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Toxic effects of chemical dispersant Corexit 9500 on water flea *Daphnia magna*

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ABSTRACT: In 2010, approximately 2.1 million gallons of chemical dispersants, mainly Corexit 9500, were applied in the Gulf of Mexico to prevent the oil slick from reaching shorelines and to accelerate biodegradation of oil during the Deepwater Horizon oil spill. Recent studies have revealed toxic effects of Corexit 9500 on marine microzooplankton that play important roles in food chains in marine ecosystems. However, there is still little known about the toxic effects of Corexit 9500 on freshwater zooplankton, even though oil spills do occur in freshwater and chemical dispersants may be used in response to these spills. The cladoceran crustacean, water flea *Daphnia magna*, is a well-established model species for various toxicological tests, including detection of juvenile hormone-like activity in test compounds. In this study, we conducted laboratory experiments to investigate the acute and chronic toxicity of Corexit 9500 using *D. magna*. The acute toxicity test was conducted according to OECD TG202 and the 48 h EC_{50} was 1.31 ppm (CIs 0.99–1.64 ppm). The reproductive chronic toxicity test was performed following OECD TG211 ANNEX 7 and 21 days LOEC and NOEC values were 4.0 and 2.0 ppm, respectively. These results indicate that Corexit 9500 has toxic effects on daphnids, particularly during the neonatal developmental stage, which is consistent with marine zooplankton results, whereas juvenile hormone-like activity was not identified. Therefore, our findings of the adverse effects of Corexit 9500 on daphnids suggest that application of this type of chemical dispersant may have catastrophic impacts on freshwater ecosystems by disrupting the key food chain network. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: Chemical dispersant; Corexit EC9500A; *Daphnia magna*; OECD TG202; OECD TG211; Short-term screening assay

Introduction

The Deepwater Horizon oil spill released an estimated 4.9 million barrels (205.8 million gallons) of crude oil into the Gulf of Mexico between April 20 and July 15, 2010, known as one of the largest marine oil spills around the world (Azwell *et al*., 2011; Sammarco *et al*., 2013). To prevent more widespread oil contamination of coastal environments, approximately 2.1 million gallons of chemical dispersant, primarily Corexit EC9500A (hereafter, Corexit 9500), were applied to the sea surface (1.4 million gallons) and at the wellhead beneath the surface (0.77 million gallons) (Kujawinski *et al*., 2011). This unprecedented large-scale application of dispersant to the marine environment in response to an oil spill has raised new questions regarding the impacts of dispersants and dispersed crude oil on marine ecosystems.

Dispersants mainly consist of solvents and surfactants that decrease the interfacial surface tension between crude oil and water, promoting the formation of tiny oil-dispersant droplets. Consequently, this allows removal of the oil from the sea surface, making it more available for biodegradation and preventing oil contamination on shorelines (Lichtenthaler and Daling, 1985; Azwell *et al*., 2011). Recently developed dispersants, such as Corexit, are believed to exhibit less toxicity compared to the conventional products, which had catastrophic impacts on marine ecosystems (Swedmark *et al*., 1973). Moreover, it has been implicated that the newly developed dispersants and the crude oil treated by those dispersants are less toxic than crude oil alone, and that they have minimal deleterious impacts on marine ecological systems (Lessard & Demarco, 2000). However, recent studies have suggested that Corexit 9500 has strong toxic effects on various metazoans such as microorganisms and zooplankton (Almeda *et al*., 2014a,b; Lively and McKenzie, 2014; Kleindienst *et al*., 2015).

Most research on the fate and effects of chemical dispersants on aquatic environments has primarily focused on marine systems, although oil spills do occur in freshwater environments as well (Walker *et al*., 1995). There is a need for greater concern about freshwater oil spills, as rivers have stronger water currents, unidirectional water flow and greater potential for floods than

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those occurring in the ocean (Walker *et al.*, 1995). Dispersants are often recommended for response to freshwater oil spills. This approach may contribute to reducing the toxicity of spilled crude oil to animals at the surface of the water, such as birds and mammals. However, it has been suggested that dispersing the oil may increase its toxicity to freshwater organisms, and dispersant alone may be toxic to marine organisms (Vindimian *et al.*, 1992). Hence, it is necessary to investigate the effects of chemical dispersants on freshwater organisms.

The cladoceran (branchiopod) crustacean water fleas, genus *Daphnia*, are well known freshwater zooplankton, which form a basic link in the food web and are widely distributed around the world. They exclusively reproduce female offspring by parthenogenesis under favorable conditions, whereas they induce production of male offspring in response to various unfavorable environmental cues such as shortened day length, low temperature, lack of nutrients, overpopulation and combinations of these cues to switch their reproductive manner from parthenogenesis to sexual reproduction (Banta & Brown, 1929; Hobæk & Larsson, 1990; Kleiven *et al.*, 1992; Smith, 1915; Toyota *et al.*, 2015). Recently, several studies have demonstrated that exposure of juvenile hormone (JH) analogs used as pesticides to daphnids induce male offspring even under female-producing conditions (Abe *et al.*, 2015a; Miyakawa *et al.*, 2013; Olmstead & LeBlanc, 2002; Tatarazako *et al.*, 2003; Toyota *et al.*, 2014). This physiological function of JH-produced male offspring has become a useful marker for detection of JH activity in chemical screening. To date, this concept was adopted by the Organization for Economic Co.-operation and Development Validation Management Group for Ecotoxicity testing (OECD VMGeco) and formalized as an endpoint in the OECD TG211 ANNEX 7 to detect chemicals bearing JH-like activity (OECD, 2012). Moreover, a short-term screening assay was recently developed for rapid detection of chemicals with JH-like activity using adult *D. magna* (Abe *et al.*, 2015a,b). In addition to aforementioned methods, many studies involved in the detection of the toxic effects of a large number of diverse chemicals on daphnids have been addressed, and the information has been accumulated based on various toxicological tests (Liess & Ohe, 2005). Like other aquatic organisms, daphnids are constitutively exposed to multiple chemicals and are sensitive to environmental chemicals. Therefore, they have been utilized extensively to assess organism- and population-based toxicological studies for environmental chemical pollutants released into freshwater ecosystems.

In this study, we performed the acute toxicity (immobilization; OECD TG202) test, short-term JH activity screening test and chronic toxicity (21 days reproduction; OECD TG211 ANNEX7) test to evaluate the toxic features of chemical dispersant, Corexit 9500, using freshwater zooplankton *D. magna*.

Materials and methods

Daphnia strain and its rearing conditions

The *Daphnia magna* strain (NIES clone) was obtained from the National Institute for Environmental Studies (Tsukuba, Japan) where it has been maintained as a single genetic stock for more than 20 years (Tatarazako *et al.*, 2003). Culture medium was prepared as dechlorinated freshwater, which was aerated and filtered through activated carbon for at least 2 weeks before use. Female neonates, less than 24 h after hatching, laid from mothers 2 weeks or older, were subcultured every week. They were used for the following experiments after acclimation over several generations. Cultures of 20 individuals per liter were incubated at 18 °C under a 14 h light/10 h dark photoperiod. A 0.01 ml suspension of 5×10^8 cells ml^{-1} chlorella (*Chlorella vulgaris*; Chlorella Industry Co., Ltd., Fukuoka, Japan) was added daily to each culture. The water hardness was 60 mg l^{-1} (as $CaCO_3$), and the dissolved oxygen concentration was 91.5%.

Chemicals and concentrations

Undiluted Corexit EC9500A (Corexit 9500) was directly obtained from Nalco Environmental Solutions LLC (Sugar Land, TX, USA). The most highly concentrated Corexit 9500 solution was prepared by direct dilution, and then serially concentrated samples were prepared by serial dilution using a common ratio of 2 (64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 μl l^{-1}; ppm) for the following assays. A stock solution of 1 mg ml^{-1} methyl farnesoate (Echelon Bioscience, Salt Lake City, UT, USA) was dissolved in dimethylformamide (analytical grade; Wako Pure Chemical Industries Ltd., Osaka, Japan) and stored at −20 °C until use.

Acute toxicity test

The acute toxicity test was conducted according to the OECD Test Guideline 202, "*Daphnia* sp. Acute Immobilization Test" (OECD, 2004). Females, less than 24 h after hatching, were used in this experiment and were exposed to various concentrations of Corexit 9500. The experiments were conducted in an incubator at 18 °C under a 14 h light/10 h dark photoperiod. Tap water filtered through activated carbon and aerated for 2 weeks was used as a culture medium (control group). Eleven concentrations of Corexit 9500, including a control group, were prepared by dilution with culture medium. Quadruplicate 100 ml glass beakers, each containing five *D. magna* neonates 24 h after hatching or younger in 50 ml of media, were used at each test concentration. All beakers were covered with Teflon caps to avoid volatilization of the test chemical. No food was provided during this experiment. The numbers of immobilized and deceased neonates were counted and recorded at 24 h and 48 h after beginning the experiment.

Short-term screening assay

Short-term screening assay was performed according to previous study (Abe *et al.*, 2015b). We used approximately 2-week-old daphnids, who had embryos inside of the brood pouch, for the short-term screening assay. Neonates released from the first brood were not used for this experiment, as male sexual fate is decided at the oocyte maturation period in the mother's ovary during the JH-sensitive period, which corresponds to 8–7 h before ovulation (Ignace *et al.*, 2011; Kato *et al.*, 2011). Each daphnid was exposed in a 100 ml glass beaker filled with 50 ml of test medium or culture medium. During this test, chlorella was provided at a concentration of 4.3×10^5 cells ml^{-1} daily. The experiment was conducted at 18 °C with a 14 h light/10 h dark photoperiod in a temperature-controlled chamber. In addition to Corexit 9500, methyl farnesoate (100 μg l^{-1}) was used as a positive control.

Chronic toxicity on reproduction

The chronic toxicity experiment was conducted according to OECD Test Guideline 211 ANNEX7, "*Daphnia magna* Reproduction Test" (OECD, 2012). Daphnids were used within 24 h after release

from their mother before the start of this experiment and were exposed to various concentrations of Corexit 9500, observed and fed (*C. vulgaris*: 4.3×10^5 cells ml^{-1}) daily for 21 days. Daphnids were maintained in 18 °C under a 14 h light/10 h dark photoperiod. Ten concentrations of Corexit 9500 and the control were prepared, with 10 replicates for each concentration. Each replicate contained an individual daphnid in a 100 ml glass beaker filled with 50 ml of test medium. The glass beakers were closed tightly with Teflon caps to prevent volatilization. The test medium was renewed every 2 days. The total number of neonates was counted daily by removing neonates from the test medium. Sex of the neonates was also observed as an indicator of JH activity in *D. magna* (Olmstead & LeBlanc, 2002; Tatarazako *et al*., 2003). Their sex was distinguished by the length and morphology of the first antennae using a stereomicroscope (Tatarazako *et al*., 2003).

Statistical analysis

EC_{50} for the acute toxicity experiment was estimated by a log-logistic model with the package "drc" in R (R Development Core Team, 2011). Statistical differences were determined by one-way ANOVA followed by Dunnett's test, except when multiple different groups were compared, in which case statistical differences were detected by one-way ANOVA followed by Tukey–Kramer *post hoc* test using Excel 2010 (Microsoft Corp., Redmond, WA, USA) fitted with add-in software, Statcel 3 (Yanai, 2011).

Results

Acute toxicity of Corexit 9500 to *Daphnia magna* offspring

An acute immobilization test was carried out according to the OECD TG202 Guideline for the Testing of Chemicals (OECD, 2004). The estimated EC_{50} values of Corexit 9500 using *D. magna* offspring were 87.83 ppm (95% confidence interval: 3.78–170.9) in 24 h exposure and 1.31 ppm (95% confidence interval: 0.99–1.64) in 48 h exposure (Fig. 1).

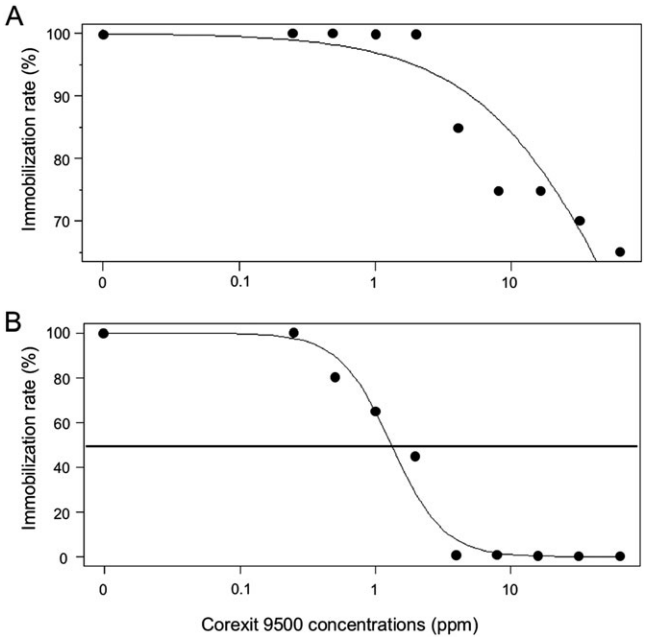

Figure 1. Dose–response curve of acute toxicity test of 24 h (A) and 48 h (B) using Corexit 9500 based on the OECD TG202. Estimated EC_{50} scores were 87. 83 ppm (95% confidence interval: 3.78–170.9) and 1.31 ppm (95% confidence interval: 0.99–1.64), respectively.

Short-term screening assay of Corexit 9500

Number of offspring and sex ratio obtained from the second brood after chemical treatment are shown in Fig. 2. Almost all mothers survived during this experiment. Mean number of offspring was not significantly changed in response to Corexit 9500 (Fig. 2A). Male offspring were not observed in the control or any Corexit 9500 treatment groups, whereas the methyl farnesoate treatment group (positive control) reached 100% male induction rate (Fig. 2B).

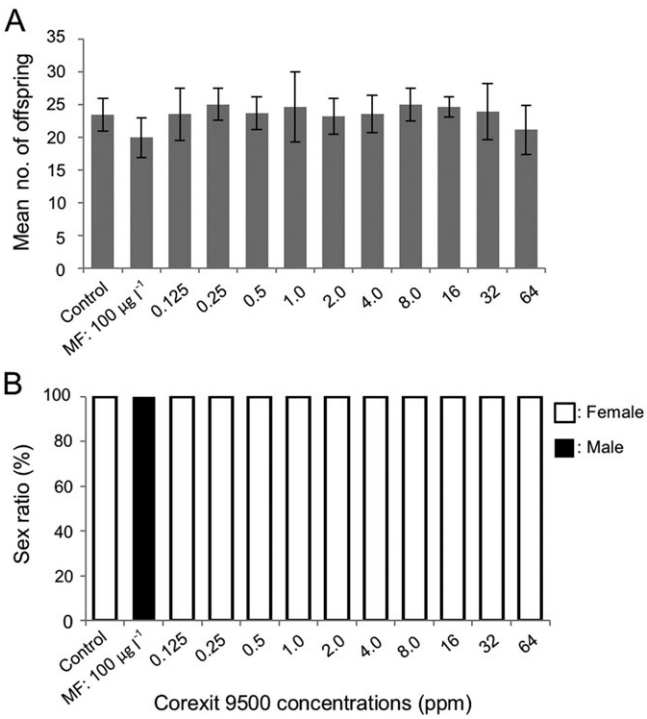

Figure 2. Short-term screening assay of Corexit 9500. Mean number (± SD) of neonates in the second brood after exposure to Corexit 9500 (n = 9–10) (A) and their sex ratio in percentage (B).

Reproduction test of Corexit 9500

OCED TG211 ANNEX7 (OECD, 2012) assay was performed to investigate the chronic effects of Corexit 9500 using the following concentration groups: 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 ppm. All daphnids died during this experiment in the 4, 8, 16, 32 and 64 ppm Corexit 9500 treatment groups (Fig 3A), although daphnids in the 4 and 8 ppm exposure groups were able to reproduce offspring once or twice before death (Fig. 3B). The durations from neonate to when the first reproduction occurred and numbers of offspring among the 0.125–8 ppm exposure groups were consistent with the control group (Fig. 3C,D). According to the protocol of OECD TG211 ANNEX7 (OECD, 2012), we removed the data from the 4, 8, 16, 32 and 64 ppm groups from the following data analysis due to anomalous lethality effects. In the 21 day reproduction test, the mean numbers of total offspring were not significantly different among 0.125, 0.25, 0.5, 1.0 and 2.0 ppm Corexit 9500 treatment

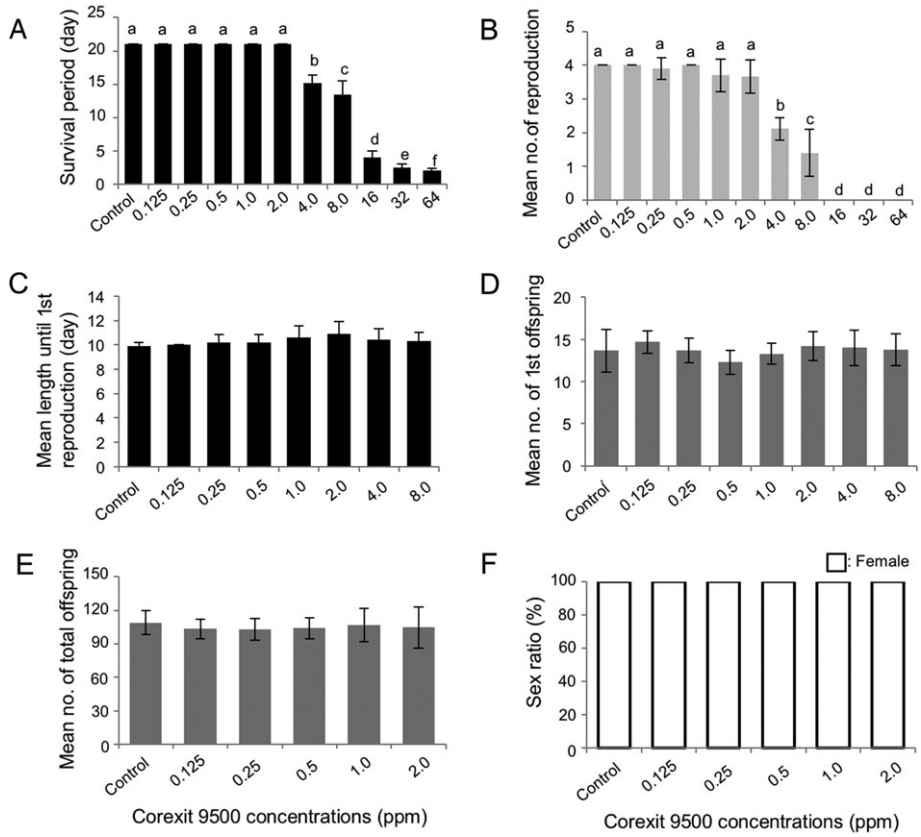

Figure 3. Corexit 9500 exposure for 21 days with OECD TG211 ANNEX7 ($n = 9$–10). Mean survival period (A); mean numbers of reproduction (B); mean length until first reproduction (C); mean numbers of first brood offspring (D); mean numbers of total offspring (E); and sex ratio of offspring (F). All bars indicate the SDs. The different letters denote significant differences (one-way ANOVA followed by Tukey–Kramer *post hoc* test, $P < 0.05$).

groups (Fig. 3E), and male offspring production was not observed among any Corexit 9500 treatment groups (Fig. 3F).

Discussion

We demonstrate that no male offspring were observed in the short-term screening assay and in the 21 day reproduction test in all Corexit 9500 exposure groups, strongly suggesting that Corexit 9500 does not have JH-like activity. Furthermore, we revealed that Corexit 9500 showed strong toxic effects (immobilized and/or lethal) on *D. magna* at concentrations of 4–64 ppm during the offspring growth period. Interestingly, unlike the result of the acute toxicity experiment, daphnids were able to normally grow up to become sexually mature adults and reproduce offspring under conditions of up to 8 ppm Corexit 9500 exposure, although daphnids in the 4 and 8 ppm exposed groups died during this experiment (after reproducing once or twice). This difference, of which concentrations displayed toxic effects between the acute and chronic toxicity tests, may depend on experimental conditions (e.g., feeding, rearing density, etc.), although further investigations are needed to clarify which factors induce the difference. These results suggest that exposure to greater than 4 ppm concentrations of Corexit 9500 alone may have huge impacts on the growth and reproduction of *D. magna*, similar to results obtained with marine zooplankton (Cohen *et al.*, 2014; Lively & McKenzie, 2014).

When a crude oil spill occurs, the bioavailable concentration in the water is quite variable, ranging from greater than 200 ppm to less than 1 ppb (Lichtenthaler & Daling, 1985; McAuliffe *et al.*, 1981). In fact, during the Deepwater Horizon oil spill, concentrations of 1–2 ppm dispersed crude oil were observed (Kerr, 2010). Likewise, in laboratory experiments, toxic effects such as death and delay in growth have been reported for various marine zooplankton as a result of exposure to Corexit 9500 in the lower range of its concentrations (less than 1 ppm to greater than 10 ppm) (Almeda *et al.*, 2014a,b; Cohen *et al.*, 2014; Lively & McKenzie, 2014). Therefore, our results demonstrate that Corexit 9500 has toxic effects on neonates of freshwater zooplankton *D. magna* at environmentally relevant concentrations that could potentially be applied after oil spills occur. However, in natural circumstances, the toxicity of chemical dispersant on zooplankton will not only depend on the dispersant concentrations, but also unidirectional water flow in the river, water stasis in the pond, exposure period, composition of zooplankton community and, particularly, coexistence with crude oil.

In this study, we only analyzed the toxic effects of chemical dispersant Corexit 9500 alone. Previous studies have demonstrated that chemically dispersed oil shows more toxic effects than non-chemically dispersed oil, as dispersant application allows the oil to mix more readily into the water column, not only in the marine environment (Almeda *et al.*, 2014a,b; Cohen *et al.*, 2014; Lively & McKenzie, 2014), but also freshwater environment (Bhattacharyya *et al.*, 2003). One possible cause of the increased toxicity of dispersant-treated crude oil to marine microzooplankton (e.g., ciliates and heterotrophic dinoflagellates) is hypothesized to be the association with both additive and/or cumulative effects of crude oil and chemical dispersant, although it may vary depending on the species and contamination levels (Almeda *et al.*, 2014b; Carro *et al.*, 2013). As an example of cumulative toxic effects of

crude oil and dispersant, it has been suggested that the dispersant increases the solubility of toxic components of crude oil in water (Greer *et al*., 2012; Wu *et al*., 2012), which may then be taken up into the cells through passive mechanisms. Further research using co-exposure to crude oil and Corexit 9500 is necessary for better understanding the effects on freshwater zooplanktonic communities. Our results support other recent studies that Corexit 9500 is more toxic than previously expected, particularly to small planktonic organisms in freshwater environments, as in marine environments (Cohen *et al*., 2014; Goodbody-Gringley *et al*., 2013). Therefore, the application of chemical dispersant as a response to crude oil spills may increase the adverse effects on key planktonic organisms in both marine and freshwater environments. Although the impacts of crude oil and chemical dispersant on zooplankton communities will depend on the specific circumstances of each incident, our data provide better prediction of the potential acute effects of crude oil pollution and dispersant application on freshwater zooplankton in aquatic ecosystems.

Author's contributions

KT, NM, DS, SK and TI conceived and designed the study. KT and NM performed experiments. KT analyzed the data. KT, NM and SK wrote a first draft. All authors participated in the modification of draft, and approved the final manuscript.

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Conflict of interest

The authors did not report any conflict of interest.

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