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Does heavy oil pollution induce bacterial diseases in Japanese flounder *Paralichthys olivaceus*?

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Abstract

As basic research for the effect of heavy oil on the fish immune system, in this study, the number of leukocyte was counted in Japanese flounder *Paralichthys olivaceus*, after exposure to heavy oil at a concentration of 30 g/8 L for 3 days. To compare the numbers of bacteria in the skin mucus between oil-exposed and control fish, viable bacteria were enumerated by counting colony forming unit (CFU). Compared with $5.79 \pm 1.88 \times 10^7$ leukocytes/mL in the controls, the exposed fish demonstrated higher counts, averaging $1.45 \pm 0.45 \times 10^8$ cells/mL. The bacterial numbers of control fish were $4.27 \pm 3.68 \times 10^4$ CFU/g, whereas they were $4.58 \pm 1.63 \times 10^5$ CFU/g in the exposed fish. The results suggest that immune suppression of the fish occurred due to heavy oil stressor, and bacteria could invade in the mucus, resulting in the increasing leukocyte number to prevent infectious disease.
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1. 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).

Crude oil consists of different types of components such as various hydrocarbons, nitrogen–oxygen compounds and heavy metals. Their solubility depends on octanol–water partition coefficients (Kow) of the components (Baršienė et al., 2006; Wake, 2005; Ramachandran et al., 2006). Amongst them, PAHs rank as relatively soluble, more soluble than alkanes having an equal number of carbon atoms (McAuliffe, 1987; Ramachandran et al., 2006) and are well-known to produce a myriad of lethal and sublethal effects in a wide range of marine biota (Kennedy and Farrell, 2005).

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 (Brown et al., 1996; Hose et al., 1996; Kocan et al., 1996; McGurk and Brown, 1996; Norcross et al.,

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1996; Carls, 1987; 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 *Paralichthys olivaceus*, one of 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.

2. Materials and methods

2.1. Fish

Japanese flounder *P. olivaceus* (average body weight is 197 g) were obtained from the Ehime Prefectural Chuyo Fisheries Experimental Station 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.

2.2. 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 a 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.

2.3. 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).

2.4. 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 for paired data.

2.5. 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 was diluted in sterilized PBS(−) from each fish 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 h of incubation at 20 °C, the numbers of colonies were enumerated. The data were analyzed and compared with the Student's t test for paired data.

2.6. 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, CA, 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.

2.7. 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, CA, USA). After electrophoresis, the gels were stained with SYBR® Green I (diluted 1:10,000 diluent, Cambrex Bio Science Rockland, ME, USA). Intensity of the band of the DGGE gel was analyzed using LAS-1000 (Fuji Film, Kanagawa, Japan).

3. 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. Supporting the observation of a decreasing trend in erythrocytes, microarray data showed that the expression levels of haemoglobin β chains in kidney of the fish used in this study were significantly down-regulated by heavy oil exposure (Nakayama et al., 2008). Regarding the effect of oil exposure on the haematological system in fish species, in previous studies, it has been reported that haematocrit (Ht) and blood haemoglobin concentration decreased in European flounder, *Pleuronectes flesus*, after 24 and 48 h exposure to 50% dilution of water soluble fraction (WSF) of crude oil in experimental conditions (Alkindi et al., 1996). They suggested that the phenomenon of a decline in Ht and blood haemoglobin concentration after 24 and 48 h exposure to WSF reflects erythrocyte haemolysis. The tendency of erythrocytes to decline in number after 74 h (3 days) exposure to heavy oil in this study corresponded to the results reported by Alkindi et al. (1996). In contrast, elevations and no change in Ht values were reported in rainbow trout, *Oncorhynchus mykiss*, and Pacific staghorn sculpin, *Leptocottus armatus*, after exposure to crude oil (Boese et al., 1982; Zbanyszek and Smith, 1984). The contradictory results in the number of erythrocytes may depend on the composition and concentration of oil used, and with fish size and species.

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	$3.17 \pm 0.39 \times 10^9$	$5.79 \pm 1.88 \times 10^7$

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

Contrary to the decreasing tendency of erythrocytes, the fish exposed to heavy oil 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 a 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 examine 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 (Fig. 1). We believe that the high numbers of bacteria in the exposed fish are strongly related to the increase in the number of leukocytes, i.e., immune suppression of the fish occurred due to the heavy oil stressor, and bacteria could easily attach, invade and/or grow in the mucus, resulting in the increasing leukocyte number to prevent infectious disease in the fish. In support of this hypothesis, we confirmed that the expression levels of some immune-related genes such as immunoglobulin (Ig) light chain and CD3 epsilon were down-regulated by heavy oil exposure in the fish used in

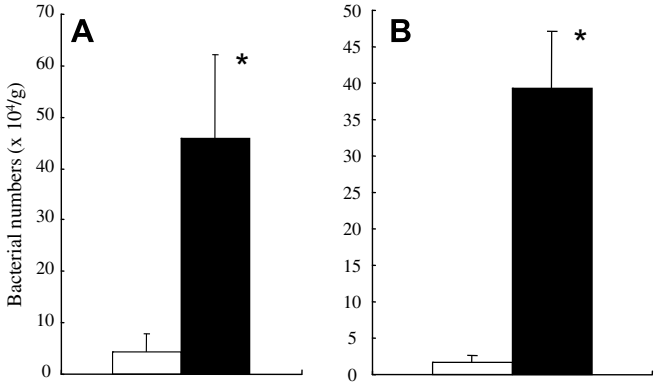

Fig. 1. Number of bacteria in the skin mucus of control (□) and oil-exposed fish (■) on marine broth 2216 (A) and brain heart infusion (B) agar. Asterisk indicates a significant difference between control and oil-exposed group at the $P < 0.05$ level.

this study by a microarray (Nakayama et al., 2008). Additionally, PAHs, the constituents of heavy oil, can have an effect on both specific and non-specific immunities of some fish species (Reynaud and Deschaux, 2006). Regarding the effects on specific immunity, in previous studies, PAHs suppressed T-lymphocyte proliferation in spot *Leiostomus xanthurus* and the antibody production against sheep red blood cells (SRBC) in chinook salmon, *Oncorhynchus tshawytscha*, (Faisal and Huggett, 1993; Arkoosh et al., 1994). On the other hand, as the effects on non-specific immunity, it was reported that non-specific cytotoxic cell (NCC) activity and macrophage phagocytic function decreased in oyster toadfish, *Opsanus tau*, exposed to 7,12-dimethylbenz[*a*]anthracene (DMBA), one of the most potent carcinogenic PAHs (Seeley and Week-Perkins, 1997). Other constituents of heavy oil, heavy metals such as cadmium, zinc and copper stressors may induce opportunistic infectious disease by infectious pancreatic necrosis virus (IPNV), as observed in grouper *Epinephelus* sp. (Chou et al., 1999). Therefore, 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.

We compared the microflora of skin mucus between control and fish exposed to heavy oil by a DGGE because the 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 disappearing 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. From the microarray data we reported elsewhere (Nakayama et al., 2008), we found that

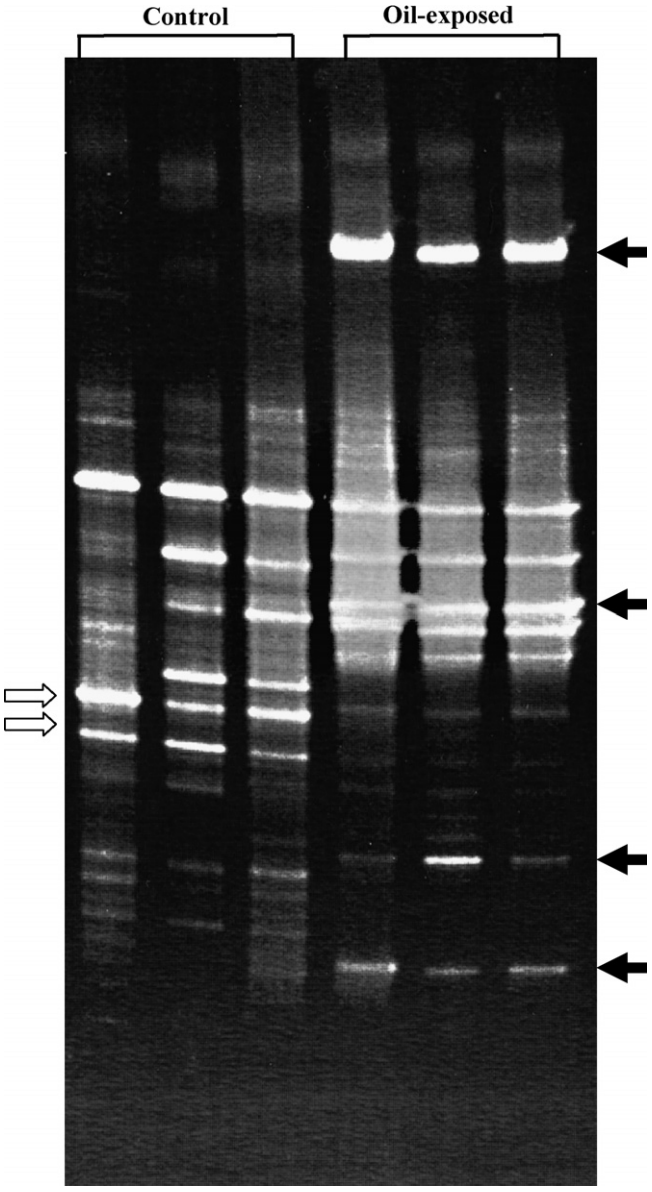

Fig. 2. DGGE profile of PCR products from microflora of skin mucus of Japanese flounder. Top of lanes indicate experimental groups. Black and white arrows show specific bands in each group.

immune-related genes were mostly down-regulated by heavy oil in the fish. Therefore, it is suggested that immune suppression of the fish occurred due to the heavy oil stressor, allowing bacteria to more easily attach, invade and/or grow in the mucus, resulting in an increasing number of leukocytes to prevent infectious disease in the fish. Since tanker accidents cause extremely high concentrations of heavy oil contamination around the accident area, the effects of heavy oil on fish, especially indirect effects such as infectious disease, may occur.

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