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Effects of ultraviolet radiation on the toxicity of water-accommodated fraction and chemically enhanced water-accommodated fraction of *Hebei Spirit* crude oil to the embryonic development of the Manila clam, *Ruditapes philippinarum*

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ABSTRACT

The purpose of this study is to evaluate the effects of oil dispersant and ultraviolet (UV) radiation on the toxicity of crude oil. The toxicity of water-accommodated fraction (WAF) and chemically enhanced water-accommodated fraction (CEWAF) of *Hebei Spirit* crude oil was investigated in the embryo of the Manila clam, *Ruditapes philippinarum* with- and without ultraviolet radiation. The WAF and CEWAF with- and without UV radiation affected significantly the embryonic development of *R. philippinarum*. The EC50s of WAF without UV, WAF with UV, CEWAF without UV, and CEWAF with UV were 2.82, 0.79, 1.60, and 0.45 g/L, respectively. CEWAF was 1.6 times more toxic than WAF. UV radiation increased crude oil toxicity to 3.6 times for both WAF and CEWAF. The oil dispersant and UV radiation did not affect the acute toxicity to the embryo but retarded the period of embryonic development up to 26%. *R. philippinarum* proved to be a sensitive species to reflect the toxic effects of oil spill combined with oil dispersant and UV radiation. It is suggested that the chemical analyses on the WAF and CEWAF is important for the identification and quantitative explanation of the phototoxic compounds in crude oil.

Key words: *Ruditapes philippinarum*, developmental toxicity, *Hebei Spirit* oil spill, ultraviolet radiation, water-accommodated fraction (WAF), chemically enhanced water-accommodated fraction (CEWAF)

Introduction

Crude oil is a complex mixture of organic compounds. The composition of spilled oil is different with the source, type of oil, and changes during weathering (Hostettler *et al*., 1992; Volkman *et al*., 1992). Since oil released into the aquatic environments contains vast kinds of toxic chemicals, analytical methods for oil constituents are limited to only parts of these chemicals, and its composition is changed by weathering process, it is quite difficult to assess and predict the effects of oil spill on aquatic ecosystems. Polycyclic aromatic hydrocarbons (PAHs) are major constituents of crude oil with high adverse biological effects (Wang *et al*., 2003) and have phototoxicity, of which the toxicity is enhanced by ultraviolet (UV) radiation (Arfsten *et al*., 1996). In the natural conditions, spilled oil is always exposed to sunlight which contains strong intensity of UV radiation attributing up to 10% of total solar energy. The modes of toxic actions in phototoxicity of PAHs vary with the chemical composition, target organisms of concern, level of biological organization, and presence/absence of oil dispersants. Oil dispersant is a

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mixture of surfactants and solvents that change the spilled oil into tiny droplets. In past, dispersants had adverse effects on ecosystems, but recently developed dispersants contains less toxic effects and accepted as safely applicable. In spite of low toxicity of oil dispersant, it changes the reactivity of hydrocarbons and increases the bioavailability of oil constituents to aquatic organisms so that the toxicity of spilled oil can be increased (Ramachandran *et al*., 2004; Schein *et al*., 2009; Wu *et al*., 2012). Therefore, the effects of UV radiation and oil dispersant must be considered in the assessment of ecotoxicity of spilled oil to aquatic organisms.

Hebei Spirit oil spill, occurred near the Taean County, west coast of Korea on December 7, 2007, released about 10,900 tonnes of crude oil and affected about 375 kilometers of shoreline along the west coast of Korean Peninsula. At least 30 beaches near the spill point were covered by oils and more than half of the sea farms lose their stocks. Many of beautiful beaches within the National Park were saturated by the spilled oil only 3 days after the accident. The government declared a state of disaster in the affected region. Cleanup campaign was activated by more than million volunteers, and scientific monitoring program and researches were organized. Thereafter, studies related to *Hebei Spirit* oil spill were performed on the chemical and biological changes in seawater and fishes (Kim *et al*., 2010; Jung *et al*., 2011; Jung *et al*., 2012; Kim *et al*., 2013), and genotoxicity, endocrine-effects, and aryl hydrocarbon receptor-mediated activity in heavily contaminated sediments (Ji *et al*., 2011; Hong *et al*., 2012). However, the combined effects of UV radiation and oil dispersant on the aquatic toxicity of *Hebei Spirit* crude oil have not been evaluated so far.

There is a limitation in representing concentration-response relationship between the amount of crude oil and the biological effect, because only the part of crude oil fraction that enters the water column has the potential to reveal toxicity, while majority of spilled oil remains as a layer above the water column. To solve this problem for practical purpose, the 'Chemical Response to Oil Spills Ecological Effects Research Forum' (CROSERF) specified the technical terms of 'water-accommodated fraction' (WAF) as a laboratory-prepared medium derived from low energy mixing of oil (Aurand and Coelho, 1996), and 'chemically enhanced water-accommodated fraction' (CEWAF) as a laboratory-prepared medium derived from the standard 20-25% vortex mixing of oil and dispersant (Aurand and Coelho, 1996). Singer *et al*. (2000) suggested the standard methods for effectively evaluate the toxicity of WAF and CEWAF capable of simulating the oil spill in wild conditions. Although there were several studies on the toxicity of WAF and CEWAF on aquatic organisms (Adams *et al*., 1999; Gulec and Holdway, 2000; Couillard *et al*. 2004; Koyama and Kakuno, 2004; Fuller *et al*., 2009; Wu *et al*., 2012), phototoxicity of oil and oil compounds (Farwell *et al*., 2006), and the combined effects of UV radiation and dispersant on toxicity of oil (Barron *et al*., 2003), most of which focused with the early life stage of fishes and shrimps. Researches pertaining to bivalve species are limited (Pelletier *et al*., 1997; Saco-Álvarez *et al*., 2008). Since, the embryo and larvae of bivalves are one of the most sensitive groups to environmental contaminants, they are more vulnerable than fishes or shrimps when oil spill occur. To this end, the purpose of present study was established to know the effects of WAF and CEWAF prepared from *Hebei Spirit* crude oil on the embryonic development of the Manila clam, *Ruditapes philippinarum* under presence and absence of UV radiation. The results from this study will provide useful information to understand the potential effects of oil spills and oil dispersants on pelagic ecosystems under natural conditions.

Materials and Methods

Adults of *Ruditapes philippinarum* were collected from a tidal flat in Yeoungheung Island, Incheon, west coast of Korea (37° 13′ 53″ N, 126° 27′ 40″ E) in June 2009. Twenty apparently most healthy individuals were selected and shells were rinsed 3 times with freshly filtered (1-μm) seawater (FSW, 32 psu) and acclimated at 20℃ for 48 hours without feeding. After acclimation, spawning was induced by exposing in air for 1 hour followed by transferring

into FSW at 25℃. *R. philippinarum* began spawning within 60 minutes. Fertilized eggs were collected on a 40-㎛ nylon mesh screen and rinsed 3 times with FSW. The density of embryos was adjusted to 5,000 embryos/mL and the suspension was kept at the experimental temperature (20℃) before use. Experiments began within 30 min after the preparation of embryo suspension.

Samples of *Hebei Spirit* crude oil and oil dispersant (HI-CLEAN, Daeil Chemical Industry, Busan, Korea) were provided from Korea Institute of Ocean Science and Technology (KIOST). The WAF and the CEWAF of *Hebei Spirit* crude oil were prepared in a 4-L glass jar according to Singer *et al*. (2000). Jar was partially filled with 2 L of FSW into which Teflon-coated magnetic stirring bar was added. Twenty g of crude oil was carefully injected onto the surface of FSW. For CEWAF preparation, additional 2 mL of oil dispersant was injected to the crude oil surface. After injecting oil and dispersant, jar was sealed by a Teflon-lined cap and sample was mixed on a stirrer with vortex depth being 20-25% of water depth at 20℃. The rpm was 190 for WAF and 180 for CEWAF. Jars were covered completely by aluminum foil to prevent light from penetrating into the jar. For WAF, mixing was stopped after 24 hours. After another 10 minutes for the vortex to become disappear, the lower layer of seawater fraction was siphoned off into an amber glass bottle. For CEWAF, mixing was stopped after 18 hours, stabilized for additional 6 hours, and then the lower layer was siphoned off into an amber glass bottle. Test water was prepared by diluting WAF and CEWAF with FSW to concentrations of 100, 50, 25, 12.5, and 6.25% according to recommendation from USEPA (2002), which are equivalent to the crude oil loading of 10, 5, 2.5, 1.25, and 0.63 g/L, respectively.

Embryo toxicity test was carried out according to Sung *et al*. (2006) with minor modification on test temperature (20℃ instead of 15℃). Five mL of each test solution was transferred to triplicate 20-mL glass scintillation vial (Wheaton), 100 ㎕ of embryo stock was injected into each vial, capped tightly, and placed in an incubator at 20℃ for 48 hours with a photoperiod of 16h light : 8h dark. Test vials were illuminated by the Oslam T5 cool white fluorescent lamp (FH 21 W/840 HE, Germany) for experiment without UV radiation, or the Sankyo Denki UV lamps (FL20SBL for UVA, G15T8E for UVB, Nagano, Japan) for experiments with UV radiation. The total light energy of UV was measured as 1.3 watt/m^2. After incubation, experiment was terminated by injecting 4% buffered formaldehyde. Hundred embryos for each vial were examined for their developmental stages and morphological conditions under an inverted microscope, and categorized into four types based on their embryonic developmental stage; (1) 'dead' - embryos retained at a fertilized egg or early cell division stages, (2) 'retarded' - larvae those were hatched but not fully developed to veliger stage, (3) 'malformed' - veliger larvae those were deformed (convex or concave hinge, incomplete shells, protruding mantle, or asymmetrical shells) or smaller than normal, and (4) 'normal' - larvae with a perfectly D-shaped (straight hinge) shell. Embryo suspended at the fertilized eggs, retarded, deformed, or small sized veliger were regarded as abnormal. Embryos for each category were counted separately. Embryo toxicity was calculated as the total proportions of dead, retarded and malformed larvae (Beiras and Albentosa, 2004).

From crude oil loading and embryotoxicity, a concentration-response relationship was established by a logistic regression model (Field *et al*., 1999).

$$E = 1 / (1 + \exp(ax + b))$$

where E is the probability of embryotoxicity, x is the logarithm of crude oil loading, a and b are estimated parameters of the logistic regression. Using estimated a and b values, median effective concentration (EC_{50}) was calculated. Toxic unit (TU) was calculated as;

TU = (crude oil loading in WAF or CEWAF) / (crude oil loading at EC_{50})

Analysis of variance (ANOVA) and t-test were applied to the toxicity data to test significance among treatments (Zar, 1984). No observed effect

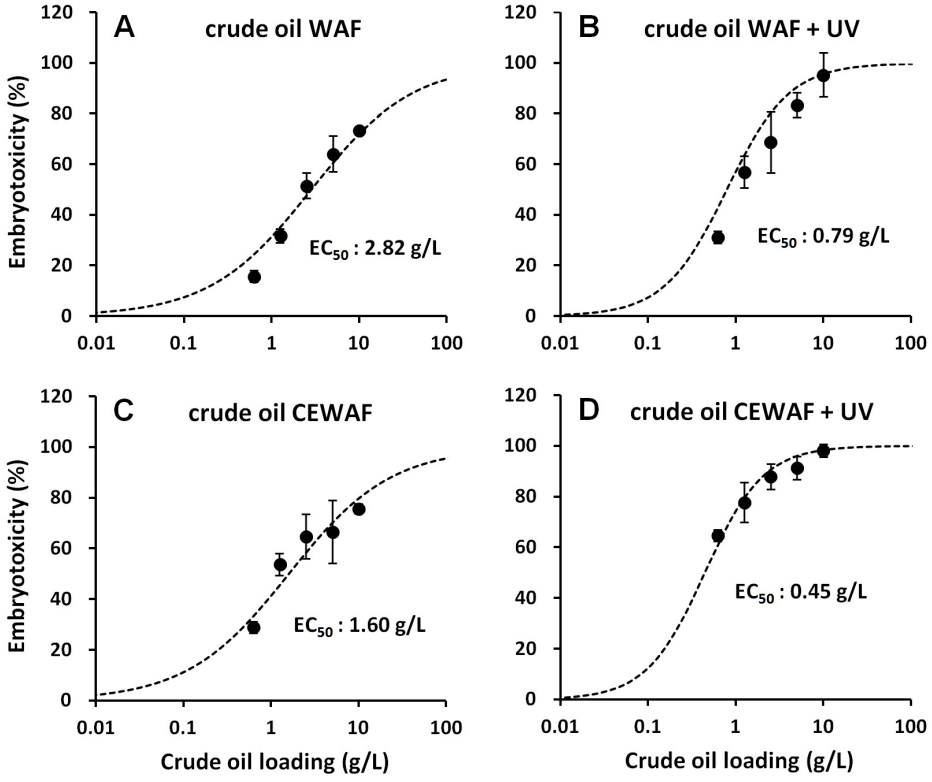

Fig 1. Concentration-response relationships between crude oil loading and embryotoxicity of *Ruditapes philippinarum* for water-accommodated fraction (WAF) without UV (A), with UV (B), chemically enhanced water-accommodated fraction (CEWAF) without UV (C) and with UV (D) of *Hebei Spirit* crude oil. Vertical bar indicates standard deviation (n = 3). Toxicity data were fitted to a logistic regression model.

concentration (NOEC) and lowest observed effect concentration (LOEC) was estimated by Dunnett's t-test (Sokal and Rholf, 1981). All statistical analyses were carried out using SPSS program (version 7.5) with a significant level of α = 0.05.

Results

The WAF of *Hebei Spirit* crude oil without UV radiation affected significantly the embryonic development of *R. philippinarum* (ANOVA, F = 109.6, p < 0.001). In FSW control, the proportion of normally developed veliger larvae (mean ± sd, n = 3) was 89.6 ± 2.3%. When crude oil loading was 0.63 g/L, embryotoxicity was low (15.7 ± 2.8%) and not statistically significant with control (p = 0.060). As the crude oil loading increased, toxicity increased gradually, and reached 73.4 ± 4.6% in 10 g/L (Fig. 1A). The NOEC and LOEC were estimated to be 0.63 g/L and 1.25 g/L. The fitting equation to the logistic regression model was E = 1 / (1 + exp (1.729x - 2.506)) (r^2 = 0.915). The EC_{50} was calculated to be 2.82 g/L.

The WAF with UV radiation affected significantly the embryonic development of *R. philippinarum* (ANOVA, F = 51.1, p < 0.001). In control, the proportion of normally developed larvae was 86.0 ± 2.4%. When crude oil loading was 0.63 g/L, significant embryotoxicity was observed (31.2 ± 6.3%, p = 0.011). As the crude oil loading increased, toxicity increased gradually, and reached 95.4 ± 6.8% in 10 g/L (Fig. 1B). Since the toxicity at lowest crude oil loading was statistically significant (Dunnett's t-test, p > 0.05), the NOEC could not be estimated. The fitting equation to the logistic regression model was E = 1 / (1 + exp (2.817x - 2.526)) (r^2 = 0.721). The

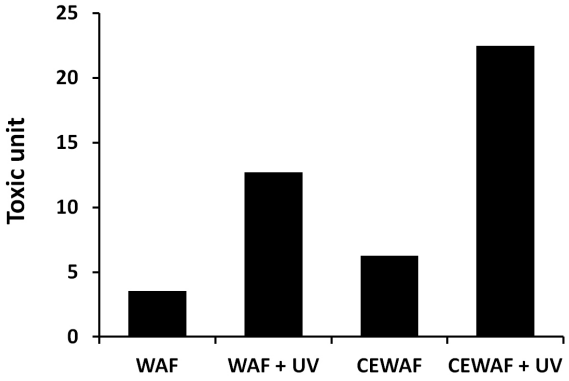

Fig. 2. Toxic unit of water-accommodated fraction (WAF) and chemically enhanced water-accommodated fraction (CEWAF) of *Hebei Spirit* crude oil with- or without UV radiation for embryotoxicity to *Ruditapes philippinarum*.

EC_{50} was calculated to be 0.79 g/L, which is lower than that of WAF without UV radiation.

The CEWAF without UV radiation affected significantly the embryonic development of *R. philippinarum* (ANOVA, F = 30.2, p < 0.001). In control, the proportion of normally developed larvae was 89.6 ± 2.3%. When crude oil loading was 0.63 g/L, significant embryotoxicity was observed (29.0 ± 4.4%, p = 0.002). As the crude oil loading increased, toxicity increased gradually, and reached 75.7 ± 10.9% in 10 g/L (Fig. 1C). Since the toxicity at lowest crude oil loading was statistically significant (Dunnett's t-test, p > 0.05), the NOEC could not be estimated. The fitting equation to the logistic regression model was $E = 1 / (1 + \exp(1.721x - 2.070))$ ($r^2 = 0.891$). The EC_{50} was calculated to be 1.60 g/L, which is lower than that of WAF.

The CEWAF with UV radiation affected significantly the embryonic development of *R. philippinarum* (ANOVA, F = 136.1, p < 0.001). In control, the proportion of normally developed larvae was 86.0 ± 2.4%. High embryotoxicity was observed (64.6 ± 7.9%, p < 0.001) even at the lowest crude oil loading (0.63 g/L). As the crude oil loading increased, toxicity increased gradually, and reached 98.2 ± 2.4% in 10 g/L (Fig. 1D). Since the toxicity at lowest crude oil loading was statistically significant (Dunnett's t-test, p > 0.05), the NOEC could not be estimated. The fitting equation to the logistic regression model

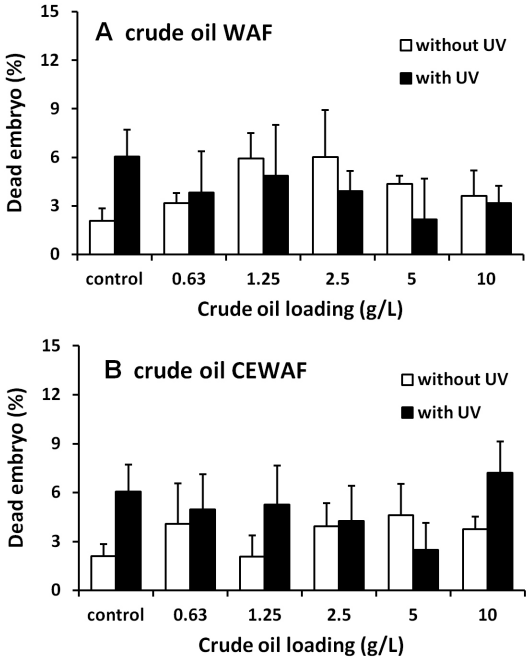

Fig. 3. Proportion of dead embryo of *Ruditapes philippinarum* at each treatment of water-accommodated fraction (WAF, A) and chemically enhanced water-accommodated fraction (CEWAF, B) of *Hebei Spirit* crude oil between with- and without UV radiation. Vertical bar indicates standard deviation (n = 3).

was $E = 1 / (1 + \exp(3.014x - 1.953))$ ($r^2 = 0.844$). The EC_{50} was calculated to be 0.45 g/L, which is lower than that of CEWAF without UV radiation.

Fig. 2 compares the TU of 4 different experiments using WAF and CEWAF of *Hebei Spirit* crude oil. The TU of CEWAF (6.3) was 1.8 times higher than the TU of WAF (3.5), which indicates that the addition of oil dispersant increased the toxicity of crude oil. There were large differences between with- and without UV radiation for both crude oil preparations. TU of WAF with UV radiation (12.7) was 3.6 times higher than that without UV radiation, and that of CEWAF with UV radiation (22.5) was also 3.6 times higher than that without UV radiation. These results indicate that the UV radiation has greatly enhanced the toxicity of crude oil. The increment of TU by UV radiation was larger than that by addition of oil dispersant.

Fig. 3 compares the proportion of dead embryo at

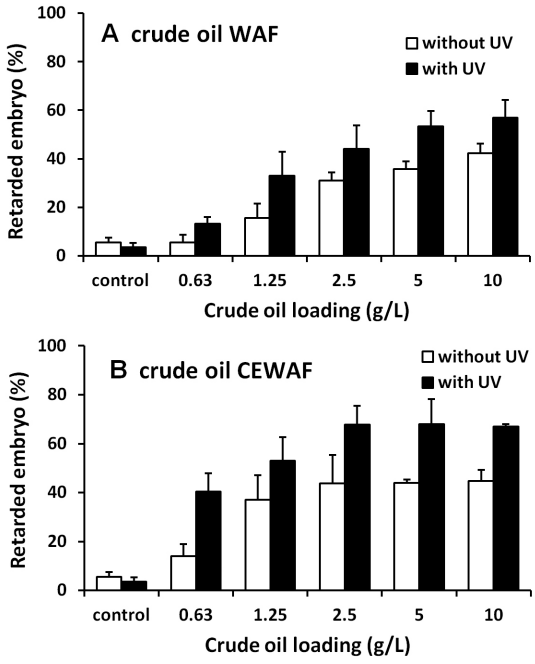

Fig. 4. Proportion of retarded embryo of *Ruditapes philippinarum* at each treatment of water-accommodated fraction (WAF, A) and chemically enhanced water-accommodated fraction (CEWAF, B) of *Hebei Spirit* crude oil between with- and without UV radiation. Vertical bar indicates standard deviation (n = 3).

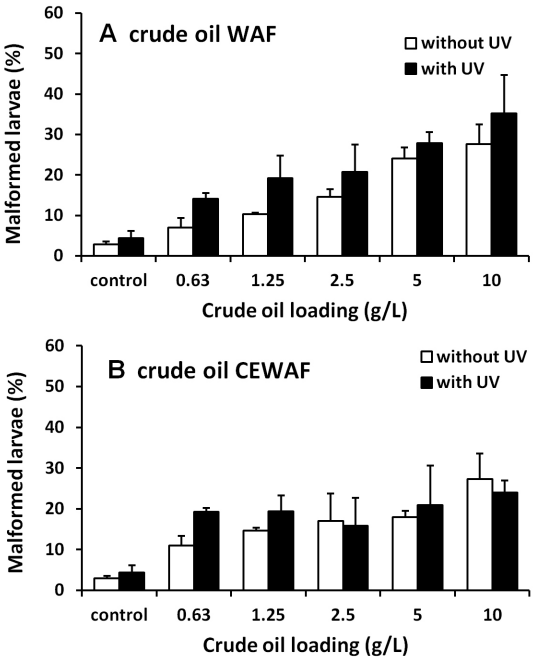

Fig. 5. Proportion of malformed larvae of *Ruditapes philippinarum* at each treatment of water-accommodated fraction (WAF, A) and chemically enhanced water-accommodated fraction (CEWAF, B) of *Hebei Spirit* crude oil between with- and without UV radiation. Vertical bar indicates standard deviation (n = 3).

each treatment of WAF and CEWAF between with- and without UV radiation. For both WAF and CEWAF, the proportion of dead embryo was lower than 10%, and concentration-response relationship could not be observed. Therefore, *Hebei Spirit* crude oil has little effect on the survival of *R. philippinarum* embryo. The differences between with- and without UV radiation were not statistically significant for all WAF and CEWAF treatments (Student's t-test, $p > 0.05$).

Fig. 4 compares the proportion of retarded embryo at each treatment of WAF and CEWAF between with- and without UV radiation. The proportion of retarded embryo increased as crude oil loading increased for both WAF and CEWAF with- and without UV radiation. It was 2 to 20% higher in CEWAF than that in WAF, indicating that oil dispersant increases the duration of embryonic development in *R. philippinarum*. The proportions of retarded embryo with UV radiation were higher than that without UV radiation. For WAF, the difference between with- and without UV radiation was 7 to 18% showing significance at three (0.63, 5, and 10 g/L) of five treatments ($p = 0.033, 0.013, 0.037$, respectively). For CEWAF, the difference was 16 to 26% showing significance at four (0.63, 2.5, 5, and 10 g/L) of five treatments ($p = 0.007, 0.041, 0.016, 0.001$, respectively). Both oil dispersant and UV radiation increased the duration of embryonic development *R. philippinarum*.

Fig. 5 compares the proportion of malformed larvae at each treatment of WAF and CEWAF between with- and without UV radiation. The proportion of malformed larvae increased as crude oil loading increased for both WAF and CEWAF with- and without UV radiation. The difference in the proportion of malformed larvae between WAF and CEWAF was less than 6% with no statistical

significance ($p > 0.05$), indicating that oil dispersant does not change the effects of crude oil on the external morphology of *R. philippinarum*. The proportions of malformed larvae with UV radiation were slightly higher than that without UV radiation. For WAF, the difference between with- and without UV radiation was 4 to 9% showing significance at only one treatment (0.63 g/L, $p = 0.011$). For CEWAF, the difference was 1 to 8% showing significance at one treatment (0.63 g/L, $p = 0.004$). Although the proportions of malformed larvae between with- and without UV radiation were numerically different, the difference was small and in most case it was not significant indicating UV radiation does not change the effects of crude oil on the morphology of *R. philippinarum*.

Discussion

The WAF and CEWAF of *Hebei Spirit* crude oil with- and without UV radiation affected the embryonic development of *R. philippinarum*. Addition of oil dispersant increased crude oil toxicity and the *Hebei Spirit* crude oil has significant phototoxicity. Under without UV radiation, the injection of oil dispersant resulted in 1.8-fold increase and the UV radiation treatment gave rise to 3.6-fold increase in crude oil toxicity. UV radiation attributed to increase in crude oil toxicity more than oil dispersant. The dispersant in this study, HI-CLEAN has low toxicity showing no acute toxicity and little chronic toxicity to benthic copepod *Tigriopus japonicus* (Lee *et al*., 2013). Nevertheless, when it was combined with crude oil, the toxicity increased to an extent more than that could be expected by simple summation of toxic units. There have been many reports that CEWAF has a higher toxicity than WAF, being consistent with this study (Singer *et al*., 2000; Barron *et al*., 2003; Lee *et al*., 2013). The increase in toxicity by dispersant can be explained as the increased seawater concentration of toxic constituents such as PAHs. The dispersed oil is surrounded by lipophilic side of dispersant, while the opposite side of dispersant is hydrophilic. Due to increased hydrophilicity, these oil-dispersant complexes are readily accessible to organisms and more easily penetrate through the cell membrane, causing increased concentration in tissues and increased toxicity. How much the concentrations of toxicants in seawater and tissues are increased by dispersant should be included in future researches.

The results of this study also indicate that the UV radiation has greatly enhanced the toxicity of crude oil, which is consistent with previous reports that hydrocarbons contained in crude oil can cause phototoxicity (Pelletier *et al*., 1997; Calfee *et al*, 1999; Little *et al*., 2000; Barron *et al*, 2003; Saco-Álvarez *et al*., 2008). Pelletier *et al*. (1997) found that the toxicity of 3 PAH compounds (anthracene, fluoranthene, pyrene) under UV radiation increased up to 50,000 times that of conventional toxicity. For most of PAH compounds, acute toxicity is rarely found because the solubility in water was very low. However, under the presence of UV radiation, these low toxic chemical may act as highly acute toxicants. The major chemicals showing phototoxicity in crude oil are based on 3-ring to 5-ring PAHs (Veith *et al*., 1995; Ribeiro and Ferreira, 2005). Since, the chemical analysis of WAF and CEWAF was not included in this study, it is impossible to identify phototoxicity-inducing constituents and to explain quantitatively their relative contributions to phototoxicity. Determination of concentrations of PAHs should be included in the future researches on phototoxicity of crude oils.

The radiation energy of UV in this study was 1.3 watt/m^2, being only 4% of UV energy in sunlight. Therefore, the phototoxicity in the wild condition in sunny days may be higher than that can be expected from the laboratory experiments. Furthermore, the ranges of wavelength of UV lamps in this study was 315 to 400 nm for UVA and 280 to 315 nm for UVB, the whole range being narrower than that of sunlight (100 to 400 nm). Although we observed significant phototoxicity of crude oil caused by UV radiation, the extent of phototoxicity may be underestimated. In order to predict qualitatively the phototoxicity, better controlled experimental design on the light energy and wavelength of UV sources will be needed.

Saco-Álvarez *et al*. (2008) measured the toxicity of WAF from *Prestige* fuel oil using various invertebrates

and vertebrates. They found that the invertebrate larvae were more sensitive than vertebrate larvae. Among them, sea urchin (*Paracentrotus lividus*) larvae was most sensitive, and bivalve (*Mytilus galloprovincialis*) embryo the second. The 96-hour survival of fish (*Cyprinodon variegatus*) larva was ca. 4 times less sensitive than the bivalve. When converting their data to equivalent crude oil loading, the EC_{50} values for sea urchin, bivalve, and fish were 4, 5.2, 19.6 g/L, respectively. In this study, the EC_{50} values of WAF and CEWAF for *R. philippinarum* were 2.82 and 1.6 g/L, which are lower than the results of Saco-Álvarez *et al*. (2008). Difference in EC_{50} can be explained by the difference in chemical composition of oils and the difference in the sensitivity of test organisms. Comparison of toxicity between *Hebei Spirit* crude oil and *Prestige* fuel oil is practically impossible. It can be only inferred indirectly by chemical analysis. Assuming that the compositions of toxicants are similar between two oils, *R. philippinarum* seems more sensitive and suitable as a bioassay organism for assessing the toxicity of crude oils.

In conclusion, both WAF and CEWAF from *Hebei Spirit* crude oil showed significant toxicity to the embryonic development of *R. philippinarum*, being CEWAF more toxic. The phototoxicity by UV radiation was also observed in both two preparations. The increase in toxicity of WAF by UV radiation was more attributed than by oil dispersant. The results from this study provide useful information for the better understanding of the impact of oil spill on marine ecosystem.

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