The effects of offshore petroleum exploitation on microbial community and antibiotic resistome of adjacent marine sediments

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ABSTRACT

The exploitation of petroleum in offshore areas is becoming more prosperous due to the increasing human demand for oil. However, the effects of offshore petroleum exploitation on the microbial community in the surrounding environment are still not adequately understood. In the present study, variations in the composition, function, and antibiotic resistance of the microbial community in marine sediments adjacent to an offshore petroleum exploitation platform were analyzed by a metagenomics-based method. Significant shifts in the microbial community composition were observed in sediments impacted by offshore petroleum exploitation. *Nitrosopumilales* was enriched in marine sediments with the activities of offshore petroleum exploitation compared to the control sediments. The abundances of function genes involved in carbon, butanoate, methane, and fatty acid metabolism in sediment microbial communities also increased due to the offshore petroleum exploitation. Offshore petroleum exploitation resulted in the propagation of some antibiotic resistance genes (ARGs), including a multidrug transporter, *smeE*, and *arnA*, in marine sediments via horizontal gene transfer mediated by class I integrons. However, the total abundance and diversity of ARGs in marine sediments were not significantly affected by offshore petroleum exploitation. This study is the first attempt to analyze the impact of offshore petroleum exploitation on the spread of antibiotic resistance.

Key words | antibiotic resistance genes, horizontal gene transfer, marine sediment, metagenomics, microbial community, offshore petroleum exploitation

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HIGHLIGHTS

- In the sediments adjacent to the petroleum exploitation platform, the archaea of *Nitrosopumilales* was significantly enriched, which can digest organic matter to produce methane.
- Offshore petroleum exploitation did not significantly change the total abundance and diversity of antibiotic resistance genes (ARGs) in the surrounding sediments.
- However, petroleum exploitation enriched specific ARGs, a multidrug transporter, *smeE*, and *arnA*, via horizontal gene transfer mediated by class I integrons.
- Our findings provide direct evidences that offshore oil exploitation can influence the microbial communities of adjacent marine sediment and improve the propagation of some specific ARGs.

INTRODUCTION

The ocean accounts for three quarters of the earth and plays a very important role in the history of human social development, providing people with various resources. With the increasing demand for petroleum and related products, the exploitation of petroleum in offshore areas developed rapidly. Owing to the offshore petroleum exploitation

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being accompanied by transportation and spill accidents, petroleum substances have been proved to be one of the most common pollutants in the offshore environment (Simons *et al.* 2013). Therefore, a lot of attention has been paid to the ecological consequences and pollution remediation of petroleum substances in the offshore environment. As the most prevalent organisms in the nature ecosystems, bacteria play a vital role in the transformation and degradation of petroleum substances (Head *et al.* 2006). It is noteworthy that the impacts of petroleum substances on the environmental microbial community have been more and more studied due to the rapid development of high-throughput sequencing technology in recent years (Wang *et al.* 2014). Most of the research on the microbial community variations associated with the offshore petroleum exploitation particularly concerned the spill accidents, because of their severe damages (Mason *et al.* 2014). However, the understanding of the variations in the microbial community composition and function in the surrounding environment of offshore petroleum exploitation platforms in normal operation is still not enough.

Antibiotic resistance genes (ARGs) in environmental settings have been considered as emerging contaminants (Pruden *et al.* 2006). The increasing emergence and dissemination of ARGs from environmental bacteria to pathogens seriously impairs the efficacy of antibiotics therapy in clinical infection (Ashbolt *et al.* 2013). ARGs are often located on mobile genetic elements (MGEs), and are assumed to be disseminated to other microorganisms via the pathway of horizontal gene transfer (HGT) (Von Wintersdorff *et al.* 2016). The marine environment has been demonstrated as a reservoir of ARGs, and ARGs in this environment have been proved to be capable of transferring to human pathogens (Wang *et al.* 2018). Moreover, many genes of the petroleum catabolic pathway have been found to reside in the MGEs, and they appear to be movable among disparate lineages of bacteria (Kweon *et al.* 2015). It has been reported that polycyclic aromatic hydrocarbons, a major component of petroleum, can increase the dissemination of ARGs in seawater through the HGT mediated by MGEs (Wang *et al.* 2017). Nonetheless, there is still a lack of evidence in the effects of offshore petroleum exploitation on ARGs at a realistic scale.

Most previous studies associated with marine petroleum contamination were primitively focused on the bacteria owing to the applied methods based on culture or 16S rRNA genes (Engelhardt *et al.* 2001; Lamendella *et al.* 2014). In this study, a metagenomics-based approach was applied to obtain the microbial community composition, microbial function profile, and antibiotic resistome simultaneously from the sediments adjacent to an offshore petroleum exploitation platform. The objectives of this study are: (1) to provide baseline data on variations in microbial community composition and function due to the offshore petroleum exploitation activity; (2) to explore the influences of offshore petroleum exploitation on the antibiotic resistome; and (3) to reveal the potential mechanism of ARGs dissemination, caused by offshore petroleum exploitation. The method of microbial taxonomy classification applied in the present study made it possible to analyze bacteria, archaea, eukaryotes, and viruses in the marine environment simultaneously. Thereby, the findings of the present study could expand the understanding of microbial communities and clarified the status of archaea and viruses in marine sediments.

METHODS

Sediment collection and DNA extraction

Three sediment samples adjacent to a petroleum exploitation platform (PEP) at Liaodong Bay, China, were collected on August 2018 (N 39.59, E 120.50). Meanwhile, three offshore sites without any petroleum exploitation activity and 5 km away from the nearest PEP were selected as control group (N 40.18, E 121.20). Surface sediment samples from each location were taken by sediment collection equipment and shipped in a 50-mL polyethylene tube. All samples were stored in a portable ice box and transported to the laboratory within 24 h.

The total DNA was extracted from each sample using the FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA) following the manufacturer's instructions. The quality and concentration of all DNA were evaluated by agarose gel electrophoresis (1.2% agarose) and spectrophotometer analysis (NanoDrop 1000, Thermo Fisher Scientific Inc., USA). All extracted DNA samples were placed in a sterile 1.5-mL polyethylene tube and stored at −20 °C for further experiments.

Metagenomic sequencing and bioinformatics analysis

All extracted DNA samples were sent to Vazyme Biotech Co., Ltd in Nanjing, China, for library construction and sequencing using an Illumina HiSeq 2500 platform with the 150 bp paired-end strategy. About 12 Gb (giga base pairs) of metagenomic data was generated for each DNA

sample, resulting in a total of ~70 Gb. The analysis process for the metagenomic data is shown in Figure 1. Briefly, sequence reads with low quality or ambiguity were removed firstly. Microbial taxonomic classification was conducted by MetaPhlAn2, which mapped metagenomic reads from bacteria DNA samples against a catalogue of clade-specific marker sequences currently spanning the bacterial and archaeal phylogenies (Segata *et al.* 2012). Metagenome sequences were assembled into contigs using SOAPdenovo (Luo *et al.* 2012) and only contigs longer than 500 bp were used for further analysis. Open reading frames were predicted from contigs using MetaGeneMark (Zhu *et al.* 2010). CD-hit was then used to remove redundant sequences among the samples (Fu *et al.* 2012). Reads were mapped back to the non-redundant gene set for each sample and the coverage for each gene was calculated as the number of mapped tags. Gene functional annotations were made by a BLASTP search in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database with the e-value $\leq 1 \times 10^{-5}$. For the ARGs annotation, the online ARGs analysis pipeline, ARGs-AOP (Yin *et al.* 2018), was applied. The sequence was considered to be an ARGs-like sequence when its best hit had similarity of no less than 90% to the reference sequences and had a query coverage of no less than 25 amino acids. Then, the normalized abundance of ARG types and subtypes (copy of ARG per bacteria cell) in bacteria DNA was also calculated by the ARG reference sequence length, the number of 16S rRNA genes, the 16S rRNA gene sequence length, and the copy number of 16S rRNA gene in the bacteria cell.

Quantification of *intI1* and 16S rRNA genes

Quantitative real-time polymerase chain reaction (qPCR) analyses were performed on an Agilent Mx3005p qPCR system (Agilent Technologies, USA) using a SYBR Green approach to quantify the bacterial 16S rRNA and *intI1* genes according to our previous study (Wang *et al.* 2017). The primers of each gene are shown in Table 1. The qPCR mixture (20 μL) consisted of 10 μL 2 × SYBR® Premix Dimer-Eraser (TaKaRa, Dalian, China), 0.2 mM of each primer (Table 1), 1 μL DNA template and nuclease-free water. To eliminate possible qPCR inhibition, 0.2 μL bovine serum albumin (10 g/L) was added to each qPCR mixture. The qPCR reactions were performed using the following protocol: 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at the specific temperatures (Table 1) for 30 s and extension at 72 °C for 30 s. A melt curve was analyzed after each qPCR run to ensure the specificity of each reaction, and non-specific amplification for all primers was not occurring. Each reaction was run in triplicate and sterile water was used as the negative control. Standard curves of each gene for the abundance estimation were also established and the results are listed in Table 2.

Statistical analysis and data visualization

To reflect the effects of offshore petroleum exploitation on sediment microbial community compositions and functions, principal coordinate analysis (PCoA) was performed using the 'vegan' package in R. Moreover, the

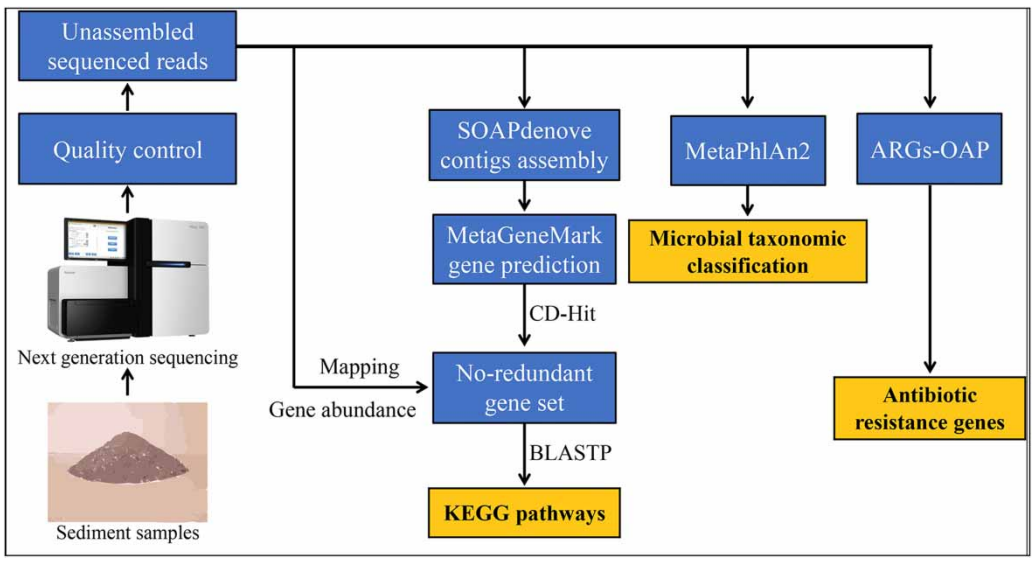

Figure 1 | The flow chart of bioinformatic analyses.

Table 1 | The primers for qPCR used in this study

Target gene	Primer	Sequence (5′–3′)	Annealing temp. (°C)	Product size	Reference
intI1	IntF IntR	GGCTTCGTGATGCCTGCTT CATTCCTGGCCGTGGTTCT	53	154 bp	Luo *et al.* (2010)
16S rRNA	1369F 1492R	CGGTGAATACGTTCYCGG GGWTACCTTGTTACGACTT	53	143 bp	Suzuki *et al.* (2000)

Table 2 | qPCR standard curves for 16S rRNA gene and class 1 integrase

Gene	Standard curves	Detection limits (copies/mg dry weight)	R^2	Efficiency
16S rRNA	$Y = -3.208*\mathrm{Log}(X) + 38.71$	1,000	0.994	105.0%
intI1	$Y = -3.341*\mathrm{Log}(X) + 41.26$	100	0.998	96.2%

adonis test was conducted to test the significance of differences in the sediment microbial communities between the PEP and control groups by 'vegan' package in R. The circular visualization for the relative abundance of dominant microbes at order level was performed using Circos software (Krzywinski *et al.* 2009). The abundances of ARG types were displayed by 'ggplot2' package in R and the significant difference of ARGs abundance and diversity between sediments with and without offshore petroleum exploitation were calculated using R. STAMP software was used to identify microbes, functions, and ARGs that were significantly affected by petroleum exploitation, based on the *t*-test with FDR (false discovery rate) adjustment (Parks *et al.* 2014). Pearson correlation was performed between the abundances of *intI1* gene and ARGs that increased due to the offshore petroleum exploitation, and the result was exhibited by 'ggplot2' package in R.

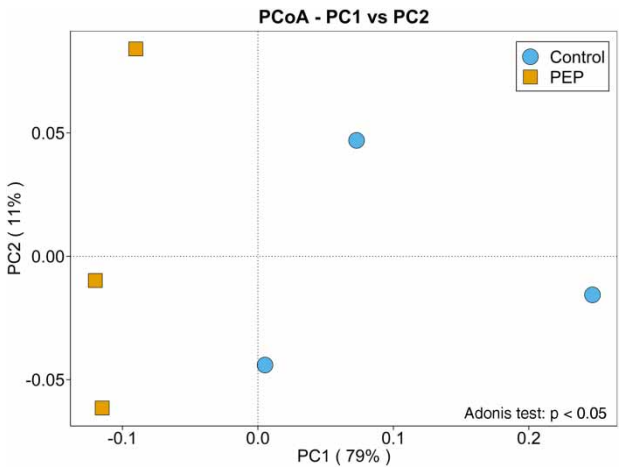

Figure 2 | The PCoA of microbial community compositions of marine sediments with and without petroleum exploitation.

RESULTS AND DISCUSSION

Effects of offshore petroleum exploitation on microbial community compositions

Offshore petroleum exploitation activity significantly altered the composition of sediment microbial communities according to PCoA and the adonis test based on Bray–Curtis distance ($p < 0.05$, Figure 2). The PEP and control groups separately lay on either side of PC1, which explained 79% of the total variations in microbial community composition. Previous studies have demonstrated that the community structure of indigenous bacteria in the coast changed considerably and accompanied the enrichment of petroleum-tolerant bacteria due to the petroleum contamination (Wang *et al.* 2014). Significant variations in microbial community composition were also found in the marine environment after oil spill accidents, which resulted in serious petroleum pollution (Mason *et al.* 2014). In the present study, there is no oil spill or severe oil pollution in the investigated area; however, the concentrations of petroleum substances in sediments adjacent to the PEP were also higher than those in control sediments (data in our unpublished research). The result indicated that the increase in the content of petroleum substances within a relatively low level due to the offshore petroleum exploitation will also significantly change the composition of the microbial community in the marine sediments.

In order to gain a deeper understanding regarding the changes in microbial abundance influenced by petroleum exploitation, the abundance of microbes in different sediments were compared at the order level (Figure 3(a)). The orders of *Xanthomonadales*, *Nitrosopumilales*, and *Caudovirales* were the dominant microbes in detected sediments, which respectively belonged to bacteria, archaea, and viruses. The relative abundance of *Xanthomonadales* was generally consistent in all sediments accounting for 35% to 40% of the total microbial communities. More importantly, *Nitrosopumilales* was significantly enriched in PEP sediments compared to controls ($p < 0.05$, Figure 3(b)). *Nitrosopumilales* is an ammonia oxidizing archaea belong to the phylum *Thaumarchaeota*, which significantly improves the nitrogen cycling in organic-rich coastal marine sediments (Toro *et al.* 2018). Meanwhile, some members of *Nitrosopumilales* can degrade organic matter to produce methane under anaerobic conditions (Poulsen *et al.* 2013).

Effects of petroleum exploitation on microbial community functions

Compared to the microbial community composition, difference in the overall function pattern of microbial communities between PEP and control groups was not significant ($p > 0.05$). The richness and diversity of the microbial community in marine sediments are extremely high; however, different microorganisms may have similar functions. Thus, the microbial composition could change significantly due to the external pressure, while the main functional structure of the microbial community remains relatively stable. Even though the petroleum exploitation activities had a small effect on the function profile of microbial communities in marine sediments, some functions with different abundances were still found (Figure 4). The abundances of genes involved in cysteine and methionine metabolism (ko00270) and aminoacyl-tRNA biosynthesis (ko00970) were significantly reduced in sediments of the PEP group ($p < 0.05$). These results implied that the offshore petroleum exploitation could reduce the production of some protein related to cysteine and methionine in microbial communities of marine sediments. In contrast, in sediments of the PEP group, the functions of pentose phosphate pathway (ko00030), fatty acid biosynthesis (ko00061), butanoate metabolism (ko00650), methane metabolism (ko00680), carbon metabolism (ko01200), and ABC transporters (ko02010) were more abundant than those in the control ($p < 0.05$). The enrichment of carbon, butanoate, and methane metabolism as well as ABC transporters in sediments from the PEP was consistent with the high abundant *Nitrosopumilales* mentioned above.

Effects of petroleum exploitation on antibiotic resistome

A total of 92 ARGs belonging to 16 types were detected in sediments of the present study, among which multidrug and tetracycline resistance genes were most diverse (Table 3). The numbers of detected ARGs in the triplicate PEP groups were 56, 57, and 40, respectively: meanwhile, 45, 43, and 34 ARGs were observed in the triplicate control groups, respectively. Although petroleum exploitation resulted in a slight increase in the number of ARGs in the sediments, no significant difference was obtained between the PEP and control groups ($p > 0.05$). The total abundance of ARGs in control sediments ranged from 0.0488 to 0.0605 copies per bacteria cell. Similar values were presented in sediments impacted by petroleum exploitation activities, which were 0.0504–0.0638 copies per bacteria cell ($p > 0.05$, Figure 5). These results indicated that offshore petroleum exploitation did not significantly change the total abundance and diversity of ARGs in the surrounding sediments. Only one previous research study examined the impact of petroleum contamination on the dissemination and persistence of ARGs at the resistome level and the results demonstrated that the total ARGs in the petroleum-contaminated soils were more abundant than those in the less-contaminated ones (Chen *et al.* 2017). Conflicting results between the previous and present researches may be due to the different type of samples and contaminants. Soil was used as the target for the investigation in the previous study, and the influencing factors were limited to polycyclic aromatic hydrocarbons, a major constituent of petroleum. However, in the present study, the marine sediment was used to evaluate the abundance of ARGs affected by the petroleum substances, which were dominated by diverse alkanes.

To better understand the influence of offshore petroleum exploitation on the ARGs in surrounding sediments, the abundances of single ARGs in PEP and control groups were compared. The genes of the multidrug transporter, *smeE*, and *arnA* were significantly enriched in marine sediments impacted by offshore petroleum exploitation (Figure 6). Moreover, significant correlations between the multidrug transporter, *smeE*, *arnA*, and *intI1* genes were discovered in the sediments of PEP and control groups ($p < 0.05$, Figure 7), suggesting that offshore petroleum exploitation could promote the HGT of ARGs, mediated by class I integrons.

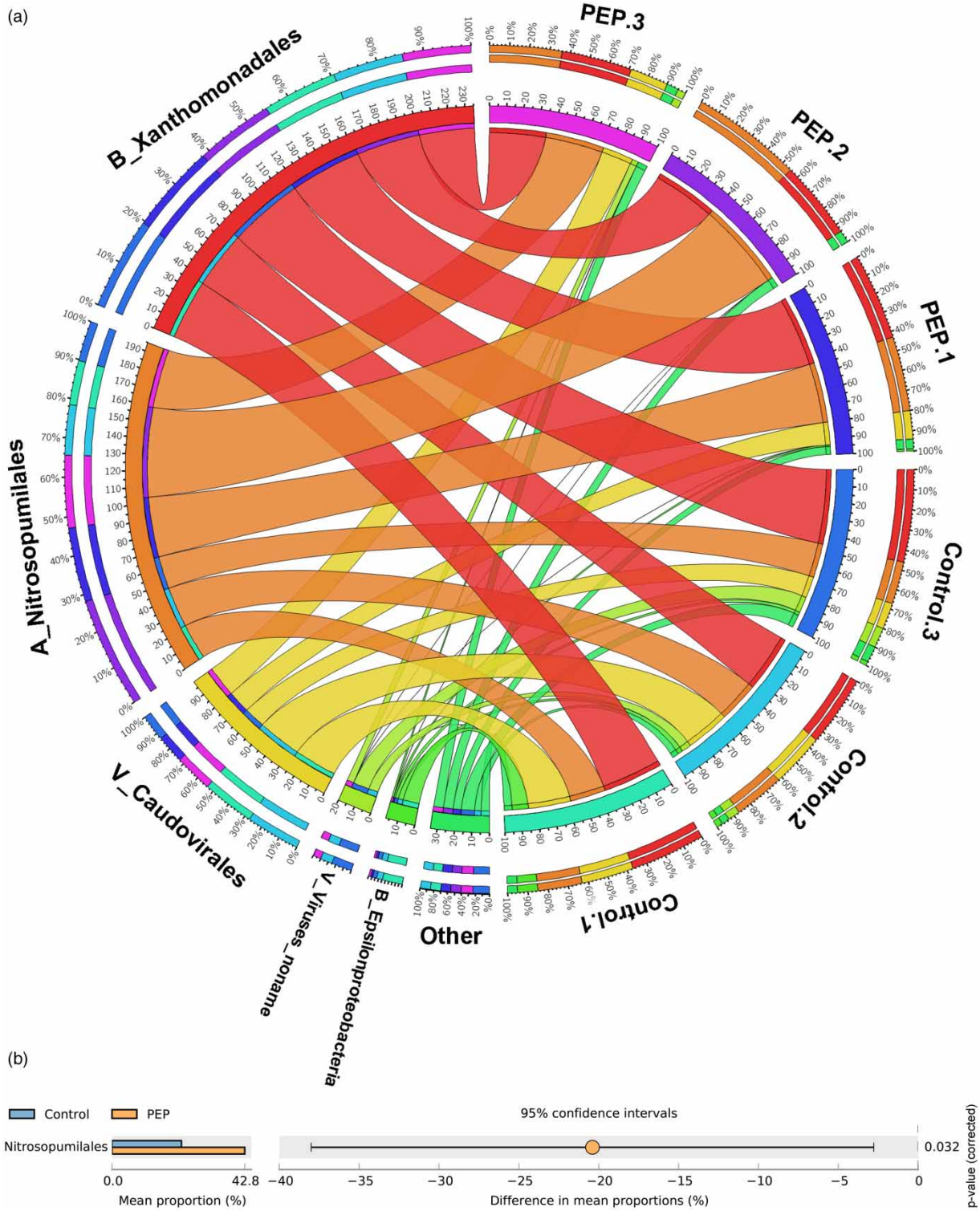

Figure 3 | (a) The composition of microbial communities at order level in all samples. (b) Microorganisms with significantly different abundances between PEP and control groups.

The prevalence of the class I integron is proposed to serve as a marker of antibiotic resistance level and anthropogenic pollution in the environment (Gillings *et al.* 2015). The 3′-conserved regions of the class I integron contains *qacΔE1* and *sul1* genes responsible for resistance to quaternary ammonium compounds and sulfonamides, respectively

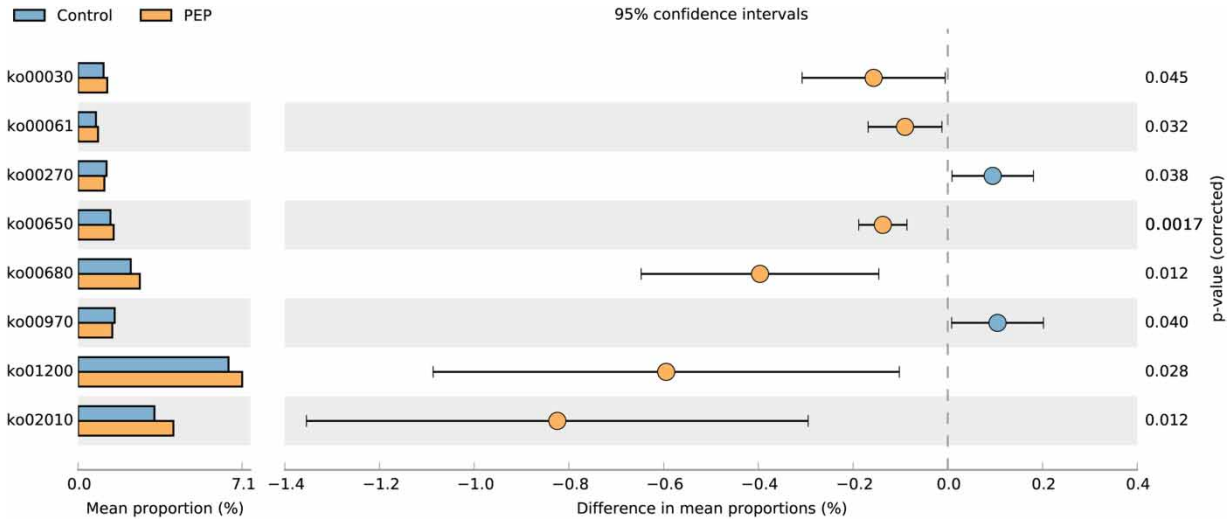

Figure 4 | Differences in the function pathways based on KEGG annotation of sediment microbial communities between PEP and control groups.

Table 3 | The number of ARG subtypes detected in different samples

	Control.1	Control.2	Control.3	PEP.1	PEP.2	PEP.3	Total
Aminoglycoside	3	1	3	2	3	1	6
Bacitracin	2	2	2	2	2	2	2
Beta-lactam	4	1	1	2	3	1	6
Chloramphenicol	1	0	0	1	1	0	1
Fosfomycin	1	1	1	1	1	1	1
Fosmidomycin	1	1	1	2	2	0	2
Kasugamycin	1	0	0	1	1	0	1
Macrolide–lincosamide–streptogramin (MLS)	2	1	1	4	2	2	5
Multidrug	14	18	13	24	24	17	34
Polymyxin	1	1	1	1	1	1	1
Rifamycin	0	0	0	0	1	0	1
Sulfonamide	0	0	0	1	1	0	2
Tetracycline	6	10	3	9	7	7	15
Trimethoprim	3	1	4	1	1	1	5
Unclassified	4	4	3	3	4	3	4
Vancomycin	2	2	1	2	3	3	6
Total	45	43	34	56	57	40	92

(Gillings 2014). Our previous study demonstrated that petroleum substances in seawater can increase the abundance of *sul1* gene through promoting the HGT mediated by class I integrons (Wang *et al.* 2017). However, to the best of our knowledge, the present study is the first to reveal the dissemination of ARGs other than sulfonamide resistance genes in the petroleum-contaminated offshore environment.

CONCLUSIONS

In summary, the effects of offshore petroleum exploitation on the composition, function, and antibiotic resistance of the microbial community in surrounding marine sediments were measured by a metagenomics-based method. Our results suggested that offshore petroleum exploitation can significantly change the microbial community compositions,

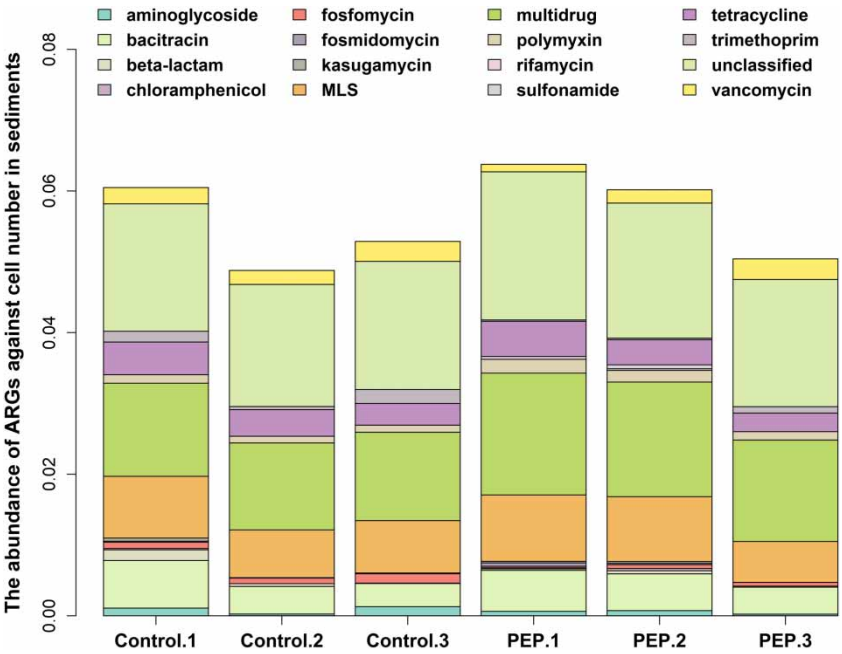

Figure 5 | The abundance of ARGs in different samples.

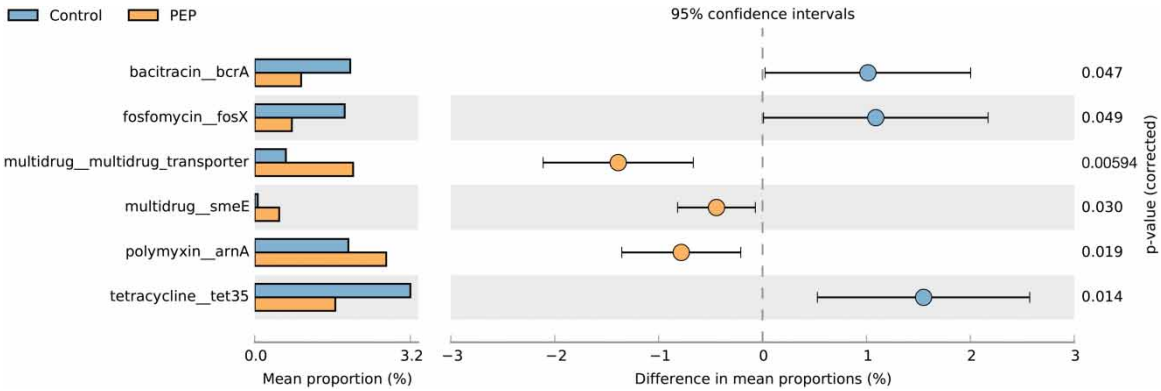

Figure 6 | Differences in the abundance of single ARGs between PEP and control groups.

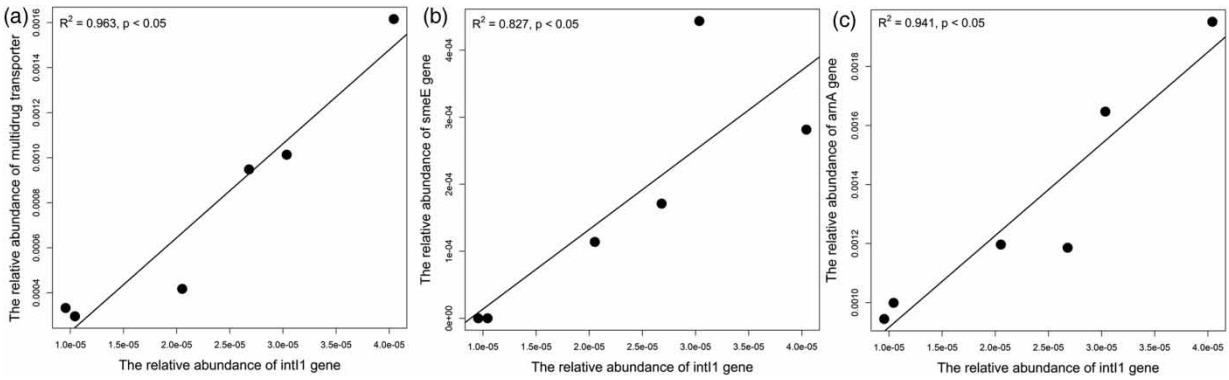

Figure 7 | The correlation results between multidrug transporter (a), *smeE* (b), and *arnA* (c) with *intI1* genes.

and partially alter the functions of microbial communities in marine sediments. In the sediments adjacent to the PEP, the archaea of *Nitrosopumilales* were significantly enriched, which can digest organic matter to produce methane. Offshore petroleum exploitation did not significantly change the total abundance and diversity of ARGs in the surrounding sediments. However, petroleum exploitation enriched specific ARGs, multidrug transporter, *smeE*, and *arnA*, via HGT mediated by class I integrons. The findings of this study suggested that a more comprehensive risk assessment on the environmental hazard of offshore petroleum exploitation is necessary.

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