

Bioremediation on the Shore after an Oil Spill from the *Nakhodka* in the Sea of Japan. I. Chemistry and Characteristics of Heavy Oil Loaded on the *Nakhodka* and Biodegradation Tests by a Bioremediation Agent with Microbiological Cultures in the Laboratory

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In January 1997, approximately 5000 tons of heavy oil was spilled from a Russian tanker, *Nakhodka*, which impacted within a 1200 km (linear km) area on the coast of Japan. We organized a joint research group to clean-up the oil on the shore with microbiological cultures for bioremediation, TerraZyme™ (Oppenheimer Biotechnology, Inc.). In this paper, we will examine the chemistry of the *Nakhodka* oil, and the oil samples treated with the bioremediation agent *in vitro*. The *Nakhodka* oil had an extremely high water content, 46.9%, and this resulted in a high oil viscosity and higher oil fluidity at a lower temperature (e.g. @ 5°C). TerraZyme™ exhibited a high potential for biodegradation of oil. Approximately 35% of the *Nakhodka* oil was degraded in 100 ml of test samples containing 1000 ppm of the initial concentration of the oil during the three-week test period. The impact of biodegradation extended to the hardest material in this contained heavy oil, asphaltum. © 2000 Elsevier Science Ltd. All rights reserved.

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Introduction

On 2 January 1997, a Russian tanker, the *Nakhodka*, broke apart and sank in the Sea of Japan. The bow-half of the ship, which floated for about five days and released approximately 5000 tons of heavy oil, ran aground on the shelf near the coast of Mikuni-town, Fukui prefecture, while the stern-half fell to the sea floor at a depth of 2500 m. The tanker was carrying 19 000 tons of heavy fuel oil. The immediate impact was that approximately 5000 tons of released oil was carried by winds to the coast of Japan. The stern-half of the ship contained 11 000 tons which submersible-robot video pictures showed was continually leaking. By 15 February, the oil had impacted the coast of 10 prefectures, from Shimane to Akita, a distance of approximately 1200 linear km, and if one included all the inlets, bays and promontories, the coastal impact may have been up to 10 times greater or 12 000 km. Such large-scale oil pollution on the shoreline has not occurred in Japan since the oil spill from the oil tank in Mizushima, Okayama prefecture, in 1975 (Okaichi and Tatsumi, 1975).

Conventional methods for the treatment of oil spills have relied on human labor, whereby a large number of volunteers attempt to collect the oil clumps and remove

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them from the substrates on the shore by hand, and on treatment with chemical agents that disperse or emulsify the oil. However, the former method imposes extremely hard labour on the volunteers, who are also exposed to the oil, which contains toxic substances. The latter method, treatment of the oil with chemical agents, is an effective measure in combating the oil spill (Nagy *et al.*, 1984; Linden *et al.*, 1985). However, the chemical agents themselves and their emulsions with oil cause toxicity in aquatic organisms (Wells and Keiser, 1975; Lönning and Hagström, 1976; Greenwood, 1983; Mori *et al.*, 1983, 1984).

Bioremediation agents have recently been applied to clean-up efforts after oil spills. These agents may contain inorganic or organic fertilizers, oil degradation bacteria and/or seed cultures of oil-degrading bacteria (Hoff, 1993; Atlas, 1995; Swannell *et al.*, 1996). In earlier studies, through the 1970s and 1980s, on the bioremediation of oil with the fertilizers, the results of the experiments with fertilizers in the laboratory and field demonstrated the feasibility of bioremediation in enhancing the degradation of oil. Most of them were, however, not statistically verified (Oliveiri *et al.*, 1978; Sendstad, 1980; Sendstad *et al.*, 1982, 1984; Sveum and Ladousse, 1989).

In the previous studies, one of the most successful treatments with fertilizer agents was done on the oil pollution following the oil spill from the *Exxon Valdez* on the shores in Alaska in 1989 (Pritchard & Costa, 1991; Bragg *et al.*, 1992, 1994). Among various fertilizers used to treat the oil, the application of an oleophilic fertilizer, Inipol EAP22™ (Société CECA, S.A.), exhibited a distinct enhancement in the biodegradation of oil on the surface of cobble on the shores within 8–14 days following the initial treatment. The surface of the cobble became almost completely free of oil. There is, however, some conflict in the literature on the effectiveness of the fertilizers used in the *Exxon Valdez* areas (Pritchard *et al.*, 1989, 1992). Pritchard *et al.* (1989) stated that in the experiment, oleophilic fertilizer was added at a rate of 0.06 lbs per square foot. This is equivalent to approximately 300 g/m², and could have affected the oil by emulsifying the oil to a water soluble end-product. The loss of the oil-fertilizer mixture could have been partially caused by tidal activity and the solubilization effect of the oleophilic fertilizer on the oil (Atlas, 1995).

Since the 1970s, microbiological cultures have also been studied in the laboratory (Jobson *et al.*, 1974; Lehtomaki and Niemela, 1975; Vecchioli *et al.*, 1990; Venosa *et al.*, 1991; Rosenberg *et al.*, 1992), and on the beach (Tagger *et al.*, 1983; Lee and Levy, 1987). The success of the biotreatment of oil in the field was reported only from the treatment with a product of Alpha Environmental, Alpha BioSea™, at the oil spill from the Mega Borg, in the Gulf of Mexico, in 1990 (New Scientist, 1990; Mauro and Wynne, III, 1990), and the Apex barge at Marrow Marsh along the Texas shoreline

(Nadeau *et al.*, 1991). Visual observations indicated the effectiveness of seeding oil-degrading bacteria in the open waters. However, no systematic monitoring had been conducted to confirm the effectiveness of the product in these applications.

Thus, the effectiveness of the oil bioremediation agents with fertilizers and microbiological cultures still remains scientifically unclear. Little evidence of their effectiveness is available (Hoff, 1993; Atlas, 1995; Swannell *et al.*, 1996; Higashihara, 1998), although we suspect their great potential for enhancing the biodegradation of oil in the field as an effective measure in eliminating marine oil pollution.

In May, 1997, we organized a joint research group, the 'Bioremediation Research Consortium for the Marine Oil Pollution by the *Nakhodka*', to confirm the effectiveness of bioremediation with microbiological cultures, TerraZyme™ (Oppenheimer Biotechnology, Inc.), on the *Nakhodka* oil spilled off the shore through the application of scientific procedures and evidence. This bioremediation agent contains almost the same bacteria cultures as Alpha BioSea™ (Alpha Environmental) (personal communication with Carl Oppenheimer). This joint research venture was designed: (1) to examine the chemistry of oil loaded on the *Nakhodka*, (2) to examine the possible toxicity of a dispersant used by the Prefecture and TerraZyme™ in some aquatic organisms, (these materials were actually used for the treatment of oil in the study areas), (3) to develop a method to quantitatively evaluate the clean-up impact of the bioremediation agent in the field, and (4) to describe the enhanced biodegradation of oil after the treatment with TerraZyme™ *in vitro* and on the shore.

In this paper, we will examine the chemistry and chemical characteristics of the heavy oil that was directly collected from the *Nakhodka* and present results of biodegradation tests on the oil with TerraZyme™ *in vitro*, and finally discuss the effectiveness of TerraZyme™ for the treatment of oil.

Materials and Methods

Chemical compositions and characteristics of oil

For the experiment, we obtained the heavy oil that remained in the bow-half of the *Nakhodka*, which ran aground on the shelf near the coast of Fukui city. These emulsified oil samples seemed to contain a large amount of water. Therefore, we first removed water from the oil samples with toluene. Subsequently, we determined the sulphur content of the remaining oil samples by the Reco method, then measured the temperature at the initial boiling point and at the distillation levels every 5% from 5% to 50% by gas chromatography (HEWLETT PACKARD, ACG2124A), and proceed a patterning analysis with an IATROSCAN TLC/FID Analyzer (IATRON, MK-5).

Degradation tests of oils with TerraZyme™

Tests were conducted to determine the biodegradation of the *Nakhodka* heavy oil, a bunker C heavy oil standardized by Japanese Industrial Standards (JIS), a diesel fuel standardized by JIS and Diisotridecyl Adipate (DITA), with a bioremediation agent of microbiological cultures, TerraZyme™ (Oppenheimer Biotechnology, Inc.). DITA has been used as a typical compound as detailed in the evaluation of biodegradable potentials with active sludge or bacteria in Co-ordinating European Council (CEC) methodology.

The procedures of the degradation tests are described below.

1. 0.19 g of the *Nakhodka* heavy oil and 0.10 g of the test samples of the bunker C heavy oil, diesel fuel and DITA were put in 500 ml flasks. Since the water content of the *Nakhodka* heavy oil was 46.9% in weight, the weight of the test samples was adjusted to be equivalent to the weight of oil in other test samples, which had very little water content. Three replicates were prepared in each test and control group.
2. 5.00 g of the TerraZyme™ was added into each flask with test samples, and it was well-mixed with the test samples of oil, using a spatula. In the control samples, no TerraZyme™ was added into the flasks.
3. A liquid medium for bacterial culture was prepared. Its ingredients are listed in Table 1.
4. 100 ml of the medium was poured into three replicates of flasks in each test and control, respectively. The concentration of oil in the medium was adjusted to 1000 ppm in all of these tests and controls.
5. All of the flasks with samples were kept in the rotary shaker at 25°C and 150 rpm.
6. The experiments were conducted for three weeks. Once a week, samples with the liquid medium were collected from each.
7. The oil residues of the samples were extracted with 50 ml of carbon tetrachloride twice. If emulsion

still formed in the samples, they were centrifuged at 3000 rpm for 10 min, and only the carbon tetrachloride solutions in the samples were removed.

8. The carbon tetrachloride solutions were dehydrated over anhydrous sodium sulphate and the hydrocarbon contents of the solutions were quantified with an infrared light spectroscope at 2950 cm^{-1} .
9. The oil residues were finally developed on a silica-gel coated glass rod using hexane, toluene and $\text{CHCl}_2\text{-MeOH}(95:5)$, and their ingredients were determined by scanning the rod with a flame ionization detector (FID), then IATROSCAN TLC/FID Analyzer (IATRON, MK-5).

Results

Ingredients and chemical characteristics of the *Nakhodka* heavy oil

The oil loaded on the *Nakhodka* was a heavy oil with a black colour, a high viscosity and a faint odour of oil. It had an extremely high water-content, 46.9%, while that of the bunker C heavy oil was only 342 ppm. This high water-content resulted in high oil viscosity and higher oil fluidity at a lower temperature (e.g. at average winter seawater temperatures @ 5°C). The elemental composition of the *Nakhodka* heavy oil is shown in Table 2. High vanadium content was noted as one of the most chemically characteristic features of this oil. It indicates that the *Nakhodka* heavy oil was produced through the addition of relatively large amounts of distillation residues of crude oil.

Fig. 1 illustrates the temperature at the initial boiling point and at distillation levels every 5% from 5% to 50% of the *Nakhodka* heavy oil, and the bunker C heavy oil. The initial boiling point of the former heavy oil, 260°C, is much higher than that of the latter one, 157°C. Fig. 2 also shows the results of the distillation analysis by gas chromatography of these two heavy oils. The patterns of relative intensity to run time in this distillation analysis indicate that the former heavy oil is poor in small hydrocarbon molecules.

Fig. 3 describes the ingredients of the *Nakhodka* heavy oil, which were determined with an IATROSCAN TLC/FID Analyzer. Half of the ingredients of the oil were made up of aromatics. The remaining half was almost evenly allocated to resin, asphaltum and saturated hydrocarbon. The ingredients of the *Nakhodka* heavy oil are close to that of the bunker C heavy oil except for a high water content.

Biodegradation tests of oils

Fig. 4 shows the results of the biodegradation tests of the *Nakhodka* heavy oil, the bunker C heavy oil, a diesel fuel and DITA with TerraZyme™. The initial

TABLE 1

Ingredients in 1 l of liquid medium for bacterial culture.

Ammonium phosphate	0.6 g
Magnesium phosphate	0.05 g
Phosphate buffer (0.1 mol)	3 ml
Boric acid	500 μg
Copper sulphate	40 μg
Ferrous sulphate	400 μg
Zinc sulphate	200 μg
Cobalt chloride	100 μg
Sodium chloride	0.1 g
Calcium chloride	0.1 g
Manganese sulphate	200 μg
Sodium Molybdenum acid	200 μg
Distilled water	1000 ml
Hydrochloric acid (0.1N)	Arbitrarily added ^a

^a Hydrochloric acid was added to adjust the pH of the liquid medium.

TABLE 2
Elemental composition of the heavy oil loaded on the Nakhodka (ppm).

Ca	Mg	P	Al	Fe	Na	Ni	Pb	Si	Sn	V	N (wt%)
1	5	2	2	3	46	10	1	1	1	22	0.204

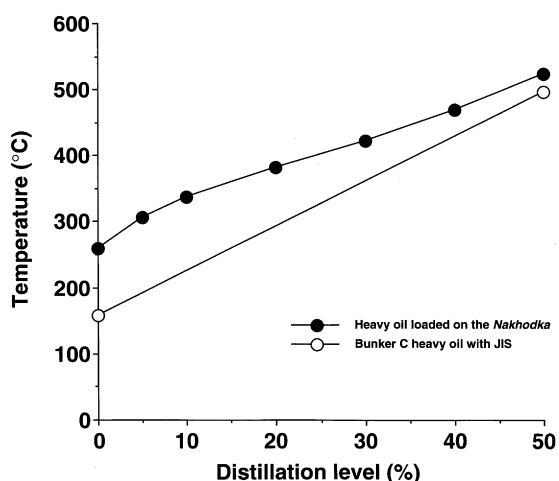


Fig. 1 The patterns of distillation levels of the *Nakhodka* heavy oil and the bunker C heavy oil.

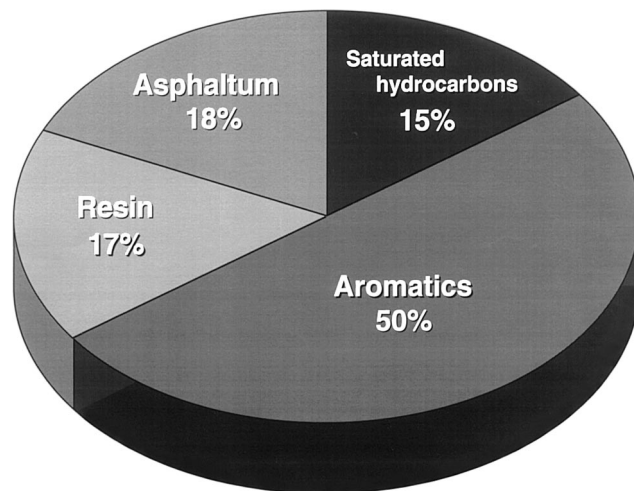


Fig. 3 The ingredients of the *Nakhodka* heavy oil as determined by an IATROSCAN TLC/FID Analyzer.

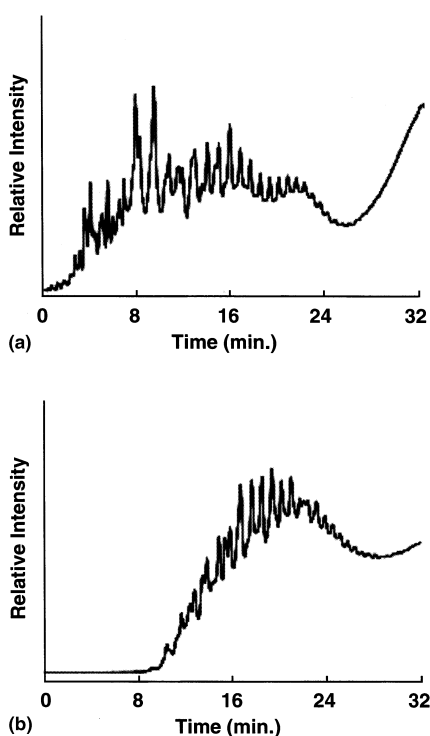


Fig. 2 The patterns of relative intensity of the *Nakhodka* heavy oil and the bunker C heavy oil determined by distillation analysis with a gas chromatography.

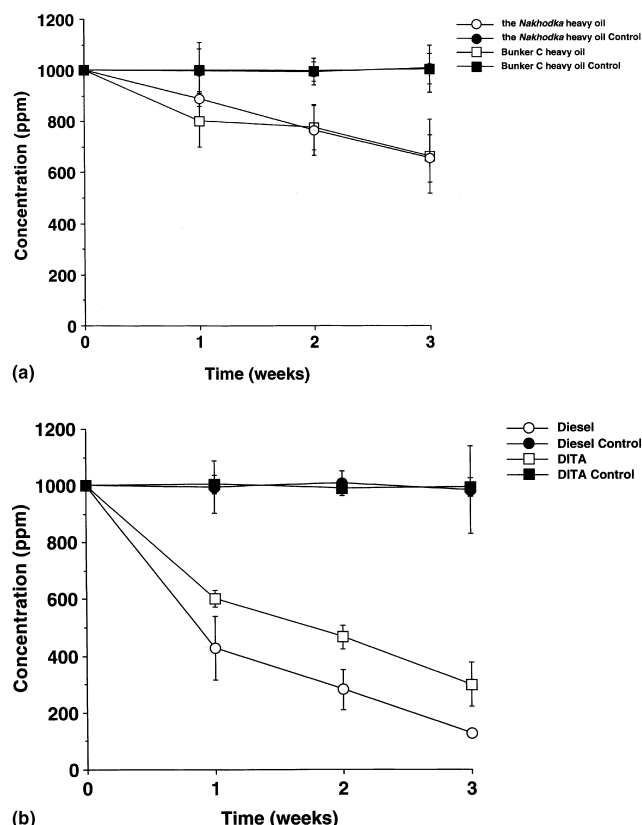


Fig. 4 The results of the biodegradation tests of the *Nakhodka* heavy oil, the bunker C heavy oil, diesel fuel and DITA, with TerraZyme™.

concentration of oil was adjusted to 1000 ppm in all of the test samples. In these biodegradation tests, very few changes from the initial concentration, 1000 ppm, were

found in all of the controls, while the concentration of oil in all of the test samples had simply decreased for three weeks. At the end of the tests, only 128 and 298

TABLE 3
Ratios of each ingredient in the test samples before and after treatment by the bioremediation agent.

	Saturated hydrocarbons	Aromatics	Resin	Asphaltum
<i>Ratios of ingredients before treatment (%)</i>				
The heavy oil from the <i>Nakhodka</i>	15	50	17	18
The bunker C heavy oil	17	40	29	14
Diesel fuel	72	28	–	–
<i>Ratios of ingredients after treatment (%)</i>				
	Saturated hydrocarbons	Aromatics	Resin	Asphaltum
The heavy oil from the <i>Nakhodka</i>	19	51	25	5
The bunker C heavy oil	16	44	32	8
Diesel fuel	75	25	–	–

ppm of oils were remained in the tests with diesel fuel and DITA, respectively. These results indicate that the test samples are able to be completely degraded by the treatment with TerraZyme™ within 4–5 weeks.

The test samples of *Nakhodka* heavy oil and the bunker C heavy oil had been more slowly degraded by the TerraZyme™ treatment than DITA or diesel fuel. Approximately 35% of the oil was lost from the test samples during the three-week test period. Table 3 compares the ratios of ingredients of the test samples before and after the treatment with TerraZyme™. Surprisingly, the ratios of the hardest portion for biodegradation in the heavy oil, asphaltum, markedly decreased from 18% to 5% in the test samples of the *Nakhodka* heavy oil and from 14% to 8% in the ones of the bunker C heavy oil after the treatment, respectively. Consequently, the degradation of asphaltum was slightly increased over the other three ingredients contained in the heavy oils. Although the degradation rate of these heavy oil samples were approximately 2.7–5.1 times slower than those of the diesel fuel and DITA samples, the results indicated that the treatment with TerraZyme™ could extensively impact on the degradation of heavy oils.

Discussion

DITA has been used as a standard to examine biodegradability with active sludge or microbes in the degradation tests conducted by the CEC. Its degradation is relatively difficult due to its chemical structure. According to the CEC decomposition tests using standardized preparations, effective sludge or microbes must be able to degrade more than 80% of 50 ppm of DITA in 150 ml of a liquid medium with 10^4 – 10^7 /ml of the initial bacterial density within three weeks. In this study, we added 5.0 g of TerraZyme™ to 100ml of the liquid medium. 1 g of TerraZyme™ contains approximately 10^8 – 10^9 cells of bacteria. The initial bacterial density of the test samples with TerraZyme™ was adjusted to be approximately less than 5×10^7 /ml, which was only slightly denser than the requirement density of CEC method. The initial concentration of DITA in our test samples was 1000 ppm, which was markedly denser than

that of the standard in the CEC method. We imposed a much harder clean-up effort on TerraZyme™ for the biodegradation of DITA. Nevertheless, approximately 70% of the DITA was degraded within only three weeks. The results of our studies indicate the high potential of TerraZyme™ for the biodegradation of DITA.

After an oil spill, the oil tends to be exposed to the activities of various natural oil-degrading bacteria in the field. Particularly, just after oil pollution occurs, the densities of these bacteria in the substrate in the field tend to markedly increase and dominate the whole bacterial community (Leahy and Colwell, 1990; Atlas, 1981, 1995; Higashihara, 1998). However, these bacterial activities result in the biodegradation of only the most easily decomposable portions of the oil, such as saturated hydrocarbons and the small molecules of aromatics. Large molecules of aromatics, resin and asphaltum in the oil are apt to be degraded extremely slowly in the natural degradation process (Leahy and Colwell, 1990; Atlas and Bartha, 1992; Higashihara, 1998). Therefore, once oil pollution occurs in the field, its influence remains for a long time, if we rely on the natural degradation process alone.

The application of the oleophilic fertilizer Inipol EAP22™ exhibited a distinct clean-up impact on the oil pollution of the treated cobble shores (Pritchard and Costa, 1991). However, Bragg *et al.* (1994) noted the limitation of this approach, since the application of the fertilizer agent was also dependent upon the oil degrading activities of bacteria that naturally occurred in the impacted areas. Asphaltum and the large molecules of hydrocarbons with a complex structure are more resistant to biodegradation by naturally occurring oil-degrading bacteria in the field and tend to be retained in the oil residue. As the content of these substances increased to approximately 60–70% of the total mass of oil residue, biodegradation slowed substantially, and in this case, nutrient availability was not the limiting factor for bacterial growth.

In previous studies on the application of bioremediation agents to oil pollution, adding seed-cultures of petroleum-degradation bacteria has proven less prom-

ising for the promotion of biodegradation of oil than adding fertilizers such as Inipol EAP22™ and ensuring adequate aeration (Atlas, 1995). The results of bioremediation tests with TerraZyme™ to the heavy oil samples in this study, however, show more extensive degradation of oil as just we had surmised. The seeding of microbiological cultures with TerraZyme™ contributed to enhancing biodegradation of asphaltum in the heavy oil samples (Table 3). TerraZyme™ is made up of various microbiological complexes, which have been collected from many different geographical localities under different environmental conditions, shown extensive clean-up impacts on oil pollution under various environmental conditions (Carl Oppenheimer, personal communication).

Unfortunately, in this study, we conducted only a short-term treatment test of heavy oils with TerraZyme™ (Fig. 4). It is still not clear how extensively the application of TerraZyme™ is able to treat the heavy oils. However, the decreasing rate of the concentration of oil residue in the tests predicts that the heavy oil samples can be completely degraded within eight weeks. Our further studies will clarify the effectiveness of the bioremediation agents with microbiological cultures including TerraZyme™ on the treatment of oil in long-term tests over several months.

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