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Synergistic toxicity of Macondo crude oil and dispersant Corexit 9500A® to the *Brachionus plicatilis* species complex (Rotifera)

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ABSTRACT

Using the marine rotifer *Brachionus plicatilis* acute toxicity tests, we estimated the toxicity of Corexit 9500A®, propylene glycol, and Macondo oil. Ratios of 1:10, 1:50 and 1:130 for Corexit 9500A®:Macondo oil mixture represent: maximum exposure concentrations, recommended ratios for deploying Corexit (1:10–1:50), 1:130 the actual dispersant:oil ratio used in the Deep Water Horizon spill. Corexit 9500A® and oil are similar in their toxicity. However, when Corexit 9500A® and oil are mixed, toxicity to *B. manjavacas* increases up to 52-fold. Extrapolating these results to the oil released by the Macondo well, suggests underestimation of increased toxicity from Corexit application. We found small differences in sensitivity among species of the *B. plicatilis* species complex, likely reflecting phylogenetic similarity. Just 2.6% of the water-accommodated fraction of oil inhibited rotifer cyst hatching by 50%, an ecologically significant result because rotifer cyst in sediments are critical resources for the recolonization of populations each Spring.

1. Introduction

The April 2010 oil spill in the Gulf of Mexico discharged 4.9 million barrels of crude oil from the Macondo well (OSAT/NOAA report, 2010). One of the first responses was to apply more than 1 million gallons of the oil dispersants Corexit 9527A® and Corexit 9500A® to the sea surface, and more than 770 thousand gallons to the sub-sea (On Scene Coordinator Report DWH, 2011). This large scale application of oil dispersants, motivated us to examine the effects of the dispersants on toxicity, especially given the limited toxicity information that is available (Judson et al., 2010).

Although oil dispersants are preapproved for this use and their deployment is widespread, there are doubts in the regulatory community about the efficacy of dispersants to ameliorate the biological impacts of oil spills because of the poor understanding of oil dispersant toxicity (Singer et al., 1998). Rigorous toxicological comparison of untreated and dispersant-treated oil is complicated by the fact that when oil, seawater, and dispersants are mixed, a complex multiphase system results. In this complex system, aquatic organisms can be exposed to many toxicants, in many forms, which can have several modes of action (National Research Council, 1989). Moreover, chemical dispersion of oil can yield: (1) dissolved petroleum hydrocarbons; (2) dissolved dispersant surfactants; (3) mixed droplets of bulk oil and surfactants (often in micellar form); and (4) nonmicellar, particulate bulk oil (Singer et al., 1998).

A second important issue for determining the effects of dispersants, is the separate and combined toxicity of the dispersant and the crude oil droplets. Toxicity became an important issue in the late 1960s and early 1970s when application of toxic products resulted in substantial loss of sea life (Fingas, 2002). Since that time, dispersants have been formulated to minimize toxicity to aquatic organisms. For example, the LC50 values of dispersants used in the early 1970s ranged from about 5 to 50 mg/L to the rainbow trout in 96 h exposures. In contrast, LC50s for dispersants available today vary from 200 to 500 mg/L and contain a mixture of surfactants and a less toxic solvent (Fingas, 2002). Nonetheless, use of oil dispersants remains a controversial countermeasure to minimize the impact of oil spills. Their ecological effects depend on whether oil dispersion increases or decreases exposure of aquatic species to toxic components of oil (Ramachandran et al., 2004). Ramachandran et al. (2004) evaluated whether fish exposure increased to polycyclic aromatic hydrocarbon (PAH) in dispersed oil relative to equivalent amounts of the water-accommodated fraction (WAF). They used fish cytochrome P4501A gene (CYP1A) induction in trout exposed to the dispersant Corexit 9500A, WAFs, and the chemically enhanced WAF dispersant of three crude oils. They concluded that Corexit 9500A® was not an inducer of CYP1A and it did not appear to affect the permeability of the gill surface to known inducers such as

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Table 1
Characteristics of the five strains of the *Brachionus plicatilis* species complex used in this work.

Description of strain	Abbreviation	Location of original collection	GenBank accession number of coxI gene sequence
Brachionus manjavacas	MAN	Sea of Azov, Russia	AY785194
Brachionus plicatilis sensu stricto	TOK	Tokyo, Japan	AY785175
Brachionus rotundiformis	HAW	Hawaii, USA	HM024708
Brachionus sp.	VER	Alvarado Lagoon, off the coast of Veracruz, Gulf of Mexico	JX644944

β-napthoflavone. Therefore, the use of oil dispersants will not increase the exposure of fish to hydrocarbons in crude oil.

The EPA required BP p.l.c. to use the *Brachionus plicatilis* acute toxicity test to assess the toxicity of oil dispersant mixtures in the Gulf of Mexico (U.S. EPA subsurface dispersant directive to BP, 2010). The species *B. plicatilis* has long been used in ecotoxicology to assess toxicity in marine waters (American Society for Testing Materials, 1998; Anon., 1998). It is one of the few cost-effective marine toxicity tests that can be replicated hundreds of times in a few days. *Brachionus plicatilis* was thought to be one species, and therefore only a single *Brachionus* marine species has been mostly used in toxicity tests, although at least 15 are believed to exist (Suatoni et al., 2006). Some of these may be more sensitive to toxicants or have other properties that make them more useful in toxicity assessments of marine waters. In light of the recent environmental catastrophe in the Gulf, it seemed prudent to systematically explore the full range of biodiversity of *Brachionus* species to identify the most sensitive species for marine toxicity assessment.

Therefore, the goals of our investigation are: 1) to study the effect of crude oil, Corexit 9500A® oil dispersant and its water-accommodated fractions on five *B. plicatilis* species complex lineages whose phylogenetic signature can be investigated and correlated with sensitivity to these toxicants, 2) assess the effects of a crude oil and Corexit 9500A® mixture at concentrations that are environmentally relevant.

2. Materials and methods

2.1. Sampling, resting egg hatching, and culturing

Geographical strains of marine *Brachionus* sp. were collected from 5 localities from several parts of the world (Table 1). The Veracruz strain is unable to produce cysts (at least under laboratory conditions) and therefore the culture was started from parthenogenetic females. Instant Ocean™ was use to prepare reconstituted seawater. Resting eggs of the other four strains were hatched in 15 psu reconstituted seawater approximately 15 cm below 40 W white fluorescent light bulbs. Rotifer were cultured in 3 mL in wells of a 9-well plastic plate filled with 15 psu reconstituted seawater, and the green alga *Tetraselmis suecica*.

2.2. Acute toxicity tests

We used the *B. plicatilis* acute toxicity test protocol described in Standard Methods (Anon., 1998) and in the American Society for Testing Materials (ASTM) protocol (ASTM, 1998). It is now understood that the species *B. manjavacas* is the species originally used to develop that protocol according to genetic analysis (Fontaneto et al., 2007). Instead of using neonates hatched from cysts (diapausing eggs) as described in protocol, we used neonates hatched from parthenogenetic eggs that were less than 24-h old. Toxicity tests with *Brachionus manjavacas* neonates hatched from cysts were also conducted to conform to the original Standard Methods and ASTM protocols and to compare results with neonates hatched from parthenogenetic eggs. A total of five independent replicates each consisting of 10 rotifer per well were conducted to obtain the Median Lethal Concentration (LC50) values for each treatment. The protocol for preparation of oil–water-accommodated-fractions (WAF) and enhanced water-accommodated-fractions (CEWAF) solutions for toxicity testing followed the recommendations of Singer et al. (2000). We stirred Macondo sweet crude oil with Instant Ocean® artificial seawater at 15 psu for 8-h with a magnetic stirrer to obtain the WAF's. LC50 values for crude oil, Corexit 9500A®, propylene glycol, which is a major component of Corexit 9500A® (Nalco Energy Services, 2012) and the Macondo oil fractions were calculated using probit models (Díaz et al., 2004).

2.3. Acute toxicity tests with Corexit 9500A:Macondo oil mix

Clark et al. (2001) suggest a 1:10 maximum exposure concentration for the Corexit 9500A®:oil mix. In contrast, the U.S. EPA (1995) recommended a 1:50 ratio. Therefore, we tested 1:10, 1:50 and 1:130 Corexit 9500A:Macondo oil ratios. This was accomplished by 8-h stirring of both the oil and the dispersant as previously described for preparing of WAF's. A different experiment consisted of adding 0.01% Corexit 9500A® (the 24-h NOEC value for Corexit with *B. manjavacas*) to a different set of Macondo oil concentrations to investigate synergistic effects during oil dispersion without stirring for 8-h. In this experiment the Corexit 9500A®:oil ratios were variable for each concentration ranging from 1:25 to 1: 500. Toxicity tests were done as described above. Five independent replicates each consisting of 10 rotifer per well were conducted to obtain the LC50 values for each treatment.

2.4. Reproductive and cyst hatching inhibition end-points

Reproductive tests were performed on neonates born from parthenogenetic *B. manjavacas* females according to the Standard Methods protocol (Anon., 1998). Twelve replicate neonates (five neonate rotifers per well), were exposed for 48 and 72-h to sublethal concentrations of Corexit 9500A® [1×10^{-6}–0.001% (v/v)], Macondo oil [0.25–5% oil (v/v)], and propylene glycol [0.1–5% (v/v)] in 1 mL volumes in a 24-well plate with 1×10^5 cells/mL of *Tetraselmis suecica*. The 24-well plates were then placed in a bioclimatic chamber under continuous light at a temperature of 25 °C for 48 and 72-h. At the end of these incubation periods, we counted the number of individuals in each well and calculated *r* (the instantaneous growth rate).

Cyst hatching inhibition assays consisted in hydrating dry *B. manjavacas* cysts for three hours, then, exposing them to same sublethal concentrations as above of Corexit 9500A®, Macondo oil WAF's, propylene glycol, or a Corexit 9500A®:Macondo oil mix for 24 or 48-h periods under fluorescent light. The number of hatching and non-hatching cysts was recorded, compared to controls in which no oil mixtures were added, in twelve replicates performed in three different dates. Each replicate consisted of ten cysts.

2.5. Statistical analysis and interpretation of data

We performed a one-way analysis of variance (ANOVA) with three independent treatments (each with four replicates) to compare five toxicant concentrations against the negative control and Dunnett's test to determine significant differences between the means of each toxicant concentration versus the no toxicity control. This allowed determination of the NOEC (no observed effect concentration) and the LOEC (lowest observed effect concentration). The EC50 values (the concentration where a 50% reduction in either the *r*-value or cyst hatching percentage, was observed) were calculated by linear regression of the different toxicant concentrations and the *r* values or cyst hatching percentages.

2.6. DNA sequencing

Genetic analyses using the cytochrome *c* oxidase subunit 1 (COI) gene were conducted to verify the species of each rotifer isolate used in these experiments. To minimize algal contamination, rotifers were incubated in 15 psu artificial seawater for 30 min to allow the rotifer guts to clear digested algal material. Genomic DNA was extracted from fresh rotifer tissue (500–1000 rotifers) using the DNeasy Tissue Extraction Kit (Qiagen). A 713 nucleotide region of the COI gene (Palumbi, 1996) was amplified via the polymerase chain reaction (PCR) using either universal COI primers LCOI1490: GGTCAACAAATCATAAAGATATTGG and HCOI2198: TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al., 1994), or (VER strain only) degenerate COI primers modified from Folmer et al. (1994), dgLCO: GGTCAACAAATCATAAAGAYATYGG and dgHCO TAAACTTCAGGGTGACCAAARAAYCA (Meyer et al., 2005). Amplifications were performed in 10 μl volume solutions with 10–50 ng genomic DNA, 1 unit *Taq* DNA polymerase and a final concentration of 0.2 mM of each dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2.5 mM $MgCl_2$, and 0.2 mM of each primer. Thermal cycling protocol conditions consisted of a denaturing step of 2 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 47 °C for 90 s and 72 °C for 90 s on an Eppendorf MasterCycler. PCR products from TOK and HAW strains were directly sequenced in both directions (Nevada Genomics Center, University of Nevada, Reno). PCR products from the VER strain were cloned using TOPO TA Cloning Kit (Invitrogen) due to amplification with degenerate primers prior to sequencing. All sequences were manually edited in BioEdit vers 7.0.5.3 (Hall, 1999) and aligned using ClustalW (Larkin et al., 2007). Similarity to other *Brachionus* species was determined in a BLAST search (Altschul et al., 1990) of sequences deposited in the NCBI GenBank nucleotide database (www.ncbi.nlm.nih.gov).