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Toxicities of Diesel Oil, Dispersant and Dispersed Oil to *Tetraselmis chuii* and *Chaetocheros calcitrans*

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ABSTRACT

Oil spillage accidents can cause severe short-term effects to marine organisms and may lead to devastating long-term impact to marine environments. This study determined the influence of water accommodated fractions (WAFs) of diesel oil, chemically enhanced WAFs (CEWAFs) of diesel oil and oil dispersant (IPAC-OCD) to the toxicity of green algae, *Tetraselmis chuii*, and marine diatom, *Chaetocheros calcitrans*. Both the phytoplankton species were exposed to different concentrations of WAFs, CEWAFs and dispersant (0%, 5%, 10%, 20%, 40%, 80%). All of the toxicants significantly reduced the growth of phytoplankton, however, the level of response varied by solution and species. The dispersant has the highest impact among all the chemicals studied. The dispersant is very highly toxic to *C. calcitrans,* but still practically non-toxic to *T. chuii.* Even though, WAFs have higher impact to *T. chuii* than *C. calcitrans,* the chemicals are practically non-toxic to both phytoplankton species and, moreover, in short-term exposure, low concentration of WAFs may stimulate the growth of *C. calcitrans*. However, the mixture of diesel oil WAFs and dispersant cause synergistic toxicity to *C. calcitrans* that increases the WAFs toxicity. This finding implies that the dispersant application to mitigate the oil spills may negatively impact the marine phytoplankton.

INTRODUCTION

Increasing oil production and transportation have caused more release of liquid petroleum hydrocarbons into the oceans and coastal waters (Burgherr 2007). Oil spills may be derived from various sources, refined petroleum products (such as diesel, gasoline) and their by-products may enter into the marine environment due to accidental spills from ships or discharges from consumption of oils.

Spilled oil can harm marine organisms because of its chemical properties, which are consisted of hydrocarbons (cycloalkanes, alkanes, aromatic hydrocarbons) and non-hydrocarbons (asphalt and resin) (Jiang et al. 2010), are poisonous. One of the major effects of the spills is on the community composition of phytoplankton and hence primary production (González et al. 2009, Hook & Osborn 2012), thereby changes in phytoplankton may cause devastating effect on the stability, structure and function of marine ecosystems (Hallare et al. 2011).

Oil dispersants are a common chemical used after oil spills to break up oil from the sea surface and disperse it into the water column as tiny (1-70 μm) droplets (Lessard & DeMarco 2000). Oil dispersants are intended to protect vulnerable coastal areas from oil coming ashore. However, oil dispersants do not reduce the amount of oil, but increase the polycyclic aromatic hydrocarbons (Lyons et al. 2011).

There have been many field and laboratory experimental studies on oil and dispersants, regarding their toxicity to different marine organisms (Almeda et al. 2014, George-Ares et al. 1999, Lee et al. 2013, Liu et al. 2006). However, different oil and dispersant types behave differently in the environment, and may result in different toxicity responses due to differences in oil and dispersant composition, water solubility, and water accommodated fractions (WAFs) component (Garr et al. 2014, Hook & Osborn 2012, Jiang et al. 2010). Moreover, oils from different oil fields within the same geographical location may differ qualitatively due to their differences in chemical characterization (Zhan et al. 2015). Besides, the impacts of oil on marine organisms also depend on the methodologies used to conduct the experiments (Singer et al. 2001).

WAFs and chemical enhanced water accommodated fractions (CEWAFs) stocks are required for the experimental exposure as it represents compounds with high bio-availability towards the test organisms. WAFs are laboratory-prepared medium made from mixing of a poorly soluble crude oil, diesel, or other petroleum products with sterilized water or sea water which should be free of bulk material particles, and CEWAFs is the mixture of WAFs and oil dispersant (Singer et al. 2001). Thus, in the current study, the objectives are to evaluate the effects of WAFs and CEWAFs, and dispersant on growth, mortality, and toxicity of green algae species *Tetraselmis chuii* and diatom species *Chaetocheros calcitrans* under different concentrations of WAFs, CEWAFs, and dispersant.

MATERIALS AND METHODS

Phytoplankton and culture conditions: Cultures of Chrysophyceae, *Chaetocheros calcitrans,* and Chlorophyceae *Tetraselmis chuii* were purchased from Balai Budidaya Air Payau (BBAP), Situbondo and Balai Besar Riset Perikanan Budidaya Laut, Gondol Bali, respectively. They were maintained in the Laboratory of Hydrobiology, Faculty of Fisheries and Marine Science, University of Brawijaya. Prior to experiment, these algae were cultured in filtered (0.45 µm) and autoclaved natural seawater, with 24-h light exposure (4000 lux) and 29±2 °C temperature in vitamin enriched De Walne's medium for *Tetraselmis chuii*, and vitamin-silica enriched diatom medium for *Chaetocheros calcitrans* (Fakhri et al. 2015, Raja et al. 2007). The dissolved oxygen (DO) and pH of the culture were 8.5 $mg.L^{-1}$ and 7.8 respectively, while the salinity was 25 psu and 30 psu for *Tetraselmis chuii* and *Chaetocheros calcitrans* respectively.

Preparation of WAFs and CEWAFs and dispersant: Diesel fuel was purchased at a PERTAMINA petrol station. IPAC-OCD was used as chemical dispersant produced and provided by CV. Pratama Abadi Chemical. The natural seawater used in the experiment was enriched with De Walne's medium for *Tetraselmis chuii*, and vitamin-silica enriched diatom medium for *Chaetocheros calcitrans*. WAFs and CEWAFs solutions were prepared according to CROSERF (Chemical Response to Oil Spills-Ecological Effects Research Forum) guidelines with minor modifications (Singer et al. 2001). To prepare WAFs, 160 mL of diesel oil was diluted with 1600 mL of 0.45 µm-filtered and autoclaved natural seawater (salinity 32 psu) in a sealed 2 L aspirator glass bottle with the height of the headspace of the bottle was approximately 20 % by volume. Then, the mixture was mixed in the dark with low energy mixing (no vortex), to minimize degradation and control volatilization of the fuel components. In CEWAFs preparation, the dispersants to oil ratio was 1 to 20 (Almeda et al. 2014). The preparation was different from WAFs preparation as 20-25 % vortex was initiated in the water mass first, then oil and dispersant were both added directly into the centre of vortex consecutively. Both WAFs and CEWAFs were stirred using a magnetic stirrer for 24 hour at 200 rpm at a room temperature. The mixture was left to settle for 1 hour before the aqueous phase of diesel WAFs and diesel CEWAFs was transferred into a flask. The WAFs and CEWAFs were then stored at ± 5 °C as the stock solution, and was used immediately (within 24 hours) for the subsequent experiments. Furthermore, dispersant stock was prepared by mixing 440 mL autoclaved and filtered natural seawater with 2 mL dispersant.

Toxicity experiment: We conducted static exposure chronic toxicity tests. In this study, WAFs, CEWAFs, and dispersant were tested at concentrations of 0 %, 5 %, 10 %, 20 %, 40 %, and 80 %. Briefly, each 10 mL of phytoplankton, taken from exponential growth phytoplankton culture, was transferred to each of total 18 Erlenmeyer flasks (100 mL flask). Filtered and autoclaved medium enriched natural seawater and WAFs, CEWAFs and dispersant stocks were then added to each Erlenmeyer flask. The total volume was adjusted to 50 mL and contained 0 %, 5 %, 10 %, 20 %, 40 % and 80 % of WAFs, CEWAFs or dispersants in each Erlenmeyer. Prior to the experiment, the phytoplankton was counted to obtain the control value of WAFs, CEWAFs or dispersant experiment. The growth and mortality of phytoplankton in each flask were then counted using haemocytometer in Olympus CX 21 LED microscope at 1 hour, 24 hour, 48 hour, and 72 hour.

Statistical analysis: Growth or mortality in each treatment was calculated using Microsoft Excel 2003. GraphPad Prims 7 (GraphPad Software, Inc.) was used to perform statistical analysis and design graphs. Data on the toxicity for each WAFs, CEWAFs and dispersant concentration and each developmental time of the phytoplankton were statistically analysed using two-way analysis of variance (ANOVA). It was followed by the use of Dunnet multiple comparison test to compare growth or mortality of phytoplankton between control and experimental treatments at each time exposure, and Sidak multiple comparison test to compare the toxicity of WAFs, CEWAFs and dispersant between both the species at each time of exposure. Furthermore, Probit analysis was applied using IBM SPSS Statistics version 23.0 to determine LC_{50} effect concentration.

RESULTS

Growth/mortality of *Chaetocheros calcitrans* and *Tetraselmis chuii* at different WAFs, CEWAFs and dispersant concentrations and time exposures: The effects of diesel oil WAFs, CEWAFs and dispersants on growth/mortality of phytoplankton varied depending on the species, exposure concentrations and time of exposure. Growth rates of C. *calcitrans* and *T. chuii* were significantly higher in the controls than all the three experimental treatments (ANOVA, Dunnet post hoc test, $p < 0.01$) at all five exposure levels (Fig. 1). In all cases, counting the phytoplankton cells at different time exposure resulted in varying phytoplankton concentration, although not always statistically significant, particularly at the highest exposure levels (Fig. 1).

Chaetocheros calcitrans was more tolerant to WAFs than that of *Tetraselmis chuii.* As in 5 % WAFs (4.5 mL L^{-1}), the former species could still be able to grow steadily, whereas the number of phytoplankton of the later species was continuously decreasing over time (ANOVA, Sidak post hoc test, $p \leq 0.01$), except at 1 hour exposure, when there were no significant differences as there was negative growth observed on both the species (ANOVA, Sidak post hoc test $p \leq 0.99$). However, in dispersant and CEWAFs exposure, the survival rate of *T. chuii* was higher than that of *C. calcitrans* (ANOVA, Sidak post hoc test, $p \leq 0.01$). The diatom's mortality was more than 90 % even in 24 hour exposure of 5 % CEWAFs (4.75 mL L^{-1}) and dispersant (0.227 mL L^{-1}) , and the later toxicant killed 100 % of the diatom in 48 hour and 72 hour exposure from the concentration of 5 % dispersant onward. Meanwhile, the concentration of the green algae decreased only 50 % in 24 hour exposure of 5 % CEWAFs, and the longer time exposure and the higher toxicant concentration led gradually decline in the phytoplankton's density, but the toxicant only swept the phytoplankton less than 90 % by the end of the experiment even in the highest CEWAFs concentration.

DISCUSSION

The aim of the study is to assess the impact of exposure to diesel oil (WAFs), chemically dispersed diesel oil (CEWAFs), and dispersant on the mortality of *C. calcitrans* and *T. chuii* as an indicator of effect of oil pollution on marine phytoplankton ecological status. According to US EPA's LC_{50} aquatic toxicity scale (National Research Council (NRC) 2014), the main findings of this study are that the dispersant is very highly toxic to *C. calcitrans* while it is moderately toxic to *T. chuii* only on the long term exposure. In addition, the long term exposure of CEWAFs is also very highly toxic to *C. calcitrans.* Consequently, these pollutants may have devastating effects on marine microbial communities and marine food webs, as planktonic metabolic activities play a role in the microbial communities (Moreau et al. 2013) and those phytoplankton are widely distributed in the marine environments (Gharib et al. 2011, Prartono et al. 2010).

Phytoplankton mortalities were reported in the exposed groups compared to control groups (ANOVA, Dunnet post hoc test, $p < 0.01$). Exposure of *C. calcitrans* and *T. chuii* to CEWAFs and dispersant resulted in mortality of experimental phytoplankton within the first hour of treatment, and it increased as the concentration of the toxicants and time of exposure increased. Constant exposure to dispersant and CEWAFs also caused dramatic declines in the exposed phytoplankton survivorship, resulting in complete mortality (100%) and near complete mortality (6 % survival) of *C. calcitrans* after exposure to lowest concentration of dispersant (0.227 mL L^{-1}) and CEWAFs (4.75 mL L^{-1}) respectively. However, *T. chuii* is more persistent to the toxicants exposure than *C. calcitrans*, they only impacted 52 % and 65 % mortality at lowest exposure concentrations of dispersant and CEWAFs respectively. Other laboratory experiments also confirmed this experiment that centric diatoms were more vulnerable to dispersant and oil-dispersant mixture than green algae (Lewis & Pryor 2013).

Furthermore, dispersant may be more or less toxic to phytoplankton than other organisms, for example, small crustacean, *Artemia* and Mysid. The 24-hours LC_{50} of Corexit 125210 to *Artemia* sp. and *Mysidopsis bahia* were 52 ppm (Wells et al. 1982) and 65 ppm (George-Ares et al. 1999) respectively. Meanwhile, in this study, dispersant was three thousand times more toxic to *C. calcitrans* than to *T. Chuii*. It was clearly shown in the probit analysis that the 24-hours LC_{50} of dispersant on *C. calcitrans* was 0.5 ppm (5×10^{-4} mL L^{-1}), while on *T. Chuii,* it was 1800 ppm (1.8 mL L^{-1}). The toxicity of WAFs, CEWAFs and dispersant was also increased through the time which is shown in the decreasing of LC_{50} from 24-hours to 72-hours exposure. The lowest concentration of dispersant in 48-hour exposure already effected 100 % mortality of *C. calcitrans*; therefore, the probit analysis of LC_{50} was not computed as no statistics could be calculated when the slopes are zero (Table 1).

It is clearly shown in this experiment that the dispersant may adversely affect marine phytoplankton, demonstrated in the present study that the chemical was more toxic for C. *calcitrans* and *T. chuii* than WAFs and even CEWAFs. This phenomena was also observed in Crustacea that dispersant was the most toxic chemical among those toxicants for *T. japonicus* (Lee et al. 2013). Furthermore, our experiment confirmed that the use of dispersant can severely alter the toxicity of diesel oil to the diatom. The mixture of dispersant to diesel oil at 24-hour exposure for example, make the oil 80 times more toxic to *C. Calcitrans* than oil alone (Table 1). However, diesel oil and dispersant do not cause the synergistic toxicity to the green algae particularly on the long term exposure. The dispersant indeed increases the bioavailability of petroleum hydrocarbons (Lessard & DeMarco 2000, Lyons et al. 2011), yet the dispersant is practically non-toxic to the green algae in our experiment. Therefore, we affirmed that the dispersant did not elevate the toxicity of CEWAFs to *T. chuii*.

Furthermore, the synergistic effects of dispersant were also noticed in an experiment using the mixture of

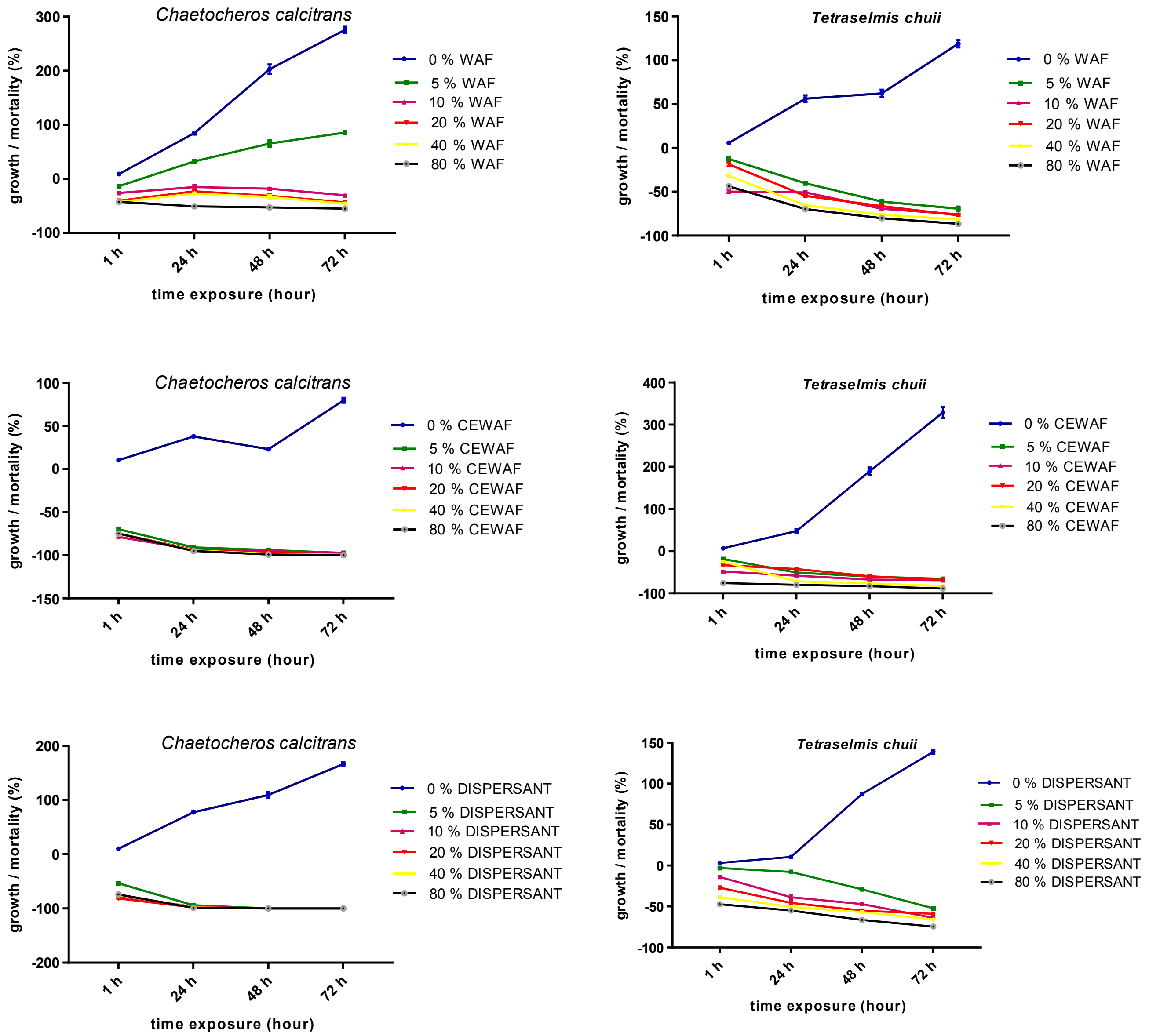

Fig. 1: Growth/mortality of *Chaetocheros calcitrans* and *Tetraselmis chuii* as percent of the initial cell abundance after 1 h, 24 h, 48 h and 72 h of exposure to 6 different concentrations (0%, 5%, 10%, 20%, 40%, 80%) of diesel oil WAFs, CEWAFs and dispersant. Error bars represent the standard deviation (n=3). There are significant differences among treatments according to the two-way ANOVA ($p < 0.01$).

COREXIT9500A® and Macondo crude oil, which showed that the toxicity to *Brachionus plicatilis* (Rotifera) increases up to 52-fold (Rico-Martínez et al. 2013). Similarly, such a strong impact of CEWAFs and dispersant has been observed on planktonic communities in the short-term marine mesocosms study that the dispersant altered the physico-chemical conditions of oil and decreased drastically most phytoplankton assemblages (Jung et al. 2012). Most toxicity of hydrocarbons as well as dispersants to phytoplankton occurs via narcosis, and dispersant along with dispersed oil cause membrane damage, while exposure to WAFs does not (Hook & Osborn 2012).

Conversely, based on LC_{50} value in the experiment, the WAFs of diesel oil are practically non-toxic (LC_{50}>100 ppm) to both the phytoplankton species. Laboratory researches and mesocosm studies demonstrated that low concentration of WAFs may induce phytoplankton growth, particularly for the microalgae that has rapid generation turnover (Jiang et al. 2010). It is reflected in our experiment that the lowest concentration of WAFs, 5 % WAFs (4.5 $mL.L^{-1}$) still

Table 1: LC_{50}, 95 % confidence interval (Cls) for *C. calcitrans*and *T. chuii* exposed to WAFs, CEWAFs and dispersant.

Time (hour)	Species	LC_{50} (mL/L)		
		WAFs	CEWAFs	DISPERSANT
24	*C. Calcitrans*	86	1.03	5×10^{-4}
	T. Chuii	8.55	7.05	1.8
48	*C. Calcitrans*	70	0.03	-*
	T. Chuii	0.82	2.03	0.83
72	*C. Calcitrans*	46	4.7×10^{-3}	-*
	T. Chuii	0.31	1.59	0.13

*No statistics were computed in 48 h and 72 h of dispersant as the slopes are zero.

promoted the growth of the microalgae C. *calcitrans*. Moreover, the 24-h LC_{50} of C. *calcitrans* exposed to WAFs was even higher (86 $mL.L^{-1}$) than the highest exposure concentration (72 $mL.L^{-1}$). The WAFs may be decomposed by the phytoplankton as some species of phytoplankton, such as *Navicula, Cyclotella* and *Nitzschia,* have capability to degrade WAFs by themselves and utilize their realized biotransformation like carbon to promote their growth (Liu et al. 2006, Semple et al. 1999).

Even though the impacts of oil on marine phytoplankton vary among species and environmental conditions (Garr et al. 2014, Huang et al. 2011), the dispersant are admittedly toxic to most marine organisms and the chemicals may elevate the toxicity of oil. Chemical dispersants are widely used to clean up and disperse oil throughout the water, thus preventing the oil from impacting shorelines. However, a number compositions of dispersant are confidential, and their proportions in the mix are not often to public knowledge (Wise & Wise Sr. 2011). In this experiment, a local dispersant was used, namely IPAC-OCD and its chemical properties are also proprietary. Therefore, the use of chemical dispersants to clean up oil spills should be evaluated, but more research should be drawn to obtain clear conclusions. Furthermore, this research helps to predict the potential acute impact of diesel oil and dispersant application on marine phytoplankton, but the impact of these toxicants on phytoplankton will depend on the specific situation of each accident.

CONCLUSION

This study demonstrates that WAFs of diesel oil, CEWAFs and dispersant significantly affect the growth of *C. calcitrans* and *T. chuii*, and the toxicity of those chemicals increase through the time. *C. calcitrans* is much more tolerant to the WAFs than *T. chuii,* and yet the WAFs are practically non-toxic to both the species. However, the dispersant is very highly toxic to *C. calcitrans,* and that chemically dispersed diesel oil is more toxic than diesel oil alone to this phytoplankton. Furthermore, even though the toxicity of dispersanit to *T. chuii* is higher than that of WAFs and CEWAFs, the toxicant is practically non-toxic to the phytoplankton. The results of this study suggest that the use of dispersants to disperse oil spills may elevate the toxicological effects to phytoplankton, particularly to the diatom, *C. calcitrans.*

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