Effects of spilled oil from tanker "Nakhodka"and dispersant on the embrionic stage of marine fish and zooplankton

EFFECTS OF SPILLED OIL FROM TANKER "NAKHODKA" AND DISPERSANT ON THE EMBRIONIC STAGE OF MARINE FISH AND ZOOPLANKTON

Tadahisa SEIKAI[1], and Katsuhiko NAKAMURO[2]

1:Department of Marine Bioscience, Fukui Prefectural University, Obama, Fukui 917-0003, Japan; e-mail: seikai@fpu.ac.jo

2:Faculty of Pharmaceutical Science, Setsunan University, Hirakata, Osaka 573-01, Japan; e-mail:

ABSTRACT

Heavy C-oil spilled from tanker "Nakhodka" polluted seriously the long coastal line facing to Japan-Sea in 1997. Laboratory experiments were conducted to reveal the effects on the early life stages of Japanese flounder and round nose flounder. Exposure to the oil suspended seawater (OSW) at unexpectedly low concentration of oil increased the deformation and the insufficient growth of larvae. Dispersant itself without oil was quite low toxic, but became very toxic at the presence of oil. Dispersed oil particles were taken into the intestine of filter feeding zooplanktons easily, suggesting the involvement of heavy C-oil to marine ecosystem.

INTRODUCTION

Russian tanker "Nakhodka" caused serious oil spill pollution from 2nd Jan., 1997 along the wide coastal areas of Japan Sea. Because of the heavy storm in the winter season, the hull was broken into two peaces, and the stern part sank at 2500m depth around Oki Island off Shimane Prefecture, but the bow part drifted and stranded on the coast of Mikuni Town in Fukui Prefecture. More than 6,000 kl of heavy C-oil was estimated to spill to the ocean from the broken hull. The spilled oil drifted with the strong winter wind and Tsushima Current, and polluted a long shoreline from Tottori Prefecture to Niigata Prefecture (Sawa, 1998). Serious damages were afraid on the various marine organisms. In such cases, impacts of oil spill on the marine ecosystem had not been concerned except intertidal zone and restricted inshore ecosystems such as seabirds, seaweed and benthos. Thereafter, we conducted the laboratory experiments to evaluate the effects of heavy C-oil spilled from tanker "Nakhodka" on the offshore ecosystems.

MATERIALS AND METHODS

Heavy C-oil of "Nakhodka"

Heavy C-oil of "Nakhodka" which had spilled and drifted on the sea surface was collected by the research vessel "Ryokuyo-maru" of Maizuru Fisheries Research Station, Field Science Education Center, Kyoto University (Maizuru City, Kyoto) around Kanmuri Island in western Wakasa-Bay on 13^{th} Jan., 1997. Already this heavy C-oil became to be water in oil emulsion including 50% seawater. In addition to this, heavy C-oil from the bow hold of the tanker stranded on the coast of Mikuni Town was provided for the present study.

Preparation of oil suspended seawater (OSW) and determination of oil content

One gram of drifted oil was added to 500*ml* seawater in a 1 *l* Erlenmeyer glass flask, and which was shaken vigorously at 10°C for 4 days (200 cycles/min. and 3cm amplitude), assuming stormy sea surface in the winter season of this area. After the settling for 30 min., the water fraction was collected as an undiluted solution of oil suspended seawater (OSW) for the further experiments. The content of oil in OSW was determined as stated below. Suspended oil particles in seawater were observed as brilliantly shining particles smaller than several micrometers in diameter under fluorescent microscope.

Chemical analyses of heavy C-oil

Each one gram of the oil collected from the ocean and the stranded bow hold was dissolved into 50*ml* of n-Hexane, and analyzed by gas chromatograph (Hewlett Packard HP5890A, Hewlet-Packerd Japan Co. Ltd.,) equipped with FID. The oil samples were wet-digested to a transparent solution with a mixture of nitric and perchloric acids. For the determination of Cd, Cu, Zn, Ni, Mn, Pb, Cr, Fe, Sr, V, the resultant solutions were then diluted to a known volume with deionized water and directly measured by flame atomic absorption spectrophotometry. These analyses were conducted by Dr. S. Kawai and Dr. Y. Yamamoto at Kobe College (Okadayama, Nishinomiya, Hyogo).

Oil in OSW was extracted with carbon tetrachloride and the content of oil was determined after JIS K0125 25 by infrared absorption photometry at Gifu Research Center for Public Health, (Akebonomachi, Gifu City, Gifu). The volatile organic substances were analyzed after JIS K0125-5.2 by GC(HP5890-II, Hewlett-Packard Japan Co. Ltd.) and MS(HP5971A, Hewlett-Packard Japan Co. Ltd.). The polycyclic aromatic hydrocarbons (PAHs) were extracted with dichloromethane, and determined by GC (Finnigan-Mat 9001, Thermo Electron Corp. Co.Ltd., Germany) / MS (Finnigan-Mat GCQ, Thermo Electron Corp. Co.Ltd., Germany).

Hatching experiments of marine fish embryos in OSW

Fertilized eggs of Japanese flounder *Paralichthys olivaceus* and round nose flounder *Eopsetta grigorjewi*, spawned naturally in captivity, were provided from Miyazu Station of Japan Sea Farming Association (Odasyukuno, Miyazu City, Kyoto) for the following hatching experiments. Undiluted solution of OSW was prepared just before each hatching experiment, and then diluted to make graded concentrations by filtrated seawater. Twenty live eggs of Japanese flounder and thirty live eggs of round nose flounder (about 24 hrs. after fertilization) were incubated in 100ml of diluted OSW with graded concentrations for 48 hrs. at 20°C and 15°C, respectively. Each of graded concentrations of diluted OSW was duplicated and triplicated for Japanese flounder and round nose flounder, respectively. Almost all of eggs in the series of diluted OSW hatched out after 24 hrs. from the start of incubation, thereafter embryos might be exposed to these solutions during the late developmental stages of egg for 24 hrs. and then during yolk sac larval stage for 24 hrs. At the end of incubation, all of unhatched eggs, live and dead larvae were counted and fixed in 5% formalin for the further study, then the dead egg rate, the hatching rate and the deformation rate of hatched out larvae were determined.

Hatching experiment in the seawater from oil polluted site

Hatching experiment in the seawater from oil-polluted site was conducted together with the former experiment of round nose flounder. On 12th and 14th Feb., 1997, the seawater was collected from the gravel shore in Mikuni Town where the bow had stranded and the coastal area had been heavily polluted. The climate condition there on 12th was so stormy that the water sampling was dangerous. Oil film spread over the surface of coastal water, and oil droplets were visible to the naked eye in the sampled water. On the other hand, the climate condition on 14th was extremely calm, and the sampled water looked clean. Five times and two times dilutions of the water on 12th were also examined because of the foreboding of the high contents of oil in the water. At the end of experiment, samples were treated as the former experiments.

Effects of dispersant with oil

Sprinkling of the dispersants to the spilled crude oil or heavy oil is popularly conducted to disperse oil film and enhance microbial or physical degradation. Because the former hatching experiments revealed the lethal and sublethal effects of suspended oil droplets on the early life stages of the two species, the effects of the dispersant with and without heavy C-oil were examined on the early life stages of fish. Heavy C-oil for this experiment was collected from the bow hold of “Nakhodka”. The dispersant used for the present study was Neos AB-3000 (Neos Co. Ltd., Kobe) which had the certificate of the Ministry of Transport and was mostly used for this oil spill accident.

Stock solution of dispersant only (0.5*ml*/1*l* seawater) and dispersant and heavy C-oil mix solution(0.5*ml* of dispersant and 2g of heavy C-oil/1*l* seawater) were prepared just prior to this experiment. The ratio between heavy C-oil and dispersant was the recommended ratio by the company. These two stock solutions were diluted by clean seawater to make graded concentrations. Each thirty fertilized eggs of round nose flounder (about 24 hrs. after fertilization) were incubated at 15°C in these 100*ml* seawater solutions with graded concentrations for 48 hrs. Each graded concentration of the solutions was not duplicated. Almost all of eggs in the experimental lots were expected to hatch out after 24 hrs. from the start of experiment, thereafter the embryos might be exposed to these solutions during late developmental stages of eggs for 24 hrs. and then during newly hatched larval stage for 24 hrs. At the end of experiment, samples were treated as the former experiments.

Effects of heavy C-oil and dispersant on the size of newly hatched fish larvae

At the end of each hatching experiment, larvae were fixed in 5% formalin, and preserved for about 1 month to complete the shrinkage by fixation, and larvae without deformation were measured their sizes in total length under Profile Projector (V-10, Nikon Co. Ltd.). For this measurement, only larvae fixed straight were used.

Observation of oil intake by newly hatched fish larvae and zooplanktons

Yolk sac larvae of Japanese flounder at 24 hours after hatching, which did not open their mouths, were immersed in the OSW for 2-3 hours, and then observed under fluorescent microscope to detect oil particles on their skin. Wild zooplankton Copepoda *Paracalanus* sp., were collected by plankton net (opening;150um) from Maizuru Bay. Rotifers *Brachionus plicatilis, which* were maintained in the culture tank at the laboratory of the station, and *Artemia* sp. nauplii, which were hatched out from the resting eggs. These zooplanktons were also immersed in OSW for 3 hrs., and then observed under fluorescent microscope to confirm the direct intake of oil particles into their intestines.

RESULTS

Characterization of spilled oil from tanker "Nakhodka"

The gas chromatogram of heavy C-oil from Nakhodka showed 270 peaks, suggesting the variety of hydrocarbons in the oil. The oil contents of OSW was determined as 3.2mg/*l*(corresponding to 3.2ppm) as the extract by carbon tetrachloride. As the volatile organic substances, *less than* 0.001mg/*l* of B*enzene, less than* 0.06mg/*l* of Toluene, and 0.04mg/*l* of Xylene were detected. Concentrations of PAHs concentrations in OSW (ug/liter) are shown in Table 1. Fluorene, Phenanthrene, Anthracene, Pyrene, Benz[a]anthracene, Chrysene, Dibenz[a,h]anthracene were detected in the certain revels.

Heavy metal concentrations in heavy C-oil sampled from the stranded bow hold and Wakasa Bay are shown in Table 2. However almost all of the heavy metal concentrations in spilled oil were less than those in the bow hold oil except Strontium and Vanadium, the unexpectedly hazardous revels were not detected in both of them.

Table 1 Concentration of Polycyclic Aromatic Hydrocarbons (PAHs) in OSW (ug/liter)

PAHs (ug/liter)	concentration
Fluorene	1.30
Phenanthrene	2.20
Anthracene	0.23
Pyrene	0.48
Benz[a]anthracene	0.30
Chrysene	0.77
Dibenz[a,h]anthracene	0.14

Table 2 Heavy metal concentration in heavy C-oil from tanker Nakhodka (ppm)

Metals	Oil from bow hold	Oil from ocean
Cd	0.139	0.023
Cu	0.570	0.070
Zn	2.220	0.230
Ni	2.960	2.210
Mn	1.500	N.D.
Pb	0.570	0.230
Cr	0.490	0.093
Fe	455.300	13.700
Sr	1.610	2.320
V	4.220	4.770

ND:not detected

Development of marine fish embryos in OSW

In Japanese flounder embryos, certain effects of OSW were detected from the 0.25 % of stock solution (corresponding to 8ppb of oil in seawater) as a slight increase of

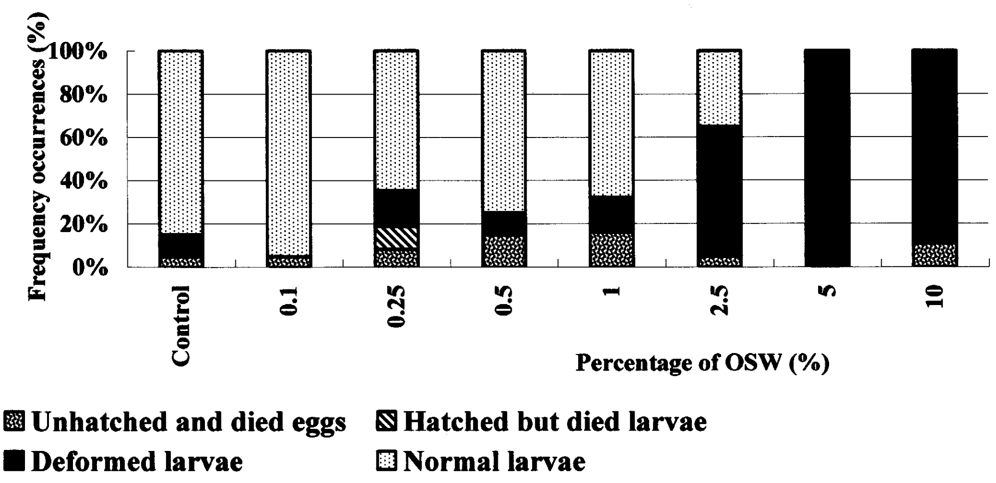

Fig.1. Effects of OSW on the early life stages of Japanese flounder *Paralichthys olivaceus* after the exposure to diluted OSW for 48hrs. during late egg and newly hatched larval stages.

unhatched eggs hatched but died larvae, and deformed larvae. But 2.5% solution (corresponding to 80ppb of oil in seawater) showed a remarkable increase of deformed larvae (Fig. 1).

In round nose flounder embryos, deformation of hatched out larvae increased remarkably from 0.25% of OSW(corresponding to 8ppb of oil in seawater) (Fig.2), and almost all of hatched out larvae deformed at 1% of OSW (corresponding to 32ppb of oil in seawater). Deformed larvae showed undeveloped tail fin fold, unusual swelling in the former part of abdominal fin fold, and bending of notochord to upward or sideways (Fig.3).

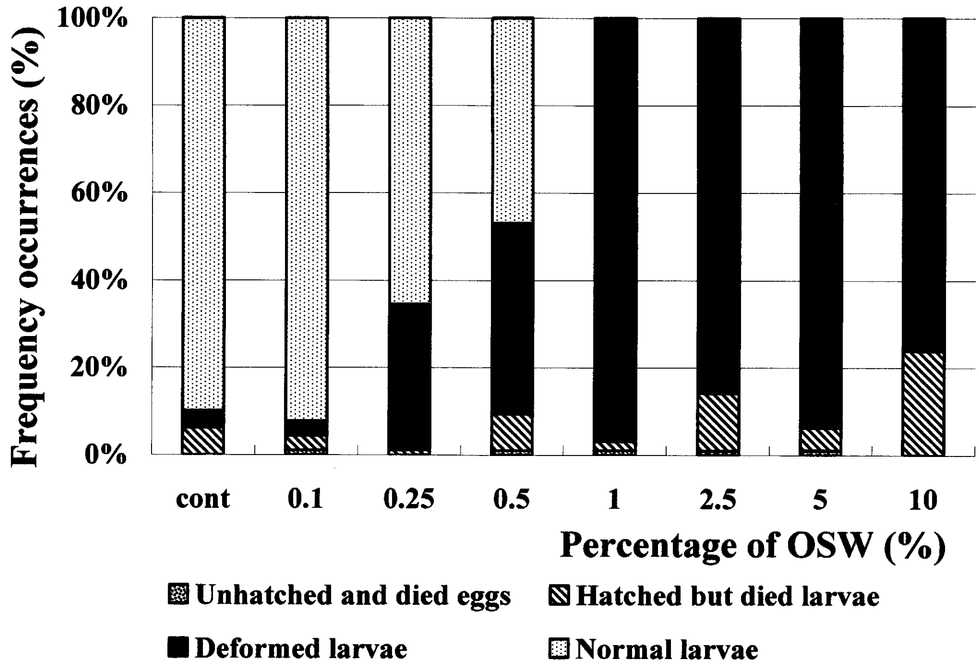

Fig.2. Effects of OSW on the early life stages of round nose flounder *Eopsetta grigorjewi* after the exposure to diluted OSW for 48hrs. during late egg and larval stages.

Effects the seawater from oil polluted site

The effects of the surface water collected on 12th and 14th, Feb. from oil polluted site in Mikuni Town were quite different on the early life stages of round nose flounder (Fig.4). The water on 14th showed the intermediate effects between 0.1% and 0.25% of

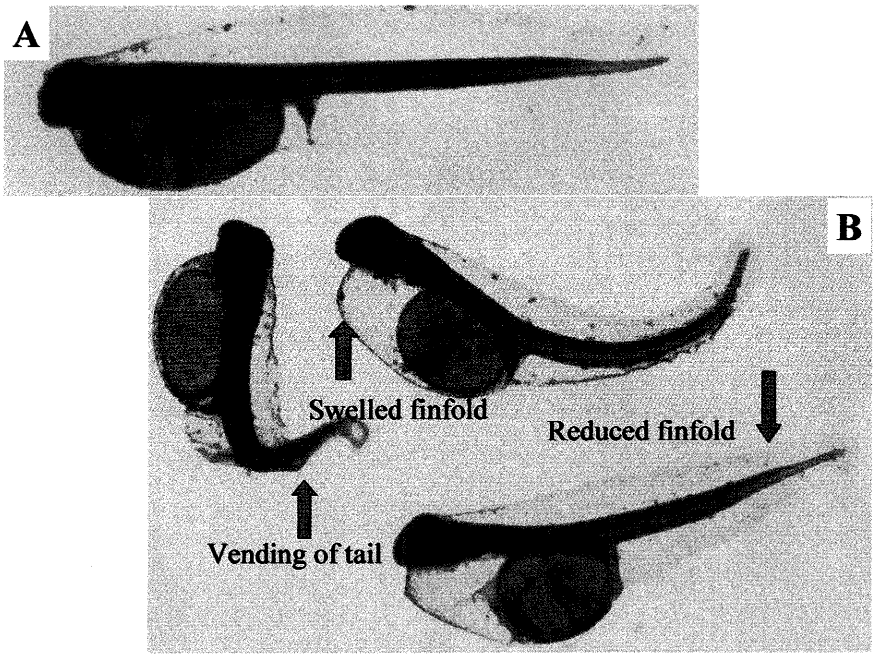

Fig.3. Deformed larvae of round nose flounder *Eopsetta grigorjewi.* Upper A shows a normally developed larva in clean seawater, and B shows deformed larvae after the exposure to 2.5% of OSW (corresponding to 80ppb of oil) for 48hrs. during egg and newly hatched larval stages.

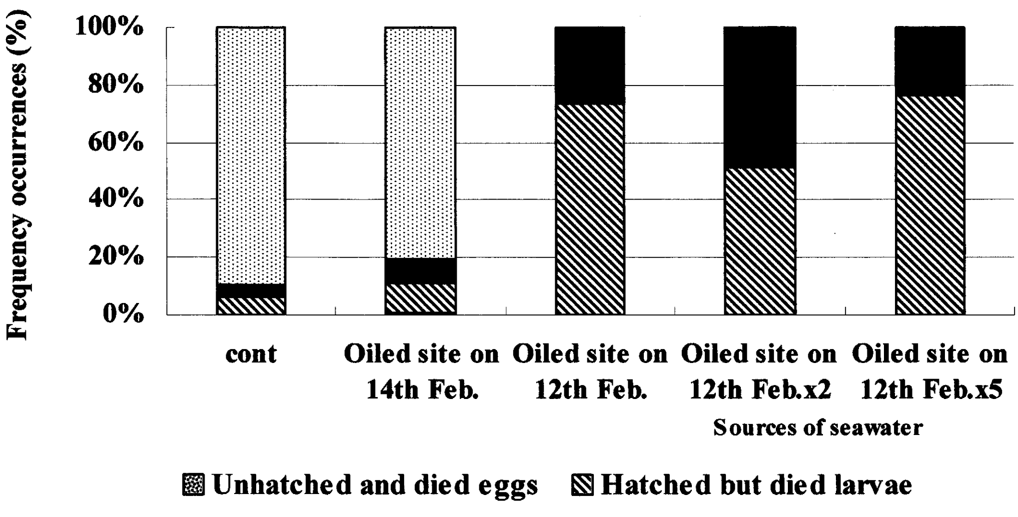

Fig.4. Effects of oiled-site seawater, collected from the same place of the heavily polluted gravel coast in Mikuni Town on 12th and 14th Feb., 1997. The surface conditions of these two days were quite different, 12th and 14th were stormy and calm, respectively. The water on 12th, Feb. was examined with no dilution, 2 times dilution, and 5 times dilution, respectively.

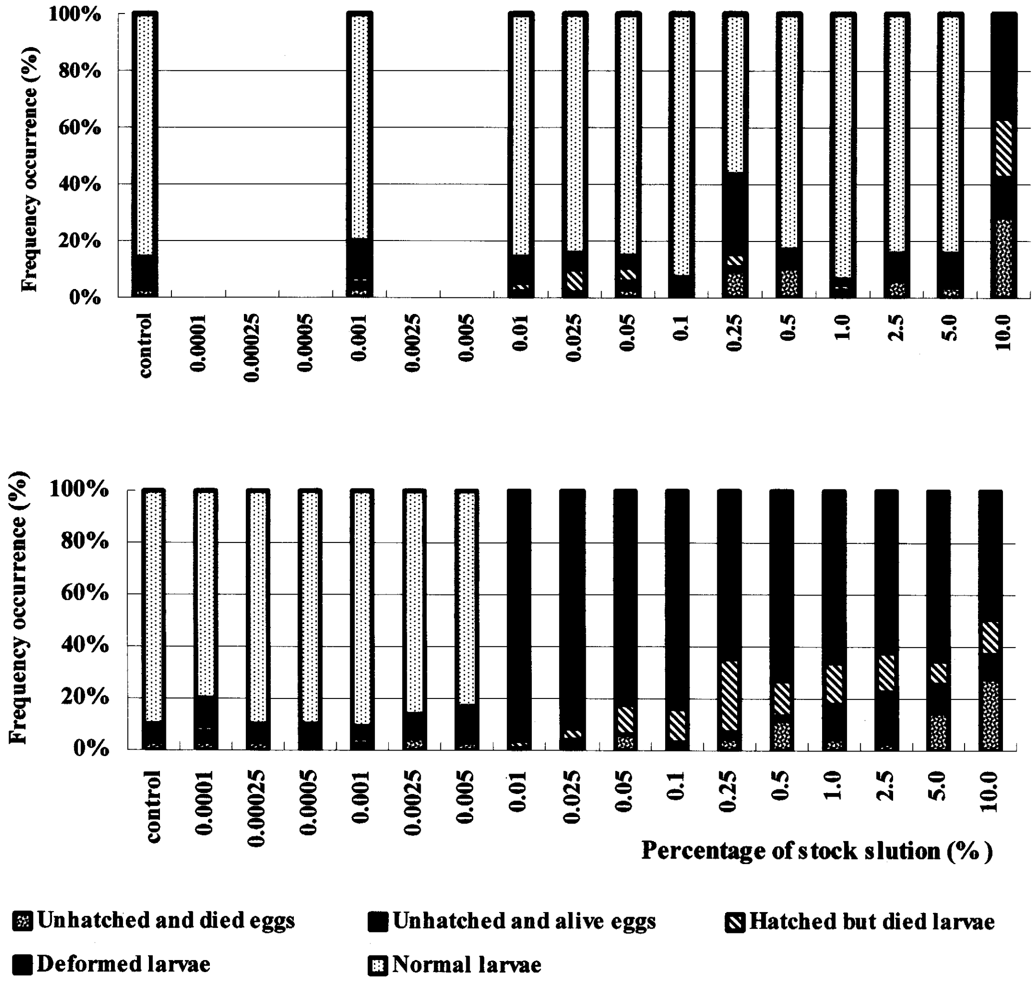

Fig.5. Effects of dispersant (upper) and dispersant + heavy C-oil (lower) on the early life stages of round nose flounder. *Eopsetta grigorjewi* after the exposure for 48hrs. during egg and larval stages.

OSW (corresponding to 3.2ppb and 8ppb of oil in seawater), but the water on 12th showed a extremely stronger effect on the early life stages than 10% of OSW (320ppb of oil in sea water). Even in 5 times dilution of the seawater, 76.5% of larvae were expected to die immediately after hatching and the rest of survived larvae deformed.

Effects of dispersant with oil

The toxicity of dispersant only was quite moderate, because only 10% of stock solution of dispersant (corresponding to 50ppm of dispersant in seawater) increased the harmful effects on the early life stages of round nose flounder (Fig.5). Mix solutions of

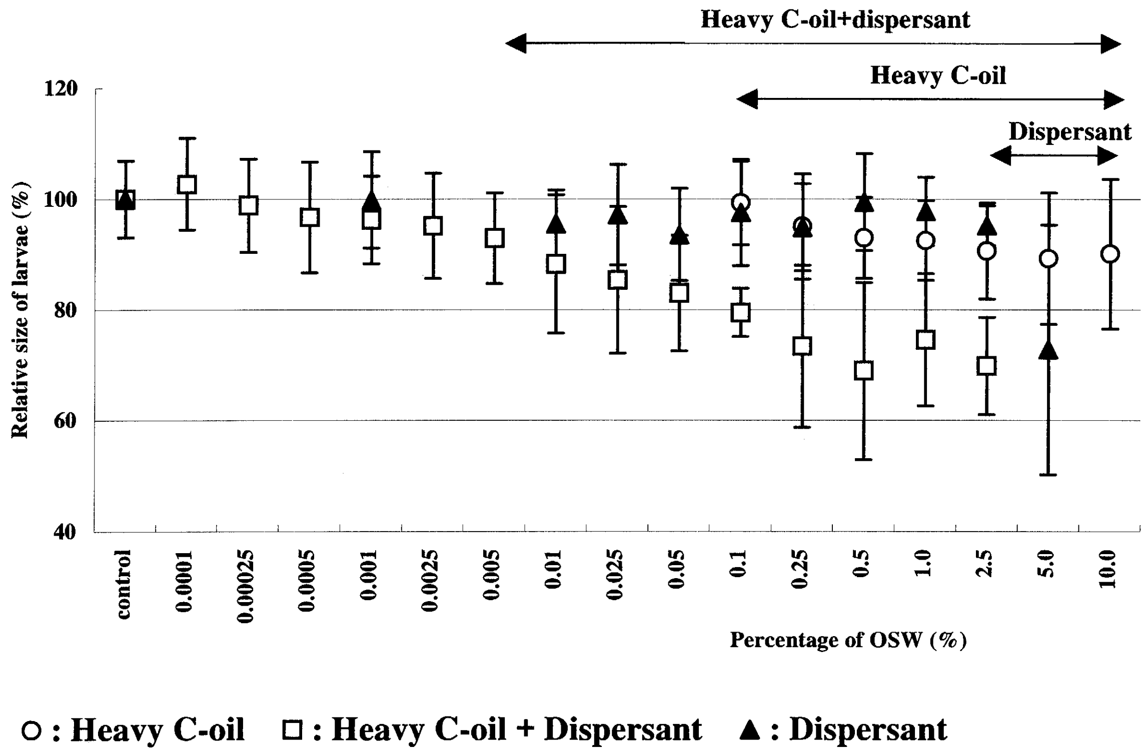

Fig. 6. Effects of oil, dispersant and their mixture on the newly hatched larval size of round nose flounder *Eopsetta grigorjewi* after the exposure of their dispersant in seawater. Arrows show the ranges of significant differences in larval length from that of control group.

dispersant and heavy C-oil showed very serious effects on the early life stages of round nose flounder at lower concentrations. In 0.01% of stock solution (corresponding to 50ppb of dispersant and 200 ppb of heavy C-oil) almost all of larvae deformed after hatching.

Effects of oil, dispersant and their mixture on the larval size after hatching

The sizes of newly hatched larvae exposed to these materials were more sensitive indicators than the deformation. As shown in Fig.6, significant differences in larval length ($p<0.01$) from the control group were detected at lower concentrations of these materials in the seawater than the changes of hatching rate or deformation rate.

Observation of oil intake by fish larvae and zooplankton

Fluorescent microscopic observation of newly hatched fish larvae exposed to OSW for 2-3 hrs. revealed that the small particles of oil attached on the surface of larval skin and fin fold, and moreover larval body emitted week fluorescence later (photograph was

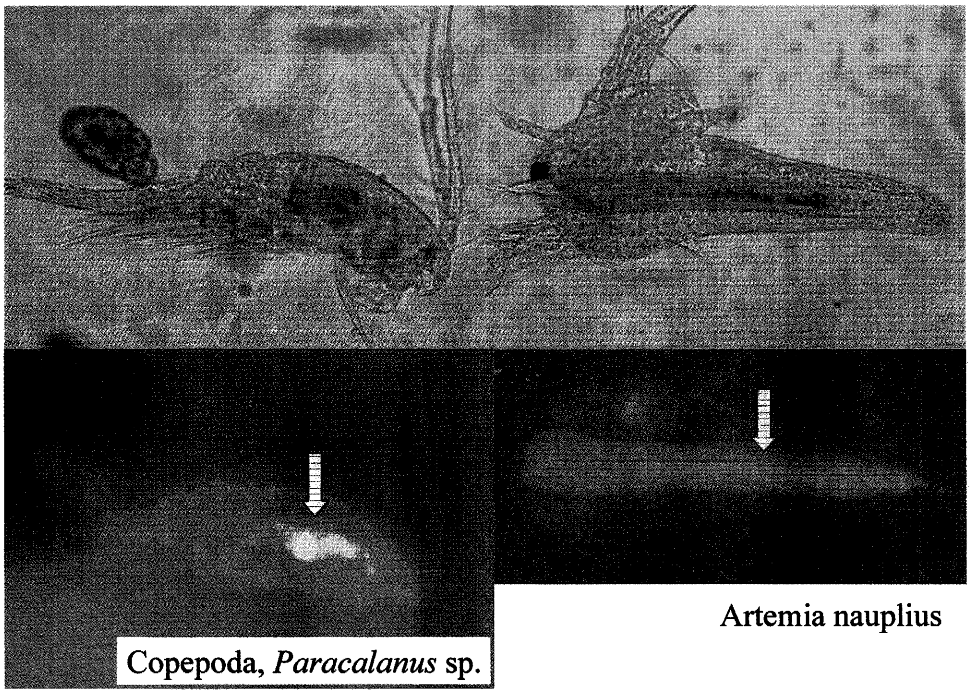

Fig.7. Oil particles intake by copepada, *Paracalanus* sp. (left), and *Artemia* sp. Nauplii (right). Dispersed oil particles were taken into the digestive tract of copepoda and *Artemia*. These particles were observed as the brilliant particles under fluorescent microscope (red arrows).

not shown). These phenomena suggested the intake of oil particles into the larval body through skin surface. Wild zooplankton copepoda *Paracalanus* sp. was immersed in the OSW for 3 hrs. (Fig.7). Then several oil droplets were observed in the digestive tract under fluorescent microscope. Also rotifers *Brachionus plicatilis* and *Artemia* salina nauplii had taken oil droplets into their digestive tracts(Fig. 7).

DISCUSSION

Characterization and the fate of spilled heavy C-oil from tanker "Nakhodka"

As heavy C-oil is the residue from dry distillation of crude oil, and consists of heavy fraction mainly, spilled oil is difficult to evaporate and becomes sticky water-in-oil emulsion easily. Moreover, spilled heavy C-oil is toxic chemicals because of the large contents of polycyclic aromatic hydrocarbons (PAHs). In this case, a certain amount of antifreeze was added to the heavy C-oil to prevent hardening because this oil should be used in the Far East Russia during cold winter season. Therefore the oil did not harden at 1°C sunken on the ocean floor of the 2500m depth around Oki Island, and continued to leak from the stern hold.

Effects of dispersed oil in seawater on the early life stage of fish

In the present study, the ratios of unhatched eggs were lower than 1.2% within the examined rage of oil concentrations. Is this revel of oil concentration reliable in the ocean at tanker accident? Higher than 50% mortality of eggs in sardine *Sardinella aurita* and herring *Clupea harengus* collected from the polluted sites of Torrey Canyon oil spill accident in 1967 (Nelson-Smith, 1977) suggests the much higher concentration of oil in the ocean than the examined revels at the present study. Higher concentrations of oil (several to 20 ppm) were detected from relatively wide areas at Julianna oil spill accident off Niigata in 1971 (Jap. Fish. Resource Cons. Associ., 1973). Regarding to the present accident, relating prefectures and Fisheries Agency conducted field surveys to detect the range of oil from ND to 104ppb in Wakasa Bay and surrounding areas (Ikeda, et al., 1998), where 60-80% anomalies were found in collected eggs maximumly (Minami and Nishida, 1998).

In the present study, hatching experiment in the seawater from oiled-site was conducted together with the hatching experiment of round nose flounder in diluted OSW. Depending on the surface conditions of the sea, effects of the seawater were quite different. If we consider the dilution rate, the concentration of oil in the seawater on 12th from the oiled-site was estimated to be higher than 1.6ppm. Another day's seawater (14th) corresponded to 3.2-8.0 ppb. These results are suggesting that the surface condition along the shore line is changing every moment, and surprisingly large amounts of oil must be diffused into the seawater under stormy conditions.

Before Exxon Valdez oil spill accident, few scientists had considered the effects of spilled oil on the early life stages of marine fishes. Because the accident occurred in the

Prince William Sound where huge number of the Pacific herring, *Clupea pallasi* migrated and started to spawn, the effects of spilled oil on the herring have been investigated from various points. As herring spawns demersal eggs on the coastal area, being close to the shore line, serious effects were worried on the early life stages of this species. Indeed, chromosome aberration (Brown, et al., 1996), delayed hatching and high percentage occurrence of deformation in newly hatched larvae (Kocan, et al., 1996a), abnormal cell proliferation in pectoral fin (Kocan, et al., 1996a), abnormal differentiation of eye during larval stages (Hose, et al., 1996) were observed in oil-water dispersion (OWD) under experimental conditions. Also field surveys revealed the deformation and delayed growth of herring larvae in the polluted areas (Norcross, et al., 1996), remaining affects on the reproduction in 3 years later (Kocan, et al., 1996b), and accumulation of PAHs in the ovary because of the exposure to the weathered oil (Carls, et al., 2000). Also, pink salmon *Onchorhnchus gorbusha* which is the target species of stock enhancement around polluted site was examined about the growth and survival rate in the ocean after egg incubation in extremely low concentration of OWD during embryonic period (Heintz, et al., 2000), and stray of homing migration was found during oceanic phase (Wertheimer, et al., 2000).

In the present study, examined eggs were pelagic, and their spawning grounds are much offshore than that of the herring. Pelagic eggs hatch out earlier at underdeveloped condition than demersal eggs. The past field surveys frequently reported higher concentration than the effective dose revel of heavy C-oil on the early life stages of these two species in the present study. Thereafter, the effects of oil spill accidents on the species, which spawn pelagic eggs offshore, also can not be ignored.

0.25% dilution of OSW, corresponding to 8ppb of C heavy oil in seawater, started to increase the morphological abnormality during the early life stages of Japanese flounder and round nose flounder. This concentration is much lower than the detectable level of officially authorized method. Such pelagic eggs must be quite useful tools to evaluate the urgent effects on the marine ecosystem as experimental materials, because of the shorter hatching period than demersal eggs, and the higher sensitivity. The present study also showed the significant size reduction of larvae at the end point of hatching experiments at lower concentration of pollutants than the significant increase of deformed larvae. As the reasons of such insufficient growth exposed to the pollutant are still unknown, further studies should be needed to reveal the mechanism.

Dispersants strengthen the toxicity of oil

The present study showed the harmful effects of suspended particles of spilled oil on the early life stages of Japanese flounder and round nose flounder. On the other hands, dispersants are popularly sprayed on the spilled oil to disperse into seawater. From the lesson of the past oil spill accidents, the dispersants have been improved to show the extremely lower toxicity on the aquatic organisms than the past ones. It has been believed that oil dispersion enhances the bacterial and physical degradation. The effects of dispersants on the early life stages of fishes were examined with and without spilled oil.

The dispersant used in the present study was one of the most popular one in Japan, and applied for this accident. Fifty ppm of dispersant in the seawater only showed the harmful effects on the early life stages of round nose flounder, but the toxicity level on the round nose flounder embryos was two digit numbers sensitive than that on the medaka *Oryzias latipe* which is the popularly used fresh water fish for toxicology. Even if the toxicity of dispersants without oil is extremely low, dispersants are used to disperse oil into seawater as small particles at the oil spill accidents. The mixture of oil and dispersant, 0.01% of stock solution (corresponding to 50ppb dispersant and 200ppb heavy C-oil) increased the appearance of deformed larvae drastically. It was suggested that dispersed oil droplets formed by the dispersant showed harmful effects on the early development of round nose flounder.

Laboratory experiment of spot (*Leiostomus xanthurus*) eggs revealed that the mortality was greater when eggs were exposed to oil associated seawater (OSW) with dispersant than OSW only (Slade, 1982). Similar tendencies were reported on *Barbus* sp. and *Clarias* sp. (Akintonwa and Ebere, 1990), and on common sea bass (*Lateolabrax japonicus*), Japanese parrotfish (*Oplegnathus fasciatus*) and also Japanese flounder (Mori. et al., 1983).

It is still uncertain how and why dispersants enhance the toxicity. The primary mechanism by which dispersants harm organisms may be the decreased surface tension at the tissue/water interface, accompanied by impairment of osmotic and ionic regulation, but the synergistic effect may also be due to enhanced biological accessibility and penetrating power of hydrocarbons in the presence of dispersants (Brown, 1990). Ironically, high performance of the present dispersants increased the toxicity of spilled oil on the marine organisms. Thereafter, from the viewpoints of acute toxicity, the use of the dispersants in the coastal areas should have a strict limitation, especially in the closed inland sea. If the dispersants do not contribute on the bacterial degradation of spilled oil, the use of dispersants at oil spill accidents will lose the theoretical background.

Impact on the ecosystem through oil intake by fish larvae and zooplankton

Spilled oil is degraded physically and biologically. Through the weathering process, it must affect the various aspects in marine life. Especially a part of heavy fraction including PAHs becomes small particles in seawater according to the wind and waves, and the sprayed dispersants sometimes. This process was confirmed by the preparation of OSW in the present study. The oil in OSW contained a certain amounts of PAHs. Newly hatched larvae were suggested to take the small oil particles through their skin, even when they did not open their mouths. Zooplankton; rotifers, *Artemia* nauplii and wild copepoda took oil droplets through their mouth like as their foods, because they are filter feeder. As the most of zooplanktons are important food items for fish larvae, ingredients of oil must be easily involved into the food web of marine ecosystem. Fat-soluble substances including PAHs will be concentrated through food web. PAHs have strong carcinogenicity and teratogenicity, and are relatively stable in the environment. Moreover, PAHs has revealed the photoenhanced toxicity (Barron and Aifue, 2001), long term influences should be concerned in the marine ecosystem. It must be one of the important subjects how to estimate the fate of spilled oil involved into the feed web by reasonable model (Jin, et al., 2001).

CONCLUSION

Spilled heavy C-oil from tanker "Nakhodka" were dispersed as small particles by the vigorous shake in the seawater (OSW), whose concentration was 3.2mg/l. Hatching experiments of Japanese flounder and round nose flounder were conducted in diluted OSW. 8ppb of oil in seawater increased deformation of larvae in these two species. The dispersant itself was not so toxic but the mixture with oil enhanced the toxicity extremely on the early life stages of these species, because the dispersant promoted the dispersion of oil into seawater. The effects on the larval size were more sensitive than the deformation in newly hatched larvae. As dispersed oil particles were easily taken into the intestine of zooplankton, which were the main food items for marine fish larvae and juveniles, we should consider the effects of PAHs on the marine ecosystem through food web.

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