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Comparative Toxicity of Louisiana Sweet Crude Oil (LSC) and Chemically Dispersed LSC to Two Gulf of Mexico Aquatic Test Species

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US EPA ARCHIVE DOCUMENT

1. INTRODUCTION

Large quantities of Louisiana sweet crude (LSC) oil have been released into the Gulf of Mexico since the explosion of the Deepwater Horizon oil exploration platform on April 20, 2010. The use of dispersants in oil spill response involves tradeoffs between effects to the shoreline and effects to pelagic and deep sea environments. In an effort to mitigate the impact of floating oil on sensitive shoreline habitats the decision was made to use dispersants listed on the U.S. Environmental Protection Agency's (EPA) National Contingency Plan (NCP) Product Schedule (EPA 2010a). Dispersants have been applied offshore on the surface as well as underwater at the source of the leak. In an effort to assess options to address this unprecedented event, the EPA conducted independent studies with eight dispersants on the NCP Product Schedule to assess the relative acute toxicity of each dispersant alone, LSC oil alone and dispersant-LSC oil mixtures.

Toxicity testing to determine the hazards of eight dispersant products has been performed in two phases. Phase one included acute toxicity tests with two Gulf of Mexico aquatic species, and *in vitro* cytotoxicity and endocrine screening assays using human cell lines. These tests were performed with each of the dispersants in the absence of oil. The results of the aquatic toxicity tests generally classified the dispersants as ranging from slightly toxic to practically non-toxic to both test species with the exception of one dispersant found to be moderately toxic to fish (Hemmer et al. 2010). The results of the *in vitro* tests showed that cytotoxicity was only observed at concentrations above 10 ppm, and none of the eight dispersants displayed biologically significant estrogenic or androgenic activity (Judson et al., 2010). Results of the *in vitro* tests were similar to the ecotoxicology tests showing generally low dispersant toxicity.

This report summarizes results of the second phase of testing obtained from acute toxicity tests conducted with LSC only and chemically dispersed LSC using each of eight commercial oil dispersants. The same eight dispersants were tested in both Phase One and Two: Corexit 9500A, Dispersit SPC 1000, JD-2000, Nokomis 3-AA, Nokomis 3-F4, Saf-Ron Gold, Sea Brat #4 and ZI-400. The approach utilized consistent test methodologies within a single laboratory which provided a means to independently assess acute toxicity estimates between LSC and dispersant-LSC mixtures for all eight dispersants.

US EPA ARCHIVE DOCUMENT

2. TEST METHODS

2.1 *Study Design*

In this study the acute toxicity of LSC and dispersant-LSC mixtures was examined using two Gulf of Mexico aquatic species: (1) the mysid shrimp, *Americamysis bahia*, an aquatic invertebrate, and (2) the inland silverside, *Menidia beryllina*, a small estuarine fish. These species are standard test organisms used in a variety of EPA toxicity test methods. The static acute toxicity test methods followed, with slight modification, EPA Test Method 821-R-02-012, *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (USEPA, 2002). Reference toxicant tests were performed and reported in Phase One (Hemmer et al., 2010).

Assessment of oil-only and dispersant-oil toxicity was determined using water accommodated fractions (WAFs) of LSC or chemically-enhanced water accommodated fractions (CE-WAFs) of dispersant-LSC as described below. All tests were conducted using an established contract testing laboratory and in compliance with the Good Laboratory Practice regulations as provided in EPA 40CFR160 (USEPA, 40CFR Part 160). Two rounds of testing of the dispersant-oil mixtures were necessary to ensure that test concentrations bracketed the CE-WAF LC50 and met test condition requirements for both mysids and *Menidia*.

2.2 *Test Organisms, Oil and Dispersants*

Larval mysid shrimp were supplied from in-house cultures maintained by the contract testing laboratory using filtered natural seawater. Larval *Menidia* were purchased from Aquatic Biosystems, Inc., Fort Collins, CO, shipped overnight to the testing laboratory and held a minimum of two days prior to testing. Culture and holding conditions for both species were 25^{o}C and 20 parts per thousand salinity. Mysids were 24-48 hours old and *Menidia* 11 or 14 days old at test initiation. All organisms for a given exposure were within 24 hours of the same age.

Non-weathered Louisiana sweet crude oil, lot # WP 681 was purchased from RT Corporation, Laramie, WY in 500 ml amber bottles and shipped directly to the testing laboratory. All dispersants were shipped directly from each manufacturer to the contract testing laboratory,

US EPA ARCHIVE DOCUMENT

logged into their test material center and maintained in accordance with GLP and Chain-of-Custody requirements.

2.3 *Preparation of Water Accommodated Fractions*

LSC WAFs and dispersant/LSC CE-WAFs were prepared following the methods of the Chemical Response to Oil Spills: Ecological Effects Research Forum (CROSERF) (Singer et al., 2000) with the variable dilution modification described by Barron and Kaaihue (2003). In brief, glass aspirator bottles containing a hose bib fitted with silicon tubing and clamp at the bottom of the vessels were used to prepare the LSC WAFs. Each bottle was filled with 19L of seawater leaving a 20% headspace above the liquid, a stir bar added and placed on magnetic stir plate. LSC was added at 25g/L seawater using a long tube attached to a glass funnel to reduce production of air bubbles in the surface slick. The stir plate was adjusted to obtain an oil vortex of 25% of the total volume of seawater which provided a similar mixing energy in each WAF preparation. The bottles were securely covered and the solutions mixed for 18 hours then allowed to settle for 6 hours. The WAF (aqueous phase) was removed from the bottom without disturbance of the oil slick remaining on the surface. The WAF was remixed after removal and 2 liters of the WAF was used for analysis of TPH with the remaining volume available for preparation of the test solutions.

The method for the preparation of each of the 8 dispersant/LSC CE-WAFs followed the LSC WAF procedure with the addition of each dispersant at a ratio of 1:10 dispersant to oil (2.5 g/L) after the 25% oil vortex was established. Mixing and settling times followed the oil-only procedures above. Dispersant manufacturers have generally recommended application rates using dispersant to oil ratios between 1:50 to 1:10 depending on oil type and sea conditions. A ratio of 1:10 is the standard recommendation for dispersant toxicity testing because it maximizes the effect of the dispersant on oil in the CE-WAF (Barron and Kaiihue, 2003).

2.4 *Preparation of Test Solutions*

Separate oil-only WAFs were used for preparing test solutions for mysids and *Menidia* with LSC. For dispersant-oil testing, each CE-WAF was divided and used to prepare solutions for both mysid and *Menidia* tests. Natural filtered seawater adjusted to 20 parts per thousand with laboratory well water was used for all static acute tests. Larval mysids and *Menidia* were

US EPA ARCHIVE DOCUMENT

treated with dilutions of LCS WAF or dispersant/oil CE-WAF plus an untreated seawater control. Six concentrations (plus control) of the oil-only WAF was tested, with the highest exposure level being 100% WAF. Each of the dispersant/oil CE-WAF tests was performed with 6 to 8 exposure concentrations (plus control) in order to bracket the median lethal concentration (Table 1).

2.5 *Exposure Conditions*

Three replicates were conducted for each exposure concentration. Test organisms were randomly assigned across exposure and control treatments with each replicate receiving 10 animals for a total of 30 animals per treatment level. One liter beakers containing 1L of test solution were maintained in 25º C temperature-controlled water baths under a photoperiod of 16 hr light:8 hr dark. All test vessels were continuously aerated (100 bubbles/min). The duration of the acute tests was 48 hrs for mysids and 96 hrs for *Menidia*. Temperature was monitored continuously using max-min thermometers; salinity and dissolved oxygen was measured once per day.

2.6 *Analytical Chemistry*

A one L sample was collected from each WAF and two replicate 1 L samples were collected from each CE-WAF for analysis of total petroleum hydrocarbons (TPH), the standard method for quantifying oil in water. Each sample was extracted with hexane, reduced to 1 mL and analyzed by GC-FID following EPA SW-846, Method 8015B –DRO. Additional analyses were performed on the CE-WAFs of Corexit 9500A and JD-2000 to provide tentative identification of single high level chemical peaks in the chromatograms. The peaks were identified as non-petroleum hydrocarbon constituents in Corexit 9500A and JD-2000 and were removed from the calculation of their respective TPH values. Final CE-WAF concentrations of TPH were determined as the average of the two replicate measured values and reported as mg TPH/L.

2.7 *Statistical Analysis*

The commercially available statistical software package, CETIS® was used for the calculation of LC50 values using an automated decision tree adapted from EPA for selection of

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the appropriate statistical method (CETIS, 2009; USEPA, 1994). The LC50 is defined as the concentration of a substance causing mortality in 50% of test organisms for a specified time interval, in this case, 48-hours for the mysid test and 96-hours for the silverside test. The non-parametric Spearman-Karber and Trimmed Spearman-Karber methods were used to calculate the LC50 values and 95% confidence intervals. LC50 values are reported as parts per million (mg/L).

3. RESULTS

3.1 *Oil-only Acute Tests with Mysid and Menidia*

3.1.1 *Test Acceptability*

Control performance met all criteria for an acceptable exposure in each test (≥90% survival) for both mysid and *Menidia* exposures. All water quality parameters in all treatments were within ranges specified in the protocol for each species.

3.1.2 *Measured TPH and Toxicity Results*

The measured TPH concentration in the LSC WAF used for the acute mysid test was 4.4 mg/L, resulting in a calculated LC50 value of 2.7 mg/L and corresponding 95% confidence interval of 2.5-3.0 mg/L. The measured TPH concentration in LSC WAF used for the acute test with *Menidia* was 2.9 mg/L. *Menidia* mortality did not exceed 7% in the highest concentration tested consisting of 100% WAF, indicating that the oil-only LC50 was greater than 2.9 mg/L. Retesting of *Menidia* with LSC WAF is scheduled and will be reported when complete.

EPA uses a five-step scale of toxicity categories to classify pesticides based on their acute toxicity to aquatic organisms which is provided in Table 2 (USEPA, 2010b). Using this toxicity classification, LSC would be classified as moderately toxic to mysids and the toxicity of LSC to *Menidia* is estimated to be within the range of moderately toxic to practically non-toxic.

3.2 Measured *TPH in Dispersant-oil CE-WAF*

TPH in 100% CE-WAF ranged from 6.8 mg/L for JD-2000 to 1800 mg/L for ZI-400 (Table 1). The CE-WAFs for Corexit 9500A and JD-2000 each contained a single large non-petroleum hydrocarbon chemical peak. The chromatogram peak areas associated with these

US EPA ARCHIVE DOCUMENT

compounds were removed from the calculation of TPH prior to determination of the LC50 values and 95% confidence intervals for Corexit 9500A and JD-2000.

3.3 <u>*Dispersant-oil Toxicity Tests - Mysids*</u>

3.3.1 *Acceptability of initial tests*

Control performance met all criteria for an acceptable exposure (≥90% survival) in all initial tests. A definitive LC50 was determined for the initial test with Saf-Ron Gold CE-WAF and all water quality parameters were within ranges specified in the protocol for all treatments. Initial tests with CE-WAFs of Dispersit SPC 1000, Nokomis 3-AA, Nokomis 3-F4 and ZI-400 had LC50s less than the lowest concentration tested which required a second round of testing. A second round of testing was also required for Corexit 9500A, JD-2000 and Sea Brat #4 because of temperature or dissolved oxygen deviations from protocols. Reference toxicant tests are reported in Hemmer et al. (2010).

3.3.2 *Acceptability of Second Round Tests*

Control performance met all criteria for an acceptable exposure (≥ 90% survival) in each of the CE-WAF tests conducted in round two. All water quality parameters were within ranges specified in the test protocol for mysids and definitive LC50 values were determined for each of the seven dispersant-oil CE-WAFs tested.

3.3.3 *Mysid Definitive Test Results*

The LC50 values for dispersant-oil acute toxicity tests with mysids ranged from 0.39 to 9.7 mg TPH/L for Nokomis 3-AA and ZI-400, respectively (Table 3). Using the EPA toxicity classification, oil-dispersant mixtures using Nokomis 3-AA would be considered highly toxic, whereas Corexit 9500A, Dispersit SPC 1000, JD-2000, Nokomis 3-F4, Saf-Ron Gold, Sea Brat #4 and ZI-400 would be classified as moderately toxic to mysid shrimp.

3.4 <u>*Dispersant-oil Toxicity Tests – Menidia*</u>

3.4.1 *Acceptability of initial tests*

US EPA ARCHIVE DOCUMENT

Control performance met all criteria for an acceptable exposure (≥90% survival) in all initial tests. Initial tests with CE-WAFs of Dispersit SPC 1000, Nokomis 3-AA, Nokomis 3-F4 Saf-Ron Gold and ZI-400 had LC50s less than the lowest concentration tested, which required a second round of tests to be conducted. Second round testing was also required for Corexit 9500A, JD-2000 and Sea Brat #4 due to temperature or dissolved oxygen deviations from protocols. Reference toxicant tests are reported in Hemmer et al. (2010).

3.4.2 *Acceptability of second round tests*

Control performance met all criteria for an acceptable exposure (≥ 90% survival) in each of the CE-WAF tests conducted in round two. All water quality parameters were within ranges specified in the test protocol for *Menidia*. In the second round of acute tests, LC50 values and 95% confidence intervals were successfully determined for all eight dispersant-oil CE-WAFs.

3.4.2 *Menidia Definitive Test Results*

The LC50 values for dispersant-oil acute toxicity tests with *Menidia* ranged from 0.64 for Dispersit SPC 1000 to 13.1 mg TPH/L for ZI-400 (Table 3). Using the EPA toxicity classification, oil-dispersant mixtures using Dispersit SPC 1000 or Nokomis 3-AA would be considered highly toxic, whereas Corexit 9500A, JD-2000, Nokomis 3-F4, Saf-Ron Gold and Sea Brat #4 would be classified as moderately toxic and ZI-400 as slightly toxic to inland silversides.

4. CONCLUSIONS

The present study provided an independent, consistent, and quantitative assessment of acute toxicities of Louisiana sweet crude (LSC) oil and eight dispersant-LSC mixtures to two aquatic species inhabiting Gulf of Mexico waters. Toxicity was determined as the LC50 (mg TPH/L or ppm) derived from standard short term acute tests using standard test species, specifically the Gulf mysid, *Americamysis bahia*, and the inland silverside, *Menidia beryllina*. LSC oil alone was found to be moderately toxic to mysids with a toxicity value (i.e., LC50) of 2.7 mg TPH/L, whereas the toxicity to *Menidia* was not definitively determined (LC50 >2.9 mg TPH/L). In the dispersant-LSC acute tests, the toxicity values for mysids and *Menidia* ranged over two orders of magnitude (0.39 to 13.1 mg TPH/L). For mysid shrimp, Nokomis 3-AA was

US EPA ARCHIVE DOCUMENT

the most toxic and ZI-4000 the least toxic. For *Menidia*, Dispersit SPC 1000 and Nokomis 3-AA were the most toxic and ZI-4000 the least toxic of the dispersant-oil mixtures. The other five dispersant/LSC mixtures had generally similar toxicity to mysids and *Menidia*, with LC50 values ranging from 1.4 to 9.7 mg TPH/L.

Overall, the dispersants/LSC mixtures were classified as being highly toxic to moderately toxic depending on the test species and dispersant. The ZI-400/LSC mixture was the exception and would be considered only slightly toxic to *Menidia*. Corexit 9500A, the dispersant that has been applied offshore at the surface and in the deep ocean, falls into the moderately toxic category for both species. For all eight dispersants in both test species, the dispersants alone were less toxic than the dispersant-oil mixture. For mysids, LSC had greater toxicity (lower LC50s) than the eight dispersants when tested alone. LSC alone had similar toxicity to mysids as the dispersant-oil mixtures, with exception of greater toxicity of the Nokomis 3-AA/LSC mixture. The LSC results for *Menidia* were inconclusive because an LC50 was not determined at the highest tested concentration. Additional testing of the toxicity of LSC to *Menidia* will be performed by EPA.

Short-term acute toxicity tests using consistent methodologies and test organisms provide important and fundamental information on oil spill dispersants and other toxicants. The comparative toxicity analysis of dispersants, sweet crude oil and dispersant-sweet crude oil mixtures on standard aquatic test species provide an improved understanding of potential toxicological effects associated with this oil spill and help inform future decision-making.

US EPA ARCHIVE DOCUMENT

6.0 References

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US EPA ARCHIVE DOCUMENT

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Table 1. Measured concentrations and visual observations, and % exposure concentrations of dispersant-oil CE-WAFs preparations used in acute toxicity tests with mysid shrimp and inland silversides.

Dispersant-LSC CE-WAF	CE-WAF Visual Observations	Measured TPH in 100% CE-WAF (mg/L)	Definitive Test Concentrations (% CE-WAF)	
			Mysid Shrimp (*Americamysis bahia*)	**Inland Silverside (*Menidia beryllina*)**
Corexit 9500A	Very dark brown	44.6	50, 25, 13, 6.3, 3.1, 1.6	100, 50, 25, 13, 6.3, 3.1
Dispersit SPC 1000	Cloudy beige	400	3.1, 1.6, 0.8, 0.4, 0.2 0.1, 0.05	6.3, 3.1, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05
JD-2000	Slightly cloudy with oil particulates	6.8	100, 50, 25, 13, 6.3, 3.1	100, 50, 25, 13, 6.3, 3.1
Nokomis 3-AA	Slightly cloudy beige	87	3.1, 1.6, 0.8, 0.4, 0.2 0.1, 0.05	6.3, 3.1, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05
Nokomis 3-F4	Dark cloudy brown	1600	3.1, 1.6, 0.8, 0.4, 0.2 0.1, 0.05	3.1, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025
Saf-Ron Gold	Cloudy pearlescent white	57 (mysid) 63 (*Menidia*)	50, 25, 13, 6.3, 3.1, 1.6	6.3, 3.1, 1.6, 0.8, 0.4, 0.2
Sea Brat #4	Slightly cloudy, brown tint	86	6.3, 3.1, 1.6, 0.8, 0.4, 0.2	13, 6.3, 3.1, 1.6, 0.8, 0.4
ZI -400	Very cloudy brown	1800	6.3, 3.1, 1.6, 0.8, 0.4, 0.2 0.1, 0.05	3.1, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025

US EPA ARCHIVE DOCUMENT

Table 2. EPA five-step scale of toxicity categories used to classify chemicals based on their acute toxicity (USEPA, 2010b).

LC50 (ppm)	Toxicity Classification
> 100	Practically Nontoxic
>10 to 100	Slightly Toxic
>1 to 10	Moderately Toxic
0.1 to 1.0	Highly Toxic
<0.1	Very Highly Toxic

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Table 3. Results of mysid 48-hr and *Menidia* 96-hr acute toxicity tests with eight dispersant-oil mixtures (CE-WAFs) and oil-only (WAF), LC50 values, 95% confidence intervals [in brackets] and their toxicity classification derived in the present study. Results of LC50s and 95% confidence intervals [in brackets] from Phase One dispersant-only acute tests are supplied for comparison. [1] *From Hemmer et al., 2010;* [2] *ppm = µL/L;* [3] *ppm = mg TPH/L*

	Mysid Shrimp (*Americamysis bahia*)				**Inland Silverside** (*Menidia beryllina*)			
Dispersant CE-WAF	**Dispersant[1] LC50 (ppm)[2] [95% CI]**	**Dispersant Toxicity Category**	**CE-WAF LC50 (ppm)[3] [95% CI]**	**CE-WAF Toxicity Category**	**Dispersant[1] LC50 (ppm)[2] [95% CI]**	**Dispersant Toxicity Category**	**CE-WAF LC50 (ppm)[3] [95% CI]**	**CE-WAF Toxicity Category**
Corexit 9500A	42 [38-47]	Slightly Toxic	5.4 [4.9-6.7]	Moderately Toxic	130 [122-138]	Practically Nontoxic	7.6 [6.2-8.5.]	Moderately Toxic
Dispersit SPC 1000	12 [10-14]	Slightly Toxic	2.1 [1.9-2.2]	Moderately Toxic	2.9 [2.5-3.2]	Moderately Toxic	0.64 [0.56-0.72]	Highly Toxic
JD-2000	788 [627-946]	Practically Nontoxic	1.4 [1.2-1.6]	Moderately Toxic	>5600 [NA]	Practically Nontoxic	4.6 [4.3-4.9]	Moderately Toxic
Nokomis 3-AA	30 [27-34]	Slightly Toxic	0.39 [0.35-0.44]	Highly Toxic	19 [17-21]	Slightly Toxic	0.96 [0.96-1.0]	Highly Toxic
Nokomis 3-4F	42 [38-47]	Slightly Toxic	7.4 [6.6-8.3]	Moderately Toxic	19 [16-21]	Slightly Toxic	9.6 [8.2-11.2]	Moderately Toxic
Saf-Ron Gold	118 [104-133]	Practically Nontoxic	2.9 [2.6-3.1]	Moderately Toxic	44 [41-47]	Slightly Toxic	1.6 [1.5-1.8]	Moderately Toxic
Sea Brat #4	65 [57-74]	Slightly Toxic	1.4 [1.2-1.6]	Moderately Toxic	55 [49-62]	Slightly Toxic	3.4 [3.0-3.7]	Moderately Toxic
ZI-400	55 [50-61]	Slightly Toxic	9.7 8.1-11.7	Moderately Toxic	21 [18-23]	Slightly Toxic	13.1 [11.2-15.3]	Slightly Toxic
Oil-only WAF			**Oil LC50 (ppm) [95% CI]**	**Oil Toxicity Category**			**Oil LC50 (ppm)**	**Oil Toxicity Category**
Louisiana Sweet Crude			2.7 [2.5-3.0]	Moderately Toxic			> 2.9	≥ Moderately Toxic