in the deep-sea sediments of both Atlantic and Pacific Oceans in our previous reports by culture enrichment (Cui et al., 2008; Shao et al., 2010; Wang et al., 2008). In this report, this is the first time by using IHTS to confirm the wide distribution in deep-sea sediments of *Cycloclasticus* bacteria. In the Arctic deep-sea sediments, bacteria

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of this genus occupied 0.2–0.5 % of the total bacteria in each sediment sample *in situ,* deduced by sequence tags of 16S rRNA gene. They must play an important role in PAHs mineralization in this environment. Interestingly, the abundance of *Cycloclasticus* increased with sediment depth and from the southern to northern sites (Table S4), in contrast to PAH concentrations that decreased with depth and from south to north. Probably, the liable carbon sources are relatively abundant in the surface sediments, and less in the old sediments (deep layers) and remote areas like BN12 close to the North Pole. In the sediments where the liable carbon source is scarce, the PAHs may turn to be a key factor in selection of the PAH-degrading bacteria that adapt to the oligotrophic circumstances.

The *Cycloclasticus* bacteria were found in all these samples, but difficult to cultivate on plate, even with simple carbon source, such as acetate and pyruvate. After many attempts, three strains were finally obtained on the ONR7a medium plates supplied with naphthalene crystals on lids, showing tiny colonies after three weeks incubation at 15 °C. Growth tests indicated that these *Cycloclasticus* strains could use naphthalene or phenanthrene as a sole carbon source but failed to utilize pyrene. In the previous study, we isolated a pyrene-degrading bacterium from Pacific deep-sea sediment, which is only one strain of *Cycloclasticus* reported by far capable to use pyrene as its sole carbon and energy source (Wang et al., 2008); while others can utilize pyrene only in the presence of other PAHs, such as phenanthrene (Geiselbrecht et al., 1998).

In addition to *Cycloclasticus*, the following PAH-degrading bacteria, which were previously described to reside in coastal environments, were also found as dominant members in some PAH-degrading consortia in this report, such as: *Pseudomonas* (Niepceron et al., 2010), *Marinomonas* (Melcher et al., 2002), *Pseudoalteromonas* (Hedlund and Staley, 2006), *Halomonas* (Garcia et al., 2005), *Alteromonas* (Jin et al., 2012), *Marinobacter* (Hedlund et al., 2001), *Vibrio* (Hedlund and Staley, 2001), and *Thalassospira* (Kodama et al., 2008). In fact, we also isolated 64 strains from the consortia enriched at 25 °C using M2 medium plate (Wang et al., 2008), which contains more kinds of carbon source than those in ONR7a medium, such as sodium acetate,

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glucose, sucrose, sodium citrate, and malic acid. Among them, only three strains, including *Marinomonas* sp. D104, *Sphingobium* sp. C100 and *Pseudomonas* sp. C39, showed good capability of PAH-degradation at 15 and 25 °C. Genome sequencing revealed the degradation genes for PAH degradation in the first two bacteria (Dong et al., 2014b, a). Particularly, strain *Marinomonas* sp. D104 can even degrade the PAH mixture of naphthalene, phenanthrene, and pyrene at 4 °C (unpublished). Although *Pseudoalteromonas* and *Halomonas* were the most predominant members in many consortia (Figs. 3 and 5) of this study, they failed to grow with the PAHs tested by far.

Pseudomonas is a common PAH-degrader in cold environments, and frequently found in the Arctic and Antarctic soils (Whyte et al., 1997; Sorensen et al., 2010; Eriksson et al., 2003; Ma et al., 2006). In this study, *Pseudomonas* occurred in situ as one of the most dominant bacteria and occupied 1.5–1.8 % of the total bacteria of the three samples (Table S4 and Fig. 3a). In many cultures enriched with PAHs, *Pseudomonas* was the dominant member and even occupied up to 70 % of the total populations in the 4BN03M consortium (Table S5 and Fig. 3b). Coincidently, three *Pseudomonas* strains were obtained from the 15 °C enrichments (Fig. S3) and were able to grow with naphthalene. However, according to our knowledge, *Pseudomonas* is less abundant in oceanic sediments (Cui et al., 2008; Wang et al., 2008; Shao et al., 2010).

In addition to the bacteria mentioned above, many other bacteria belonging to *Dietzia*, *Alcanivorax*, *Colwellia*, *Thalassolituus*, *Oceanobacter, Hahella,* and *Roseovarius* were also relatively dominant in some of the PAH-degrading communities. *Dietzia* (Alonso-Gutierrez et al., 2011), *Alcanivorax* (Schneiker et al., 2006), *Colwellia* (Baelum et al., 2012), *Thalassolituus* (Yakimov et al., 2004) and *Oceanobacter* (Teramoto et al., 2009) have been reported as hydrocarbon-degrading bacteria, but not as a PAH degrader yet. It is noteworthy that bacteria of *Dietzia* were abundant in situ, particularly in the middle or lower layers of all sediments, and occupied 1.65–7.8 % of the total tags (Fig. 3a, Table S4). These organisms might thrive on alkanes in these environments, but are not likely to thrive on PAHs because they only occupied 11.25 % of the total tags in one consortium (the upper layer of site BN03, enriched at 15 °C) (Fig. 3c,

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Table S5). Interestingly, bacteria of *Thalassolituus* occurred as the rare species (abundance < 0.01 %) in all in situ sediments. They turned predominant in the 4 °C cultures of site BN12 enriched with oil containing PAHs (Fig. 6a), but diluted from the PAH-enriched cultures at both 15 and 25 °C (Figs. 6b and 5). These results were quite consistent with their aliphatic hydrocarbon-degrading and psychrotolerant characteristics (Yakimov et al., 2004).

It is worthy to mention that the once frequently reported PAH-degrading bacteria, *Novosphingobium* spp., were not detected in the sediments of the Arctic Ocean. They are common PAH degraders in both marine and terrestrial environments (Gan et al., 2013). In our previous reports, they occurred as a predominant PAH degrader in nearly all PAH enrichment cultures, such as those from deep-sea column of the India Ocean (Yuan et al., 2009) and those from hydrothermal sediment of the Lau Basin (Dong et al., 2011). Other reports also proved the widespread of *Novosphingobium* bacteria as an effective PAH degrader (Balkwill et al., 1997; Sohn et al., 2004; Yuan et al., 2009; Notomista et al., 2011). Similar to *Novosphingobium*, bacteria of *Sphingomonas* and *Neptunomonas* were neither found in the sediments of the Arctic Ocean, while they are also PAH-degraders in marine environments and detected quite often (Demaneche et al., 2004; Hedlund et al., 1999).

In summary, various PAHs and the degrading bacteria are ubiquitous in the Arctic deep-sea sediments. In general, the total PAH concentrations decreased with sediment depths and as well as from the southern to northern, and ranged from 2.0 to 41.6 ng g^{-1} dry weight. Correspondingly, various bacteria involved in PAH degradation exist in the deep-sea sediments, including the obligate marine hydrocarbon-degrading bacteria *Cycloclasticus*, *Alcanivorax*, and *Thalassolituus,* as well as *Pseudomonas*, *Pseudoalteromonas, Marinobacter*, *Marinomonas, Acinetobacter*, *Bacillus*, *Colwellia*, *Dietzia*, *Halomonas*, *Rhodovibrio*, *Salinisphaera* and *Shewanella*. Among them, *Cycloclasticus*, *Pseudomonas*, *Pseudoalteromonas, Marinomonas, Halomonas* and *Dietzia* may play a more important role in PAH degradation in situ of the Arctic Ocean. Bioat-

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tenuation of PAH pollutant occurs whilst bacteria survive in the remote deep-sea areas, cold, dark, oligotrophic, high pressure, and ice covered perennially.

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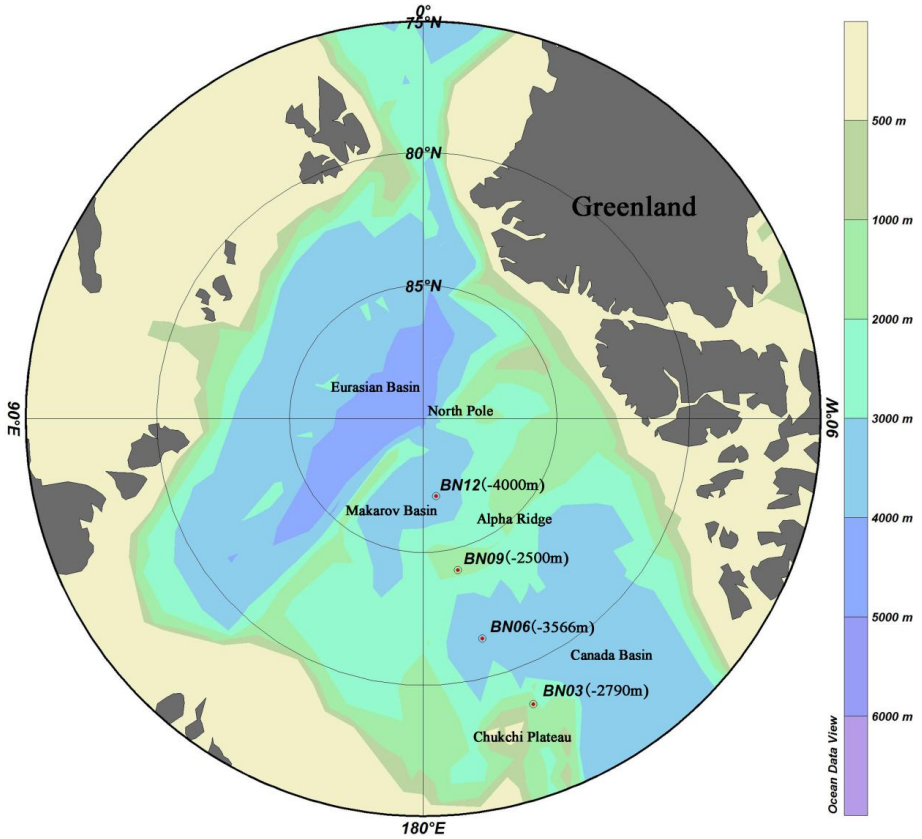

Figure 1. Locations of the deep-sea sediment sampling sites in the high-latitude Arctic Ocean.

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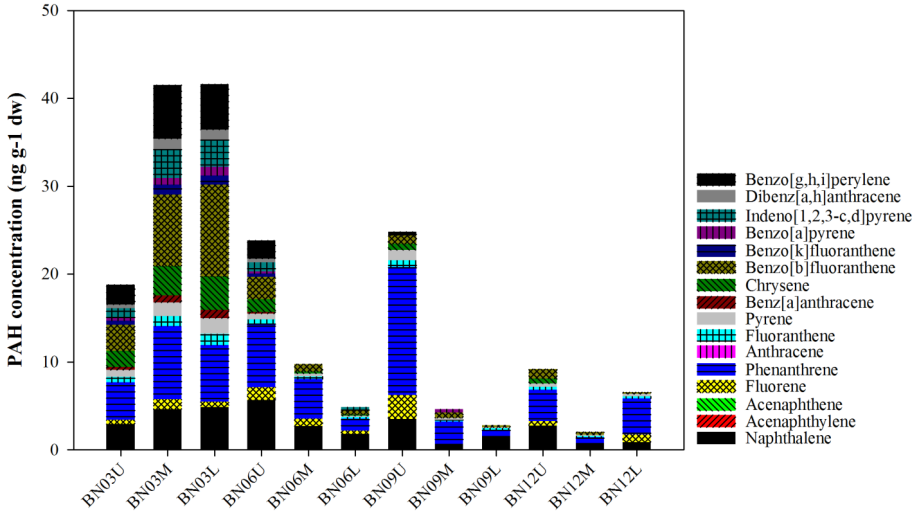

Figure 2. The distribution and concentration of 16 EPA-priority PAHs in the sediments of the Arctic Ocean. U, M, and L in the sample names refer to the upper, middle, and lower layers of the sediments, respectively.

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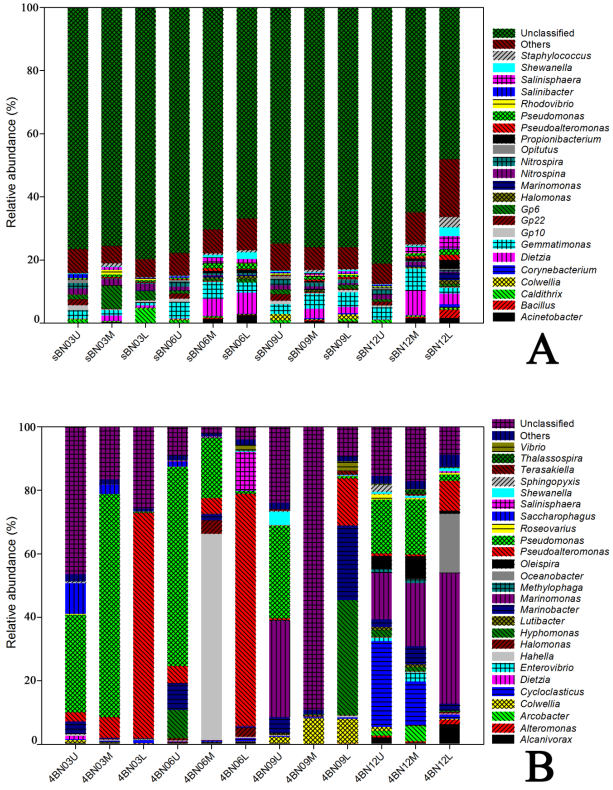

Figure 3. Relative abundances of bacteria (genus level) in the sediments and enriched consortia from the Arctic Ocean. **(A)** Sediment; **(B)** 4 °C consortia; **(C)** 15 °C consortia; **(D)** 25 °C consortia. The genera of abundance more than 1 % of the total tags at least in one consortium were listed in each plot. "Others" refer to the genera constituting less than 1 % of the total tags of a sample for all samples.

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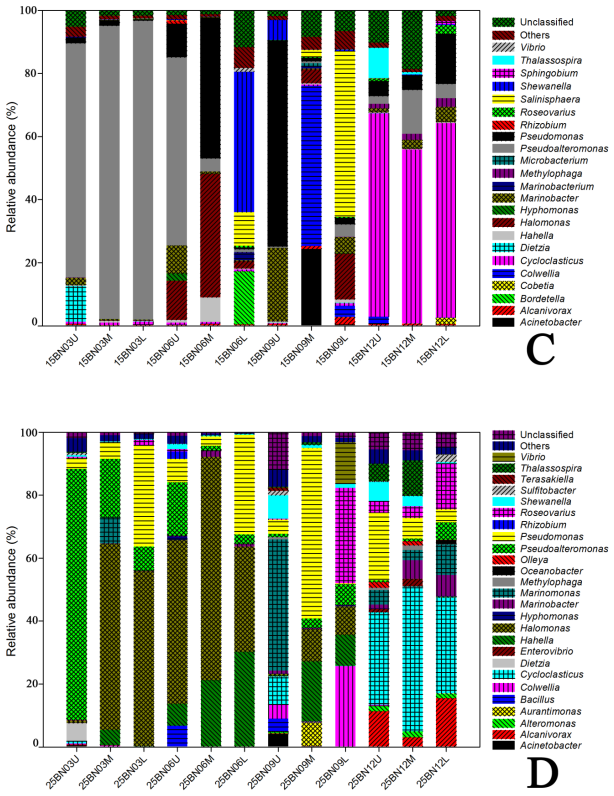

Figure 3. Continued.

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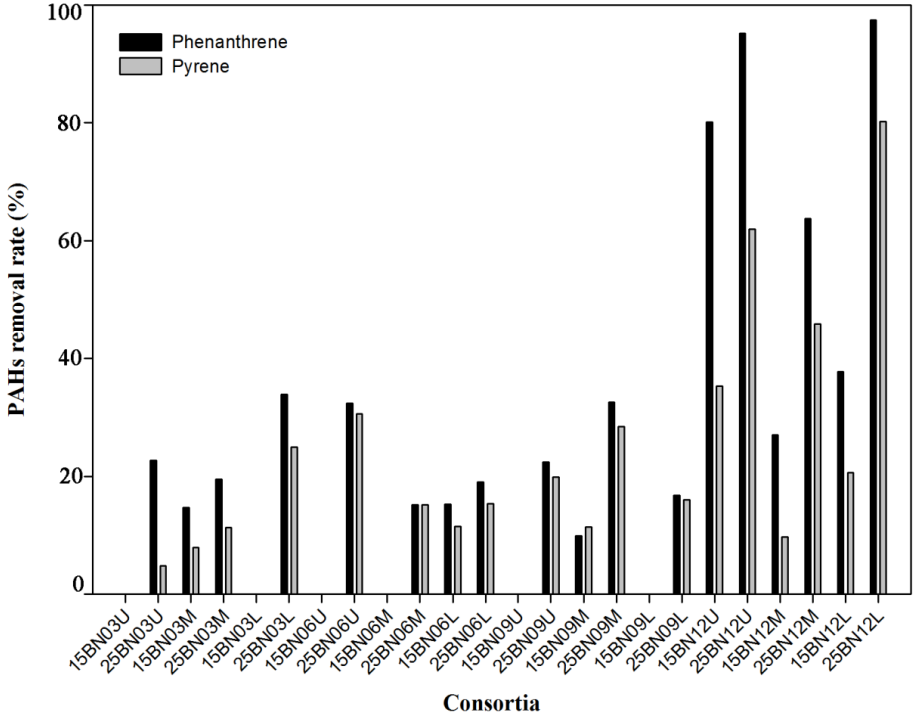

Figure 4. PAH-removal rates of the consortia that were enriched from the sediments of the Arctic Ocean. The consortia were grown with a mixture of PAHs (naphthalene, phenanthrene, and pyrene) as the sole carbon and energy source. PAHs were quantified using GC/MS after pretreatments. Naphthalene was not included due to an error caused by its apt volatility. Consortia names with prefixes "15" or "25" indicated they were enriched at 15 or 25 °C, respectively.

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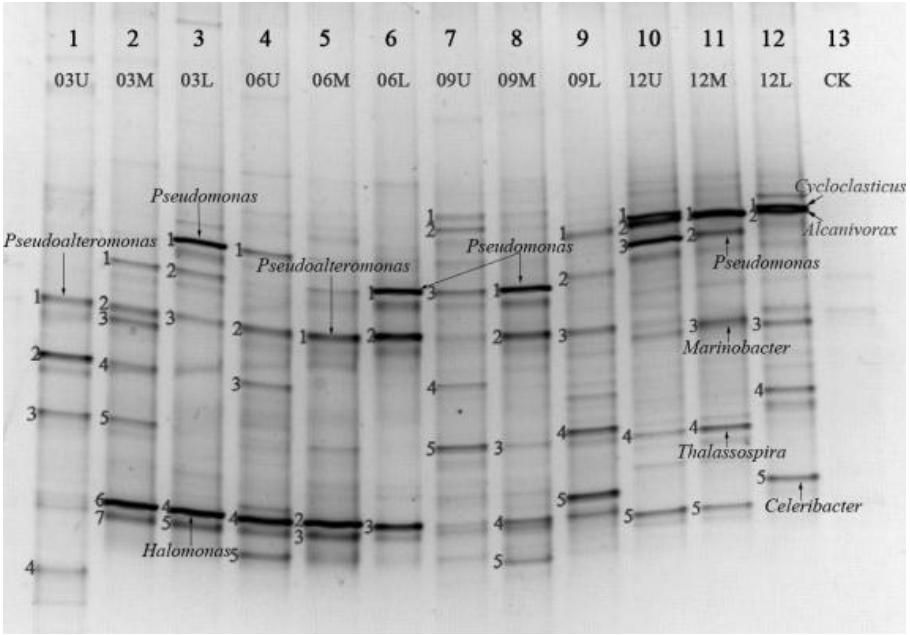

Figure 5. PCR-DGGE profiles of 12 PAH-degrading consortia that were enriched at 25 °C. Lanes 1–3, site BN03; Lanes 4–6, site BN06; Lanes 7–9, site BN09; Lanes 10–12, BN12; and Lane 13, negative control.

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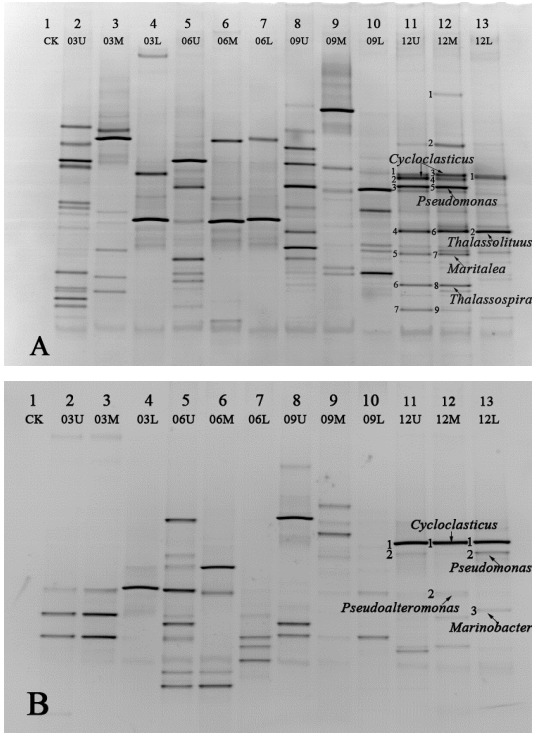

Figure 6. PCR-DGGE profiles of PAH-degrading consortia that were enriched low temperatures. Lane CK, negative control; **(A)** consortia enriched at 4 °C; and **(B)** consortia enriched at 15 °C.

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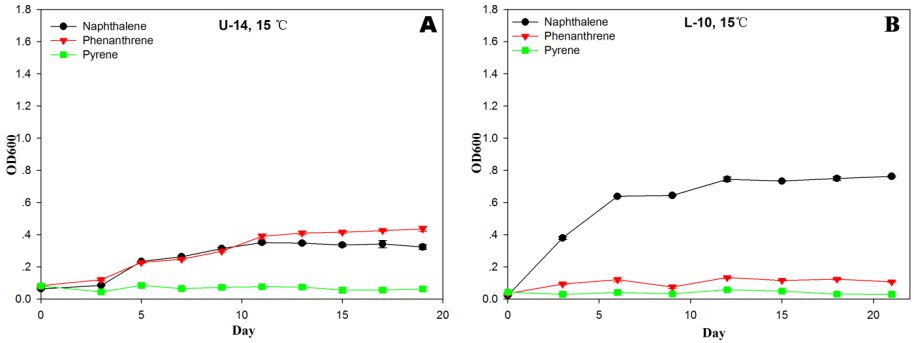

Figure 7. Growth curves of strains 15BN12U-14 and 15BN12L-10 using individual PAHs as the sole carbon and energy source in ONR7a medium. (**A** and **B**) Strain U-14 and L-10 cultivated at 15 ˚C, respectively. OD_{600} was defined as meaning optical density at 600 nm.

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