Characterization of heavy oil degrading bacterial groups isolated from the Nakhodka oil spill

CHARACTERIZATION OF HEAVY OIL DEGRADING BACTERIAL GROUPS ISOLATED FROM THE NAKHODKA OIL SPILL

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ABSTRACT

We have characterized two bacterial groups, ODB-G1 and ODB-G2, isolated from the spilled oil from the tanker Nakhodka. ODB-G1 contained two genera. One was classified as *Caulobacter* sp. on the basis of its morphogenesis during the cell cycle, and the other was classified as an *Alcanivorax* sp. based on the sequence of its 16S rRNA gene. ODB-G2 also contained two genera, *Alcanivorax* sp. and *Halomonas* sp., as identified by the BiOLOG carbon substrate utilization profile and the sequence of 16S rRNA gene. All of these bacteria were gram-negative. Their abilities to degrade oil components were examined by the TLC/FID method or by the change in the dry weight of the heavy oil with which they were incubated. The results obtained by the TLC/FID method showed that ODB-G1 and ODB-G2 degraded the saturate fraction and the aromatic fraction very well. They were also able to degrade the resin fraction and the asphaltene fraction, although the quantity was little. The *Alcanivorax* sp. degraded heavy oil well, but the *Caulobacter* sp. and the *Halomonas* sp. were found unable to degrade heavy oil as assessed by the change in the dry weight of heavy oil. However, ODB-G1 (a mixture of *Caulobacter* sp. and *Alcanivorax* sp.) and ODB-G2 (a mixture of *Halomonas* sp. and *Alcanivorax* sp.) displayed a higher degradation rate than *Alcanivorax* sp. alone. The effect of heavy oil on their growth was examined. Cell density of both *Alcanivorax* sp. in the stationary phase in the medium contained heavy oil was about 10 times higher than that in the medium without heavy oil. On the other hand, such difference could not be observed in *Caulobacter* sp. and *Halomonas* sp. This result suggests both of *Alcanivorax* sp. have utilized the heavy oil component(s).

INTRODUCTION

The oil tanker Nakhodka wrecked and split into two halves on the open sea in Shimane Prefecture in 1997. The front part, which had drifted afterwards, ran aground on the coast of Mikuni Town, Fukui Prefecture. About 5,000 kl or more was spilled from its front portion and washed up the entire shore from Shimane Prefecture to Yamagata Prefecture. There were several large-scale spill incidents in the past and sequel in Japan. One example is the accident of the oil tanker Juliana in Niigata Prefecture in 1971, when about 7,000 kl crude oil was spilled. Another is the disruption of the oil tank in an accident of Mitsubishi Oil Mizushima refinery in Okayama prefecture in 1974; 7,500-9,500kl C heavy oil was spilled into the sea. Also, a large number of small-scale spill accidents occur daily. Such oil pollution has become a worldwide problem, since it not only gives serious damage on fisheries but also causes adverse effects on the natural environment and ecosystem.

These accidents have activated developments of methods for microbiological degradation (i.e. bioremediation) of oil. The accident of the Nakhodka attracted the attention in bioremediation in our country. An attempt for bioremediation of oil was first seriously carried out in the Exxon Valdez spill accident in 1989. It was shown at that time that indigenous bacteria were useful for oil degradation (Swannell *et al.*, 1996). Here, following that line of study, we have tried to isolate indigenous bacteria that can degrade heavy oil from the Nakhodka spill oil. Because we supposed that indigenous bacteria were already adhered to the spill oil while it was drifting in the sea, we looked for bacteria in the oil spill itself. In fact, we were able to isolate six bacterial groups and two bacteria. In the study presented here, we have characterized two of these bacterial groups. A preliminary report of some of these results appeared previously (Ishiyama *et al.*, 1999).

MATERIALS AND METHODS

Isolation and cultivation of bacterial groups and bacteria

Bacteria were isolated from the heavy oil floating on the sea around the Noto Peninsula or recovered on the beach of Neagari Town in Ishikawa Prefecture. We first added 5 grams of those heavy oil samples to 100 ml of NSW culture medium (Higashihara *et al.*, 1978) and kept the mixture shaking for 5 - 7 days at the room temperature. Part of the culture solution including proliferated bacteria was then inoculated on NSW agar plates (1.5%) containing 5mg/ml heavy oil.

Colonies formed were isolated and transferred into NSW liquid medium containing 5mg/ml heavy oil and kept shaking for 5 - 10 days. Those bacterial clones that emulsified the oil were preserved in NSW agar slants medium containing 5mg/ml heavy oil. Those bacteria were used in this study.

Aliquots of experimental cells were taken at suitable time intervals and fixed with 1% glutaraldehyde in culture medium. Cell densities were measured for each samples with a phase contrast microscope (Olympus IX70) using a bacteria counting chamber (Erma Inc., Tokyo). The measurement images were enlarged with an image processor system Argus-20 (Hamamatsu Photonics K.K., Shizuoka).

Observation of the bacterial cell

The morphology and motility of the bacteria were first observed by the dark-field microscopy (Nakamura *et al.*, 1990). They were also examined ultra-structurally by electron microscopy, using negative staining with 1% uranyl acetate. For ultra-thin section, cells were fixed with 2% glutaraldehyde in 0.1M sodium cacodylate buffer for 1 hr and post-fixed with 1% OsO4 in the same buffer for 1hr. After being dehydration with an ethanol-series the samples were embedded in epoxy resin (Epok 812, Oken Shoji Co., Ltd, Tokyo). Sections were cut with a diamond knife on a Sorvall MT1 Porter-Blum ultramicrotome, and stained with uranyl acetate and lead citrate. A transmission electron microscope (JEOL 100 SX) was used for observation at 80 kV.

Measurements of heavy oil degradation rate

The heavy oil degradation rate was measured by TLC/FID method after Goto et al. (1994) as described elsewhere (Ishiyama *et al.*, 1999). The data obtained by the TLC/FID method should be regarded as only semi-quantitative. This is because we could not separate the peak of the internal reference, stearyl alcohol, from those of resins + asphaltenes as clearly as Goto et al. reported, and therefore we had to use stearyl alcohol as an external reference. For a more reliable estimate of the degradation rate, we therefore examined the change in the dry weight of heavy oil before and after incubation. Two sets of samples, with and without bacterial inoculation, were incubated at 25°C for 7 days with shaking. After incubation, the heavy oil remaining in each sample was extracted with chloroform, and the extracted samples were dried with Na_2SO_4, and then evaporated. Each sample was dissolved with a small amount of chloroform, and reheated at 80°C for additional 3 hr to evaporate chloroform, then allowed to cool

in a desiccator, and weighed. Heavy oil degradation rate was estimated from the dry weight difference between of two sets of samples.

Identification of bacterial species

To identify the species of bacteria, two methods were mainly used. One is based on the BiOLOG microplates system (BiOLOG, Hayword, CA, USA) which examined utilization of the carbon source of various types, and the other is based on the sequence of the16S rRNA gene (Ishiyama *et al.*, 1999). Gram staining was carried out by FAVOR-G SET-S (Nissui Pharma-ceutical Co., Ltd., Japan).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of whole bacteria was performed on 7.5% acrylamide gels (Laemmli, 1970) and stained with silver (Morrisey, 1981).

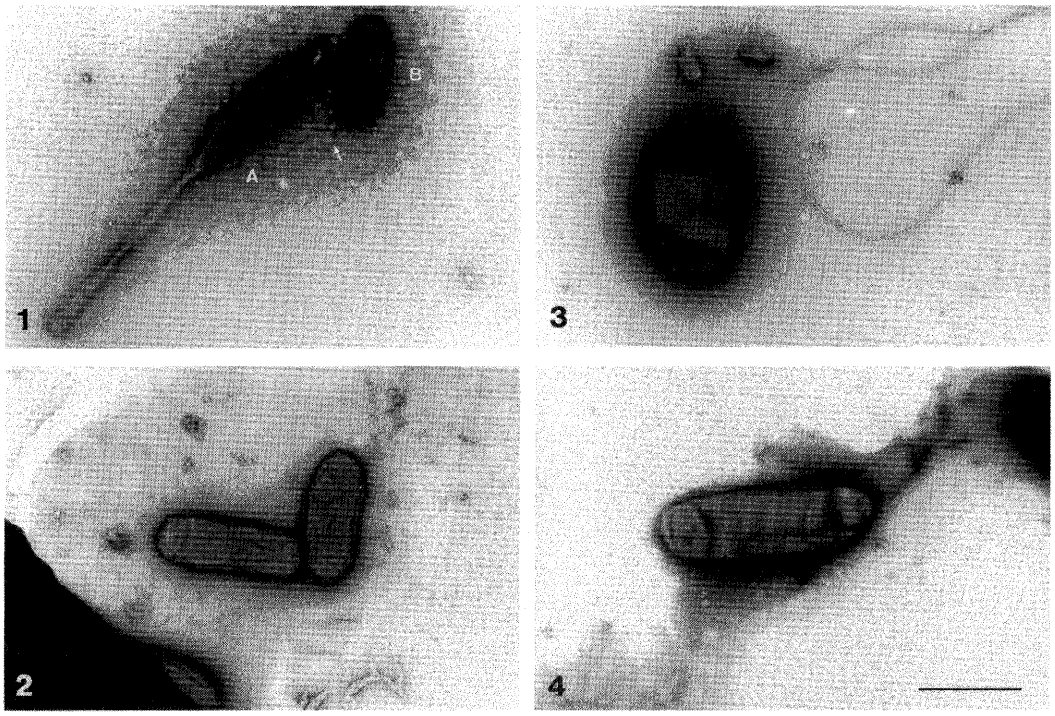
RESULS AND DISCUSSION

We isolated two bacterial groups with the activities to emulsify the heavy oil in NSW medium. We designated them Oil Degradation Bacterial Group (ODB-G) 1 and 2. In this study, we characterized them. They were isolated from the heavy oil that flowed on the sea around Noto Peninsula or recovered on the beach of Neagari Town respectively. We could not isolate similar bacterial groups or bacteria from the heavy oil sampled directly from the Nakhodka. These results suggest that the oil degrading bacterial groups or bacteria are distributed in the sea around Noto Peninsula.

Morphology of isolated bacteria

Initially, we isolated ODB-G1 and ODB-G2 as a single colony. However, dark-field microscopy indicated that each of them contained 2 - 3 different types of bacteria (data not shown). In ODB-G1, there were small rod bacteria without motility, stalked bacteria (like a tennis racket), and motile rod bacteria. By electron microscopy, the motile rod bacterium was found to have a single polar flagellum and a body shape typical of stalked bacteria (Fig. 1A, B). From these observations we concluded that the stalked bacteria and the motile rod bacteria are the same species of *Caulobacter*, a Gram negative bacterium that displays two cell types during life cycle, a motile swarmer cell and a sessile stalked cell. The other non-motile bacteria had no flagellum and different cellular morphology (Fig. 2). Similarly, ODB-G2 contained non-motile rod bacteria and motile rod bacteria. Electron microscopy showed the coexistence of bacteria with many flagella (Fig. 3) and those with no flagella (Fig. 4). These bacteria differed from each other in

cellular morphology also.



Figures 1- 4 Electron micrographs of negatively stained bacteria in ODB-G1 (Figs. 1, 2) and G2 (Figs. 3, 4). Fig. 1: (A) a stalked bacterium and (B) a motile rod bacterium with a single polar flagellum (an arrow). We concluded that both type bacteria are the same species of *Caulobacter* sp. Fig. 2: These rod bacteria had no flagella and width of the cell body was longer than *Caulobacter* sp. in Fig. 1. Fig. 3: A rod bacterium with many flagella was observed. Fig. 4: A rod bacterium with no flagella was also observed in ODB-G2. Bars, 1 μm.

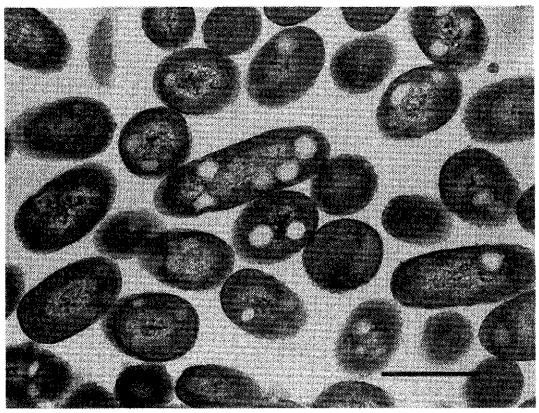


Figure 5 A typical ultrathin section of G1ST (*Alcanivorax* sp.). Both G1ST and G2ST have several vacuoles in their cytoplasm. Bar, 1 μm

From the observation of the ultra-thin sections, we could confirm G1ST and G2ST have several vacuoles in their cytoplasm (Fig. 5). Mrikawa and Imanaka (1993) reported that when *Pseudomonas* sp. HD-1 cells were grown on n-alkane anaerobically they formed many oil vacuoles in its cytoplasm. Therefore, we have to examine the vacuoles formation in

G1ST and G2ST in several growth conditions.

Identification of bacterial species

All bacteria in ODB-G1 and ODB-G2 were cloned again from colonies formed on NSW agar plates. White opaque colonies and transparent colonies were formed. The transparent colonies were difficult to find on the plates. We temporarily designated the bacterium forming opaque colonies in ODB-G1 as G1 and those in ODB-G2 as G2, and the bacteria forming transparent colonies in ODB-G1 as G1ST (ST: stealth) and those in ODB-G2 as G2ST. All of these bacteria were Gram-negative. G1 corresponds to the one that was morphologically identified as a *Caulobacter* sp (see above). G2 was a motile rod bacterium in ODB-G2 and was identified as *Halomonas* sp. based on its carbon substrate utilization in the BiOLOG profile. Both G1ST and G2ST were non-motile rod bacteria, and they were identified as *Alcanivorax* sp. from their sequences of 16S rRNA genes.

SDS-PAGE showed that their protein band patterns were clearly different among *Caulobacter* sp. (G1), *Halomonas* sp. (G2), and *Alcanivorax* sp. (G1ST, G2ST). The band patterns of G1ST, G2ST, were similar, but there were some differences (Fig. 6). From these results, we speculate that G1ST and G2ST are distinct strains of *Alcanivorax* sp.

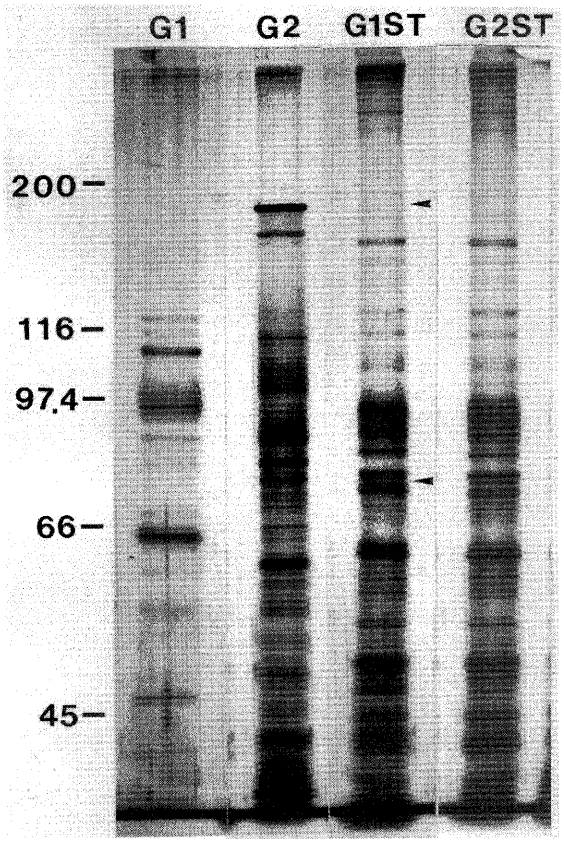


Figure 6 SDS-polyacrylamide gel electrophoresis of whole bacterial cells. A 7.5% separation gel and a 3% stacking gel were used, and then stained with silver. Arrowheads show the bands in G1ST, which could not observe in G2ST. The apparent molecular masses of marker proteins are given in kilodaltons.

Degradation rate of heavy oil

The ability of ODB-G1 and ODB-G2 to degrade heavy oil was examined by the TLC/FID method. The method measures the heavy oil components by roughly separating into saturates, aromatics, resins and asphaltenes. Although the rate of degradation varied from experiment to experiment, both bacterial groups were found to degrade all components (Table 1). Especially, saturates and aromatics were degraded very well. Resins and asphaltenes were also degraded although the quantity was little. However, the degradation rate of ODB-G1 in commercial type C heavy oil (not weathered) was lower than that in spilled oil (weathered naturally for about 2 weeks), and ODB-G1 could not degrade resins and asphaltenes in it. From this result, we speculate that ODB-G1 may be easily damaged by some volatile components contained in the commercial type C heavy oil. On the other hand, the results shown in Table 1 indicate ODB-G2 appears to be fairly resistant to the volatile components.

Table 1 Degradation rates of heavy oil in bacterial groups

	ODB-G1		ODB-G2	
	Spilled Oil	Commercial Oil	Spilled Oil	Commercial Oil
Saturates	46.3±15.5*	17.0±8.0	37.5±7.5	38.5±9.5
Aromatics	26.0±19.2	15.0±1.0	28.0±13.0	35.5±16.5
Resins	19.0±5.4	0.0	25.0±16.0	17.0±10.0
Asphaltens	22.7±16.5	0.0	6.5±6.5	20.0±17.0
Total	27.7±12.5	9.0±0.0	19.0±8.0	30.0±14.0

*Average±standard deviation (%) in 2-3 measurements.
Degradation rates were measured by the TLC/FID method.

We think these results obtained by the TLC/FID method were rather qualitative and not quantitative, as stated in the Materials and Methods. To measure degradation rate quantitatively, we examined dry weight change of heavy oil in each bacteria and bacterial groups (Table 2). The results show that *Alcanivorax* sp. (G1ST, G2ST) degraded heavy oil very well, but *Caulobacter* sp. (G1) and *Halomonas* sp. (G2) were unable to degrade it. However, degradation rate in the group of ODB-G1 and ODB-G2 was higher than *Alcanivorax* sp. alone. From these results, we suppose that *Caulobacter* sp. (G1) and *Halomonas* sp. (G2) can help and accelerate the degradation of heavy oil by *Alcanivorax* sp (G1ST and G2ST). Although many petroleum-degrading bacteria were isolated (Austin *et al.*, 1990; Atlas, 1981; Leahy and Colwell, 1990), to our knowledge this is the first report that bacteria that cannot degrade heavy oil by themselves can promote oil

degradation by other bacteria. We speculate that *Caulobacter* sp. and *Halomonas* sp. may promote heavy oil degradation by degrading some metabolites that *Alcanivorax* sp. produces through degradation of heavy oil. Alternatively, those bacteria may help *Alcanivorax* sp. to degrade oil by producing favorable conditions. Although its mechanism remains to be established in future studies, our present study thus indicates that such an inter-species cooperativity is an important factor to consider in search for efficient means for bioremediation of oil.

Table 2 Degradation rates of heavy oil in each bacteria or bacterial groups

	ODB-G1	ODB-G2	G1	G1ST	G2	G2ST
Total	10.7±3.4*	13.1±1.1	0.0±0.1	4.9±1.3	0.9±0.4	7.8±0.3

*Average±standard deviation (%) in 2-3 measurements.
Degradation rates were examined the change in the dry weight of heavy oil before and after incubation. In this experiment, commercial type C heavy oil was used.

Effects of heavy oil on growth

Optimum growth temperature of every species in ODB-G1 and ODB-G2 was 30°C. Then the effect of heavy oil on the growth of these bacterial species was examined at 30°C (Fig. 7). The culture medium with heavy oil increased cell densities about 10 times or more in comparison with it without heavy oil in G1ST and G2ST. This result indicates that G1ST and G2ST can utilize heavy oil component(s). On the other hand, for G1 and G2, there was no difference whether the medium contained heavy oil or not.

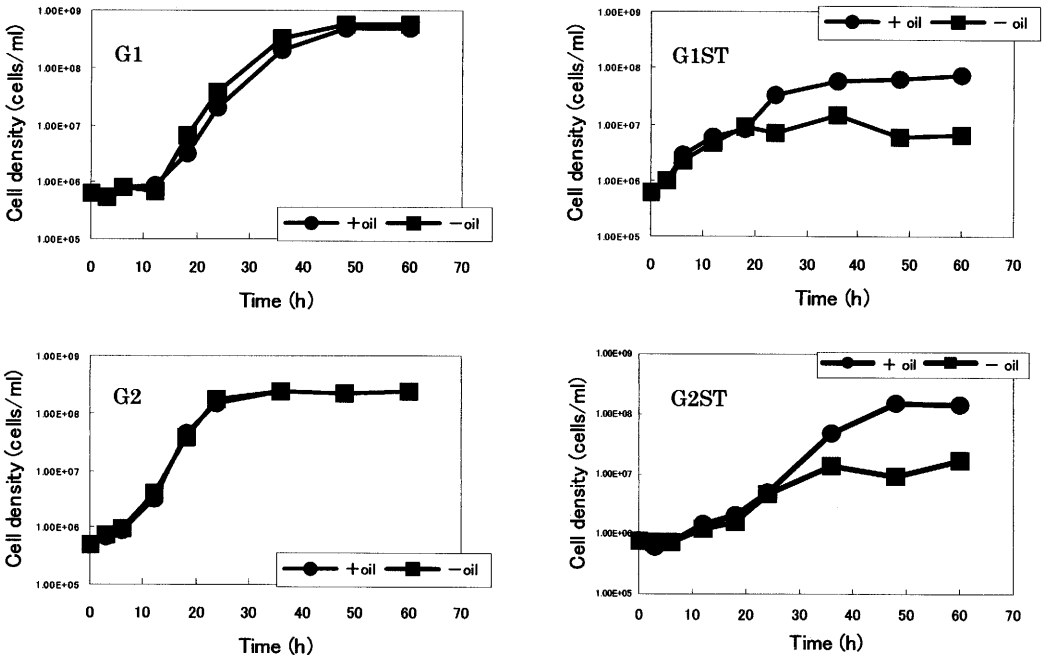


Figure 7 Effect of heavy oil on growth of each bacterial species at 30°C.

ACKNOWLEGEMENTS

We would like to thank Dr. R. Kamiya (University of Tokyo) for his helpful comments and critical reading of this paper. We also thank Dr. T. Takahashi (Toho University), Dr. T. Kusui (Toyama Prefectural University), and Dr. K. Tazaki (Kanazawa University) for supplying spilled oil or direct drawn oil from the Nakhodka. This study was supported in part by Grants-in-Aids from the JSPS to S.N. (No.12680564).

REFERENCES

Atlas, R. M. (1981). Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiol. Rev.*, **45**, 180-209.

Austin, B., Calomiris, J. J., Walker, J. D., and Colwell. (1977). Numerical t axonomy and ecology of petroleum-degrading bacteria. *Appl. Environ. Microbiol.*, **34**, 60-68.

Goto, M., Kato, M., Asaumi, M., Shirai, K., and Venkateswaren, K. (1994). TLC/FID method for evaluation of the crude-oil-degrading capability of marine microorganisms. *J. Mar. Biotechnol.*, **2**, 45-50.

Higashihara, T., Sato, A., and Shimizu, U. (1978). An MPN method for the enumeration of marine hydrocarbon degrading bacteria. *Nippon Suisan Gakkaishi*, **44**, 1127-1134.

Ishiyama, M., Matsui, N., Kunii, K., Kubo, K., Sato, C., and Nakamura, S. (1999). Degradation of heavy oil by bacteria isolated fro the NAKHODKA oil spill. *Proceeding of the International Symposium "Earth-Water-Humans"*, Kanazawa University, 97-103.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, **227**, 680-685.

Leahy, J. G. and Colwell, R. R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev.*, **54**, 305-315.

Morikawa, M. and T. Imanaka (1993). Isolation of a new mixotrophic bacterium which can fix CO2 and assimilate aliphatic and aromatic hydrocarbons anaerobically. *Journal of Fermentation and Bioengineering*, **76**, 280-283.

Morrisey, J. H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.*, **117**,

307-310.

Nakamura, S., Watanabe, M., Hatase, K., and Kojima, M. K. (1990). Light inhibits flagellation in a *Chlamydomonas* mutant. *Plant Cell Physiol.*, **31**, 399-401.

Swannell, R. P. J., Lee, K., and McDonagh, M. (1996). Field evaluations of marine oil spill bioremediation. *Microbiol. Rev.*, **60**, 342-365.