Effect of Temperature on Hydrocarbon Bioremediation in Simulated Petroleum-Polluted Seawater Collected from Tokyo Bay

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Abstract

This study focused on the effect of temperature on biological removal of petroleum hydrocarbons. Seawater samples were collected from two stations in Tokyo Bay seasonally, and were made into petroleum-polluted seawater by adding mixture of n-alkanes in a fixed concentration in laboratory. A 28 days of bioremediation incubation experiment was carried out with and without additional nutrients supplement in each season, and the remained hydrocarbons during incubations were measured by GC–MS. alkB gene, the functional gene responsible for alkane degradation, was also recorded throughout the incubation. Our results showed that bioremediation efficiency of petroleum hydrocarbon was highest in summer, followed by spring, autumn, and winter respectively. Alkanes with as much as 34 carbon atoms can be significantly degraded within 28 days of incubation in summer, and in contrast, no obvious degradation can be recognized in winter regardless of the number of carbon atoms. N-alkanes degradation efficiency was generally associated with, but not necessarily correlated to the copy number of alkB gene.

Key words: Bioremediation, Petroleum pollution, Hydrocarbon degradation, alkB gene

INTRODUCTION

With growing needs for humans’ daily life, more and more crude oil has been explored and exploited worldwide. However, releases of crude oil, due to the spills from ship tankers, offshore platforms, and drilling wells, are now threatening the marine and coastal ecosystems. Recently, the crude oil spills that occurred in America, Korea, Spain, Singapore, and many other places have brought severe damage to the local ecosystems. Crude oil and other petroleum by-products are mainly composed of hydrocarbons of various molecular weights, such as alkanes, cyclo-

alkanes and aromatic hydrocarbons. These organic compounds are difficult to be metabolized into lighter forms or to be degraded, and are toxic to most animals and humans. Nowadays, bioremediation, which uses microbial metabolism to breakdown petroleum hydrocarbons with additional nutrients supply, has been proven to be an effective method to remove petroleum pollution after crude oil spills.

Although bioremediation has already been introduced into some oil spill accident scenes, and increasing knowledge has been accumulated in bioremediation processes, information about biodegradation associated
with carbon numbers of hydrocarbons as well as effects of temperature and other environmental conditions remains to be limited. Temperature, one of the important environmental factor affecting the growth of microorganisms, also influenced the petroleum degradation not only affecting the activities of petroleum degrading microorganisms, but also changing the properties of petroleum itself\(^1\)-\(^3\). Activities of petroleum-degrading microorganisms could increase with the increase of temperature\(^4\)\,-\(^5\), however, toxicity of petroleum components may also increase with temperature and the biodegradation of the fraction of recalcitrant petroleum components may still be poor\(^6\)\,-\(^8\). Although bioremediation efficiency has been tested on subpolar soil, estuary and seawater samples on low temperatures down to \(-1^\circ C\)\(^9\)\,-\(^15\), further discussion on the influence of temperature is still needed, in aspects like seasonal bioremediation dynamics in the temperate zones, and degradation efficiency associated with hydrocarbon structures, and so on.

In this study, we investigated seasonal changes of crude oil degradation efficiencies in simulated petroleum-polluted seawater collected from Tokyo Bay, and for the first time, demonstrated degradation dynamics of individual hydrocarbons in different carbon number. At meantime, we also connected seasonal petroleum degradation to the shifts of potential functional genes of alkane degrading bacteria. This study provides an integrated insight into the effect of temperature on the bioremediation of petroleum hydrocarbons in different carbon number.

**MATERIALS AND METHODS**

**Sampling** Seawater sample was collected in two stations in Tokyo Bay (St.1 Inner Tokyo Bay and St.2 Central Tokyo Bay) seasonally (Fig. 1). One liter of surface seawater was collected in each station, and stored in polyethylene bottles on ice before use.

**Water quality measurements** Water temperature, pH and DO were measured simultaneously *in situ* by using alcohol thermometer (AS ONE), pH/conductivity meter D-54 (HORIBA), DO meter (Pro DO, YSI Nanotech) respectively. Nutrients concentration (nitrate, nitrite, ammonium and phosphate) were determined in laboratory within 24 h after sampling. Nitrate and nitrite concentrations were measured by using a colorimetric procedure described by Saito et al.\(^16\); ammonia concentration was measured by indophenol blue method; phosphate concentration was measured by molybdenum blue method\(^17\).

**Bioremediation experiment**

**Preparation of microcosm and incubation experiment** Seawater samples were first filtered through 3.0 µm pore size membrane filter (Nuclepore Membrane Filter, Whatman) to prevent reduction of petroleum-degrading bacteria due to predation pressure of larger microorganisms. Then, 20 mL of filtrated seawater was added into a 100 mL of glass vial bottle (Nichiden Rika).

The above seawater samples were then subjected to two different treatments: (1) NT; additional nutrients were supplied (20 mg/L NH\(_4\)NO\(_3\) and 10 mg/L KH\(_2\)PO\(_4\))\(^10\), and then loaded with an oil sheet (made by adding 20 µL of vacuum distilled petroleum (prepared as petroleum of C19 to C36 alkanes, Cosmo Oil Co., Ltd.) to pre-sterilized glass fiber mesh (cut in circle with a diameter of 10 mm)). (2) CK; control blank without additional nutrients supplement, only loaded with an oil sheet. To exclude the possible loss of petroleum hydrocarbons due to reasons other...
than bioremediation, (3) WK; water blank, which is sterilized artificial seawater loaded with an oil sheet, was prepared in each incubation period. The artificial seawater (salinity: 33‰) was made by the ingredients of artificial seawater (Marine Art SF-1, Tomita Seiyaku Co.).

These microcosms were sealed with PTFE coated rubber stopper and an aluminum cap, and incubated in an incubator for 28 days under dark condition (incubation temperature was controlled at 10°C (winter), 23°C (spring), 31°C (summer), and 17°C (autumn), respectively).

**Determination of petroleum component**

Determination of petroleum component was carried out on 0, 7, 14, 21 and 28th day of incubation and the procedures are described as followed. First, 20 mL of incubation sample was flowed through a pre-conditioning SPE (solid phase extraction) tube (Supelclean™ ENVI-18 SPE tube, SIGMA-ALDRICH). Then the microcosm vial bottle and oil sheet were rinsed with sterilized MiliQ water (for 1 time) and mixed solution of hexane and dichloromethane (1:1) (for 3 times). The rinsed solvents were also flowed through the SPE tube. All solvents that passed through the SPE tube were collected in a test tube, and dehydrated by gas injection (blow nitrogen gas onto the top of solvents for 1 h) in a draft under room temperature. Petroleum component that attached to the test tube was then dissolved in 1.5 mL of mixed solution of hexane and dichloromethane (1:1), transferred to a 2 mL vial bottle (Mighty vial, Maruemu), and stored at −30°C before analysis.

Concentrations of petroleum hydrocarbons in the extract were quantified by GC-MS (GC-MS 2010 plus, SHIMAZU). For standard curve, a mixture of n-alkanes ranging in different carbon number from C9 to C40 dissolved in n-hexane (Wako Pure Chemicals) were diluted in 20 times, 100 times, 200 times and 1000 times. And the above extracted samples were then diluted in 50 times before measure by GC-MS. The GC-MS conditions were listed in Table 1.

**Determination of total bacterial cell number**

Samples for cell count were also collected throughout the incubation period in all groups (on 0, 7, 14, 21, and 28th day). For these samples, paraformaldehyde was added into each microcosm (final concentration vol/vol: 2.5%). Then 10 mL of each microcosm was filtered through 0.2 µm pore size polycarbonate filter (Whatman). These filters were then loaded with 2 µL/mL of adjusted DAPI solution (DAPI, PBS, Vectershild, PBS Glycerol) and dyed for 5 min at room temperature. The total bacterial cell was counted under fluorescent microscope (BX-50, OLYMPUS).

**Nucleic acid experiments**

20 mL of incubation samples on day 0, 14 and 28 in each incubation group was filtered through 0.2 µm pore size polycarbonate filter (Whatman),

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**Table 1 Measurement condition of GC/MS.**

| GC                     | MS                |
|------------------------|-------------------|
| **Column**             | Rtx-5 MS (manufactured by RESTEK) Length 30 m × inner diameter 0.25 mm × thickness 0.5 µm |
| **Sample introduction method** | Splitless method (high pressure injection mode 250 Kpa, 1.5 min)     |
| **Sample injection volume** | 1 µL              |
| **Sample vaporization chamber temp.** | 330°C            |
| **Oven**               | 85°C (2 min - 20°C / min to 330°C (25 min) |
| **Carrier gas**        | He (5.19 cm/s)    |
| **Interface temperature** | 230°C            |
| **Ion source temperature** | 200°C           |
| **Ionization method**  | SIM method        |
| **Event time**         | 0.3 s             |
and the filters were stored at −80°C before nucleic acid extraction. DNA samples were then extracted from these frozen filters using ISOIL for Beads Beating DNA extraction kit (Nippon Gene), and dissolved in TE buffer. To record the responsible petroleum-degrading bacteria during incubation, the copy numbers of alkB gene (which encodes for alkanes monooxygenase enzyme, is the biomarker of n-alkanes-degrading bacteria) were measured by real-time PCR. Real-time PCR assays were performed using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) equipped with ABI 7500 software (ver. 1.4.1). Thermal cycle conditions were first optimized by adjusting annealing temperature and primer concentration to ensure the best assay specificity and efficiency. The copy numbers of alkB gene were determined in triplicate on nondiluted DNA samples, using primer set designed by Bell et al.18) in an optimized concentration of 0.2 µM (final concentration of both forward and reverse primer) and the Thunderbird Probe qPCR Mix (TOYOBO) according to the manufacturer’s instruction. Known concentration of alkB gene amplification of environmental seawater samples was used as the standard DNA in real-time PCR analysis. Real-time PCR thermal cycles were conducted as follows: initial denaturation at 95°C for 5 min; amplification for 35 cycles of 95°C for 30 s, primer annealing at 57.5°C for 35 s, and extension at 72°C for 30 s, with a plate read between each cycle; melting curve analysis at 60°C to 94°C with a plate read every 0.5°C and held for 1 s between each read.

RESULTS

Environmental parameters In situ environmental data during the seawater sample collection are shown in Table 2. Among all seasons, water temperature was highest in summer, and then in the order of spring, autumn, and winter; while DO was highest in winter, lowest in summer. Relatively high nutrients concentrations were observed at the two stations, which are considered as the result of large amount domestic wastewater inflow19).

Dynamics of total bacterial cell density

Except the WK group, all the other groups showed observable cell growth during incubation (Fig. 2), while the groups with nutrients addition demonstrated much more considerable and continuous growth compared to without nutrients addition. All groups with nutrients addition in 4 seasons reached approximately 2.0×10^8 cells/mL after 28 days of incubation, showing no obvious seasonal difference.

Degradation of total petroleum hydrocarbons (TPH)

In winter, TPH degradation was 37 ± 8.94% and 40 ± 3.89% after 7 days of incubation for central and inner Tokyo Bay respectively; and gradually reached 53 ± 2.47% for central Tokyo Bay, and 46 ± 18.42% for inner Tokyo Bay after 28 days incubation. In contrast, the TPH degradation (here may also consider as TPH loss in control groups) was closed to 24% (SD: 1.54 to 19.97) in all

| Station | Water Temp. (°C) | DO (mg/L) | pH | NH4-N (mg/L) | NO3-N (mg/L) | NO2-N (mg/L) | PO4-P (mg/L) |
|---------|------------------|-----------|----|--------------|--------------|--------------|--------------|
| Winter  |                  |           |    |              |              |              |              |
| St.1    | 10.8             | 12.1      | 8.03| 0.050        | 0.93         | 0.042        | 0.059        |
| St.2    | 8.9              | 11.5      | 7.82| 0.092        | 0.86         | 0.019        | 0.077        |
| Spring  |                  |           |    |              |              |              |              |
| St.1    | 22.0             | 8.1       | 8.56| 0.042        | 0.51         | 0.018        | 0.023        |
| St.2    | 21.0             | 9.0       | 7.84| 0.043        | 0.42         | 0.003        | 0.033        |
| Summer  |                  |           |    |              |              |              |              |
| St.1    | 28.6             | 7.8       | 8.56| 0.217        | 0.32         | 0.073        | 0.081        |
| St.2    | 31.2             | 6.8       | 7.97| 0.058        | 0.18         | 0.010        | 0.026        |
| Autumn  |                  |           |    |              |              |              |              |
| St.1    | 17.5             | 8.9       | 7.85| 0.085        | 0.94         | 0.038        | 0.040        |
| St.2    | 17.9             | 8.2       | 7.77| 0.146        | 0.72         | 0.031        | 0.057        |

Winter: 2nd Feb, Spring: 4th May, Summer: 6th Aug, Autumn: 17th Nov; all in the year of 2015.
three control groups (two groups without nutrients addition, one for artificial seawater) after 28 days incubation (Fig. 3. upleft).

Increased by 13°C on temperature, TPH degradation was more efficient in spring than in winter. After 28 days of incubation, TPH degradation was 86 ± 4.54% and 87 ± 1.32% for central and inner Tokyo Bay respectively.

![Graphs showing changes in cell density and degradation of TPH over time](https://via.placeholder.com/150)

**Fig. 2** Changes of total bacterial cell densities during 28 days incubation.

![Graphs showing degradation of TPH and abundance of alkB copy number over time](https://via.placeholder.com/150)

**Fig. 3** Degradation of TPH (shown as lines and symbols, left Y-axis), and abundance of alkB copy number (shown as bars, right Y-axis) during 28 days incubation.
in contrast to 20 ± 8.36% and 24 ± 9.18% in respective groups without nutrients addition (Fig. 3. upright).

Summer season showed the highest TPH degradation in all seasons. TPH degradation suddenly reached to nearly 70% after 7 days in both groups with nutrients addition, when the rates were only 8% to 17% in the control groups. After 28 days, the TPH degradation increased to 80% (central) and 87% (inner) with nutrients addition, and 3% to 12% without nutrients addition (Fig. 3. downleft).

Dynamics of TPH degradation in autumn was similar to that in spring, despite 6°C of difference in water temperature. After 28 days, TPH degradation was 66 to 67% for groups with nutrients addition, and 21 to 31% for groups without nutrient addition (Fig. 3. downright).

Degradation of \( n \)-alkanes (ranged from C20 to C36) In general, bioremediation efficiency decreased with the increase of carbon number in all seasons.

The changes in the percentage of remaining alkanes (separated by its carbon number) during bioremediation in each season are shown in Figure 4. In winter, degradation could be observed between groups with nutrients (NT treatment) addition and groups without nutrient addition (CK and WK treatment) when carbon number is less than 25. However, the degradation rates in winter were much slower when compared to other seasons. No significant difference can be observed when carbon number is more than 25 neither with nor without nutrient addition.

In spring and summer, significant difference can be found between nutrients groups and no nutrients groups when carbon number is up to 36. Nevertheless, bioremediation in summer was more active than in spring, as most alkanes were effectively degraded within 7 days in summer, and this took place for 14 to 21 days in spring.

In autumn, alkanes with more than 30 carbons were not significantly degraded within 28 days of incubation. Bioremediation rates for C20 to C29 were also not as fast as rates in summer, but close to the rates in spring.

Dynamics of \( alkB \) gene \( alkB \) gene was detected in all samples during incubation in 4 seasons. In the beginning of incubation, \( 5.1 \times 10^2 \) to \( 1.1 \times 10^4 \) copies per L of \( alkB \) gene were detected, and increased to \( 2.6 \times 10^6 \) to \( 2.3 \times 10^8 \) copies per L in the 28th day of incubation (Fig. 3). The most rapid reproductivity of \( alkB \) gene in all seasons was found in the first 14 days, while no or less significant growth in their copy number can be found from 14th day to 28th day. In general, copy number in winter was nearly 1 order of magnitude lower than in other three seasons.

DISCUSSION

Previous field studies and laboratory researches have revealed that bioremediation was cost-effective method to accelerate petroleum hydrocarbons degradation in oil-polluted seawater and soils, such as in the Sea of Japan\(^{20}\), subpolar areas\(^{12,13}\), Norwegian coastal seawater samples\(^{14,15}\), Chinese soil sample\(^{21,22}\), Kuwait oil lake sediments\(^{23}\), Brazil dystrophic red argisol\(^{24}\), and so on. It is also found that the petroleum hydrocarbon degrading bacteria ubiquitously distributed in the marine environment\(^{25-29}\), and the population and the community of such microbes shift rapidly when the environment is contaminated with petroleum\(^{25-29}\). Although the population of responsible microorganisms appears to grow rapidly when sudden crude oil inflow even without extra nutrient supplement, bioremediation process can be accelerated with proper nutrients addition\(^{30,31}\). In our study, TPH were deduced more rapidly by NT treatment than by CK or WK treatment, indicating that groups with nutrients addition can lead to faster petroleum hydrocarbon degradation. As a limiting factor in natural marine environment, nutrients concentration largely regulates the growth of most microorganisms. Our results showed that total bacterial cell numbers in NT treatment groups (with nutrients addition) were significantly larger than in CK or WK treatment groups (without nutrients addition), indicating that nutrients addition accelerated the growth of microorganisms in the microcosms. On the other hand, considerable increased in \( alkB \) gene was also observed during incubation, showing microbial response to the sudden environ-
Fig. 4 Degradation of n-alkane during 28 days incubation.
mental change (when added or contaminated with petroleum compounds). Numerous studies have reported this kind of microbial response to the oil pollution^26-29, 32, 33^, and this response can be observed in both microbial abundance and its community. Although microbial community has not been discussed in this study, remarkable increased of total bacterial cell as well as $alkB$ gene were detected in groups with nutrients addition, suggested that, nutrients addition could promote the growth of microorganisms in microcosm, and when accompanied with petroleum contamination, this significant increase in cell number are mainly because of the growth of petroleum-degrading bacteria.

Although $pH$$^0, 34^$, salinity$^{35}$, $DO$$^0, 36^$, nutrients concentration in the indigenous environments$^{37-40}$ and temperature$^{11, 14}$ have been reported to affect bioremediation, this study focuses on the effect of temperature which generally covers the temperature range in all seasons in temperate areas, and demonstrates the biodegradation of petroleum hydrocarbons individually in its carbon number. Bioremediation efficiency was highest in summer, followed by spring, autumn, and winter. As similar trends were also detected in the total bacterial cell number and $alkB$ gene abundance, this seasonal difference showed in bioremediation efficiency can be explained by microbial activities under different temperature, most possibly, the growth of these responsible microorganisms thrive in warm temperature (like summer and spring) but might be constrained in cold temperature (like winter). Our results showed that, bioremediation in winter were not as effective as in other seasons, especially for degradation of hydrocarbon with more than 25 carbon atoms. However, significant bioremediation can still be observed when water temperature was as low as 0 to $5^\circ C$^11, 14, which is even lower than in our winter experiment ($10^\circ C$). In the study conducted by Venosa et al.$^{11}$, enriched degrading bacterial culture was used, which indicate a higher responsible microbial density compared with our study; and Brakstad et al.$^{14}$ use seawater samples collected from a high latitude marine area (Norwegian sea) for microcosm preparation, where the naturally distributed microbial community tends to have higher cold tolerance or cold adaptation than our study (which is collected from temperate zone). Thus, the differences showed in bioremediation efficiency in low temperatures are more likely to be the consequences of responsible microbial density and biological divergence of microorganisms in the indigenous environments.

Bioremediation of n-alkanes have been confirmed up to $C_{44}$-alkanes$^{43}$, our results showed that degradation was becoming difficult when the carbon number increased. In particular, degradation of $>C_{33}$-alkanes was more difficult than its lighter companions in summer and spring; and this ‘threshold’ was $>C_{30}$ in autumn, and $>C_{27}$ in winter. These findings generally follow the finding by testing high-latitude originate enrichment$^{11}$ and subpolar soil$^{13}$, both of which reported more readily degradation of lighter n-alkanes than heavier n-alkanes.

REFERENCES

1) Nedwell, D. B.: Effect of low temperature on microbial growth: lowered affinity for substrates limits growth at low temperature, FEMS Microbiol. Ecol., 30, 101–111 (1999).
2) Northcott, G. L., and Jones, K. C.: Experimental approaches and analytical techniques for determining organic compound bound residues in soil and sediment, Environ. Pollut., 108, 19–43 (2000).
3) Rowland, A. P., Lindley, D. K., Hall, G. H., Rossall, M. J., Wilson, D. R., Benham, D. G., Harrison, A. F., and Daniels, R. E.: Effects of beach sand properties, temperature and rainfall on the degradation rates of oil in buried oil/beach sand mixtures, Environ. Pollut., 109, 109–118 (2000).
4) Bossert, I., Kachel, W. M., and Bartha, R.: Fate of hydrocarbons during oily sludge disposal in soil. Appl. Environ. Microbiol., 47, 763–767 (1984).
5) Cooney, J. J.: “The fate of petroleum pollutants in fresh water ecosystems,” in Petroleum Microbiology, Atlas, R. M. Ed., Macmillan, New York, NY, USA, 399–434 (1984).
6) Leahy, J. G., and Colwell, R. R.: Microbial degradation of hydrocarbons in the environment, *Microbiol. Rev.*, 54, 305–315 (1990).

7) Whyte, L. G., Hawari, J., Zhou, E., Bourbonnière, L., Inniss, W. E., and Greer, C. W.: Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp., *Appl. Environ. Microbiol.*, 64, 2578–2584 (1998).

8) DeFoe, D. L., and Ankley, G. T.: Evaluation of time-to-effects as a basis for quantifying the toxicity of contaminated sediments, *Chemosphere*, 51, 1–5 (2003).

9) Colwell, R. R., Mills, A. L., Walker, J. D., Garcia-Tello, P., and Campos-P, V.: Microbial ecology studies of the Metula spill in the Straits of Magellan, *J. Fish. Res. Board Can.*, 35, 573–580 (1978).

10) Coulon, F., McKew, B. A., Osborn, A. M., McGentry, T. J., and Timmis, K. N.: Effects of temperature and biostimulation on oil-degrading microbial communities in temperate estuarine waters, *Environ. Microbiol.*, 9, 177–186 (2007).

11) Venosa, A. D., and Holder, E. L.: Biodegradability of dispersed crude oil at two different temperatures, *Mar. Pollut. Bull.*, 54, 545–553 (2007).

12) Camenzuli, D., and Freidman, B. L.: On-site and in situ remediation technologies applicable to petroleum hydrocarbon contaminated sites in the Antarctic and Arctic, *Polar Res.*, 34, 24492–24511 (2015).

13) Akbari, A. and Ghoshal, S.: Effects of diurnal temperature variation on microbial community and petroleum hydrocarbon biodegradation in contaminated soils from a sub-Arctic site, *Environ. Microbiol.*, 17, 4916–4928 (2015).

14) Brakstad, O. G., and Bonaunet, K.: Biodegradation of petroleum hydrocarbons in seawater at low temperatures (0–5°C) and bacterial communities associated with degradation, *Biodegradation*, 17, 71–82 (2006).

15) Brakstad, O. G., Throne-Holst, M., Netzer, R., Stoeckel, D. M., and Atlas, R. M.: Microbial communities related to biodegradation of dispersed Macondo oil at low seawater temperature with Norwegian coastal seawater, *Microb. Biotechnol.*, 8, 989–998 (2015).

16) Saito, Y., Sugimoto, K., and Hagino, K.: Spectrophotometric determination of nitrate and/or nitrite using brucine sulfate, *Bunseki Kagaku*, 20, 542–549 (1971) (in Japanese).

17) Parsons, T. R., Maita, M., and Lalli, C. M.: A manual of chemical and biological methods for seawater analysis, Pergamon Press, New York, NY, USA., 1–173 (1985).

18) Bell, T. H., Yergeau, E., Martineau, C., Juck, D., Whyte, L. G., and Greer, C. W.: Identification of nitrogen-incorporating bacteria in petroleum-contaminated Arctic soils by using [15N]DNA-based stable isotope probing and pyrosequencing, *Appl. Environ. Microbiol.*, 77, 4163–4171 (2011).

19) Matsumura, T., and Ishimaru, T.: Freshwater discharge and inflow loads of nitrogen and phosphorus to Tokyo Bay (from April 1997 to March 1999), *Oceanogr. Japan*, 13, 25–36 (2004) (in Japanese).

20) Maki, H., Hirayama, N., Hiwatari, T., Kohata, K., Uchiyama, H., Watanabe, M., Yamasaki, F., and Furuki, M.: Crude oil bioremediation field experiment in the Sea of Japan, *Mar. Poll. Bull.*, 47, 74–77 (2003).

21) Wu, M., Dick, W. A., Li, W., Wang, X., Yang, Q., Wang, T., Xu, L., Zhang, M., and Chen, L.: Bioaugmentation and biostimulation of hydrocarbon degradation and the microbial community in a petroleum-contaminated soil, *Int Biodeterior. Biodegradation*, 107, 158–164 (2016).

22) Wu, M., Ye, X., Chen, K., Li, W., Yuan, J., and Jiang, X.: Bacterial community shift and hydrocarbon transformation during bioremediation of short-term petroleum-contaminated soil, *Environ. Pollut.*, 223, 657–664 (2017).

23) Al-Mutairi, N., Bufarsan, A., and Al-Rukaibi, F.: Ecotoxic evaluation and treatability potential of soils contaminated with petroleum hydrocarbon-based fuels, *Chemosphere*, 74, 142–148 (2008).

24) Cerqueira, V. S., Maria do Carmo, R. P., Camargo, F. A., and Bento, F. M.: Comparison of bioremediation strategies for soil impacted with petrochemical oily sludge, *Int Biodeterior. Biodegradation*, 95, 338–345 (2014).

25) Atlas, R. M.: Bioremediation of petroleum pollutants, *Int Biodeterior. Biodegradation*, 35, 317–327 (1995).
26) MacNaughton, S. J., Stephen, J. R., Venosa, A. D., Davis, G. A., Chang, Y. J., and White, D. C.: Microbial population changes during bioremediation of an experimental oil spill, *Appl. Environ. Microbiol.*, **65**, 3566–3574 (1999).

27) Shahi, A., Aydin, S., Ince, B., and Ince, O.: Evaluation of microbial population and functional genes during the bioremediation of petroleum-contaminated soil as an effective monitoring approach, *Ecotox. Environ. Safe.*, **125**, 53–160 (2016).

28) Scoma, A., Yakimov, M. M., Daffonchio, D., and Boon, N.: Self-healing capacity of deep-sea ecosystems affected by petroleum hydrocarbons: Understanding microbial oil degradation at hydrocarbon seeps is key to sustainable bioremediation protocols, *EMBO Rep.*, **18**, 868–872 (2017).

29) Liu, J., Techtmann, S. M., Woo, H. L., Ning, D., Fortney, J. L., and Hazen, T. C.: Rapid response of Eastern Mediterranean Deep Sea microbial communities to oil, *Sci. Rep.*, **7**, 5762 (2017).

30) Rahman, K. S. M., Rahman, T. J., Kourkoutas, Y., Petsas, I., Marchant, R., and Banat, I. M.: Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients, *Bioresour. Technol.*, **90**, 159–168 (2003).

31) Tellez, G. T., Nirmalakhandan, N., and Gardea-Torresdey, J. L.: Performance evaluation of an activated sludge system for removing petroleum hydrocarbons from oilfield produced water, *Adv. Environ. Res.*, **6**, 455–470 (2002).

32) Hazen, T. C., Dubinsky, E. A., DeSantis, T. Z., Andersen, G. L., Piceno, Y. M., Singh, N., Jansson, J. K., Probst, A., Borglin, S. E., Fortney, J. L., and Stringfellow, W. T.: Deep-sea oil plume enriches indigenous oil-degrading bacteria, *Science*, **330**, 204–208 (2010).

33) Hu, P., Dubinsky, E. A., Probst, A. J., Wang, J., Sieber, C. M., Tom, L. M., Cardinale, P. R., Banfield, J. F., Atlas, R. M., and Andersen, G. L.: Simulation of Deepwater Horizon oil plume reveals substrate specialization within a complex community of hydrocarbon degraders, *Proc. Natl. Acad. Sci. U. S. A.*, **114**, 7432–7437 (2017).

34) Foght, J. M., and Westlake, D. W. S.: “Biodegradation of hydrocarbons in freshwater,” in *Oil in Freshwater: Chemistry, Biology, Countermeasure Technology*, Vandermeulen, J. H., and S. R. Hrudey, S. R. Eds., Pergamon Press, New York, 217–230 (1987).

35) Zobell, C. E.: Microbial degradation of oil: Present status, problems and perspectives. *The Microbial Degradation of Oil Pollutants*, Louisiana State University Publication, 3–16 (1973).

36) Head, I. M., and Swannell, R. P.: Bioremediation of petroleum hydrocarbon contaminants in marine habitats, *Cur. Opin. Biotechnol.*, **10**, 234–239 (1999).

37) Al-Awadhi, H., Dashti, N., Kansour, M., Sorkhoh, N., and Radwan, S.: Hydrocarbon-utilizing bacteria associated with biofouling materials from offshore waters of the Arabian Gulf, *Int Biodeterior. Biodegradation*, **69**, 10–16 (2012).

38) Baelum, J., Borglin, S., Chakraborty, R., Fortney, J. L., Lamendella, R., Mason, O. U., Auer, M., Zemla, M., Bill, M., Conrad, M. E., and Malfatti, S. A.: Deep-sea bacteria enriched by oil and dispersant from the Deepwater Horizon spill, *Environ. Microbiol.*, **14**, 2405–2416 (2012).

39) Head, I. M., Jones, D. M., and Röling, W. F.: Marine microorganisms make a meal of oil, *Nat. Rev. Microbiol.*, **4**, 173 (2006).

40) Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., and Ruan, R. R.: Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp., *Bioresour. Technol.*, **101**, 2623–2628 (2010).

41) Atlas, R. M., Boehm, P. D., and Calder, J. A.: Chemical and biological weathering of oil, from the Amoco Cadiz spillage, within the littoral zone, *Estuar. Coast Shelf Sci.*, **12**, 589–608 (1981).

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