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Nutrient depletion is the main limiting factor in the crude oil bioaugmentation process

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ABSTRACT

The biodegradation was considered as the prime mechanism of crude oil degradation. To validate the efficacy and survival of the crude oil-degrading strain in a bioremediation process, the enhanced green fluorescent protein gene (*egfp*) was introduced into *Acinetobacter* sp. HC8–3S. In this study, an oil-contaminated sediment microcosm was conducted to investigate the temporal dynamics of the physicochemical characterization and microbial community in response to bacterium amendment. The introduced strains were able to survive, flourish and degrade crude oil quickly in the early stage of the bioremediation. However, the high abundance cannot be maintained due to the ammonium (NH_4^+-N) and phosphorus (PO_4^{3-}-P) contents decreased rapidly after 15 days of remediation. The sediment microbial community changed considerably and reached relatively stable after nutrient depletion. Therefore, the addition of crude oil and degrading cells did not show a long-time impact on the original microbial communities, and sufficient nitrogen and phosphorus nutrients ensures the survive and activity of degrader. Our studies expand the understanding of the crude oil degradative processes, which will help to develop more rational bioremediation strategies.

Introduction

Petroleum pollution from both natural and man-made sources poses significant threats to the environment. Masses of petroleum entered coastal zones, surface runoffs and soil, endangering resident organisms. As oil is spilled into the sea, its form would experience changes caused by wave effects, sunlight and inherent microbial community over time (Fingas, 2017). Hydrocarbons were fully mineralized into CO_2 and water by the natural metabolic processes induced by microbial communities in seawater and sediments.

Bioremediation is an economical technique utilizing biological organisms to remove hazardous substances (Marchut-Mikolajczyk et al., 2020). Bioaugmentation is a method of adding oil-degrading microorganisms into the oil-contaminated area, thus enhancing the microbial populations

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Table 1 – Bacterial strains, plasmids, and primers used in this study.

Strain, plasmid, or primers	Description	Source or reference
Strain		
Acinetobacter sp. strain HC8–3S	Aerobic, G-; degrades crude oil	Our lab preserved
E. coli Trans-T1	$F^{-}\phi80$ (lacZ) ΔM15 ΔlacX 74 hsdR(r_k^{-} m_k^{-}) ΔrecA 1398endA1 tonA	Tiangen
Plasmid		
pTn-oKm	pMB1 replicon, Tn5 inverted repeat; Km^R	Dennis and Zylstra., 1998
pMp2444	Gm^R, Plasmid containing the *egfp* gene	Bloemberg et al., 2000
PUC57	Amp^R, Plasmid containing CP25 promoter	Zhang et al., 2010
Primers(5′–3′)		
F1	ACGCACGCGTCGACATCGATTCGAG	This study
R1	CCGTAGGTCAGGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTT	This study
F2	GCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCG	This study
R2	CGGCGGGGTACCTTACTTGTACAGC	This study

Amp^R, ampicillin resistant; Kan^R, kanamycin resistant; Gm^R, gentamicin resistant.
Restriction sites in the sequences of the primers are underlined.

and reducing the lag period to initiate the bioremediation process. This method would be suggested when the native microbial community is inefficient of degrading complex mixtures (i.e. crude oil), or when the community is suffering because of exposure to the spill. In this way, the potential for the degradation of petroleum hydrocarbon can be enhanced by selective enrichment of natural bacterial populations. If this approach is applicable in the field, the introduced microorganisms should meet the following conditions: i) smoothly degrading most petroleum components; ii) maintaining genetic viability and stability during bioaugmentation; iii) surviving in foreign and polluted environments and competing with indigenous microbe.

In our previous studies, marine bacterium *Acinetobacter* sp. HC8–3S was isolated from a petroleum hydrocarbon culture system. Its biodegradation efficiency of the saturated hydrocarbon was 94% in 5 days (Lin et al., 2014). In this study, the effect of *Acinetobacter* sp. HC8–3S on crude oil degradation in sediments was investigated.

To verify the efficacy of the *Acinetobacter* sp. HC8–3S inoculum in bioremediation profiles, we monitored the survival and activity of these cells specifically during the actual degradation process. However, observing a specific inoculum in a complex system (i.e. marine sediment) would be much more complicated due to the massive presence of indigenous microorganisms. Moreover, microbial inoculants grown on agar media after long-term lab incubation may fail to be cultured in the environment, which confines the feasibility of culture-based detection techniques. One of the ways to monitor the survival and activity of the inoculated cells is adding a biomarker to the genome, encoding a unique phenotype so that they can be specifically detected without cultivation (Dai et al., 2020). Enhanced green fluorescent protein (EGFP) is an artificial red-shift variant of GFP, which would fluoresce under visible light. Its fluorescent intensity is 35 times (488 nm) stronger than GFP (Cormack et al., 1996). Because of its small molecular weight, stable fluorescent, species-specific, and cell security, EGFP has been used widely in the dynamic testing study in microorganisms.

Our study aims were, (i) to investigate chromosomally marked *Acinetobacter* sp. HC8–3S cells with *egfp* biomarkers; (ii) to observe the survival and activity of the marked cells during crude oil degradation in marine sediment microcosm.

1. Material and methods

1.1. *Bacterial strains, plasmids, and culture conditions*

The strains, primers, and plasmids used in this study are listed in Table 1. The wild strain used in this study, *Acinetobacter* sp. HC8–3S, was previously isolated from petroleum-contaminated sea sediment from Bohai Bay, China (Lin et al., 2014). Precultures of *Acinetobacter* sp. were grown for 24 hr in Zobell 2216E growth medium at 30°C. T4 DNA ligase, λDNA/HindIIIMarker, MarkerDL2000 and restriction enzymes for DNA manipulations were purchased from TaKaRa Biotechnology Co. Ltd. (China) and Sino-American Biotechnology Co. Ltd. (China). Ampicillin, kanamycin and gentamicin were produced from Sino-American Biotechnology Co. Ltd. (China). A physical map of the plasmids constructed for this study is shown in Appendix A Fig. S1.

1.2. *Experimental microcosm setup*

To evaluate the responses of microorganism communities to the introduction of crude oil and external strains, we set up five marine microcosms: (1) Control group: non-sterilized sediment and seawater, (2) ON group: non-sterilized sediment and seawater + crude oil, (3) HN group: non-sterilized sediment and seawater + *Acinetobacter* sp. HC8–3S-9, (4) HSO group: sterilized sediment and seawater + *Acinetobacter* sp. HC8–3S-9 + crude oil, (5) ONH group: non-sterilized sediment and seawater + *Acinetobacter* sp. HC8–3S-9 + crude oil. The bacterial strains used in this study were *Acinetobacter* sp. HC8–3S-9 (the transformant). Solutions and instruments were sterilized, and all steps were performed in a sterile cabinet. Sediment and seawater samples were obtained from Bohai Sea

(37.713°N, 119.873°E). Large objects in the sediment, such as stones and debris, were removed manually in the laboratory before use. The sediment samples were then homogenized and prepared for oil degradation treatment. The crude oil was collected from Shengli oilfield, Dongying, China. The API (American Petroleum Institute) gravity was 25.6, and the viscosity was 4896 mPa-s (Shanghai Changji Instrument Tech. Co., Ltd, China).

Each experimental group consisted of a glass jar (45 cm length × 30 cm width × 30 cm height) containing 2 kg of sediment and 2 L seawater. Microcosms were incubated at room temperature (20°C), and an air pump was used to provide oxygen for the microorganisms. The final concentration of *Acinetobacter* sp. HC8–3S-9 and the crude oil were 1×10^6 CFU/mL and 200 mg/L respectively. Surface sediment samples from each treatment were obtained for chemical analysis and DNA extraction on day 0, 5, 15, 30, 45 and 60 after incubation.

1.3. *Preparation of oil samples*

Experimental samples were prepared for oil analysis using the following steps. Firstly, the freeze-dried sediment samples were homogenized, pulverized, and extracted with 15 mL dichloromethane (DCM) in an ultrasonic wave cleaner for 15 min. Then 0.5 g of anhydrous sodium sulfate was added into the triangle flask and oscillated sufficiently. The extraction procedure was repeated three times and then combined. The extract was concentrated to 10 mL after the solvent was exchanged to hexane and then fractionated by column chromatography (Cleanert Florisil). The residual oil was eluted under gravity with 10 mL n-hexane and analyzed by ultraviolet spectrophotometer method. The extraction of petroleum from water samples was also carried out in accordance with the above steps.

1.4. *Chemical analysis and DNA extraction*

A YSI 6600 device (YSI Incorporated, USA) was used to measure dissolved oxygen (DO), temperature, pressure, salinity, pH value, electrical conductivity and oxidation–reduction potential (ORP) *in situ*. Sediment samples were acidified with dilute HCl (1 mol/L) before total organic carbon (TOC)/total organic nitrogen (TON) analysis to remove carbonates, and then subsequently rinsed with deionized water three times before drying overnight at 60°C. TOC and TON were determined on a CHNS Vario E1 III elemental analyzer. Samples collected in vacuum tubes (10 mL) were frozen at 4°C for nutrient (NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}, and SiO_3^{2-}) analysis using an Auto-Analyzer (Bran+ Lubbe K.K, Model TRAACS 2000 and Alliance, Model Futura). DNA was extracted from 0.5 g (dry weight) of sediments using Fast DNA® SPIN Kit for Soil Kits (MP Biomedicals) in accordance with the manufacturer's specifications. DNA samples were amplified using the 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 907R (5′-CCGTCAATTCMTTTRAGT-3′) primer set that amplified the V4-V5 region of the 16S rRNA gene and analyzed using the Illumina MiSeq platform.

Real-time PCR was used to quantify the *egfp* gene from samples on a 7500 Fast Real-Time PCR System (Thermo-Fisher, Waltham, MA, USA) using primers 5′-CTCGTGACCACCCTGACCTACGGCG-3′ and 5′-CGGCGGGGTACCTTACTTGTACAGC-3′ according to (Wang et al., 2018). Real-time PCR of *egfp* was modified with an initial denaturation (95°C for 30 sec) followed by 40 cycles of 95°C for 5 sec, 60°C for 34 sec before a final plate read (30 sec). The amplification efficiency was 118% with an R^2 value of 0.990 for the *egfp* gene.

1.5. *16S gene sequence analysis and data availability*

16S gene sequence analysis was performed using the online platform of Majorbio Cloud Platform (www.majorbio.com). Sequence data determined in this study are available at NCBI under SRA Accession PRJNA513087 (https://www.ncbi.nlm.nih.gov/sra/PRJNA513087).

2. Results

2.1. *Introduction and expression of the* egfp *gene into bacteria*

The transformant of *Acinetobacter* sp. HC8–3S-9 emitted strong green fluorescence when viewed under a laser confocal scanning microscope (Appendix A Fig. S2). Extrachromosomal DNA from transformed clones was isolated and analyzed by agarose gel electrophoresis, which confirmed the existence of the fusion protein CP25-EGFP gene (Fig. 1). In addition, this result indicated that the strain NO.9 was able to induce the expression of the recombinant protein (red arrow). Thus, this strain was chosen for all subsequent experiments.

Fluorescence microscopy was used to detect the fluorescence and survival of *Acinetobacter* sp. HC8–3S-9 in microcosms and bright green fluorescence dots had been witnessed. No colored dots in the control samples could be detected in the microscope (data not shown). As shown in Appendix A Fig. S3, *Acinetobacter* sp. HC8–3S-9 bacteria had good fluorescence and abundance in the early stages of the microcosms. The abundance of the bacteria decreased but remained stable in the microcosms at the late stage of degradation.

Real-time PCR was applied to detect the copy numbers of the *egfp* gene. The results showed that the copy numbers decreased in three treatments, and the final gene copy number did not differ from the initial gene copy number by order of magnitude, as shown in Appendix A Fig. S4. ONH group (oil + non-sterilized seawater and sediment + *Acinetobacter* sp. HC8–3S-9) had a high level of *egfp* gene copies in the 5th and 15th days, which could be related to the multiplication of the transformant after induction of oil pollutants. These results showed that the *egfp* gene can expressed stably in *Acinetobacter* sp. HC8–3S-9 engineering bacterium and monitored the abundance and survival of petroleum hydrocarbon-degrading bacteria.

2.2. *Oil removal and physicochemical characterization during the bioaugmentation process*

To examine the degradation activity of crude oil, a complex mixture of thousands of individual compounds, we used the UV photometry at the wavelength 260 nm as an alternative

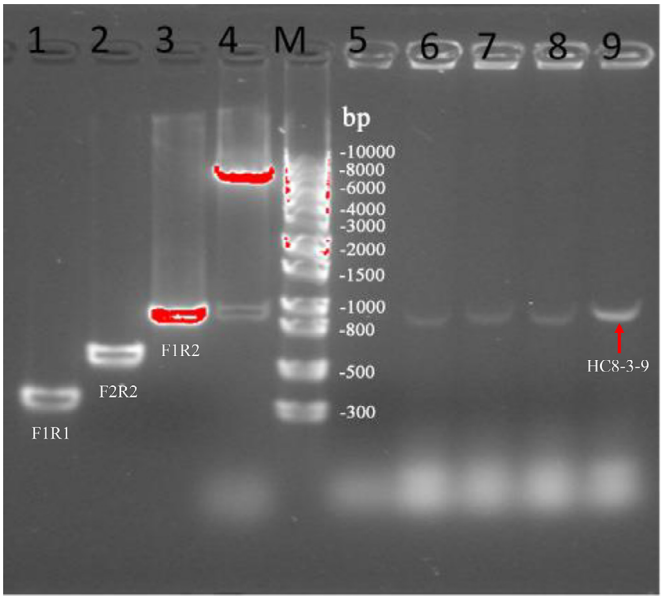

Fig. 1 – Identification of pTnMod-oKm-CP25-*egfp* plasmids. Genomic DNA was isolated from bacterial strains according to the manufacturer's instructions. Initial characterization was carried out by PCR with F1R1 and F2R2 primers. For Southern blot analysis, undigested and digested (SalI and KpnI) plasmid DNA and genome DNA from *Acinetobacter* sp. parent strains were run on a 1% (W/V) agarose gel. Lanes: 1, F1R1 (CP25 promoter gene, 331 bps); 2, F2R2 (*egfp* gene 551 bps); 3, F1R2 (SalI-CP25-egfp-KpnI, 882 bps); 4, pTn-oKm-F1R2 restriction enzyme fragment (5980 bps); M, marker; 5–9, Colony PCR of HC8–3S.

speedy analytical method (Mao and Han, 2014). Most of the crude oil was concentrated in sediments, and the concentration in water was only about 20 mg/L in this study (Appendix A Fig. S5). The crude oil concentration significantly decreased over 60 days in all treatments. The removal efficiency of ONH treatment was not significantly ($p>0.05$) higher than that of the other treatments, which may indicate that the limitation of the microcosm was the bioavailability of nutrients and the population of indigenous degraders. The mean total concentrations of crude oil were 27.29, 32.91 and 21.71 mg/L in the ON, HSO and ONH treatments after 60 days of culture, and the removal efficiencies reached 85.76%, 83.82% and 89.31%, respectively (Fig. 2).

The physicochemical parameters of the microcosms sample were shown in Appendix A Table S1. Temperature and dissolved oxygen remained changeless during the experiments. The average temperature of the overlying water was 20°C, and the average of dissolved oxygen concentration was above 75% as the results of constant aeration. The pH of the water was close to neutral seawater (8.3), while the salinity was 29.6 PSU. The values for total nitrogen (TN), total organic nitrogen (TON), total carbon (TC), and total organic carbon (TOC) were 0.049%, 0.043%, 1.42%, 0.352%, respectively. In our study, ammonium (NH_4^+-N) and phosphorus (PO_4^{3-}-P) concentrations in microcosm decreased significantly in the initial 15 days. They then remained at a relatively stable and low level for the remaining period of the 60-day experiment. While the values of nitrate (NO_3^--N, NO_2^--N) followed the trend of decreasing first, then increased, and declined to a stable level finally. The silicate content was relatively stable in the microcosm (Fig. 2).

2.3. *Overall structure of bacterial communities across samples*

Total 24 samples were sequenced using an Illumina MiSeq system, and 149,857,5 high-quality sequences with an average length of 372 bp were recovered for downstream analysis after quality trimming and chimera checking. Good's estimator of coverage was 97.63%, indicating the 16S rRNA sequences identified in this research represented most bacterial sequences present in the samples. Shannon, Sobs, Simpson, Abundance-based Coverage Estimator (ACE), and Chao indices were employed to estimate the α-diversity of the bacterial communities (Appendix A Table S2).

To examine the identifiable common core microbiome, we defined a core as the group with shared members among the microbial community at 97% identity and then represented by overlapping areas in a Venn diagram (Appendix A Fig. S6). We identified 4754, 4985, 4731, and 4704 OTUs in the Control, ON, ONH, and HN groups, respectively. There exists 3622 core OTUs (99 core genera) among different groups, which can be regarded as the core microbiome. The six most abundant genera were *Acinetobacter* (14.75% of total abundances), *Sulfurovum* (7.20%), *Anaerolineaceae* (4.88%), *Bacillus* (3.63%), and *Desulfobulbus* (3.49%). Common elements in ON and ONH groups included 14 genera: *Luteimonas, Nubsella, Rhizomicrobium, Mesorhizobium*, norank_f__Bradyrhizobiaceae, *Bradymonas, Robertkochia, Acidibacter*, norank_f__A0839, *Atopobium, Arenimonas, Nordella, Microbacterium*, and *Phenylobacterium*, which may relate to oil degradation.

Unique representative sequences were classified into 5552 OTUs at a 97% similarity level, from which 58 phyla, 844

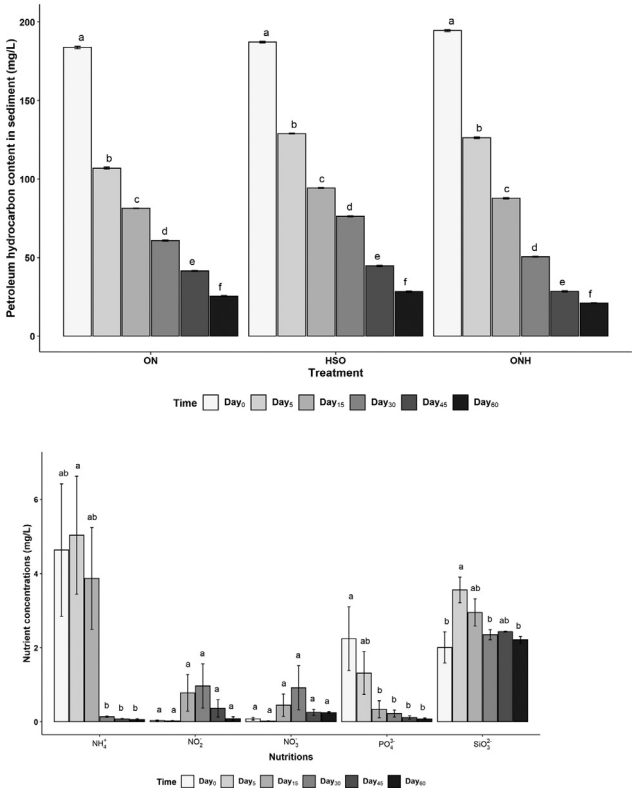

Fig. 2 – Petroleum hydrocarbon (left) and average nutrients concentrations (rigth) in microcosms. ON = Oil+ non-sterilized sediment and seawater; HSO = *Acinetobacter* sp. HC8–3S-9+ sterilized sediment and seawater + Oil; ONH = Oil+ non-sterilized sediment and seawater+ *Acinetobacter* sp. HC8–3S-9. The mean values with the different letters within the same group show significant differences at the 0.05 probability level, and those with the same letters within the same group are not significantly different.

genus and 1697 species were detected. The top five most abundant phyla, Proteobacteria, Firmicutes, Chloroflexi, Acidobacteria and Bacteroidetes, together comprised 87.55% of all sequences. Proteobacteria was the most abundant phylum, accounting for 53.67% of sequences. This phenomenon was mainly caused by the addition of *Acinetobacter* sp. HC8–3S-9. At the genus level, *Acinetobacter, Sulfurovum, Anaerolineaceae, Fusibacter* and *Desulfobulbus* were the five most abundant genera, comprising 16.78%, 6.76%, 4.25%, 3.29% and 3.26% of sequences, respectively (Fig. 3).

In the control group, comparing the community structure at the beginning and end of the experiment, it can be concluded that: the relative abundance of *Acinetobacter* decreased from 18.51% to 1.09%, *Bacillus* from 10.92% to 5.42%, and *Sulfurovum* from 7.64% to 4.92%, while, *Desulfobulbus* increased from 2.5% to 4.6%, norank_Cloftridiaceae from 0.49% to 5.86%, and *Tepodibacter* from 0.21% to 3.51%.

In the ON group (Oil+ non-sterilized sediment and seawater), the relative abundance of *Bacillus* and *Acinetobacter* decreased from 12.33% to 5.00%, and from 12.39% to 0.04%, respectively. The relative abundance of *Sulfurovum* remained stable, from 8.66% to 7.67%, while that of *Desulfobulbus* and *Marinobacter* increased in the presence of oil, from 2.60% to 8.43%, and from 0.25% to 3.32%, respectively.

As for the ONH group with introduced strains, it can be seen from Fig. 3 that the *Acinetobacter* sp. HC8–3S-9 strains maintained high abundance at day 0 and day 5, respectively 69.09% and 66.70%. But on day 15, the abundance dropped to 3.89%, and by the end of the experiment, it was 0.37%. Besides, the HN group changed similarly in community structure with the ONH group, and the relative abundance of *Acinetobacter* sp. HC8–3S-9 strains decreased sharply on day 15.

There were 6 statistically significant differences between in incubation time at the genus level (Appendix A Fig. S7). The relative abundance of *Acinetobacter* (36.08% at day 0 versus 0.45% at day 60, the same below, $p \leq 0.01$) and *Bacillus* (7.12% versus 4.11%, $p \leq 0.05$) decreased, while relative abundance of *Desulfobulbus* (2.11% versus 5.34%, $p \leq 0.01$), *Fusibacter* (0.68% versus 2.64%, $p \leq 0.05$), norank_f_Clostridiaceae (0.27% versus 3.25%, $p \leq 0.01$) and *Photobacterium* (0.14% versus 3.10%, $p \leq 0.01$) increased during incubation.

To describe relationships of the microbial community in the different microcosms, we analyzed the banding patterns quantitatively by using non-metric multidimensional scaling (NMDS) (Appendix A Fig. S8). NMDS analysis based on Bray-Curtis similarity distance showed that microbial community at day 15, 30, and 45 and 60 clustered more closely together, while samples from day 0 and 5 were further apart from each other on the ordination. Moreover, NMDS analysis showed that the other samples clustered more closely, except for the four samples of d5-ONH/d0-ONH/d5-HN/d0-HN. In particular, the microbial communities of the HN and ONH experimental groups were like that of the Control and ON experimental group after 15 days.

Partial Least Squares Discriminant Analysis (PLS-DA), a supervised analysis suitable for high-dimensional data, was performed (Appendix A Fig. S9). The bacterial communities in the ON samples and the control group clustered separately, indicating the overall structures of the bacterial communities in these groups were of a wide discrepancy. However, the HN and ONH samples could be clustered together, indicating that the addition of crude oil would not cause an obvious difference of the bacterial communities, under the condition of *Acinetobacter* sp. HC8–3S-9.

2.4. *Environmental factors and main microbial community abundance trend*

Changes in environmental factors may affect microbial community composition and diversity. To further explore the relative importance of individual environmental variables, we measured the variance inflation factor (VIF) among different environmental factors to judge the multicollinearity. The VIF values of PO_4^{3-}, NO_3^-, NH_4^+, NO_2^- SiO_3^{2-}, WTPH (total petroleum hydrocarbons in water) and STPH (total petroleum hydrocarbons in sediment) were lower than ten and maintained.

The Spearman correlation heatmap showed the relationship between environmental factors and microbial phyla (Fig. 4). From the clustering results, NH_4^+, NO_2^- SiO_3^{2-}, WTPH

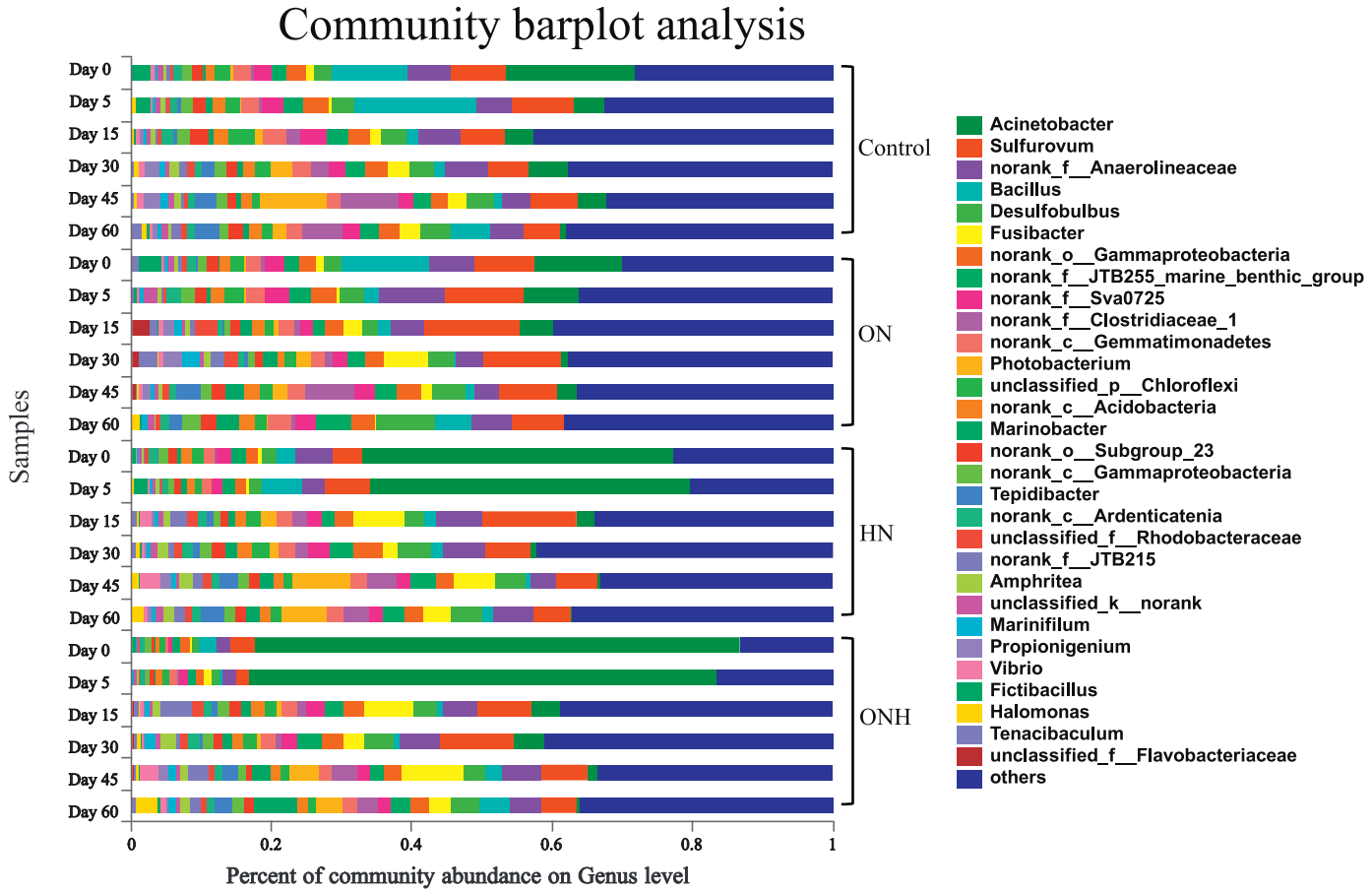

Fig. 3 – Distribution of the predominant bacteria at genus taxonomic levels. The predominant taxa (>2% relative abundance) in each level are shown.

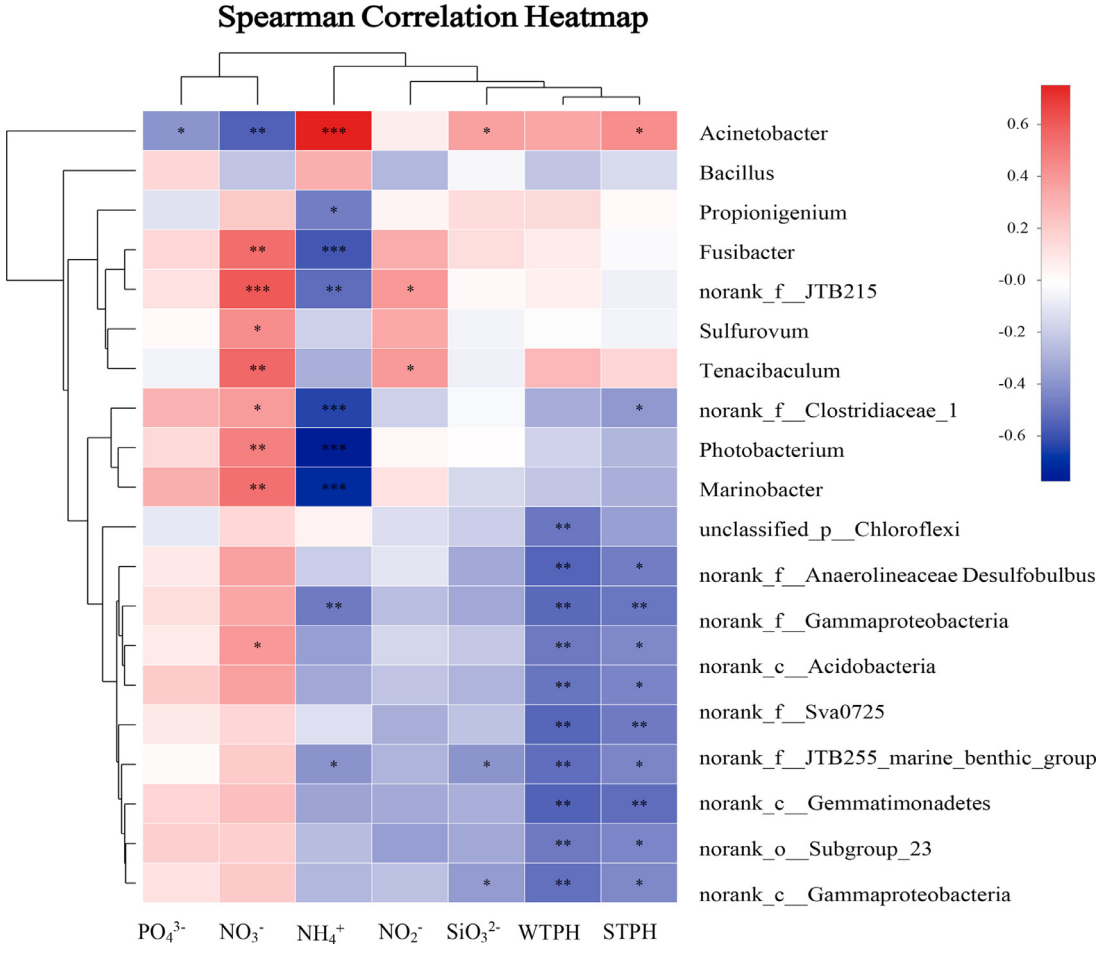

Fig. 4 – Correlation heat map of the top 20 genus and environmental factors of microcosm. X and Y axis are environmental factors and genus. *r* in different colors to show, the right side of the legend is the color range of different *r* values, with $p <$ 0.05*, 0.01, and 0.001***, respectively.**

and STPH clustered into one group, with PO_4^{3-} and NO_3^- into another.

Acinetobacter showed significant positive correlations with NH_4^+ ($p<0.001$, Spearman correlations $r = 0.75$), SiO_3^{2-} ($p<0.05$, $r = 0.37$) and STPH ($p<0.05$, $r = 0.44$), and demonstrated a negative correlation with PO_4^{3-} ($p<0.05$, $r = -0.39$) and NO_3^- ($p<0.01$, $r = -0.55$). *Fusibacter*, norank_f_JTB215, norank_f_Clostridiaceae, *Photobacterium* and *Marinobacter* showed similar correlations with environmental factors, that is, negative correlations with NH_4^+ and positive correlations with NO_3^-. The last ten undefined genera all showed negative correlations with WYPH and STHP, suggesting that they were slowly accumulating in the late stages of oil incubation and may be involved in the degradation of petroleum hydrocarbons.

3. Discussion

The plasmid pTnMod-oKm-CP25-*egfp* in which EGFP was placed under the control of the CP25 promoter was constructed and introduced into *Acinetobacter* sp. HC8–3S by electroporation. EGFP is a stable and useful marker, which can be used to detect the labelled *Acinetobacter* cells conveniently, quickly and cheaply.

3.1. Nutritional limitations for biodegradation of crude oil

In our study, ammonium (NH_4^+-N) and phosphorus (PO_4^{3-}-P) concentrations in microcosm decreased significantly in the initial 15 days and then remained at a relatively stable and low level for the remaining period of the 60-day experiment (Fig. 2). In addition, the spearman correlation heatmap (Fig. 4) showed that *Acinetobacter* presented significant positive correlations with NH_4^+ ($p<0.001$, Spearman correlations $r = 0.75$). This relationship implied that the relative abundance of *Acinetobacter* decreased sharply after 15 days, possibly due to the decline in the NH_4^+ concentration.

There are always limited or lacking inorganic nutrients, especially for the macronutrients in typical soil, resulting in slow degradation rate even in the presence of the carbon and energy required for growth (Chettri et al., 2016). In this study, nutrients concentrations in ONH group decreased sharply after 15 days (Appendix A Fig. S10), and showed a strongly positively correlation with *egfp* gene copy number and petroleum hydrocarbons concentration (Appendix A Fig. S11). The *egfp* gene

copy number was represented the abundance of *Acinetobacter* sp. HC8–3S-9. According to these results, we speculated that the rapid depletion of nutrients caused the decrease of *Acinetobacter* sp. HC8–3S-9 abundance. It had been reported that the limitation of organic pollutant degradation was the shortage of nitrogen and phosphorus, where physicochemical parameters such as temperature and pH were constant and optimal (Imron et al., 2020; Wu et al., 2020). It is a common phenomenon that when the native microbial populations grow vigorously through supplement nutrient, and petroleum hydrocarbons will be degraded more effectively.

Degradation process could take place without intervention, but the degradation speed was slightly improved by the added remediation cells in the present research. However, the inorganic nutrients did not sustain the petroleum hydrocarbon degraders in the seawater and sediment microcosm to the end of the 60-day experimental period. In this study, the mean total concentrations of crude oil were 27.29, 32.91 and 21.71 ppm in the ON, HSO and ONH treatments after 60 days of culture and the removal efficiencies reached 85.76%, 83.82% and 89.31%, respectively (Fig. 2). This phenomenon suggested that indigenous microorganisms were able to degrade crude oil in the sediment microcosm. A similar result was also observed that control treatment showed the same degradation capacity even though *Acinetobacter baumannii* T30C was not augmented into the soil (Lee et al., 2011). Addition of the petroleum hydrocarbon-degrading bacteria did not deliver the best performance, maybe indicated that the limiting factors of the microcosm were the bioavailability of N and P. Decline in the populations at the later part of the remediation process may be attributed to the depletion of the N and P that were utilized during the early stages (day 1 to day 15). Indigenous bacteria needed to be "enhanced" and "nourished" when nutrients deficient, especially for NPK deficient (Haleyur et al., 2019; Roy et al., 2018). Meanwhile, the addition of nutrients could be detrimental and limiting the degradation process if not applied in an appropriate way (Stein et al., 2018). Suitable amounts of trace elements for microbial growth can be added in an available form (Adams et al., 2015), which also serves as an electron donor, to stimulate bioremediation. By adding nutrients to adjust the C/N/P ratio of microcosm to 100:10:1, which is closer to the requirements of indigenous microorganisms, both the microbial activity and petroleum hydrocarbon biodegradation are improved. In general, biostimulation strategies involve the single application of N and P. However, nutrient deficiency is likely to occur in the late stages of remediation, as nutrients are gradually consumed for bacterial growth and hydrocarbon metabolism processes, especially when a higher initial TPH contamination level in the microcosm (Xu and Lu, 2010). In all, valid implementation of bioremediation required a consideration of the indigenous microorganisms, nutrient availability as well as other physicochemical parameters necessary.

3.2. *Microbial community dynamic of dominant bacteria in microcosms*

In our study, *Acinetobacter, Sulfurovum, Anaerolineaceae, Fusibacter* and *Desulfobulbus* were the five most abundant genera. Bacteria of the genus *Acinetobacter* are anaerobic, non-fermentative, Gram-negative bacilli, and ubiquitously distribute in hydrocarbon-contaminated environments (Percival, SL and Williams, 2014). In the previous paper, *Acinetobacter* sp. HC8–3S could degrade saturated hydrocarbon fractions effectively, and the biodegradation efficiency of immobilized strains increased by 30% compared with planktonic cells. Furthermore, *Acinetobacter* sp. HC8–3S maintained growth at pH ranging from 5.6 to 8.6 and salinity from 30 to 70 g/L and successfully enriched on industrial-scale fermenters (Liu et al., 2016). All the above features indicated that *Acinetobacter* sp. HC8–3S is a potentially useful industrial strain with strong tolerance and good degradation effect.

In this study, *Acinetobacter* sp. HC8–3S-9 strains maintained high abundance at day 0 and day 5 then decreased sharply on day 15, which was consistent with the Real-time PCR results (Appendix A Fig. S4). This phenomenon indicated that the abundance of introduced microorganisms decreased during bioremediation. A similar result was also observed by Muratova et al. (2018), who reported that *Acinetobacter* sp. S-33 introduction into oil-contaminated soils had a significant effect on TPH degradation but reduced the total number of culturable heterotrophic microorganisms by 46%. In addition, bioaugmentation of a TPH-contaminated microcosm with *Acinetobacter baumannii* S30 pJES showed that the population of strains to be $6.5 \pm 0.13 \times 10^8$ CFU/g just after bioaugmentation and $2.09 \pm 0.08 \times 10^8$ CFU/g after 90 days of incubation (Mishra et al., 2004). In addition, the copy number of *egfp* decreased more rapidly in sterilized microcosms than in non-sterilized microcosms (Figure S4), possibly due to the great impact of autoclaving on the sediment geochemistry and mineralogy (Otte et al., 2018).

In this study, results from Illumina MiSeq suggested that the simulated oil-contaminated microcosms showed shifts in bacteria communities after addition of heavy oil, in which some species, such as *Fusibacter, Photobacterium* and *Desulfobulbus*, were benefited from the oil introduction (Fig. 3). Zheng et al. (2016) also observed the natural succession of bacterial communities in oil-contaminated groundwater-soil environment, including the increase of hydrocarbon-degrading indigenous flora. Bacterial groups which metabolized more recalcitrant oil compounds afterwards replaced the groups that have limited hydrocarbon-degrading ability.

In this study, anaerobic sulfur-oxidizing bacteria such as *Sulfurovum* sp., as well as sulfate-reducing bacteria (SRBs) *Desulfobulbus* sp. have been detected. This phenomenon indicated the presence of an active sulfur cycle in the hydrocarbon pollution sediment. High input of organic matter, such as oil pollution, could stimulate sulfur cycle (Wei et al., 2018). This view has also been confirmed in the last few years by some bioremediation studies (Santisi et al., 2019; Abed et al., 2018; Tan et al., 2013). Bacteria of the genus *Fusibacter* anaerobic, fermentative bacilli that isolated in an oil-producing well might be involved in acetate production by fermentation (Folwell et al., 2016). Hasegawa et al. (2014) identified the microorganisms that were responsible for anaerobic souring in oil reservoirs, and the *Fusibacter* species was enriched. Anaeroineaceae comprises obligate anaerobes, as a majority in our crude oil-degrading microcosm is not a surprise; they were ubiquitously observed in many oil-contaminated environments (Sun et al., 2015), especially *n*-alkanes biodegrada-

tion process (Ji et al., 2020). Liang et al. (2015) suggested that Anaerolineaceae turned to be dominant strains in alkanes-dependent methanogenic culture after 1300 days and 1750 days (Liang et al., 2016) incubation.

Petroleum hydrocarbons are usually maintaining a structure with molecular oxygen, requiring an electron acceptor. However, the heavy oil added in the microcosms covered the surface of the seawater and sediment layer, which limits the oxygen penetration and leading to anoxia in subsurface layers (Bonaglia et al., 2020). Furthermore, the decomposition of hydrocarbon substances induced by aerobic microorganisms would consume dissolved oxygen. These results, therefore, suggested that both aerobic and anaerobic hydrocarbon degradation occurred in all the microcosms. Nitrate, bicarbonate, nitrous oxide, iron, and sulfate have been shown to act as alternate electron acceptors during hydrocarbon degradation in the absence of molecular oxygen (de Rezende et al., 2020).

3.3. *Influence of foreign microorganisms on microbial community*

Foreign microorganisms have been applied widely, but the efficiency depends on the competitiveness with indigenous microorganisms, predators and various abiotic factors (Zeneli et al., 2019;Tribedi et al., 2018). The toxicity of a high concentration of hydrocarbons, the limitation in nutrient sources, low numbers of the principal oil degraders in the sediment and seawater, and by-products of degradation to the bacterial community can affect the rate and effectiveness of natural biodegradation (Ossai et al., 2020).

In our study, the abundance of *Acinetobacter* in ONH treatment reached 69.09% at day 0, 66.70% at day 5, and decreased gradually after that to 12.39% at day 45, after 60 days, the abundance ratio was 8.34% (Fig. 3). This result suggested that the native indigenous microbial population might resist or compete with the introduced species. This inhibition probably resulted from various biotic and abiotic factors. Foreign strains need to compete with the indigenous microorganisms and predatory protozoa. At the same time, the impact of microscale distribution on the potential relationship between inoculated strains and indigenous microbial niche cannot be ignored (Trabelsi and Mhamdi, 2013).

Studies have indicated that bioremediation strategy may cause changes in the indigenous community. For example, Wu et al. (2019) reported that the microbial community structure greatly changed in the first week of bioaugmentation, and then remained basically stable in the twelfth week. In addition, the bioaugmentation treatment led to the lowest microbial diversity among all soil samples. Similar findings have been reported that bioaugmentation with a consortium for the oil-polluted soil caused more drastic changes in the microbial community, which can compromise the ulterior functionality of the soil (Festa et al., 2016). Our results of NMDS (Appendix A Fig. S8) and PLS-DA (Appendix A Fig. S9) showed that the addition of crude oil would not cause an obvious difference of the bacterial communities, under the condition of *Acinetobacter* sp. HC8–3S-9. More importantly, the addition of *Acinetobacter* sp. HC8–3S-9 cannot significantly affect the original bacterial community structure and maintain a relatively stable bacterial ecological network.

3.4. *Strategies for oil removal*

The concentration and chemical structure of crude oil affect the growth of microorganisms used for bioaugmentation, to some extent, determinate exploitability of pollutants to microorganisms (Ribicic et al., 2018). In addition, the size and structure of the microbial population and the environmental parameters should also be taken into consideration. Different microbial species have different metabolic abilities and preferences for the petroleum compound degradation. Some microorganisms degrade branched, cyclic or linear alkanes, others prefer mono- or polynuclear aromatics, and others jointly degrade both alkanes and aromatics (Ossai et al., 2020).

Our results further authenticate, the necessity of bioaugmentation and other bioremediation techniques in expediting the biodegradation of hydrocarbon pollutants, provide that inoculation protocol and inoculum size are designed to permit the growth and activity of the introduced strain.

In order to achieve a better degradation effect in the environment, we recommend: (i) using indigenous bacterial consortium; (ii) adding nitrogen and phosphorus; (iii) applying immobilization. The reasons are as follows, (i) indigenous bacteria consortium has a good potential for application in bioremediation of crude oil contaminated soils, which can make the crude oil biodegradation close to the optimal conditions, as compared to the individual strains; (ii) in the petroleum-contaminated environment, N and P are generally acknowledged to be insufficient to support the growth of *in situ* microorganisms (binti Che Abdul Rahim et al., 2019). Additionally, considering the high carbon content of crude oil, the low levels of N and P are the limitation for degradation in toxic sediment; (iii) compared to the planktonic bacteria, immobilized bacteria could not only shield perturbations of environmental conditions (such as toxic compounds) but also stay away from predators and natural competition with the indigenous microorganisms (Lin et al., 2014).

4. Conclusions

Acinetobacter sp. HC8–3S cells were successfully tagged with *egfp* biomarkers in this study. And the application of EGFP-labeled strain allows us to stably, rapidly, easily and economically detect the survival of petroleum hydrocarbon-degrading bacteria.

The ammonium (NH_4^+-N) and phosphorus (PO_4^{3-}-P) contents decreased rapidly after 15 days post inoculation, and the abundance of the strain also decreased at the same time. In addition, crude oil and foreign microorganisms did not show a long-time impact on the original microbial communities. A balanced bacterial community established *in situ* after bioaugmentation. Our studies validate that nutrient depletion was the main limiting factor in the crude oil bioaugmentation process.

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Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.07.025.

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