



# Bioremediation on the Shore after an Oil Spill from the *Nakhodka* in the Sea of Japan. II. Toxicity of a Bioremediation Agent with Microbiological Cultures in Aquatic Organisms

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We conducted two laboratory tests in order to investigate the potential toxicity of a seed culture of petroleum-degrading bacteria, TerraZyme™ (Oppenheimer Biotechnology), in aquatic organisms. One test involved the fertilization of sea urchin eggs in solutions of the bioremediation agent with five different concentrations (1, 5, 10, 50, 100 ppm). We also applied the same toxicity tests with solutions of an oil dispersant and a neutral detergent. The results indicated a far weaker negative impact of TerraZyme™ in the fertilization of gametes of a sea urchin than those of the oil dispersant and the neutral detergent. A second test dealt with the potential toxicity of TerraZyme™ in fish. We reared fish (*Chromis viridis*) in water containing TerraZyme™ for a week, and in separate experiments, fed 'sweet fish' (*Plecoglossus altivelis*), food containing TerraZyme™ for a week in one experiment, and for a month in another experiment. In all cases, we were unable to discover a distinct negative impact on the aquatic organisms. © 2000 Elsevier Science Ltd. All rights reserved.

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## Introduction

In accidental oil spills in the sea, chemical agents such as oil dispersants, surface washing agents and surface collecting agents have been widely used to treat oil spills. Oil dispersants are the most popular agents used. Their ingredients are hydrocarbon solvents such as paraffin, surfactant or mixtures (cf. Fujii, 1992). They emulsify the oil and disperse its emulsion into the water. The emulsification of the oil increases the surface area of the oil and promotes biodegradation, evaporation and oxidation (Wells and Keizer, 1975; Nagy *et al.*, 1984; Linden *et al.*, 1985; Okuno, 1997). The oil dispersants themselves (or oil dispersions), however, often have a lethal effect on various aquatic organisms at concentrations of 1–1000 ppm (Nelson-Smith, 1970; Wells and Keizer, 1975; Wu, 1981; Scott, 1984). The effects on fertilization and consequent early growth of various aquatic organisms are more acute. Even exposure to less than 10 ppm of the oil dispersants or their emulsion with oils disturbs the fertilization of gametes and the development of embryos and larvae (Nelson-Smith, 1970; Lönning and Hagström, 1976; Morton and Wu, 1977; Wilson, 1976; Greenwood, 1983). Oil dispersants have been improved year to year, and more recent compounds, e.g., Corexit 7664™, BP1100X™ etc., have exhibited weaker toxicity to organisms (Wilson, 1977; Tokuda, 1998). Clean-up efforts which utilize chemical agents are, however, not necessarily the only appropriate measures to control oil pollution following accidental oil spills.

Bioremediation agents, widely used commercially for soil and aquifer hydrocarbon biodegradation, have been recently applied in clean-up efforts following oil spills. They consist of fertilizers to activate naturally occurring

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oil-degradation bacteria in the field and promote their growth, or contain natural oil-decomposing bacteria which have been massively cultured (Hoff, 1993; Atlas, 1995; Swannell *et al.*, 1996). In the latest 'National Contingency Plan Product Schedule (NCP)' published from US Environmental Protection Agency (EPA) in February 1999, nine microbiological cultures, one enzyme additives and three nutrient additives appear as bioremediation agents for oil (US EPA, 1999).

In this study, prior to the application of TerraZyme™ to treat the heavy oil adhered to the rocks and the concrete of seawalls on the shore by the oil spill from the *Nakhodka*, we conducted two laboratory tests to confirm TerraZyme™'s potential toxicity in aquatic organisms. One test involved the fertilization of sea urchin eggs in solution containing the bioremediation agent in five different concentrations. The ministry of Environment of Canada and the US EPA have recently adopted this test to bioassay the quality of seawater (Environment Canada, 1992; Kobayashi, 1997). We similarly applied the two toxicity tests to both solutions of an oil dispersant (which was actually used for the treatment of oil on the shores after the oil spill from the *Nakhodka*), and to those of a neutral detergent with 20% of surfactant. The dispersant results were compared with those of the tests with TerraZyme™ solutions. A second test dealt with the potential toxicity of TerraZyme™ to fish. We reared two types of fish in water containing TerraZyme™ and/or fed them food containing TerraZyme™. In this paper, we will report the results of these tests, and discuss the toxicity potentials of TerraZyme™ in the aquatic organisms.

## Materials and Methods

### *Test 1: fertilization test of sea urchin eggs*

Fertilization tests were conducted on sea urchins, *Toxopneustes pileolus*, in seawater, which were collected from the Tomioka Bay, Reihoku-cho, Amakusa, Kumamoto, Japan. Prior to the tests, the seawater was filtered through 0.45 µm membrane filters to remove suspended particulates. Just before we started the test at the laboratory, we removed the Aristotle lantern at the bottom of each sea urchin with forceps, and added several drops of saturated potassium chloride solution inside its body through the hole with a pipette. These sea urchins soon released sperms or eggs into the seawater of beakers through the stimulation of potassium chloride. In this test on the fertilization of sea urchin eggs, we examined the influence of: (1) the bioremediation agent with microbiological cultures TerraZyme™ (Oppenheimer Biotechnology), (2) an oil dispersant, YCC Blue Clean™ (Exeno Yamamizu), which was used for the treatment of oil from the *Nakhodka* in Kasumi-cho, Kinosaki-gun, Hyogo Prefecture, Japan, from January to February 1997, and, (3) a neutral detergent for domestic use with 20% of surfactant (Fresh Lemon,

Nissan Sekken). Three different seawater solutions containing the above test materials were prepared with five different concentrations (1, 5, 10, 50, 100 ppm). For a control, we used unadulterated seawater.

The sea urchin eggs were selected with a pipette at random from a stock in the beakers, then placed into the test solutions and seawater control solutions in 300 ml beakers at 25°C. A drop of sperm was added to each to start the fertilization of the gametes. At least 5 min later, the eggs were removed from each beaker, mounted on a glass slide, and the number of fertilized eggs that formed a fertilized membrane per 100 eggs were counted under a microscope. We repeated these counting procedures four times, and adopted their mean number as a single datum determining the ratio of fertilization. All of tests have four replicates.

### *Test 2: toxicity tests of the bioremediation agent on fish*

(a) *Short-term toxicity test on Chromis viridis*. We prepared two 20 l fish tanks, and filled them with artificial seawater, Marine Art™ (Senju Pharmaceutical). In one of these two tanks, we added the bioremediation agent, Formula 1™ (Oppenheimer Biotechnology), to the seawater, and adjusted its concentration in the seawater to 0.1%. Another tank was used as a control. The seawater in each tank was filtered with coral sand on the bottom of the tank and gently aerated with an air pump. We used *C. viridis*, whose mean body length and weight are approximately 3 cm and 1 g, respectively, in the test. These fish were provided by Nikkai Center. In each tank, we put 10 individuals, and kept them at 28°C water temperature and fed them Tetra Marine Large Flakes™ (Tetra Werke) to them thrice in a day. One week later from the start of the test, we counted the number of living fish in each tank.

(b) *Short-term toxicity test on sweet fish, Plecoglossus altevelis*. We prepared 200 l fish tanks, and provided underground water to them via a pump at the exchange-rate of water eight times per day. The DO of the water was kept in saturated conditions. In each tank, we reared 20 juvenile individuals of a 'sweet fish', *P. altevelis* (mean body size 8 cm in length and 5 g in weight, provided from Hamana Fisheries), at 19–20°C water temperature, for one week. Thrice in a day, we fed the fish Nosan Larval Diet Flakes™ for Japanese river trout No. 2 (Nihon Nousan Kogyo). In one of these two fish tanks, we mixed the bioremediation agent, Formula 1™ (Oppenheimer Biotechnology), with the fish food at 0.1% of its total weight. One week following the start of the test, we counted the number of living fish in each fish tank.

(c) *Long-term toxicity test in sweet fish, P. altevelis*. As in the same manner of Test 2(b), we prepared two 80 l tanks, and provided underground water to them with a

pump at the exchange-rate of eight times per day. DO of the water was kept in saturated conditions. In each fish tank, we reared 20 'sweet fish' juvenile individuals at 19–20°C water temperature, and fed the fish Nosan Larval Diet Flakes™ for Japanese river trout No. 2 (Nihon Nousan Kogyo), thrice in a day. In one of these two fish tanks, we mixed the bioremediation agent, TerraZyme™ (Oppenheimer Biotechnology), with the fish food at 0.1% of its total weight. TerraZyme™ is a bioremediation agent with microbiological cultures in which the bacterial density is diluted to one hundredth of that of Formula 1™. One month following from the start of the test, we counted the number of living fish in each fish tank.

## Results

### Test 1: fertilization test of sea urchin eggs

In the control tests, the ratio of fertilized eggs to unfertilized eggs of the sea urchin reached  $95.3 \pm 2.6\%$  (mean  $\pm$  S.D.). It indicated that almost all of the eggs were potentially fertilizable. The ratio of fertilized eggs in three different solutions with a bioremediation agent 'TerraZyme™', an oil dispersant, and a neutral detergent are shown in Fig. 1, respectively. The bioremediation agent solution had the weakest impact on the fertilization of eggs. Up to the concentration of 10 ppm of the solution, the mean ratio of fertilized eggs ranged between 91.8% and 95.8%. Although the ratio of fertilized eggs decreased to  $79.8 \pm 17.1\%$  (mean  $\pm$  S.D.) in the solution with a concentration of 50 ppm, no significant differences were found between these values and those of the control test (ANOVA,  $p < 0.01$ ). In the solution with a concentration of 100 ppm, the ratio of fertilized eggs was further decreased to  $67.8 \pm 18.8\%$  (mean  $\pm$  S.D.), significantly lower than those of the control test (ANOVA,  $p < 0.01$ ). However, these ratio

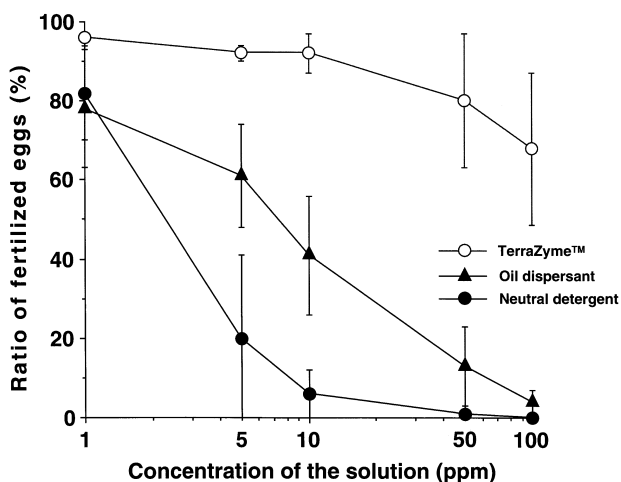


Fig. 1 The ratios of fertilized eggs in the solutions of a bioremediation agent "TerraZyme™", an oil dispersant and a neutral detergent.

TABLE 1

The results of toxicity tests (Test 2) of the bioremediation agent 'TerraZyme™' in fish.

	No. of alive fishes	No. of dead fishes
(a) Short-term toxicity in <i>C. viridis</i>		
Water with the bioremediation agent	10	0
Control: water without any agent	10	0
(b) Short-term toxicity in sweet fish, <i>P. altevelis</i>		
Food with the bioremediation agent	20	0
Control: food without any agent	20	0
(c) Long-term toxicity in sweet fish, <i>P. altevelis</i>		
Food with the bioremediation agent	16	4
Control: food without any agent	15	5

were quite a bit higher than the  $4.0 \pm 2.8\%$  (mean  $\pm$  S.D.) ratio of the oil dispersant solution and the 0.0% ratio in the neutral detergent solution.

The oil dispersant and neutral detergent had much stronger impacts on the fertilization of sea urchin eggs than the bioremediation agent. The ratio of fertilized eggs in the solutions of the oil dispersant and the neutral detergent were significantly lower than those of the control test from the concentrations of 1 and 5 ppm, respectively (ANOVA,  $p < 0.05$ ).

### Test 2: toxicity tests of the bioremediation agent in fish

The results of Test 2 were shown in Table 1.

(a) Short-term toxicity in *C. viridis*. All the 10, *C. viridis*, individuals were alive and in healthy condition in both of the tests with the bioremediation agent solution of seawater and controls throughout the test, until its completion, after one week. No toxicity due to the bioremediation agent was observed in the fish in this test.

(b) Short-term toxicity in sweet fish. All the 20, *P. altevelis*, individuals were alive and in healthy conditions throughout one-week tests in which we fed the fish food containing 0.1% of the bioremediation agent in weight and in the control experiment in which the fish were fed food without any admixture of bioremediation agent. No toxicity from the bioremediation agent was observed in the fish in this test.

(c) Long-term toxicity to sweet fish, *P. altevelis*. In the month-long test in which we fed food with 0.1% of the bioremediation agent in weight to the fish, three of 20 individuals died. The number of survivors in the test community was, however, greater by one individual than that of the control tank. No significant toxicity due to the bioremediation agent was observed in the fish in this test.

## Discussion

The results of Test 1 exhibited a far weaker negative impact of TerraZyme™ in the fertilization of sea urchin gametes than those of either an oil dispersant, or YCC Blue Clean™, a neutral detergent for domestic use (Fig. 1). For example, in solutions with a concentration of 50 ppm, the ratio of fertilized eggs in the TerraZyme™ solution was  $79.8 \pm 17.1\%$  (mean  $\pm$  S.D.). These values were not significantly different from those of the control (ANOVA,  $p < 0.05$ ). However, the ratio of fertilized eggs in the oil dispersant solution and the neutral detergent solution were markedly decreased to  $13.3 \pm 9.6\%$  (mean  $\pm$  S.D.) and only  $1.3 \pm 1.9\%$  (mean  $\pm$  S.D.), respectively.

The negative impact of YCC Blue Clean™ on the fertilization of sea urchin gametes was almost at the same level as that of COREXIT9527™ (Lönning and Hagström, 1976), which appears in the latest NCP of the U.S. EPA as one of the oil dispersants for treating the oil pollution (US EPA, 1999). TerraZyme™ is also noted in the NCP as one of the bioremediation agents with microbiological cultures. Although the results of similar tests with other four oil dispersants in the NCP were not available, we have deduced that the negative impact of TerraZyme™ in the early development of aquatic organisms is much weaker than that of the low-toxic oil dispersants detailed in the NCP list.

The results of Test 2 provide additional verification on the extremely low toxicity of TerraZyme™ in aquatic organisms. Only in Test 2(c), three test animals fed food with the bioremediation agent died. However, in the control test tank, four individuals died. The death of all these animals occurred during the first week of the one-month test. No affected parts were found on the body surface. The postmortem of these fish showed no signs of the typical effects due to toxic substances, such as internal bleeding and abnormal bending. We deduced that the fish died due to physiological stress from the changes of rearing conditions in Test 2(c).

Thus, from the results of this study, we were able to find no distinct negative impact to the aquatic organisms, due to TerraZyme™ exposure. TerraZyme™ thus reliably possesses a low toxicity with regard to aquatic organisms, and has a good potential for enhancing the biodegradation of oil. This is partly because the petroleum-degrading microorganisms are contained within a bentonite substrate in TerraZyme™. The majority of the weight of the bioremediation agent is occupied by the non-reactive substrate. Although TerraZyme™ possessed a high potential for enhancing the biodegradation of all of the ingredients of heavy oil, including resin and asphaltum (Hozumi *et al.*, 2000), only a portion of TerraZyme™, limited as to weight-percentage is actually reactive with the oil and the organisms in the environment. It is noteworthy that TerraZyme™ has both a much lower environmental impact and a much

greater ability to bio-degrade oil than surfactant agents now in common use.

The fertilization test of sea urchin eggs was done with the collaboration of 27 students in a 'Biological experiments' class, in the Faculty of Human Life Sciences, Prefectural University of Kumamoto, in the first semester, 1997. This test was conducted with financial support from a special research grant of the 'Supporter's Organization', Prefectural University of Kumamoto. All of the fish tests were carried out at the laboratory of Nihon Chlorella Co., Ltd. We would like to express our thanks to all those who collaborated in this study. We also would like to thank Tsuneaki Terakawa, Takehiko Manabe, Toyoharu Hozumi, Koji Takai, Masakazu Kono, Makoto Haraguchi and Isamu Yamamoto for their advice and suggestions. We would also like to thank Carl Oppenheimer and Richard Gilbert for their critical reading of the manuscript.

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