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Responses of bacterial communities in seagrass sediments to polycyclic aromatic hydrocarbon-induced stress

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Abstract The seagrass meadows represent one of the highest productive marine ecosystems, and have the great ecological and economic values. Bacteria play important roles in energy flow, nutrient biogeochemical cycle and organic matter turnover in marine ecosystems. The seagrass meadows are experiencing a world-wide decline, and the pollution is one of the main reasons. Polycyclic aromatic hydrocarbons (PAHs) are thought be the most common. Bacterial communities in the seagrass *Enhalus acoroides* sediments were analyzed for their responses to PAHs induced stress. Dynamics of the composition and abundance of bacterial communities during the incubation period were explored by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) and quantitative PCR assay, respectively. Both the incubation time and the PAHs concentration played significant roles in determining the microbial diversity, as reflected by the detected DGGE bands. Analysis of sequencing results showed that the *Gammaproteobacteria* were dominant in the seagrass sediments, accounting for 61.29 % of all sequenced bands. As PAHs could be used as carbon source for microbes, the species and diversity of the PAH-added groups (group 1 and 2) presented higher Shannon Wiener index than the group CK, with the group 1 showing the highest values almost through the same incubation stage. Patterns of changes in abundance of the three groups over the experiment time were quite different. The bacterial abundance of the group CK and group 2 decreased sharply from 4.15×10^{11} and 6.37×10^{11} to 1.17×10^{10} and 1.07×10^{10} copies/g from day 2 to 35, respectively while bacterial abundance of group 1 increased significantly from 1.59×10^{11} copies/g at day 2 to 8.80×10^{11} copies/g at day 7, and then dropped from day 14 till the end of the incubation. Statistical analysis (UMPGA and PCA) results suggested that the bacterial community were more likely to be affected by the incubation time than the concentration of the PAHs. This study provided the important information about dynamics of bacterial community under the PAHs stress and revealed the high bacterial diversity in sediments of *E. acoroides*. Investigation results also indicated that microbial community structure in the seagrass sediment were sensible to the PAHs induced stress, and may be used as potential indicators for the PAHs contamination.

Keywords South China Sea · Xincun Bay · Seagrass · PAH · Bacterial communities · PCR-DGGE · *q*PCR

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Introduction

The seagrass is the only true flowering plants that grow and reproduce underwater in shallow coastal waters, being an important feature of coastal systems worldwide. There are a total of 72 species seagrass belong to 12 genera in six families distributing all over the world (Short et al. 2011).

Tropical seagrass ecosystems are highly productive and important for providing food and shelters for many marine creatures, stabilizing sediment, clearing water and linking sediment and water column nutrient cycles (Heather 2010). They are capable of fixing significant amounts of carbon dioxide from the atmosphere, hence potentially helping to alleviate rising carbon dioxide level (Heather 2010; Raja et al. 2012). Bacteria in the seagrass sediment could function both as important contributor and transformers of primary production, and consequently could provide an important link between detritus and the aquatic food web in the seagrass meadow (James et al. 2006). Furthermore, some of these bacteria are being nitrogen fixer by introducing the new nitrogen into the ecosystem to lessen the nitrogen limit of productivity in the oligotrophic areas (Raja et al. 2012). Therefore, the seagrass meadow is of great environmental and ecological importance.

However, seagrass ecosystem is one of the world's most threatened tropical ecosystems. The disappearing rate have accelerated from less than 1 % per year before 1940 to 7 % per year since 1990, and about half of the world's the seagrass meadows are threatened (Waycott et al. 2009). Moreover, seagrass is disappearing at rates similar to coral reefs and tropical rainforests. Pollution was one of the main causes for seagrass decline and polycyclic aromatic hydrocarbons (PAHs) are thought to be the most common. PAHs are great environmental and human health concerns due to their widespread occurrence, persistence and carcinogenic properties. They are also oligotrophic and able to pass through lipid membranes and inclined to be accumulated in the thylakoid membranes of the chloroplasts (Ren et al. 1994). Thorhaug (1986) reported that the dispersed oil had some negative effects on the growth of *Halodule wrightii*, *Syringodium filiforme* and *Thalassia testudiumthan.* Inestigations have also been conducted to evaluate the capability of seagrass in PAHs contamination and they found that *Posidonia oceanicais* was able to accumulate PAHs in its tissues. Most of PAHs analyzed exhibited a higher concentration in the vicinity of contamination source (Larkum et al. 2006).

Bacterial communities comprised the large part of biomass and chemical activity in sediments. Besides, they also take part in energy flow, nutrient biogeochemical cycle and organic matter turnover. Dynamics of the bacterial communities were more applicable to be used to investigate the effect of PAHs by molecular techniques (Nealson 1997). Denaturing gradient gel electrophoresis (DGGE) was firstly employed in molecular ecology by Muyzer et al. (1993) for profiling complex microbial populations. It has become a popular technique to investigate the bacterial community variations in microbial ecology, such as Three Gorges Reservoir, heavily creosote-contaminated soil, coastal bay, PAHs biodegradation process and so on (Viñas et al. 2005; Yan et al. 2008; Ling et al. 2012; 2013; González et al. 2011; Muyzer and Smalla 1998; Ortmann and Ortell 2014). Quantitative PCR (*q*PCR) with 16S rRNA gene targeted species-specific primers was extensively used to evaluate the changes in bacterial abundance in different PAHs-contaminated ecosystems (Cébron et al. 2009; Di Gennaro et al. 2009; Guazzaroni et al. 2013; Ren et al. 2015). However, the information to date available regarding response of the bacterial community structure in the seagrass meadow to the stress induced by PAHs is scare.

The present study was aimed at investigating the effects of three mixed PAHs [naphthalene (Nap), fluorene (Flu) and pyrene (Pyr)] on bacterial communities of the seagrass sediments, and laboratory-incubated experiments have been designed to minimize the environmental complexity and the responses of microbial communities to PAHs were been compared. Results would provide a better understanding of their interactions, which can provide more reasonable explanation for the seagrass decline and enhance efforts in the seagrass management and restoration.

Materials and methods

Study sites and sample collection

Samples were obtained from the Xincun Bay (18°24′34″N–18°24′42″N, 109°57′42″E–109°57′58″E) of Hainan Island in China. Xincun Bay with an area of more than 13.1 km^2 is an enclosed bay with only one narrow channel connected with the open sea. The seagrass is widely distributed at the south of the lagoon, with the dominant species is *Enhalus acoroides* (Jiang et al. 2014). Triplicate surface sediment samples of *E. acoroides* (around 0–4 cm) were collected randomly at low tide from the mid-intertidal zone of Xincun Bay on April 24th, 2012. All sediment samples were thoroughly homogenized using the sterilized spoon. Then the sediment samples were stored at −20 °C in sterile polyethylene bags until analysis.

Experimental setup

The sediments were divided into three groups according to the recommendations of Zhou et al. (2008) and Wu et al. (2013) with some modifications, with the first group being the control (group CK, named 0), while the second (low concentration, named 1) and third groups (high concentration, named 2) receiving low and high PAHs contamination, respectively. In the control, 50 g of fresh sieved sediment was added to 50 mL sterilized mineral salt medium (MSM) in a 150-mL Erlenmeyer flask with a layer of glass beads. For the low PAHs contaminated group (group 1), PAHs were added to the flask with a final

concentration of 100 mg/kg mixed PAHs (33.3 mg/kg each for Nap, Flu and Pyr) dissolved in acetone prior to the addition of sediment and water. For the high-contaminated group (group 2), PAHs concentrations were at 1000 mg/kg with mixed PAHs (333 mg/kg each for Nap, Flu and Pyr). While the first group as the control treatment, the sediment was treated with acetone mixed with sterilized water. All flasks were wrapped with aluminum foil during the incubation period on an orbital shaker (150 rpm). The temperature and salinity were adjusted to 30 ‰ and 25 °C in accordance with the in situ temperature and salinity of the sampling site. The slurry samples (5 mL) were collected from each flask at days 2 (A0, A1 and A2), 7 (B0, B1 and B2), 14 (C0, C1 and C2) and 35 (D0, D1 and D2) with a plastic cut-off-tip pipette, and then stored at −20 °C prior to be analyzed.

DNA extraction and PCR amplification

Total community DNA was extracted using a Soil DNA Kit (Omega, USA) according to the manufacturer's protocol. The DNA of triplicate samples from the same treatments was mixed together gently and equally. Nested PCR approach was employed to amplify the targeted 16S rDNA sequences. The 16S rDNA fragments were firstly amplified using the primers 27F and 1492R (Suzuki and Giovannoni 1996) and then with primers 338F (5′-CCTA CGGGAGGCAGCAG-3′) and 518R (5′-ATTACCGCG GCTGCTGG-3′) for the second round amplify bacterial 16S rDNA gene-V3 segments (Muyzer and Smalla 1998). Each reaction was performed in a 25-μL volume containing 2 μL of DNA template, 1 μL of each primer (5 pM, 338F and 518R), 2.5 μL of 10× PCR buffer (Takara, Japan), 1.6 μL of dNTP (2.5 mM), 0.125 μL of Taq (5 U/μL) and 16.775 μL of ddH_2O. The PCR cycle was started with 2 min at 50 °C and 10 min at 95 °C, followed by total of 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. In order to reduce the possible inter-sample PCR variation, all PCR reactions were run in triplicate and pooled together before loading on DGGE gel. PCR products were analyzed by electrophoresis in 1.5 % agarose gels after ethidium bromide staining.

Quantitative PCR assay

The copy numbers of 16S rDNA gene in all samples were determined in triplicate by quantitative real-time PCR (Q-PCR) (Bio-Rad Real-Time PCR System PTC220) using SYBR Green as a fluorescent dye (Takara Bio, Tokyo,. Japan). All PCR runs started with an initial enzyme activation step with performing at 95 °C for 10 min. Each reaction was performed in a 20-μL volume containing 2 μL of DNA template, 2 μL of each primer (1 μM, 338F and 518R), 10 μL of 2 × 2 × SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) (Takara, Japan), 0.4 μL of ROX Reference Dye and 3.6 μL of ddH_2O. A melting curve was then generated using a program of 95 °C for 10 s, 55 °C for 30 and a subsequent temperature increase to 95 °C. A tenfold serial dilutions of a known copy number of the plasmid DNA were subjected to qPCR assay in triplicate to generate an external standard curve. Standard plasmid carrying 16S rDNA gene fragment was generated by amplifying 16S rDNA gene from extracted DNA of sediments and cloning into pEASY-T Vector (Axygen, USA). The plasmid DNA concentration was determined a nanodrop (NanoDrop Technology, Wilmington, Delaware, USA) and the copy number of target gene was calculated directly from the concentration of the extracted plasmid DNA (Hong et al. 2014).

DGGE analysis

DGGE was performed using Dcode universal mutation detection system as described in the manufacturer's manual (Bio-Rad, Hercules, CA, USA). Equal amounts of the PCR products (approximately 300 ng/mL) from the different samples were loaded onto an acrylamide gel (8 % acrylamide). The 100 % denaturing solution consisted of 7 M urea and 40 % formamide (v/v). Optimal separation of cyanobacterial DNA in the samples was achieved using a 40–70 % denaturing gradient. DGGE was performed at 60 °C for 10 h at a constant voltage of 100 V. After electrophoresis, DGGE gels were stained with ethidium bromide, visualized and photographed under UV light in an Alpha Imager imaging system (Alpha innotech, Japan).

Sequencing and phylogenetic analysis

Individual DGGE bands of intense DNA bands were excised from the gel, transferred to 20 μL PCR quality water and left at 4 °C overnight to elute DNA, thus they were subjected to be reamplified under previously described reaction conditions. For the bands with same distance from the well in different lanes, only one band was cut representing the same bacterial strain. PCR products were loaded again on the DGGE gel to confirm the mobility. Bands showing the expected melting position were again amplified using 338 F (without GC clamps) and 518R primers, purified and then be ligated into a pMD18-T cloning vector, and subsequently transformed into *Escherichia coli* DH5α according to the manufacturer's instructions (Takara Shuzo Co, Ltd, Otsu, Japan). Positive recombinants were identified by PCR amplification with primer pairs T7 and M13 (Tabor and Richardson 1987) and then submitted for sequencing on an ABI3730 DNA Sequencer (USA) at the Shanghai Invitrogen Biotech Co.,

Ltd. The obtained sequences were analyzed against sequences in the Ribosomal Database Project (RDP) using the Classier tool, and against GenBank sequences using the BLASTN program available on the NCBI website (http://www.ncbi.nlm.nih.gov) (Altschul et al. 1997; Maidak et al. 1999). Phylogenetic trees of 16S rDNA partial sequences were generated using the neighbor-joining algorithms in Mega VI software with bootstrap analyses for 1000 replicates (Tamura et al. 2011). The evolutionary distances were computed using the maximum composite likelihood method and expressed as the number of base substitutions per site (Saitou and Nei 1987).

Statistical analysis

Quantity One 4.6.2 (Bio-Rad, USA) have been employed to analyze the DGGE profile according to the method prescribed by Zhang et al. (2009). Both the intensities and positions of the bands were taken into account. Similarities of the bacterial communities were analyzed through the unweighted pair group method with arithmetic means (UPGMA). Shannon index of different communities were analyzed according to Zhang et al. (2008) and Ling et al. (2012). Based on the intensities and positions of bands of one lane in the DGGE profile, principal component analysis (PCA) plot was generated using MVSP 3.1 software (http://www.kovcomp.co.uk/mvsp/index.html) to determine the shifts of the bacterial community under different conditions over the incubation time. Statistical analysis on the apparent bacterial diversity (calculated as numbers of DGGE bands) in different group of different incubation stage t were also be conducted by MVSP.

Results

DGGE patterns the bacterial communities under PAHs stress

The DGGE profile of 12 samples collected at four different incubation phases were shown in Fig. 1. The banding patterns among all the samples were distinct from each other. Analysis of DGGE gel yielded a total of 215 detectable bands in 64 different positions with the range of the bands detected per sample from 16 to 32, indicating that bacterial comminutes composition have changed dramatically under the PAHs stress. Most of species numbers detected in the CK group were less than group 1 and 2. It may due to that PAHs could be used as carbon and energy source for some bacteria in the sediment and stimulated the growth of some stains of microbial community. Differences in the compositions of bacterial communities were observed over the whole incubation period, with some bands existed though the period, whereas some bands were

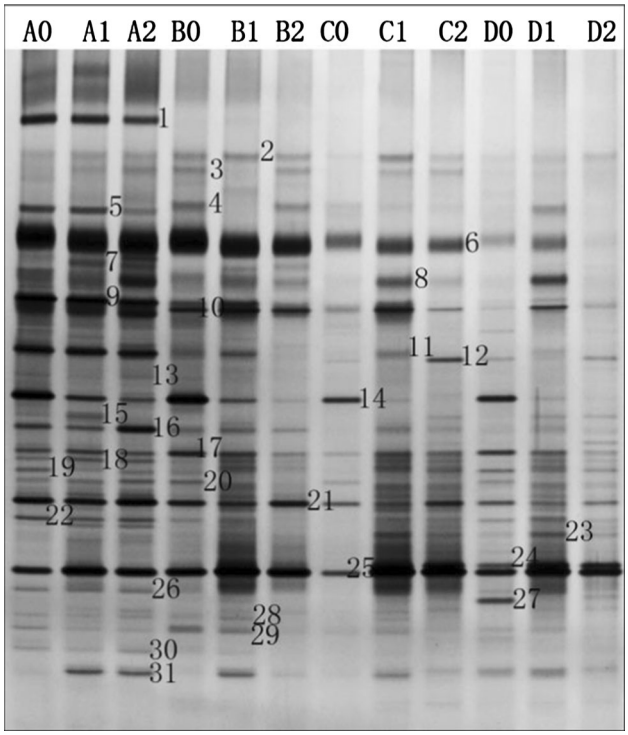

Fig. 1 DGGE profiles of sediment bacterial community exposed to different concentration of PAH contamination of different incubation stage (*A* day 2, *B* day 7, *C* day 14, *D* day 35; *CK* control without no PAH addition; *group 1* 100 mg/kg; *group 2* 1000 mg/kg)

present only at a certain stage. For example, bands 1, 5, 11 and 26 only existed at the beginning days, and they disappeared in the following incubation time while bands 21 and 25 could be detected through the 35 days incubation time. Some of the bands, such as band 16, were induced at day 2 of group diminished in intensity or even disappeared by day 35. Interestingly, band 31 was induced in the group 1 and group 2 at day 2 while it could not be detected in the group CK at day 2. However, it only can be found in all groups including group CK at the 35-days incubation stage. Although some bands existed in all groups, but the intensities were quite different. For instance, band 14 could be found in the group CK with high intensities while in the PAHs contaminated sediments (group 1 and 2) it become much lower or even disappeared by day 35. It may be suppressed in the environment with the presence of PAHs. Based on the whole DGGE profile, it could be seen that all the bacterial community varied significantly with time, even in the control.

Sequencing and phylogenetic analysis of DGGE fragments

In all, a total of 31 different bands were excised from the gel, purified and sequenced. The detailed information based on the sequencing of the band is listed in the Table 1.

Table 1 Summary of 16S rDNA sequence obtained from the respective bands in DGGE gels and the closest match to the sequence from GenBank database

Band no.	Phylogenetic group	Batabase match with accession number in parentheses	Similarity (%)	Sources
6	*Alpha-proteobacteria*	*Novosphingobium* sp. (KF559238)	100	Pak soil enrichment culture
11		*Nitratireductor* sp. 2–9 (JN942135)	100	Crude oil-contaminated surface water and sponges China: Yellow Sea at Dalian
26		*Phaeobacter* sp. SSK6-1 (KF554505)	99	Water from junction between ocean and freshwater spring
29		*Bradyrhizobium canariense* GV159 (KF483534)	100	Nodule
30		Uncultured marine bacterium S24-3 (JX406090)	99	Seawater
31		*Ruegeria* sp. (GU551937)	100	South Korea: Yellow Sea
1	*Gamm-proteobacteria*	*Idiomarina* sp. CF5 (KC200266)	100	Seaweeds from intertidal zone of Zhoushan Sea area, East China Sea
2		Uncultured *Idiomarina* sp. DVASD_J147 (KF464134)	98	Alang–Sosiya ship breaking yard
5		*Idiomarina* sp. CF5 (KC200266)	99	Seaweeds from intertidal zone of Zhoushan Sea area, East China Sea
7		*Idiomarina* sp. CF5 (KC200266)	99	Seaweeds from intertidal zone of Zhoushan Sea area, East China Sea
8		*Idiomarina* sp. CF5 (KC200266)	99	Seaweeds from intertidal zone of Zhoushan Sea area, East China Sea
9		Uncultured *Idiomarina* sp. DVASD_J123 (KF464110)	100	Alang–Sosiya ship breaking yard
10		*Idiomarina* sp. CF5 (KC200266)	100	Seaweeds from intertidal zone of Zhoushan Sea area, East China Sea
12		*Idiomarina* sp. CF5 (KC200266)	100	Seaweeds from intertidal zone of Zhoushan Sea area, East China Sea
14		Uncultured *Idiomarina* sp. DVASD_J123 (KF464110)	100	Alang–Sosiya ship breaking yard
16		*Idiomarina tainanensis* PIN1 (NR_044493)	100	Seawater samples collected from the shallow coastal region of An-Ping Harbour, Tainan
17		Uncultured *Idiomarina* sp. DVASD_J123 (KF464110)	99	Alang–Sosiya ship breaking yard
18		*Pseudidiomarina* sp. MOLA 425 (AM990692)	99	Seawater
19		Uncultured *Idiomarina* sp. DVASD_J123 (KF464110)	100	Alang–Sosiya ship breaking yard
20		*Idiomarina tainanensis* PIN1 (NR_044493)	99	Seawater samples collected from the shallow coastal region of An-Ping Harbour, Tainan
21		*Pseudidiomarina* sp. MOLA 425 (AM990692)	99	Sea water
22		*Pseudidiomarina* sp. MOLA 425 (AM990692)	100	Sea water
23		*Idiomarina* sp. CF5 (KC200266)	100	Seaweeds from intertidal zone of Zhoushan Sea area, East China Sea
25		*Idiomarina* sp. CF5 (KC200266)	100	Seaweeds from intertidal zone of Zhoushan Sea area, East China Sea
28		*Idiomarina* sp. CF5 (KC200266)	99	Seaweeds from intertidal zone of Zhoushan Sea area, East China Sea
13	*Bacteroidetes*	*Lacinutrix* sp. SS16.30 (KC160949)	99	Antarctic sea sediment
15		*Mesoflavibacter zeaxanthinifaciens* (AB681705)	100	Type of Mesoflavibacter zeaxanthinifaciens
24		Uncultured bacterium (AB369186)	99	Riser drilling mud fluid
27		Uncultured Bacteroidetes (FJ902264)	99	Water column sample from 32 m deep in cenote Caracol

Table 1 continued

Band no.	Phylogenetic group	Batabase match with accession number in parentheses	Similarity (%)	Sources
3	*Tenericutes*	Anaerobic bacterium MO-XQ (AB598274)	100	Subseafloor sediments
4		Anaerobic bacterium MO-XQ (AB598274)	99	Subseafloor sediments

The 16S rDNA gene sequences determined in this study was deposited in the GenBank database with the following accession numbers from KJ588733-KJ588763. The closest relatives with most of the sequences obtained in this study were marine sources, such South Korea: Yellow Sea, East China Sea and An-Ping Harbor, Tainan so on. The percentage similarity with the clone and its closest blast hits ranged from 99 to 100 %, respectively.

Taxonomic analysis based on top BLAST hits to the GenBank showed that all the DGGE bands sequenced were identified as *Gammaprotebacteria* (61.29 %). The rest of the phylogenetic groups were less abundant and identified included *Alphaprotebacteria* (19.35 %), *Bacteroidetes* (12.9 %) and *Tenericutes* (6.45 %) (Figs. 2, 3). According to the band intensities and positions in the DGGE profile, sequencing results of all DGGE bands showed that marine bacteria from the genera of *Idiomarina* (Band 25) and *Ruegeria* (Band 31) were most abundant after PAHs exposure. Phylogenetic analysis results revealed that sediments of *E. acoroides* harbored a great diversity of bacteria, with numerous sequences lying within phylum *Protebacteria*.

Dynamics of Shannon index and bacterial abundance analysis

The Shannon index determined by DGGE band numbers of all group changed significantly though 35 days incubation period. Although the apparent bacterial richness in group 1 was lower than group CK at day 2, while all the contaminated groups (1 and 2) were both higher than group CK at the end of experimental time. In the group CK, it decreased sharply from 3.18 to 2.40 in the first 14 days incubation and then increase to 2.6 in the end. Group 1 showed the similar tendency of Shannon index with the CK group. However, the Shannon index of group 2 dropped in the former 7 days incubation, and then increased slowly from 2.7 to 2.8 during day 7 to day 14 and then fall to 2.7 again at the end of incubation (Fig. 4).

The abundance of bacterial communities as indicated by the number of 16S rDNA copies measured using *q*PCR was shown in Fig. 5. It is obvious that abundance of all bacterial communities of group CK and group 2 dropped significantly during the incubation period, with the abundance from 4.15×10^{11} and 6.37×10^{11} to 1.17×10^{10} and 1.07×10^{10} copies/g, respectively. On the other hand, the bacterial abundance of group 1 increased significantly from 1.59×10^{11} copies/g at day of 2 to 8.80×10^{11} copies/g at day 7, and then droped to 1.89×10^{11} copies/g at day 14. The patterns of the shannon index and the abundance of the bacterial communities presented different tendencies.

UPGMA cluster and PCA analysis

Incubation time and concentration of PAHs were both drivers of bacterial communities based on the UPMGA analysis based on comparison of DGGE patterns. Figure 6 indicated that the highest community similarity was recorded between samples A1 and A2 (about 78 %) while bacterial community of A0 and D0 only share a similarity of 61 %. However, after 35 days' incubation, the similarities between the two bacterial communities decreased sharply to about 62 %.

PCA was employed to investigate the dynamic change of the microbial community composition based on the intensities and positions of bacterial species (Fig. 7). The first two principal components of the PCA could explain 34.6 and 17.2 % of the observed variation, respectively. The cumulative percentage variance was 74.3 %. All the samples could be divided into two clusters with one cluster comprising of A0, A1, A2, B0, B1 and B2, and the other cluster including the samples of C0, C1, C2, D0, D1 and D2. It may be inferred that the incubation time rather than doses of PAHs accounted for large variations of bacterial communities.

Discussion

Due to the high productivity and the other ecological functions, the ecological importance of the seagrass meadows has been realized. Microbes drive various biogeochemical cycles in the seagrass meadows which are critical to plant growth and health has been poorly studied with only few investigations focusing on the seagrass

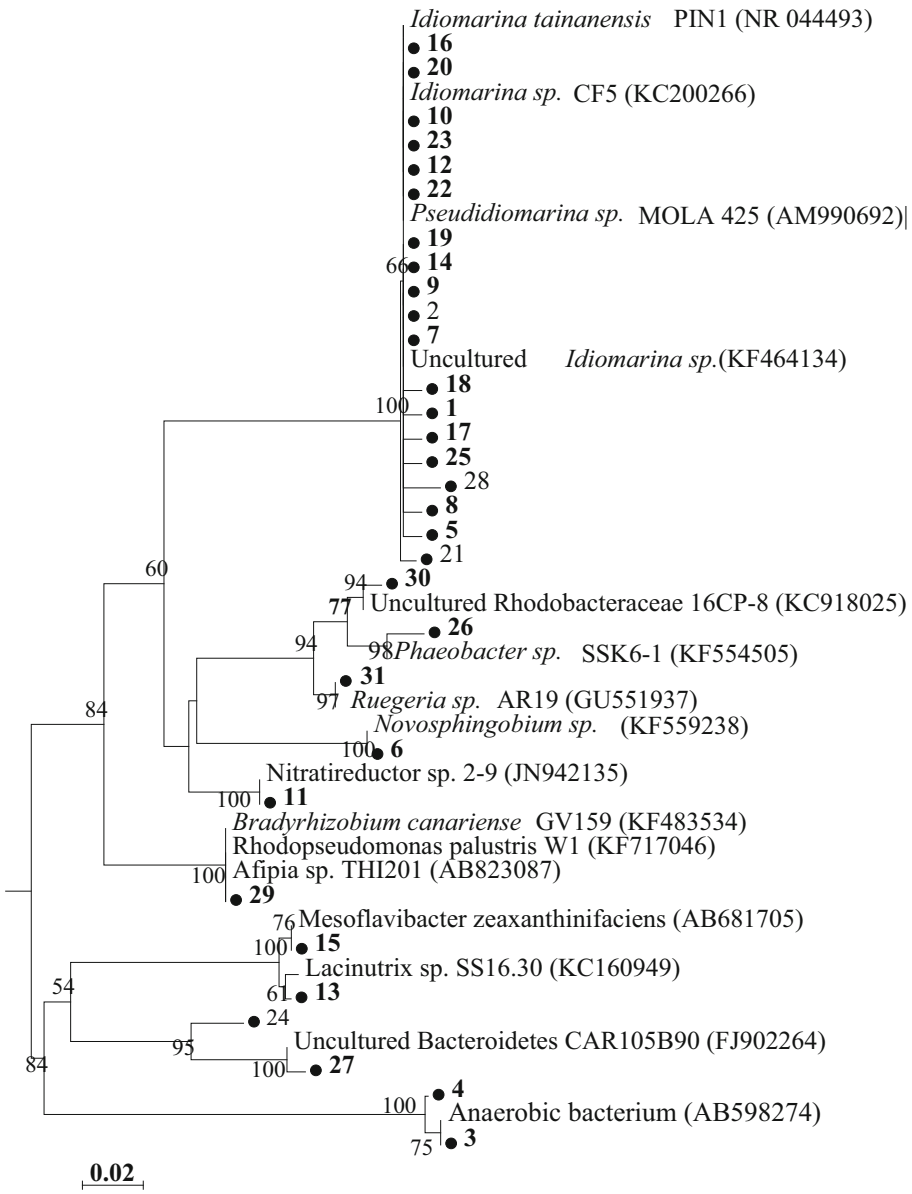

Fig. 2 Phylogenetic tree of 16S rDNA genes about selected DGGE bands. Bootstrap values represent 1000 replicates and only values above 50 % are shown. The *scale bar* represents the expected number of changes per nucleotide position (2 % nucleotide substitution)

epiphyte, phyllophere, rhizosphere, and meadow sediments (Bagwell et al. 2002; Fourquerean and Zieman 2002; Jones et al. 2003; Duarte et al. 2005; Jensen et al. 2007; Uku et al. 2007; Ikenaga et al. 2010; Green-García and Engel 2012; Wu et al. 2012). The previous study demonstrated that there was a strong link between the mineralogy and geochemistry of sediment and microbiology in that the abundance and distribution of bacteria in the sediments varied with the seagrass species, depth of sediments and locations. However, bacterial distribution in the seagrass meadow was specific to some extent. Because Green-García and Engel (2012) reported that the similarities of the composition of bacterial communities in the different seagrass meadow sediments were much higher than that with other marine habitats such as mangrove sediment. This may be explained by unique plant–microbe symbiotic associations, especially the lucinid-microbe chemosymbiotic association within the seagrass bed (Green-García and Engel 2012).

This study showed the successive changes of microbial community composition in *E. acoroides* sediments, which has been exposed to the mixed PAHs. Results showed that both the incubation time and concentration of PAHs played significant roles in altering the microbial diversities determined by the number of detectable DGGE bands. The diversity of microbial community is one of the main indicators for assessing the effect of organic pollutants on the bacterial community structure (compositions and abundance). However, the effects of different organic pollutants induced or the same pollutant with different concentrations were quite different. They could function as the stimulator

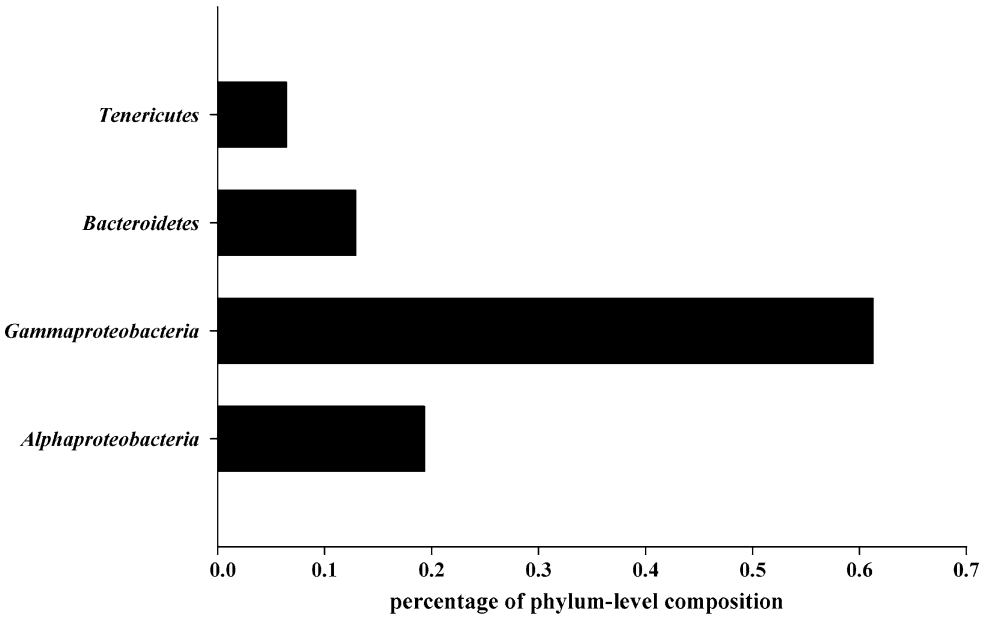

Fig. 3 Phylum level compositions of bacteria from the seagrass sediment based on 16S rDNA fragment DGGE analysis

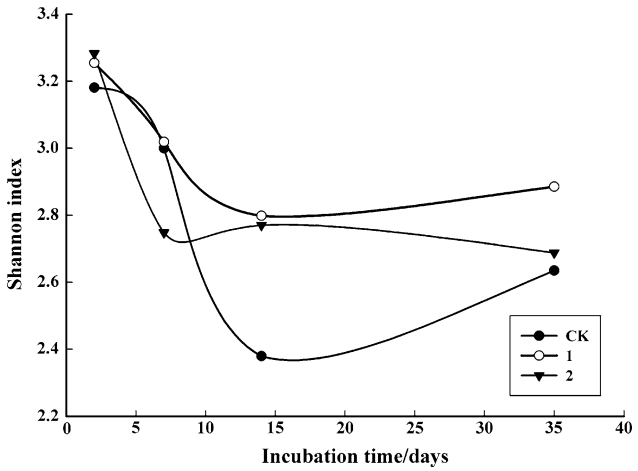

Fig. 4 Shannon index for the bacterial community based on DGGE profile

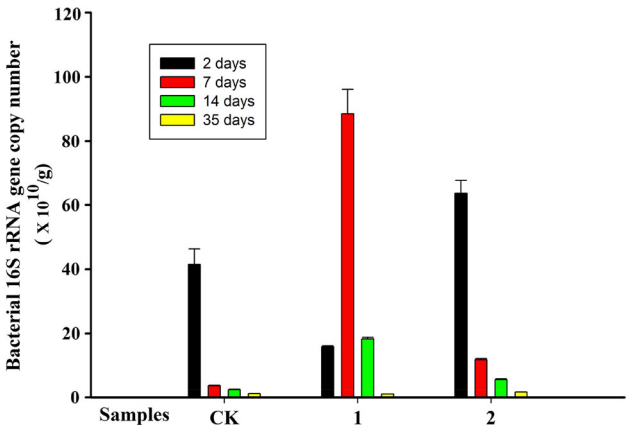

Fig. 5 The dynamics of bacterial abundance 16S rDNA gene copy number as measured by qPCR during the incubation. *CK* contol without no PAH addition; *group 1* 100 mg/kg; *group 2*: 1000 mg/kg; *black columns* represented the bacterial community after 2-day incubation; *red columns* represented the bacterial community after 7-day incubation; *green columns* represented the bacterial community after 14-day incubation; *yellow columns* represented the bacterial community after 35-day incubation (Color figure online)

by promoting the bacterial growth for some strains which were capable of utilizing the PAHs as a carbon and energy source while they also could decrease the bacterial abundance or even caused death of the bacteria by posing an a toxic threat to microorganisms (Zhou et al. 2008; Sun et al. 2012; Wu et al. 2013).

Reponses of different bacterial strains and different bacterial communities to the exposure to the PAHs were quite different. In the present study, all the Shannon index in group CK were lower than that of group 1 and 2 (except the A0 and D2). The reason for this phenomenon was mentioned above that PAHs could promote the growth of some rare bacterial species as the incubation went on. From Fig. 5, it could be concluded that the group 1 with lower concentration significantly stimulated the bacterial growth by compared with group CK and 2. On the contrary, Sun et al. (2012) found that higher concentration of PAHs had larger impact on the nitrogen-fixing bacterial community of mangrove *Sonneratia apetala* sediment.

Band 14 was intense in group CK over the incubation period while the abundance of species 14 of the processed group 1 and 2 was much lower as the incubation time went longer. It almost disappeared in the sample of D1 and D2 at the end of incubation period. However, the band 31 was not obvious in sample A0 while in the A1 and A2, it showed high densities. All of this indicated that the species 14

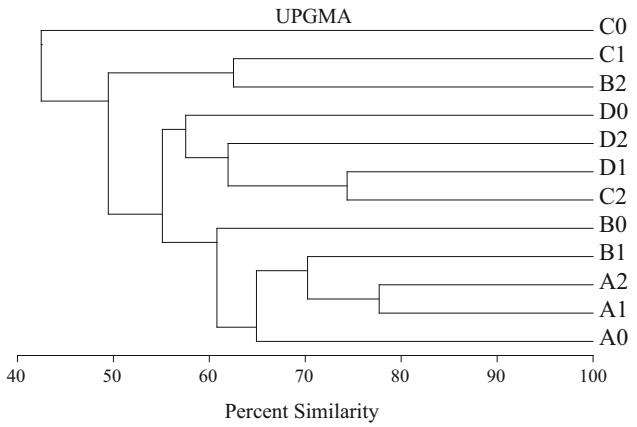

Fig. 6 Cluster analysis of DGGE gel pattern showing the amplified 16S rDNA gene fragments from the samples. Cluster analysis was performed based on the percent similarity correlation and the Ward dendrogramming method

could not adapt to stress of PAHs while bacteria 31 have been stimulated by the PAHs, and it responded quickly to the PAHs addition. Moreover, the bacterial abundance and the diversity of one community did not generate the same response at the same time. It could be referred from the patterns and trends in their curves of Figs. 2, 5.

The UPGMA cluster analysis and PCA based on the DGGE dendrogram have been used to investigate the variations of microbial communities induced by the addition of PAHs. Results suggested that most of the shifts of the bacterial communities were caused by the addition of PAHs (Fig. 1). In other words, the bacterial communities of seagrass sediments were sensitive to the PAH contamination. This result was consistent with results of PCA analysis. Similarly, all the bacterial communities on the plot of PCA could be divided into two parts almost based on the exposure time. In laboratory based incubated experiment of other organic pollutant, such as xenobiotic, the results also indicated that the pollutant concentrations and incubation times were both significant factors influencing the microbial community structure (Zhou et al. 2008; Wang et al. 2009; Peng et al. 2010). According to the report of Sun et al. (2012) investigation on the mangrove sediment, results indicated that different types of PAHs generated different effects. For instance, Nap owned the greatest short term toxicity, Pyr had the strongest long-term impact whereas Flu has relatively higher longtime effect with the others.

Most of the bacteria identified based on the sequencing results of the DGGE bands in this study showed that the dominant phenotypes belonged to phylum *Gamma-*

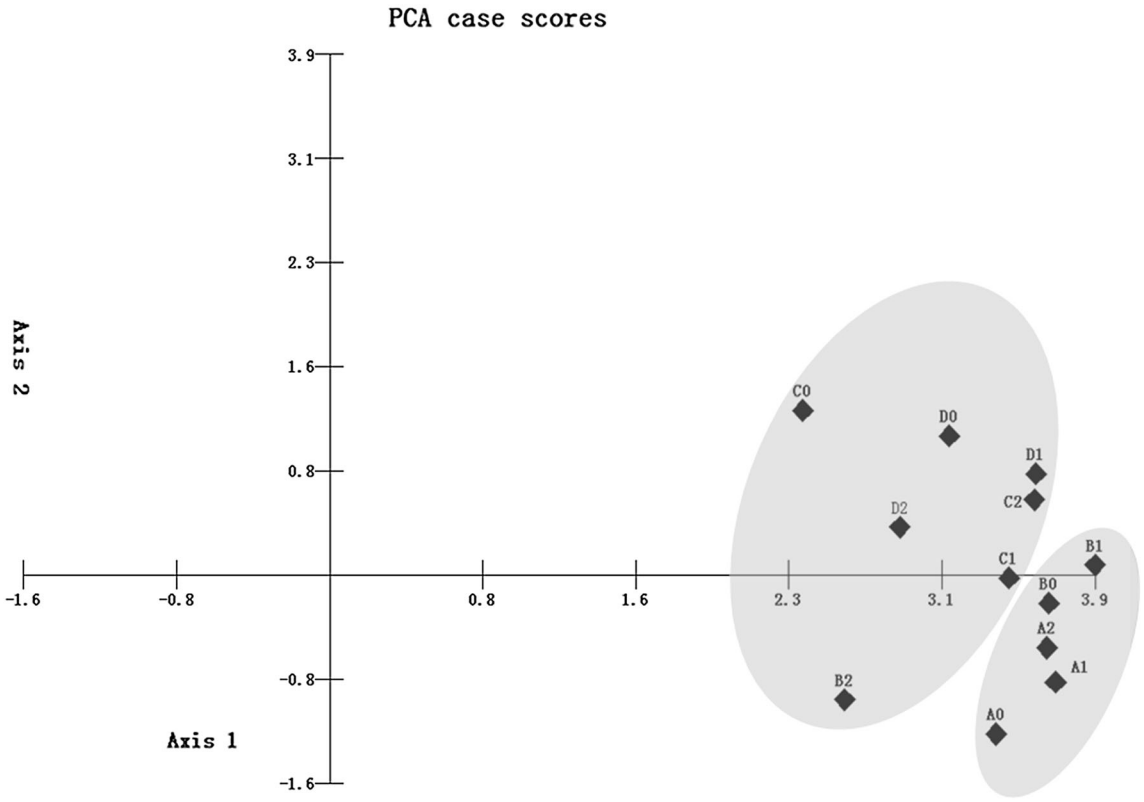

Fig. 7 Principal-component analysis scatter plot of denaturing gradient gel electrophoresis profiles (Fig. 2) over the course of the incubation period; 0, 1, and 2 indicated group CK, group 1 and group 2, respectively; The numbers of days after treatment are also indicated; *A*, *B*, *C* and *D* indicates incubation on day 2, 7, 14 and 35, respectively

protebacteria, which accounted for almost 61.29 % of all sequenced bands. The other bacteria obtained in this study have been identified as *Alphaprotebacteria Bacteroidetes* and *Tenericutes.* This result was in support with the report of Green-García and Engel (2012) that *Proteobacteria* were predominant in the seagrass meadow sediment. But, bacterial diversity in a *Thalassia testudinum* meadow with 25 major taxonomic groups (excluding candidate divisions) retrieved, including *Alpha-*, *Delta-*, and *Gamma-proteobacteria*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, *Spirochaetes*, and *Firmicutes,* was much higher than sediment of *E. acoroides* investigated in this study. Bactreria from genus *Diomarina* was the dominant specie and it was ubiquitously distribution in the marine environment, such as north-western Pacific Ocean at a depth of 4000–5000 m, deep seawater of Ridge hydrothermal areas of Southwest India Ocean and the surface seawater around the Xiamen Island (Lai et al. 2012). *Idiomarina xiamenensis* sp. was isolated from a crude oil-degrading consortium enriched from surface seawater collected around Xiamen Island supplemented with sterilized crude oil as a carbon source, and it has been reported to be capable of PAH-degrading with high efficiency (Wang et al. 2011). Bacteria belonging to the genus *Novosphingobium* are known to be metabolically versatile and live in different habits (Gan et al. 2013). Many stains from this genus have the ability to degrade PAH and their derivatives, such as nitrobenzene, 4-chlorobenzene, phenanthrene, pyrene, and dibenzofuran, and they are widely distributed in estuarine sediment, coastal sediment and marine aquatic environments (Sohn et al. 2004; Gan et al. 2013).

This work also revealed potentially novel bacterial taxonomic and functional diversity. Bacterium of phylum *Tenericutes* had not been reported to be existed in marine seagrass sediments. Yet, the closest match obtained from subsea floor sediment in the NCBI of band 3 and 4 was *Tenericutes*-related species (Guazzaroni et al. 2013). Phylum *Tenericutes* lack a cell wall and therefore are gram negative. Bacteria of this pylum may have potential function of degrading naphthalene in the contaminated environments. Some of bacteria belong to this phylum are plant pathogenic bacteria (Guazzaroni et al. 2013).

Conclusions

Bacterial communities in the sediments of *E. acoroides* meadow took place dramatically changes under the PAHs stress. Investigation analysis showed that there existed high bacterial diversity in the seagrass sediments. Both the incubation time and concentration of PAHs played important roles in determining bacterial community shifts, which indicated that suggesting that the bacterial communities in the seagrass sediments were sensitive to PAHs and could easily affected by PAHs contamination. The effects of PAHs with low (100 mg/L) and high concentration (1000 mg/L) on the bacterial community structure in the sediment of *E. acoroides* meadow were quite different. Furthermore, responses of the diversity and abundance in the same bacterial community were quite different to different stresses. The predominant bacteria obtained in this study were *Gammaprotebacteria* (61.29 %), *Alphaprotebacteria* (19.35 %), *Bacteroidetes* (12.9 %) and *Tenericutes* (6.45 %). Complex microbial community interactions occurred during the whole incubation period in the seagrass sediment.

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Conflict of interest The authors declare that they have no conflict of interest associated with this publication.

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