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Degradation of crude oil in a contaminated tidal flat area and the resilience of bacterial community

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ABSTRACT

Crude oil spills, Hebei Spirit in South Korea, is considered as one of the worst environmental disasters of the region. Our understanding on activation of oil-degrading bacteria and resilience of microbial community in oil contaminated sites are limited due to scarcity of such event. In the present study, tidal flat sediment contaminated by the oil spill were investigated for duration of 13 months to identify temporal change in microbial community and functional genes responsible for PAH-degradation. The results showed predominance of previously known oil-degrading genera, such as *Cycloclasticus*, *Alcanivorax*, and *Thalassolituus*, displaying significant increase within first four months of the accident. The disturbance caused by the oil spill altered the microbial community and its functional structures, but they were almost restored to the original state after 13 months. Present study demonstrated high detoxification capacity of indigenous bacterial populations in the tidal flat sediments and its resilience of microbial community.

1. Introduction

Tidal flats, a type of coastal wetlands, regulate extensive influx of nutrients (organic and inorganic) and pollutants from open sea to terrestrial environments and vice versa (Ridgway and Shimmield, 2002). Thus, tidal flat ecosystems are considered as highly productive and ecologically important habitats for marine environment (Wilms et al., 2006; Kim, 2010). One of the valuable function of tidal flats (i.e., estimated as 6696 $\$\cdot ha^{-1}\cdot yr^{-1}$) is self-purification, which includes recovery of nutrients, pollution control, and detoxification (Costanza et al., 1997). Despite the large self-purification capacity of tidal flats, an environmental disaster, particularly crude oil spill, is a major threat to marine environment (Halpern et al., 2008).

In December 2007, oil tanker, Hebei Spirit, collided with a barge spilling 10,900 tons of crude oil into the Yellow Sea (Sim et al., 2010; Yim et al., 2012; Hong et al., 2012; Kim et al., 2013). The catastrophe, which happened just 9 km off the west coast of South Korea, is recorded as the one of the worst environmental disaster to this day. The spill, mostly Iranian heavy crude containing aliphatic/aromatic hydrocarbons, polar compounds, heavy metals and volatile organic compounds, polluted >375 km of the coastline (ESTC, 2006; Sim et al., 2010). Although the spilled oil was quickly cleared by the effective emergency response with help from large number of volunteers, residual oil trapped within the coastal sediments was found even after five years (Kim et al., 2013; Lee et al., 2013). Majority of the spilled oil can be removed through various weathering processes, such as evaporation, dissolution, dispersion, emulsification, photooxidation, adsorption, and biodegradation (Yim et al., 2012; McGenity et al., 2012). However, once spilled oil migrates into the subsurface, especially into tidal flats that have high proportion of clay, only biodegradation remains as a feasible option to degrade the spilled oil, because physical clean-up processes are challenging in those areas due to high adsorption capacity of the clay (Atlas and Hazen, 2011; McGenity et al., 2012).

Up to date, diverse species oil-degrading bacteria have been isolated from marine environments. Even though these bacteria usually exist in low abundance in marine environments, influx of petroleum hydrocarbons stimulates substantial growth of the oil-degrading bacteria and changes microbial community structures (Caruso et al., 2004; Atlas and Hazen, 2011). Marine oil-degrading bacteria have been found in a wide variety of genera, including *Alcanivorax* (Yakimov et al., 1998; Liu and Shao, 2005), *Cycloclasticus* (Dyksterhouse et al., 1995; Chung and King, 2001), *Thalassolituus* (Yakimov et al., 2004), *Marinobacter* (Gauthier et al., 1992), *Oleiphilus* (Golyshin et al., 2002), *Oleispira* (Yakimov et al., 2003), *Neptunomonas* (Hedlund et al., 1999), *Planococcus* (Engelhardt et al., 2001), and *Pseudomonas* (Das et al., 2006). Although the extent of crude oil biodegradation in marine environment depends on the combination factors such as of oil composition, abundance of oil-degrading bacteria, weathering processes, and environmental conditions (McGenity et al., 2012), it has been proven that

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indigenous oil-degrading bacteria play a significant role to reduce the overall environmental impact (Atlas and Hazen, 2011). A notable pattern that many researchers have recognized in oil-contaminated marine environments is the bloom of certain bacterial populations, particularly *Alcanivoranx* spp. and *Cycloclasticus* spp. (Kasai et al., 2002; Maruyama et al., 2003; Cappello et al., 2007; Yakimov et al., 2007; McGenity et al., 2012). Harayama et al. (2004) demonstrated that *Alcanivorax* and *Cycloclasticus* were key organisms that degraded petroleum hydrocarbons in oil-contaminated seawater (Harayama et al., 2004). Kostka et al. (2011) also revealed that members of the genera *Alcanivorax* and *Marinobacter* were responsible for oil-degradation in coastal beaches after the Deepwater Horizon oil spill (Kostka et al., 2011). Generally, *Alcanivorax* strains are known to grow on straight-chain and branched alkanes, whereas *Cycloclasticus* strains degrade polycyclic aromatic hydrocarbons (PAHs) (Harayama et al., 2004; Head et al., 2006; Yakimov et al., 2007; McKew et al., 2007a, b; Niepceron et al., 2010; Kostka et al., 2011).

After the accident, many studies have focused on the effect of oil spill on public health, macroorganisms (Sim et al., 2010; Lee et al., 2011a, b), response and recovery of coastline ecosystem (Lee et al., 2009; Jung et al., 2011; Jung et al., 2012, Yu et al., 2013), and residual oil in environment media (i.e., distribution, concentration, toxicity, and composition) (Ji et al., 2011; Hong et al., 2012; Yim et al., 2012; Kim et al., 2013, Lee et al., 2013). Although previous oil spill incidents revealed the importance of indigenous oil-degrading bacteria in minimizing the overall environmental impact (Atlas and Hazen, 2011), their presence in contaminated costlines and contribution in remediation is barely investigated for Hebei Spirit accident. Jin et al. (2012) suggested that the members in the genus *Alteromonas* could be responsible for the degradation of PAHs in an oil-contaminated tidal flat through enrichment culture experiments and a fingerprinting method (i.e., Denaturing Gradient Gel Electrophoresis (DGGE)). However, there have been limited efforts to monitor the effect of crude oil spill on the indigenous microbial community over time and to identify bacterial species that contributed to in situ biodegradation in the contaminated area.

Therefore, the present study aimed (i) to investigate indigenous oil-degrading bacteria responsible for in situ biodegradation of crude oil contaminated tidal flat, (ii) to monitor dioxygenase genes which were possibly involved in PAH-degradation, (iii) to evaluate the influence of crude oil spill on the microbial community of tidal flat sediments and resilience of the community. The concentrations of petroleum hydrocarbons were measured over time and 454 amplicon pyrosequencing were conducted using tidal flat sediment sampled periodically for 13 months from the area contaminated by the Hebei Spirit accident. Bacterial 16S rRNA genes and aromatic ring hydroxylating dioxygenase (ARHD) genes were investigated as the target gene the evaluate changes in the microbial community and PAH degrading populations over time, respectively.

2. Material and methods

2.1. Sample collection

Tidal flat sediment samples were collected every 4–5 months for 13 months (i.e., December 2007 to January 2009) from a non-vegetated intertidal zone located in Uihang-ri, Taean, South Korea (36°50′28″N, 126° 9′49″E). Unlike a nearby docking area, where extensive chemical and physical treatments were conducted, active clean-up processes could not be applied to the sampling area due to the high portion of muddy clay in the sediment. An experimental plot (2×2 m^2) was established and three PVC cores (7.5 cm internal diameter, 50 cm long) were inserted in the sediment. To avoid edge effects, the sediment samples for this experiment were taken at two different depths, labeled as the top (5–10 cm depth) and the bottom (15–20 cm depth), using 50 mL conical tubes (BD Biosciences, USA) and stored at −80 °C until DNA extraction. The extra sediments at same depth in the core were sieved to determine soil properties. The range of sand, silt and clay fraction content at each depth were 11–13%, 31–36%, 53–57%, respectively.

2.2. Laboratory methods

2.2.1. TPH measurement

The sediment samples (5 g) from three cores at each sampling period were extracted with 10 mL of methanol for 2 min using a shaker, centrifuged for 5 min at 4000 rpm, and then 0.1 mL of the extract was diluted with 1.9 mL of distilled water. The diluted samples were used for headspace solid phase micro-extraction (HS-SPME) procedure as previously suggested (Doong et al., 2000). Total petroleum hydrocarbons and those fractions were quantified using an Agilent 6890 gas chromatograph equipped with an Agilent 5795C mass detector and a capillary column (60 m × 0.25 mm × 0.25 μm). The instrumental conditions were as follows: splitless; injector temperature, 320 °C; electron impact ionization mode; energy of electron, 70 eV; initial oven temperature, 40 °C (for 5 min), increased to 110 °C at a rate 50 °C/min, subsequently increased to 320 °C at a rate of 12 °C/min, and then held for 10 min. Helium at 1.0 mL/min was used as carrier gas. Three different concentrations of mixtures of three target PAHs (naphthalene, phenanthrene and pyrene) and 17 target alkanes (C8–C40) were used as external standards for determination of components of extracts.

2.2.2. DNA extraction and PCR amplification

Total DNA was extracted from 0.5 g of tidal flat sediment using the Power Soil DNA Isolation Kit (MoBio, USA) following the manufacturer's instructions. Both depths of sediment in each of three cores were thoroughly mixed together to produce a composite samples for each depth. Biological replicate subsample (0.5 g) from the homogenous mixture from each depth were used in each DNA extraction procedure. To improve the coverage, DNA extractions and PCR reactions were also performed in technical replicates for each sample. The 16S rRNA gene (27F/518R) and dioxygenase gene (888F/300R) were PCR amplified with primer. The primers for PCR amplifications and PCR conditions were followed in previous study (Lee et al., 2011). Before pyrosequencing process, the PCR products obtained from the replicates of each sample were pooled and double purified using the QIAquick PCR Purifiation Kit (QIAGEN, USA) and the QIAquick Gel Extraction Kit (QIAGEN, USA). DNA concentrations were measured using a NanoDrop 1000 (Thermo Scientific, USA) and combined in equimolar ratios. Purified amplicons were then sequenced using a 454 pyrosequencing Genome Sequencer FLX titanium (Roche Diagnostics, Germany).

2.3. Data analysis

2.3.1. Sequence analysis

After sorting raw sequence reads using barcodes to distinguish each sample, primer and barcode sequences were trimmed out. Sequences contained ambiguous bases, 8 bp or longer homopolymers, and < 250 bp length were removed using Mothur v1.21 (Schloss et al., 2009). The chimeric sequences produced during PCR amplification were removed by Uchime implemented in Mothur (Edgar et al., 2011). After quality sequence filtering, the sample with higher than 1000 sequence reads were used (Caporaso et al., 2011). The one of replicates for 2007 Dec and 2008 Sep were <1000 sequences and those were not used to reduce sequencing efforts on further analysis. The reads for 16S rRNA genes were clustered into operational taxonomic units (OTUs) at 97% sequence identity, and the representative sequences were classified using the RDP training set ver. 10 implemented in Mothur and a minimum bootstrap support of 50%. The reads for dioxygenase genes were translated into their HMM model produced from the dioxygenase curated database (Iwai et al., 2010) using the toll FrameBot of RDP's FunGene Pipeline (http://fungene.cme.msu.edu/FunGenePipeline) (Fish et al., 2013). The translated sequences were aligned and clustered using the MCCLUST in FunGene Pipeline.

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