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Immune Gene Expression Levels Correlate with the Phenotype of Japanese Flounder Exposed to Heavy Oil

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Abstract—Heavy oil pollution caused by tanker accidents is one of the most important environmental issues in the world. The pollution is related to the impact on marine organisms, since heavy oil consists of toxic substances such as polycyclic aromatic hydrocarbons (PAHs), heavy metals and benzol. However, the effect of heavy oil on the fish immune system is not well understood. In the present study, therefore, we exposed Japanese flounder (*Paralichthys olivaceus*) to heavy oil and measured the immune response of the fish. For the exposure experiment, six Japanese flounder (mean body weight: 197 g) were acclimated under laboratory conditions at 19–20°C. Three fish were exposed to heavy oil C (bunker C) at a concentration of 3.8 g/L for 3 days, and the others were kept in seawater without heavy oil and used as control. Then, the blood, kidney and skin mucus were collected from the tested fish. Results showed the number of leukocytes in flounder blood increased after heavy oil exposure. The bacterial numbers in skin mucus of heavy oil-exposed fish were significantly higher than that from the control. Gene expression analyses displayed immune system-related genes were mostly down-regulated by heavy oil exposure, while interleukin-8 and lysozyme, involved in proinflammatory processes, were up-regulated. Considering the data on phenotypic effects and gene expression profiling, pathogen resistance in flounder may be compromised by heavy oil exposure, which can cause bacterial infection and subsequent induction of proinflammatory genes as a defensive response against the infection.

Keywords: heavy oil, Japanese flounder, immunity

INTRODUCTION

Anthropogenic sources of many organic trace pollutants, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polychlorinated dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs) have been produced during the 20th century and, in part, released into the aquatic ecosystems as well as terrestrial environments (Stegeman and Hahn,

1994; van der Oost *et al.*, 2003). Especially, oil spills associated with marine transport such as tanker accidents and offshore production facilities are main routes for the release of crude oil into the open sea (Ramachandran *et al.*, 2006). The released oil may result in large numbers of small oil droplets in the water column which may reach the benthos and enter the sediment, thus exposing sediment-dwelling organisms to hydrocarbon contamination (SEEEC, 1998).

The acute toxicity of oil and its components have been well documented for several teleosts (Anderson *et al.*, 1974; Rice *et al.*, 1987), causing morphological and histopathological changes and genetic damage in larval and juvenile stages (Carls, 1987; Brown *et al.*, 1996; Hose *et al.*, 1996; Kocan *et al.*, 1996; McGurk and Brown, 1996; Norcross *et al.*, 1996; Carls *et al.*, 1999; Heintz *et al.*, 1999). Exposure of adult fish to parts per million (ppm) concentrations of crude oil has been reported to elevate plasma adrenaline, noradrenaline and plasma cortisol concentrations (Thomas and Rice, 1987; Alkindi *et al.*, 1996). Thus, oil pollution can be significant stressor for fish, and it is thought to lead to immune suppression in fish. However, the effect of crude oil to fish immune system is not well resolved.

As basic research for the effect of oil on the fish immune system, in this study we measured the number of blood leukocyte and erythrocyte in Japanese flounder *Paralichythys olivaceus*, one the most important benthic fish species in Asia, after exposure to heavy oil (bunker C). We also counted the number of bacteria in the skin mucus of fish to understand whether marine bacteria can more easily attach to the fish and/or grow in the mucus after the exposure stressor. Furthermore, the changes of microflora in the mucus were investigated by denaturing gradient gel electrophoresis (DGGE) analysis. In order to understand immune status of the fish after heavy oil exposure at molecular levels, we constructed a cDNA microarray spotted with 309 immune system-related genes, and alteration of gene expression profiles was monitored in the kidneys of the fish.

MATERIALS AND METHODS

Fish

Japanese flounder (*Paralichthys olivaceus*) were obtained from the Ehime Prefectural Chuyo Fisheries Experimental Station, Japan and placed in a 125 L holding tank supplied with re-circulating filtered seawater at 20°C. The fish were acclimatized for one week before an experiment. During acclimation, the fish were fed daily with a commercial fish food. Feed was withheld throughout the exposure period. Post-acclimation the fish were weighted, and then transferred into experimental aquarium for the exposure.

Exposure to heavy oil

Heavy oil (bunker C) was obtained from an oil company and used for exposure experiments. Experimental treatments comprised of two tanks with three fish per 8 L of seawater in a 13 L tank supplied with filtered seawater with

continuous aeration at 20°C. One was added heavy oil at a concentration of 30 g/8 L for 3 days and another was control tank without any exposure. After heavy oil exposure for 3 days, fish were reared in filtered seawater without heavy oil for 4 days and then collected for the analysis.

Sampling

After treatment for 7 days, approximately 1 ml of blood sample from each fish was collected from the caudal vein using a syringe. The blood was immediately transferred to an Eppendorf tube treated with 2.7% EDTA solution, shaken gently and kept at 4°C. This sample was used for the enumeration of blood leukocytes and erythrocytes. Next, mucus of each fish was collected by scraping the skin surface with a sterilized surgical blade to analyze mucus-contained bacteria. A portion of mucus was immediately diluted 10 times in sterilized PBS(–) for bacterial count and the remainder was stored at –80°C until use for denaturing gradient gel electrophoresis (DGGE).

Blood leukocytes and erythrocytes count

The treated blood cells with EDTA were stained with Natt-Herrick's staining solution (Natt and Herrick, 1952) at room temperature for about 20 min. After staining the blood leukocytes and erythrocytes were enumerated on a cell counting chamber. The data were analyzed and compared using the Student's *t*-test.

Mucus included bacterial count

To compare the number of bacteria in the mucus between oil-exposed and control fish, viable bacteria were measured by counting colony forming unit (CFU). The mucus from each fish was diluted in sterilized PBS(–) and thoroughly mixed by vortexing. Next, 100 μl of the diluted mucus was spread on brain heart infusion (BHI) agar containing 1.5% of NaCl and marine broth 2216 (MB) agar. After 24 hours of incubation at 20°C, the numbers of colonies were enumerated. The data were analyzed and compared with the Student's *t*-test.

Polymerase chain reaction amplification of 16S rDNA

Bacterial 16S rDNA fragments containing the V3 region were amplified by PCR with forward primer f341-GC-clamp (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3′) and reverse primer r534 (5′-ATT ACC GCG GCT GCT GG-3′), which are specific for domain bacteria (Muyzer *et al*., 1993). Amplification reaction was performed in 100 μL of reaction mixture containing 0.25 μM of each primer, 2 mM $MgCl_2$, 1 × PCR Gold buffer, 0.2 mM dNTPs, 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, California, USA), and 1 μL of DNA template solution. Touchdown thermal cycling was used for the amplification, which consisted of the following steps: initial denaturing at 95°C for 10 min, annealing at 65°C for

0.5 min, extension at 72°C for 0.5 min; then 14 cycles of 94°C for 0.5 min, 64–55°C (reduced by 2°C each two cycles) for 0.5 min, 72°C for 0.5 min; followed by 11 cycles of 94°C for 0.5 min, 55°C for 0.5 min, 72°C for 0.5 min; final extension at 72°C for 5 min and then held at 4°C.

Denaturing gradient gel electrophoresis (DGGE) analysis

Polymerase chain reaction products were visualized by electrophoresis on 1.5% agarose gels with ethidium bromide to confirm the specific single band. A 2.8 μg of each PCR product was used for DGGE. DGGE was performed on 8% polyacrylamide (acrylamide/bisacrylamide, 37.5:1) gels with 40–80% gradient of urea-formamide denaturant, where 100% denaturant solution contained 7 M urea and 40% (v/v) formamide. Electrophoresis was performed using 40 mM Tris-acetate with 1 mM EDTA (pH 8.0) at 55°C for 10 min at 20 V and subsequently for 12 h at 100 V using DCode System (Bio-Rad Laboratories, California, USA). After electrophoresis, the gels were stained with SYBR® Green I (diluted 1:10,000 diluent, Cambrex Bio Science Rockland, Maine, USA). Intensity of the band of the DGGE gel was analyzed using LAS-1000 (Fuji Film, Kanagawa, Japan).

Microarray experiment

Japanese flounder EST library was established by Digital Genomics (Seoul, Korea). All sequences were subjected to BLASTX homology search against a non-redundant protein database using the BLASTALL program. To construct a cDNA array, 309 selected clones were spotted onto glass slides (GenePlorer™ Twinchip™, Digital Genomics).

After the exposure test, the three fish in each treatment were dissected and their kidneys were collected. The kidney samples were immediately frozen in liquid nitrogen and were stored at –80°C until use. Total RNA was extracted from the kidney with TRIzol reagent (Invitrogen, Carlsbad, CA). RNA samples from control fish were pooled to use as a common reference. Second strand cDNA was synthesized and antisense RNA (aRNA) was amplified using a MessageAmp™ II aRNA Amplification Kit (Ambion, Austin, TX). Purified aRNA was reverse-transcribed with amino allyl-dUTP using an Amino Allyl cDNA Labeling Kit (Ambion), in which amino allyl cDNA was coupled with Cy3 (control or heavy oil-exposed) or Cy5 (common reference) monofunctional reactive dye (GE Healthcare UK, England, UK) and then mixed together. Excess Cy dye was removed using a QIAquick PCR purification kit (QIAGEN K. K., Tokyo, Japan). For hybridization, the following solution was used: Cy dye-labeled DNA solution in 5× SSC, 0.1% SDS, 0.5 mg/ml PolyA (Invitrogen), 0.1 mg/ml salmon sperm DNA (Sigma-Aldrich Japan K. K., Tokyo, Japan), and 25% formamide. The solution was heated at 95°C for 2 min, and then placed on a glass slide. Three slides were used for each sample, and the slides were incubated at 60°C for 17 hrs. Following hybridization, the slides were washed twice with 0.1% SDS in 2× SSC at 58°C, and four times with 0.1% SDS in 0.1× SSC at room temperature, rinsed

Table 1. Numbers of erythrocytes and leukocytes in peripheral blood of oil-exposed and control fish (n = 3).

Treatment	Erythrocytes (cells/mL)	Leukocytes (cells/mL)
Oil exposure	$1.77 \pm 1.09 \times 10^9$	$1.45 \pm 0.45 \times 10^8$*
Control	$3.17 \pm 0.39 \times 10^9$	$5.79 \pm 1.88 \times 10^7$

*Statistically significant ($P < 0.05$).
Data represent as mean ± SD.

three times with 0.1× SSC, and then dried by centrifugation at 800 rpm for 3 min. The washed slides were then scanned using a fluor-image analyzer (FLA-8000, Fuji Photofilm Co. Ltd., Tokyo, Japan) at 532 nm (Cy3) and at 635 nm (Cy5).

Data analyses

Fluorescent intensities were quantified by ArrayGauge 2.1 (Fuji Photofilm). The intensities of the surrounding areas of each spot were used as background. Expression levels of each gene were represented as Cy3 (control or heavy oil-exposed): Cy5 (common reference) ratios, following normalization by the Locfit (LOWESS) function using TIGR MIDAS (version 2.19; Saeed *et al*., 2003). The expression data of each spot were validated by checking the cumulative variations among three slides, which should be lower than 20%, and only data with acceptable variation were used for subsequent analyses.

Differences in gene expression levels between heavy oil-exposed and control groups were analyzed by the Student's t-test. Prior to analysis, gene expression levels were logarithmically transformed. All statistical analyses were performed using SPSS 15.0J (SPSS Japan, Tokyo, Japan). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

After exposure to heavy oil, increased respiration, excess mucus secretion, and cephalic reddish were observed in all three Japanese flounder, although no acute mortality of the fish occurred (data not shown). The symptoms continued during the experimental period without recovery, even after the fish were reared in seawater with no heavy oil. This suggests that constitutive parts of heavy oil affected the nervous, circulatory and/or respiratory systems of the fish.

The numbers of erythrocytes and leukocytes in peripheral blood of the fish are summarized in Table 1. Erythrocyte counts in the control and exposed fish were $3.17 \pm 0.39 \times 10^9$ and $1.77 \pm 1.09 \times 10^9$ cells/mL, respectively. Although there was no significant difference between them the numbers tended to decrease after heavy oil exposure. As supporting data for the decreasing tendency of erythrocytes, the expression of hemoglobin (Hb) beta chain was significantly

Table 2. Numbers of bacteria in skin mucus of oil-exposed and control fish on marine broth (MB) and brain heart infusion (BHI) agar plates.

Treatment	MB (CFU*/g)	BHI (CFU/g)
Oil exposure	$4.58 \pm 1.63 \times 10^5$**	$3.94 \pm 0.79 \times 10^5$**
Control	$4.27 \pm 3.68 \times 10^4$	$1.59 \pm 1.17 \times 10^4$

*Colony forming unit.
**Statistically significant ($P < 0.05$).
Data represent as mean ± SD.

suppressed by heavy oil exposure ($p = 0.032$). Additionally, Hb alpha chain expression in heavy oil-exposed fish was also 2.19-fold lower than that in control fish, although the difference was not statistically significant ($p = 0.057$). Previous studies on gene expression profiling in rainbow trout (*Oncorhynchus mykiss*) have demonstrated down-regulation of both Hb alpha and beta chain mRNAs in the kidney after exposure to carbon tetrachloride or pyrene (Krasnov *et al.*, 2005), and of Hb beta in the liver after BaP exposure (Hook *et al.*, 2006). Moreover, a decline in hemoglobin was found in winter flounder (*Pleuronectes americanus*) collected from PAH- and PCB-contaminated areas compared with those from a cleaner reference site (Khan, 2003). Thus, heavy oil exposure may suppress hemoglobin mRNA expression and subsequently result in declines in red blood cell counts.

Contrary to the decreasing tendency of erythrocytes, the heavy oil exposed fish showed significantly higher counts of leukocytes, averaging $1.45 \pm 0.45 \times 10^8$ leukocytes/mL, compared with $5.79 \pm 1.88 \times 10^7$ cells/mL in the control (Table 1). Generally, it is well known that the numbers of leukocytes dramatically increase for immune defense against bacterial infection. External and internal epithelial surfaces of fish were covered with mucus layer providing protection against environmental factors like microorganisms, toxins, pollutants, acidic pH and hydrolytic enzymes (Ræder *et al.*, 2007). Bacterial adhesion to the body surface of fish is the most important step in the initial stage of infection. Therefore, we counted the numbers of bacteria in the skin mucus of Japanese flounder employed in this study to know whether marine bacteria inhabiting environmental seawater can easily attach to the fish and/or grow in the mucus after the exposure stressor. Results from bacterial counts in fish mucus showed significantly higher numbers in the heavy oil exposed fish to those of control fish; $4.58 \pm 1.63 \times 10^5$ colony forming unit (CFU)/g and $3.94 \pm 0.79 \times 10^5$ CFU/g on MB and BHI plates in the exposed fish, respectively, and $4.27 \pm 3.68 \times 10^4$ and $1.59 \pm 1.17 \times 10^4$ CFU/g in the control fish (Table 2). We believed that the high numbers of bacteria in the exposed fish are strongly related to the increasing leukocytes number i.e., immune suppression of the fish occurred due to heavy oil stressor, and bacteria could easily attach, invade and/or grow in the mucus,

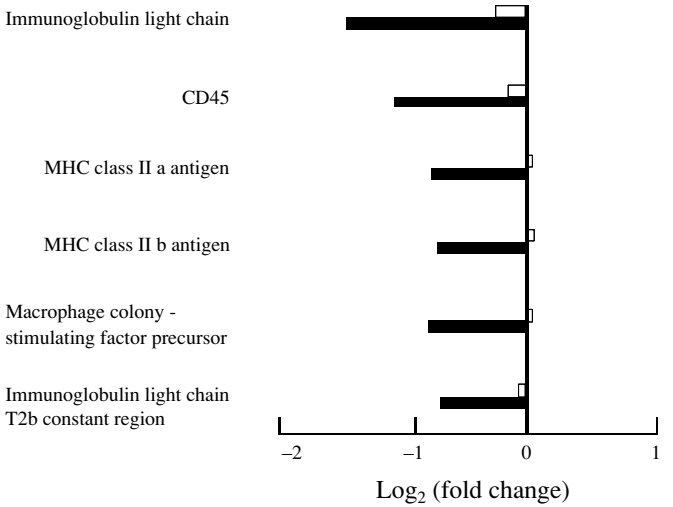

Fig. 1. Identification and fold change of immune system-related genes whose expression was significantly ($p < 0.05$) altered by heavy oil exposure. □; Control group, ■; Heavy oil exposure group.

resulting in the increasing leukocyte number to prevent infectious disease in the fish. In support of this hypothesis, several immune system-related genes, including immunoglobulin light chain, CD45, major histocompatibility complex (MHC) class II antigens, and macrophage colony-stimulating factor precursor, were down-regulated more than 1.5-fold compared with those of control group (Fig. 1). These results suggest that heavy oil exposure may suppress the immune function of the fish, especially the antibody generation and antigen presentation processes.

Additionally, PAHs, the constituents of heavy oil, suppressed both specific and non-specific immunities of some fish species (Reynaud and Deschaux, 2006). Other constituents of heavy oil, heavy metals such as cadmium, zinc and copper may induce opportunistic infectious disease by infectious pancreatic necrosis virus (IPNV), as observed in grouper *Epinephelus* sp. (Chou *et al*., 1999). From this evidence, heavy oil used in this study might have caused immune suppression in Japanese flounder. Further investigation on the causative agents of immune suppression in heavy oil used in this study should be identified.

On the other hand, the expression of beta-2 microglobulin, lysozyme and interleukin-8 (IL-8) were increased in the kidney of heavy oil-exposed flounders (data not shown). This pattern suggests that heavy oil exposure may not suppress overall immune function in flounder. Up-regulation of IL-8 might be a defensive response against bacterial infection, since bacterial counts increased in skin mucus of the fish. Chen *et al*. (2005) reported that bacterial infection induced IL-8 mRNA in the spleen of channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*). It is well known that IL-8 can stimulate chemotaxis of neutrophils,

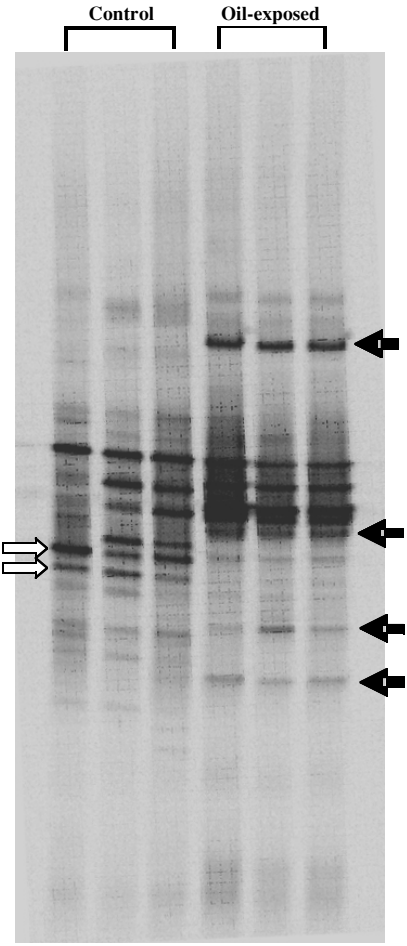

Fig. 2. DGGE profile of PCR products from microflora of skin mucus of Japanese flounder. Top of lanes indicate experimental groups. Black and white arrows show specific bands in each group.

which have a phagocytotic function. Since neutrophils produce lysozyme, increased IL-8 may attract neutrophils as a defensive response to infection, followed by up-regulation of lysozyme. From another viewpoint, since neutrophils cause cellular damage, excessively induced IL-8 might cause cell damage in the kidney of flounders exposed to heavy oil. From these data, we hypothesize that heavy oil may suppress the pathogen resistance of exposed fish, leading to bacterial infection, and that the immune system may react against the infection, but it is unclear whether this response is ultimately effective.

We compared the microflora of skin mucus between control and heavy oil exposed fish by a denaturing gradient gel electrophoresis (DGGE), since the

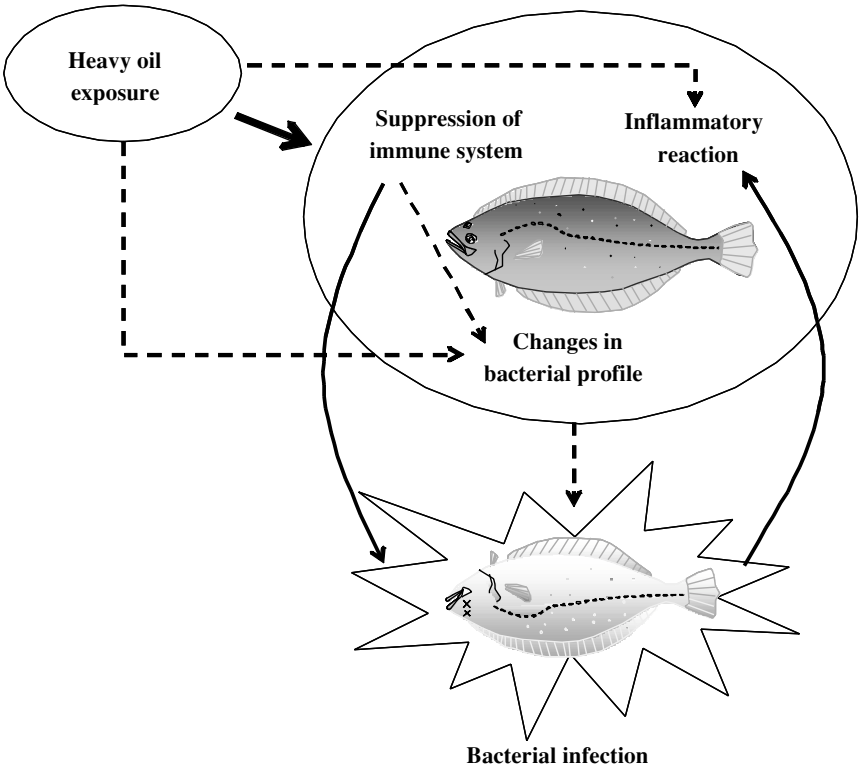

Fig. 3. Predictable scheme of heavy oil effects on Japanese flounder from viewpoint of immune system of the fish.

mucus layer plays an important role in the prevention of infectious diseases by mechanisms of competition with the microflora present in the mucus (Westerdahl *et al.*, 1991). The method is useful for separation of the same size of PCR products amplified by universal primer set, resulting in each band theoretically representing a different bacterial population in the community. From the result of DGGE, we found that bacterial profiles were different between the control and experimental group, although it was fairly similar among each group (Fig. 2). Especially, at least four bands appeared and two bands disappeared in the heavy oil exposed group compared with control group. The disappeared bands indicated bacteria susceptible to heavy oil. This is the first report of clear changes in the microflora profile in fish skin mucus after chemical exposure. It is interesting whether the disappeared bacteria due to heavy oil exposure play a role in the prevention of infectious diseases for the fish because some bacteria can produce immunostimulating, anti bacterial and anti viral substances. In addition, the identification and characterization of bacteria appearing after heavy oil exposure is needed to know whether the bacteria are pathogenic for Japanese flounder.

In this study, we investigated the toxicity of heavy oil to Japanese flounder from the viewpoint of immune response and susceptibility to bacterial disease. In conclusion, the numbers of leukocytes in peripheral blood of the fish significantly increased after heavy oil exposure. The numbers of bacteria in mucus of the exposed fish were also higher than those of control fish. The present study demonstrated transcriptome responses to heavy oil exposure in the kidney of Japanese flounder using our custom-made cDNA microarray. Immune system-related genes were mostly down-regulated by heavy oil, while IL-8 and lysozyme, which are involved in proinflammatory processes, were up-regulated. Considering the data obtained in this study, pathogen resistance may be weakened in heavy oil-exposed fish, causing a subsequent bacterial infection, and then proinflammatory genes may be induced as a defensive response against the infection (Fig. 3). Major tanker accidents have spilled tens of thousands of oil (Stagg *et al.*, 1998; Hayakawa *et al.*, 2006; Davoodi and Claireaux, 2007), which may cause extremely high concentrations of oil contamination around the accident area. Even if dispersed oil were removed from the area soon after an accident, the effects of heavy oil on fish, especially indirect effects such as infectious disease, may occur.

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