

Toxicity to freshwater organisms from oils and oil spill chemical treatments in laboratory microcosms

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“Capsule”: *Toxicity of oil and diesel fuel to freshwater biota may be increased by use of oil spill cleaning agents.*

Abstract

Toxicity and temporal changes in toxicity of freshwater-marsh-microcosms containing South Louisiana Crude (SLC) or diesel fuel and treated with a cleaner or dispersant, were investigated using *Chironomus tentans*, *Daphnia pulex*, and *Oryzias latipes*. Bioassays used microcosm water (for *D. pulex* and *O. latipes*) or soil slurry (for *C. tentans*) taken 1, 7, 31, and 186 days after treatment. SLC was less toxic than diesel, chemical additives enhanced oil toxicity, the dispersant was more toxic than the cleaner, and toxicities were greatly reduced by day 186. Toxicities were higher in the bioassay with the benthic species than in those with the two water-column species. A separate experiment showed that *C. tentans*' sensitivity was intermediate to that of *Tubifex tubifex* and *Hyallolella azteca*. Freshwater organisms, especially benthic invertebrates, thus appear seriously effected by oil under the worst-case-scenario of our microcosms. Moreover, the cleaner and dispersant tested were poor response options under those conditions.

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1. Introduction

Most research on the fate and effects of oil entering the aquatic environment has focused on marine systems, as most of the large oil spills that have received much attention and evoked public outcry have occurred in marine environments. Parallel concern for the freshwater environment has lagged behind. However, oil spills do occur in freshwaters as a consequence of the many oil-related activities in this environment (Green and Trett, 1989). Hence, it is important to study impacts and cleanup options for petroleum spills in freshwater habitats.

Oil is retained much longer in marshes and other low-energy environments than on wave-swept coasts (Baca et al., 1985). Oils have been found in sediments at low-energy sites as much as 5 years after the occurrence of spills (Burns and Teal, 1979; Sanders et al., 1980), and they may be released into the water column long after

the initial spill. Thus water-column species as well as species inhabiting the sediment may be affected by oil spilled in low-energy environments. Oil may have various chronic effects on water-column species. These effects include neurosensory disruption, behavioral and developmental abnormalities and reduced fertility (Green and Trett, 1989). Oil spilled on the water surface may also limit oxygen exchange, coat the gills of aquatic organisms, cause pathological lesions on respiratory surfaces, and thus cause problems for aquatic organisms with their oxygen-supply and respiration. Effects of oil on freshwater benthic organisms may result from oil settling on the sediment surfaces and accumulating in the sediment. This can prevent invertebrate colonization or results in various lethal and sublethal effects (Hoehn et al., 1974). For example, exposure to crude Dubai oil caused 100% mortality in the benthos-dwelling isopod *Asellus aquaticus*, with severe toxicity observed after only a few hours at concentrations at 9.8 mg/l and above (Ramusino and Zanzottera, 1986). Complete recovery of benthic communities may be a matter of years, with communities in the mean time consisting solely of pollution-tolerant organisms (Harrel, 1985).

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Oil spill cleanup treatments aim to minimize ecological damage, and information regarding the effect of chemical treatments on acute and chronic effects of an oil spill for both sediment-inhabiting and water-column organisms is essential for arriving at an optimal response to oil spills. Chemical additive treatments for clean-up of oil spills can involve the use of cleaners and dispersants. Dispersants are used to promote the break-up or dispersion of an oil slick into small droplets that distribute into the water column (Clayton et al., 1992). Cleaners allow oil to be washed from surfaces, such as rocks or vegetation, and thereby also facilitate the recovery of the oil.

In comparison to the number of marine studies on the effects of surfactants and dispersants, relatively few investigations have been done in freshwater systems (Green and Trett, 1989). Under marine conditions, wave action and surface turbulence are usually high, the volume and depth of water treated is usually large and the land/water interface comparatively small, while the opposite is true in freshwater systems (Green and Trett, 1989). Mixing and dilution are therefore usually poor in freshwater systems and infiltration into marginal ecosystems may be accelerated. Also, fundamental differences exist between the physiology of marine and freshwater organisms. The increased osmotic challenges experienced by freshwater species and their need to conserve salts has led to the reduction in permeability and surface area of exposed membranes. Surfactants and dispersants can have marked effects on the integrity of these membranes and may disrupt vital transport mechanisms (Green and Trett, 1989). For example, dispersants were highly toxic to bluegill sunfish (*Lepomis macrochirus*) and channel catfish (*Ictalurus punctatus*).

Early hydrocarbon-based surfactant formulations proved to be highly toxic and have been superseded by less toxic concentrates and water miscible compounds (USEPA, 1993). However, these newer formulations can still exert their toxic effects on aquatic organisms. In invertebrates, effects of dispersants include reductions in brood size, offspring size, and duration of reproductive periods, and changes in larval molting patterns (Shcherban, 1980; Van Emden et al., 1974). Synergistic interactions in toxicity between oils and dispersants appear common (Sprague and Carson, 1970; Mitchell and Bennett, 1972; McCarthy and Lindblom, 1978; Allen, 1984). The presence of these toxicity interactions may differ among oils. For example, toxicity of Light Arabian Crude and No. 2 fuel oil to mangroves was increased by Corexit 9527, while it was decreased for Bunker C oil (Getter and Baca, 1984).

Another option for chemical treatment of an oil spill is the use of a cleaner such as Corexit 9580 (BioVersal USA, 1990; Fiocco et al., 1991). Their toxicity appears to be fairly low (BioVersal USA 1990; Fiocco et al., 1991). However, very little research has been done on

the effectiveness and toxicity implications for the use of cleaners in freshwater oil spills.

The project described here investigated the toxicity and temporal changes in this toxicity of two oils (South Louisiana Crude or SLC, and diesel) and two chemical additive treatments (the dispersant Corexit 9500, and the cleaner Corexit 9580) in laboratory microcosms containing fresh marsh soils, during a 6-month period following addition of the hydrocarbon mixtures. The project used soils from two different freshwater marsh sites in Louisiana that share dominant vegetation (*Panicum hemitomon*) but differ in soil composition. Toxicity was assessed using three freshwater animals, i.e. two water column species (*Daphnia pulex* and the fish *Oryzias latipes*), and a benthic species (the chironomid *Chironomus tentans*).

Since encountered toxicities were very high for *C. tentans*, a side project compared the sensitivity of this species to that of two other freshwater benthic species (the amphipod *Hyallolella azteca*, and the oligochaete *Tubifex tubifex*). This experiment used SLC and the cleaner (Corexit 9580).

2. Materials and methods

For the main project, soil samples were collected from two freshwater marsh sites in Louisiana and were used to set up 144 microcosms per site. At specific time points following the addition of oil and/or treatment chemicals, the microcosms were taken apart and water and soil portions were used in the toxicity experiments. Toxicity tests for each marsh site were done at different times, for logistical reasons. A side project was conducted to compare toxicity among three different benthic species. This project was set up with soils from only one marsh site, toxicity was determined at only one time point, and the design consisted of 28 microcosms.

2.1. Soil collection and microcosm preparation

For the main project, soils were collected from Big Burn (near Cameron Prairie National Wildlife Refuge, Louisiana, USA) on July 11 1997; and on October 24 1997 from Forked Islands (Louisiana, USA). Both marshes share the same dominant vegetation (*Panicum hemitomon*). Soil characterization was done on two soil cores from each collection site, and consisted of determining bulk density, mineral density, organic density, organic content, and water content. Cores were sectioned into 3-cm increments. Each increment was weighed to determine wet density, dried (at 100 °C) to a constant mass, and weighed to determine dry bulk density. Sub-samples of each increment were combusted at 400 °C for 12 h to determine organic matter content. Characterization of the soils revealed considerable differences with

respect to mineral content and organic composition. For the 23 Big Burn soil samples, mean bulk density was 0.075 g/cm^3 (range $0.05\text{--}1.0 \text{ g/cm}^3$), mean organic content was 69% (range 53.0–85.1%), mean mineral density was 0.026 g/cm^3 (range $0.005\text{--}0.047 \text{ g/cm}^3$) and mean water content was 88.9% (range 87.3–90.5%). For the 24 Forked Island soil samples, mean bulk density was 0.050 g/cm^3 (range $0.037\text{--}0.063 \text{ g/cm}^3$), mean organic content was 89% (range 82.4–95.6%), mean mineral density was 0.006 g/cm^3 (range $0.001\text{--}0.011 \text{ g/cm}^3$), and mean water content was 91.6% (range 89.8–93.4%). Hence the Big Burn soil had a higher bulk density and contained much more mineral matter, and hence less organic matter, than the Forked Island soil. Upon return to the laboratory, the soil was cut up with knives to promote the breakdown of bulky plant material and destruction of the living root network. Each bulk sample was homogenized, large roots of *Panicum hemitomon* were removed and smaller roots were cut up. No additional water was added to the bulk soil samples. The soil slurries thus obtained were used to prepare the microcosms.

When the homogenized soil was being placed in microcosms, six 100-ml subsamples were set aside and used to characterize the soil in the microcosms. Variables and methodology were the same as those used for the characterization of the soil cores. Differences in soil characteristics between samples from the two collection sites were less pronounced, though the same trends still existed. A total of 144 microcosms was prepared for each marsh site. Individual microcosms were prepared in 1-l erlenmeyer flasks which had been thoroughly rinsed with dichloromethane. Each flask was filled with 480 ml of the soil slurry, the sides were then rinsed down and the final volume in each flask was brought up to 800 ml with well water. The sides of the flask were wrapped in aluminum foil to limit the amount of light entry, but the mouths of the flasks were kept uncovered to allow natural evaporation. The microcosms were allowed to acclimate to the laboratory conditions for 5 months before initiation of the exposures. Deionized water was added to the microcosms once every two weeks during the aging process in order to maintain the 800-ml level in each flask.

The side-project used the same laboratory microcosm design described for the main project. The soil used for preparing these microcosms was collected from Forked Island at the same time as that used for the main project. A total of 28 laboratory-microcosms were prepared similarly to those used for the main experiment.

2.2. Treatment of microcosms with oils and dispersants

For the main project, two oils (South Louisiana Crude and diesel fuel) and two chemical additives (the cleaner Corexit 9580 and the dispersant Corexit 9500)

were employed for the exposures, in a design resulting in the following nine different test treatments: control, cleaner by itself, dispersant by itself, SLC by itself, SLC + cleaner, SLC + dispersant, diesel by itself, diesel + cleaner, and diesel + dispersant. Since bioassays were conducted at four different times (on day 1, day 7, day 31, and day 186 after addition of test treatments) and sampling of microcosms for conducting the bioassays was destructive, a different microcosm was set up for each time point. We used four replicates for each treatment \times time combination. Treatments with only chemical additives were not weathered, but treatments with oil and oil + chemical additives were weathered in deionized water before they were used for the exposures. The weathering was done to more closely approximate environmental conditions following an oil spill, and the “chemical treatments only” chemicals were not weathered to reflect the fact that chemical additives are applied fresh and thus initially encountered by the organisms in that state. The oil treatments without chemical additives were weathered using a 1:3 oil:water ratio by volume, and treatments with both oils and chemical responses were weathered using a 5:1:15 oil:chemical additive:water ratio by volume. Weathering was done prior to addition to the microcosms in 1000-ml beakers containing 600 ml deionized water. Each beaker was continuously stirred at a uniform speed under a fume hood for 17.5 h. The stirring speed was kept such that there was a vortex, but the vortex was not pulled down towards the stirrer bar. The weathering was done for all six treatments that contained oil. After weathering, oil fractions were separated from water fractions using a separatory funnel. The mixtures were allowed to sit until the phases had clearly separated (10 min for oil + water mixtures, 60 min for oil + water + dispersant/cleaner mixtures). Separated fractions were weighed to determine loss during weathering and to allow the later addition to microcosms on weight rather than volume basis. Loss in weight of SLC by itself due to weathering was 12.72%. Each fraction was stored in an amber glass jar under refrigeration until both oil and aqueous fractions were applied to the microcosms. Since not all exposures were started at the same time, the separation was required to allow the addition of similarly-composed treatments to replicate microcosms.

For treatments with oil, specific amounts (by weight) of oil and water fractions were added to microcosms, with the weights equivalent to 6 ml unweathered oil, 18 ml of water and 1.2 ml of dispersant or cleaner per 800-ml microcosm. Treatments with only chemical additives received additives and water by weight, equivalent to 18 ml of deionized water and 1.2 ml of cleaner/dispersant, while controls received only 18 ml of deionized water. Each fraction was added separately in order to maintain the 3:1 oil:water ratio used for weathering. The specific volumes of oil added to microcosms were based on trials

in other experiments that indicated that this amount of oil would produce approximately 75% coverage of the surface area in our microcosms. After addition of the oils and chemical additives, microcosms were left undisturbed during the treatment period (except for the addition of deionized water to compensate for loss of volume due to evaporation).

The side-project used seven different test treatments: control, cleaner by itself (two concentrations), SLC by itself (two concentrations), and SLC+cleaner (two concentrations). There were again four replicate microcosms per treatment. The following combinations were weathered in 1000-ml beakers containing 600 ml deionized water: (1) SLC (200 ml); and (2) SLC+cleaner (200 ml oil + 40 ml COREXIT 9580). Each beaker was continuously stirred at uniform speeds under a fume hood for 17.5 h. The oil and water fractions were separated and stored using the procedures utilized for the main project. Treatments with only the cleaner received specific amounts of additives and water by weight, equivalent to 18 ml of deionized water and 1.2 ml of cleaner per microcosm for one test concentration, and 9 ml of water and 0.6 ml cleaner for the other concentration. Controls received only 18 ml of deionized water. For treatments with oil, oil and water fractions were added to the appropriate microcosm in a specific oil:cleaner:water ratio by weight (equivalent to 6 ml unweathered oil, 18 ml of water and 1.2 ml of cleaner per microcosm for one test concentration, and 3 ml of unweathered oil, 9 ml of water and 0.6 ml of cleaner for the other test concentration). After addition of the oils, microcosms were left undisturbed for seven days (except for the addition of deionized water to compensate for loss of volume due to evaporation), at which point the bioassays were started.

2.3. Use of microcosms in bioassays

Prior to conducting bioassays for the main project, the volume of the microcosms to be used that day was brought up to 850 ml using dechlorinated tap water. Each microcosm was divided into two 425-ml portions, with one portion used for hydrocarbon analyses (methodology and results reported elsewhere) and the other portion used for the bioassays as follows: (1) A 200-ml subsample (soil/water slurry) was used for bioassays with the benthic invertebrate *Chironomus tentans*; (2) The remainder of the sample was divided among two 250-ml centrifuge bottles (teflon coated) and centrifuged at 4000 rpm for 15 min at room temperature. The supernatant was used to perform the bioassays on the Japanese medaka (*Oryzias latipes*) and the *Daphnia pulex*.

For the side-project, microcosm volumes were again brought up to 850 ml. Most of the microcosm contents (600 ml of soil slurry) was distributed evenly over six test chambers for duplicate bioassays for each of the three test species (see below).

2.4. Bioassays

Bioassay procedures used throughout were based on the guidelines set forth in ASTM Standards on Aquatic Toxicology and Hazard Evaluation (American Society for Testing and Materials, 1993). Bioassays were conducted in duplicate for each of the four microcosm replicates. Since it was not feasible to conduct all 216 exposures (nine test treatments with four replicate microcosms, three test species, and two replicates per bioassay) for a specific time point simultaneously, the experiments for each of the two soil types were conducted three months apart, while experiments with the four microcosm replicates were staggered over 16 days. However, the 54 bioassays for a single replicate for one specific time point were conducted simultaneously for all treatments. Bioassays were conducted at room temperature (22–25 °C).

Chironomus tentans was cultured from egg-masses obtained from a culture at the Department of Fisheries and Wildlife, Michigan State University. Chironomids were cultured in 4-l glass culture bowls with about 5-cm substrate (shredded and acetone-leached paper towel) and culture water (dechlorinated tap water). Larvae were fed a suspension of finely ground fish food in dechlorinated tap water. Experiments were conducted with 28 to 30-day old larvae. Each set of experiments (one replicate of one specific time period for all test treatments) was conducted with chironomids from one culture (started with first instar larvae from at least three eggmasses). For every microcosm, two 250-ml exposure beakers were set up with the soil and water from that microcosm, each beaker containing 100 ml of test slurry. Six larvae of uniform size were added to each beaker. The tops of the test chambers were covered with aluminum foil perforated to allow air exchange. No aeration of test chambers or feeding of larvae was done during the bioassay. The mortality was assessed on day four, when the beaker contents were sieved. Larvae not recovered were assumed to be dead (since dead larvae break down rapidly under these conditions). The results of the two replicates for each microcosm were averaged, and only the means were used in the data analyses.

Medakas (*Oryzias latipes*) were obtained from Ward's. The adults were kept under 14-h light and 10-h dark conditions and fed twice a day with fish food flakes or brine shrimp nauplii. Eggs were collected on a daily basis and kept in dishes with dechlorinated tap water with some methylene blue (a fungicide). Newborn fry were removed daily from the culture dishes and fed *Paramecium* once a day, but were not fed during the bioassays. The bioassays used fry less than 72 hours old. For each test treatment, exposures were conducted in duplicate for each microcosm, in 50-ml beakers each containing 40 ml of the supernatant obtained from centrifugation of a portion of the contents of an individual

microcosm. Five randomly-chosen medaka fry were placed in each test chamber. No aeration was supplied during the bioassay. The toxicity endpoint was mortality, which was assessed on days 1, 2 and 4. Dead fish were removed from the test chambers. The survival counts for the duplicates of each microcosm were averaged over the 3 days and the overall mean for each microcosm was used in the data analyses.

Daphnia pulex was obtained from Carolina Biological Supply, maintained in the laboratory in 2-l culture bowls with dechlorinated tap water, supplied with gentle aeration, and fed once every two days with *Chlamydomonas*. New-born daphnids (0–1 day old) were used in the bioassays. These bioassays were set up in 24-well tissue culture plates, with duplicate wells containing five daphnids and 1 ml of supernatant from the centrifuged microcosm contents. The number of surviving daphnids in each chamber was monitored on days 1 and 2. The counts for the duplicates of each treatment were averaged over the two days, and the overall means were used in the data analyses.

For the bioassays in the side-project, three different freshwater benthic invertebrate species were used: the chironomid *Chironomus tentans*, the amphipod *Hyallolella azteca*, and the oligochaete *Tubifex tubifex*. The chironomid source and culturing methods were identical to those for the main experiment. The amphipods were obtained from Chesapeake Cultures and tubifex worms were obtained from Ward's. Both species were maintained in the laboratory in dechlorinated tap water with gentle aeration. They were fed finely ground fish food flakes. Juvenile amphipods (about 2–3mm long and 4–5 days old) were used for the bioassays, while the tubifex worms used were not limited to a specific age or size group. The bioassays in the side-project were done only at one time, i.e. on day 7 after addition of the test treatments. Duplicate test chambers (400-ml beakers) were set up for each of the three different bioassay species, each test chamber holding 100 ml of the test slurry. Six organisms were added to each test chamber, and chambers were covered with aluminum foil with perforations allowing air exchange. No aeration of test chambers or feeding of the organisms was done during the bioassay. Mortality of the test organisms was used as the biological endpoint, and was assessed on day four. The organisms were separated from the sediment using a 500- μ m standard size sieve. The animals not recovered were assumed to be dead. The number of surviving animals was averaged for the two duplicates for each microcosm, and these means were used in the data analyses.

2.5. Statistical analyses

Results of all bioassays of the main project were analyzed using a 4-way ANOVA SuperANOVA (Statview). The dependent variable was the arcsine \sqrt{p} of survival. The four factors tested were oil scenario, chemical

additive, microcosm time, and species. The effect of the site factor was not considered for the analyses, as the two different sites were used merely to extend the validity of the results over more than just one marsh site and one soil collection time. Hence any variability due to the two soil collections was considered to be random variability. This made for a more conservative test for investigating effects of oil treatment, chemical additive, bioassay type/species, and treatment duration.

Results of the side-project were analyzed using a three-way ANOVA using SuperANOVA (Statview). The dependent variable was arcsine \sqrt{p} of survival, and the factors tested were oil, chemical response, and bioassay species.

3. Results

3.1. Main project

The four-way ANOVA showed significant effects for each main factor (i.e. oil scenario, chemical additive, time, and species), as well as for all interactions between the factors (Table 1). The effects of the nine different test treatments on the medaka, daphnids, and chironomids are shown in Figs. 1–3 respectively.

3.1.1. Variation in toxicity among bioassays

Toxicities differed appreciably among the three bioassays which varied in test species and microcosm component used (Figs. 1–3). Mortality was especially high in the chironomid bioassay. For example, 100% mortality occurred on day 1 for all treatments except the control (Fig. 3). In general, mortality was as follows: medaka < daphnid < chironomid, with overall least square means for survival of these three species being 60.9, 52.2, and 18.3%, respectively.

3.1.2. Toxicity of oils, chemical responses, and oil/response combinations

For all three species, the diesel was generally more toxic than South Louisiana Crude, with overall least square means of survival being 12.2 and 43.4%, respectively. For all three species, treatments with the dispersant were more toxic than the treatments with the cleaner, which in turn were more toxic than those without any chemical additive. Over all experiments combined, least square means for survival in the three additive treatments were 26.6, 44.1, and 60.9% for dispersant, cleaner, and no chemical additive respectively. The chemical additives showed some toxicity in the absence of oil, but toxicities were especially high when both oil and chemical additives were present. This interactive effect was again stronger for the dispersant than for the cleaner, with the highest mortalities in all three species being observed for the diesel + dispersant treatment (Figs. 1–3).

Table 1
Summary of the four-way ANOVA results for survival (arcsine \sqrt{p} transformed) in the main project^a

Effect	df	Sum of squares	Mean square	F value	P value
Oil scenario	2	134.973	67.487	1513.74	<0.0001
Additive	2	40.707	20.354	456.54	<0.0001
Time	3	36.196	12.065	270.63	<0.0001
Species	2	55.388	27.694	621.18	<0.0001
Oil scenario×additive	4	6.814	1.703	38.21	<0.0001
Oil scenario×time	6	3.722	0.620	13.92	<0.0001
Oil scenario×species	4	12.224	3.056	68.55	<0.0001
Additive×time	6	2.979	0.496	11.14	<0.0001
Additive×species	4	2.861	0.715	16.05	<0.0001
Time×species	6	3.293	0.549	12.31	<0.0001
Oil scenario×additive×time	12	9.149	0.762	17.10	<0.0001
Oil scenario×additive×species	8	10.609	1.326	29.75	<0.0001
Oil scenario×time×species	12	12.238	1.020	22.88	<0.0001
Additive×time×species	12	1.089	0.091	2.04	0.0191
Oil scenario×additive×time×species	24	6.295	0.262	5.88	<0.0001
Residual	754	33.615	0.045		

^a This experiment compared toxicity for three oil scenarios (none, diesel, SLC), three additive treatments (none, cleaner, dispersant), three freshwater species (daphnid, medaka, chironomid) and four time points (day 1, 7, 31, 186) after the treatment of the microcosms. ANOVA results are shown for the main effects and all interactions.

3.1.3. Temporal changes in toxicity

Toxicity of the treatments generally decreased over time (Figs. 1–3). Overall, the least square means for survival on day 1, day 7, day 31, and day 186 were 31.1, 35.4, 46.0, and 62.9% respectively. For all three species, treatments without oil (i.e. dispersant or cleaner by itself) became much less toxic over time, and were virtually non-toxic by day 186. The two oil treatments showed a similar pattern, but considerable toxicities remained by day 186, especially for the diesel treatments. However, this reduction in toxicity over time was more marked in case of the medaka (Fig. 1) and daphnids (Fig. 2) than for the chironomids (Fig. 3). For the medaka and daphnid bioassays, survival increased markedly with time, and by day 186 the toxicities of most treatments were reduced drastically (Figs. 1 and 2). However, the temporal change in toxicity was very slow for the chironomid bioassay (Fig. 3). For all time point tested, there was 0% survival in the SLC treatments with the cleaner and dispersants, and all of the diesel treatments. Only the cleaner and dispersant by themselves slowly lost their toxicity for the chironomids, and became non-toxic by day 186. Treatments with only SLC did not show any survival till day 31, and survival increased slightly more by day 186.

3.2. Side project

3.2.1. Comparison of the sensitivities of the three benthic test species

The results of the three-way ANOVA on the sediment bioassays with the benthic invertebrates (chironomids, amphipods, and tubifex worms) revealed highly sig-

nificant main effects, as well as significant interactions between all factors (Table 2). Overall, the amphipod was the most sensitive to the test treatments, while the tubificid was the most tolerant. Overall least square means for survival of amphipods, chironomids, and tubifex were 26.5, 33.4, and 90.8%, respectively. In the presence of SLC, survival was only seen in the tubifex worms (Fig. 4). Similarly to the results of the main project experiments, survival was reduced in the presence of oil or cleaner, while survival was further reduced when both oil and cleaner were present (Fig. 4).

4. Discussion

As always, the limits to the general applicability of an experiment's results are dictated by the experimental design. We used one concentration of two specific oils and two chemical treatments, and tested toxicity of the microcosm contents to three freshwater species at four specific time points. The fact that microcosms soils were obtained from two different marsh sites expands the applicability beyond one specific soil type. The conditions of our laboratory microcosms probably represented a "worst-case scenario" for an oil spill in a freshwater marsh, with no wind, water exchange, or wave actions to disperse or dilute the oil and chemical treatments. While those effects are likely to play a role in oil degradation and removal, wind and wave actions are less pronounced in marshes and estuaries than in open marine environments. For example, it was shown that the presence of numerous mud banks limited the flushing effects of tides in a Florida bay (Holmquist et al., 1989).

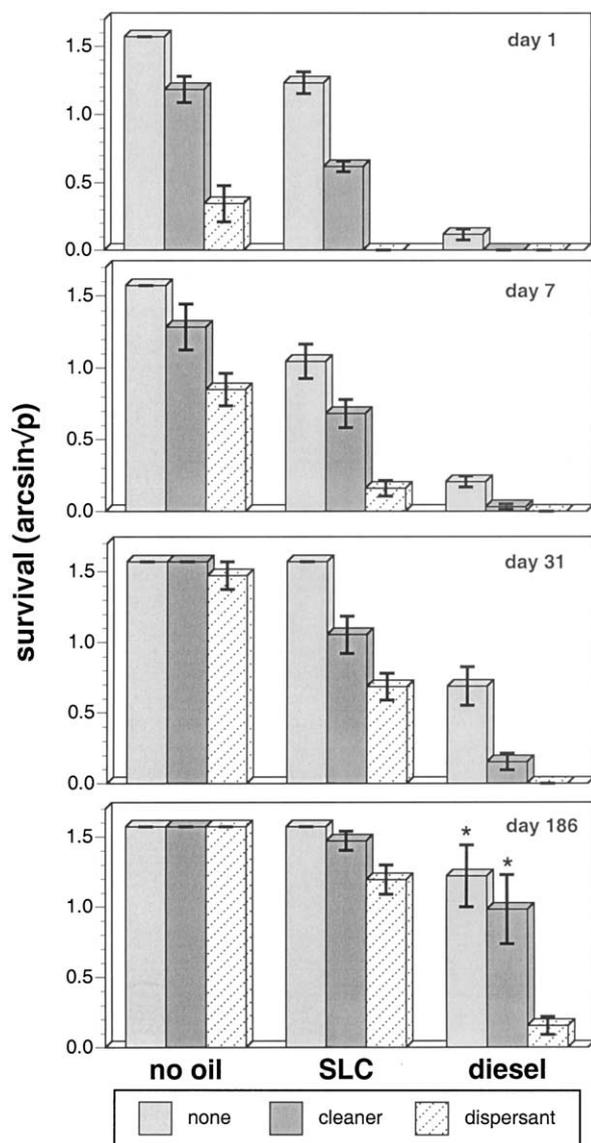


Fig. 1. Survival (least square mean \pm S.E.) showing oil and chemical additive treatment effects for exposures with the medaka. The bioassays for medaka used only the water from the microcosms. Samples sizes are $n=8$ (with exception of for $n=7$ for two means, indicated by *). Results are shown separately for each of the four different time points (day 1, 7, 31, and 186) on which bioassays were started. A 1.57 value for arcsine \sqrt{p} corresponds to 100% survival.

In some cases, chronic oil toxicity persists in the environment despite the initial flushing and weathering of oil as seen in case of the Bahía Las Minas oil spill on the Caribbean coast of Panama. Despite initial weathering and tidal flushing following the oil spill, anoxic mangrove muds served as long-term reservoirs for toxic hydrocarbons and caused chronic oiling and contamination of contiguous coastal communities for over five years (Levings et al., 1994). Thus, while being a worst-case scenario, results obtained in the microcosms provide insight into effects on organisms in oiled freshwater marshes.

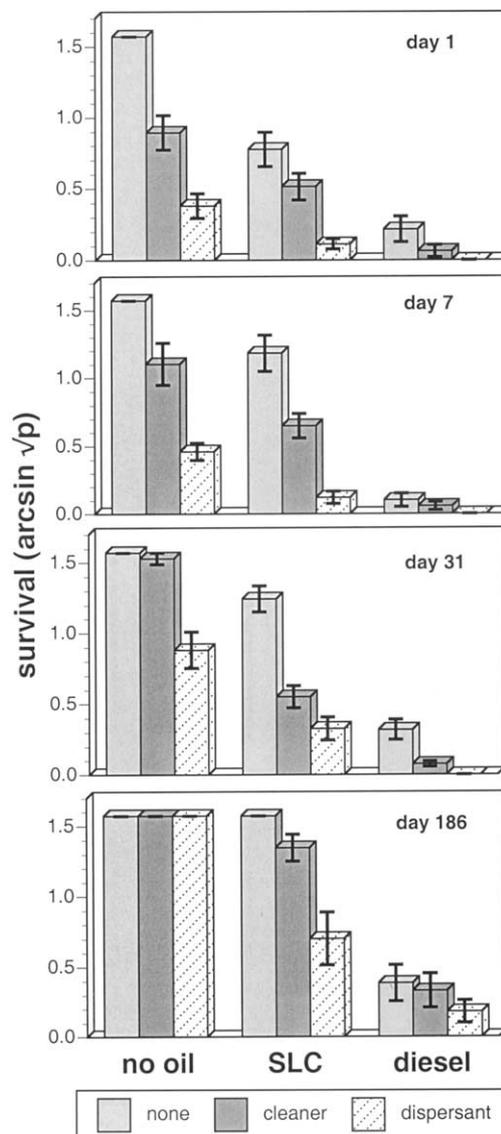


Fig. 2. Survival (least square mean \pm S.E., $n=8$) showing oil and chemical additive treatment effects for exposures with the daphnia. Exposures for daphnids used only the microcosm water. Results are shown separately for each of the four different time points (day 1, 7, 31, and 186) on which bioassays were started. A 1.57 value for arcsine \sqrt{p} corresponds to 100% survival.

The results revealed that South Louisiana Crude is less toxic than diesel, which is consistent with other studies which showed that diesel fuels are generally more toxic than the heavier crude oils (Moore and Dwyer, 1974; Michael, 1977; Rice et al., 1977).

Both cleaner and dispersant exhibited toxic effects, even in the absence of oil. The toxicity of the cleaner was less than that of the dispersant. Moreover, when the cleaner was used at half of the concentration used in the initial experiment, toxicity on day 7 after treatment was relatively minor in the chironomid bioassay. Previous research has shown that cleaners are less toxic than dispersants (BioVersal USA, 1990; Fiocco et al., 1991). Cleaners have been used effectively for treating oil spills;

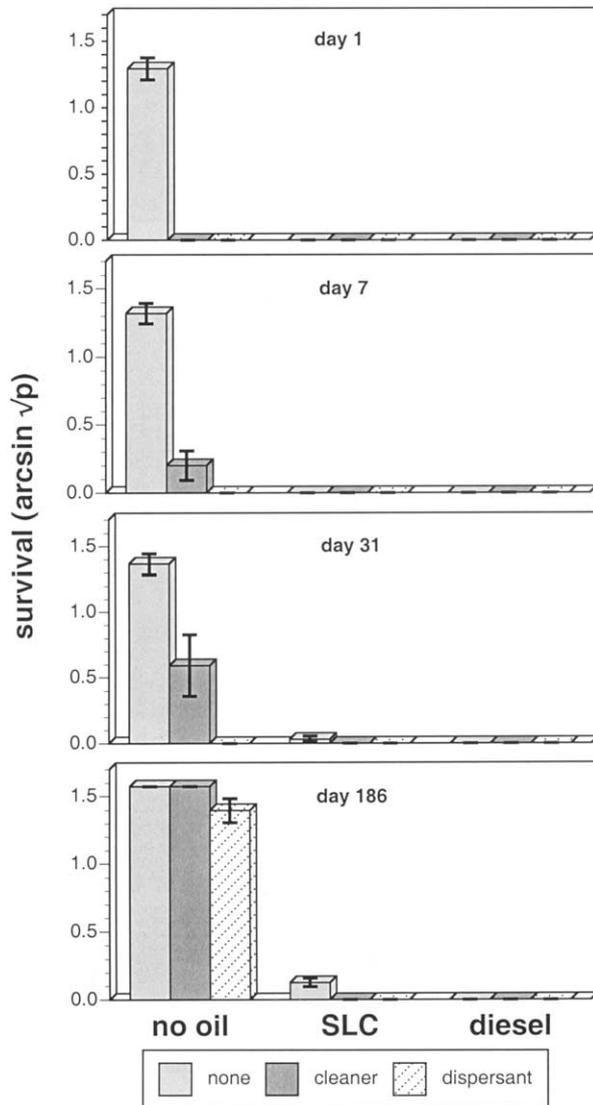


Fig. 3. Survival (least square mean \pm S.E., $n=8$) showing oil and chemical additive treatment effects for exposures with the chironomids. Tests with chironomids were performed with the microcosm soil slurry (water + oil). Results are shown separately for each of the four different time points (day 1, 7, 31, and 186) on which bioassays were started. A 1.57 value for arcsine \sqrt{p} corresponds to 100% survival.

for example, a cleaner prevented mortality of oiled freshwater marsh grasses in greenhouse experiments (Pezeshki et al., 1995), and a new non-dispersing cleaner was effective in restoring oiled mangroves without any toxic effects on the biota (Teas et al., 1993).

The most important result of this study is that the toxicity of both SLC and diesel was enhanced on addition of the dispersant and cleaner. This enhancement occurred for both water-column species (exposed to water-only of the treated microcosms) and benthic species (exposed to complete microcosm subsamples), and remained throughout the 186-day period following the oil and chemical treatments. These findings are consistent with those of other studies, where the toxicity of

Table 2
Summary of the three-way ANOVA results for survival (arcsine \sqrt{p} transformed) in the side-project^a

Effect	df	Sum of squares	Mean square	F value	P value
Oil scenario	2	12.776	6.388	292.55	<0.0001
Additive	2	3.220	1.610	73.73	<0.0001
Species	2	17.707	8.854	405.47	<0.0001
Oil scenario \times additive	2	0.626	0.313	14.33	<0.0001
Oil scenario \times species	4	2.028	0.507	23.22	<0.0001
Additive \times species	4	0.252	0.603	2.89	0.0292
Oil scenario \times additive \times species	4	2.920	0.730	33.43	<0.0001
Residual	63	1.376	0.022		

^a This experiment compared toxicity for three oil scenarios (none, SLC, SLC diluted), three additive treatments (none, cleaner, cleaner diluted), and three benthic species (chironomid, oligochaete, amphipod). ANOVA results are shown for the main effects and all interactions.

oils generally increased on application of dispersants (Sprague and Carson, 1970; Mitchell and Bennett, 1972). It was recently shown that such an enhancement may be dependent on toxicity endpoint, exposure duration and bioassay species (Singer et al., 1998). The interaction in toxicity between oil and dispersant is probably due to the dispersant causing the incorporation of the highly toxic volatile liquid hydrocarbons (VLHs) from the oil slick into the water column. These VLHs are readily transferred through biological membranes (McDonald et al., 1984). For example, experiments with No.2 fuel oil and the dispersants Corexit 7664 and Corexit 9527 showed that either of the dispersants combined with the oil increased the total water-soluble fraction of No.2 fuel oil in seawater and, consequently, toxic and teratogenic responses in larvae of inland silversides (Midgaugh and Whiting, 1995). The dispersion of VLHs into the water column may thus increase the toxicity to both water column as well as benthic species, as the dispersed hydrocarbons in the water column may sink and ultimately become incorporated in the sediment (Green and Trett, 1989). Also, benthic organisms may accumulate water-column hydrocarbons directly from the water column or from the interstitial water in the sediment as a consequence of chemical exchange between the water column and the interstitial water. In the current study, the oil + cleaner treatments were less toxic than the oil + dispersant combinations. This is likely to be due to the fact that most commercial cleaners do not disperse hydrocarbons from an oil slick into the water column like the dispersants do (BioVersal USA 1990; Fiocco et al., 1991). While one could hypothesize that the addition of cleaner or dispersant could speed up microbial degradation and therefore reduce toxicity at later time-points, our study following toxicity over a 6-month duration did not find any evidence for such a scenario. In support of this conclusion, results from hydrocarbon analyses conducted as a companion-study to this one,

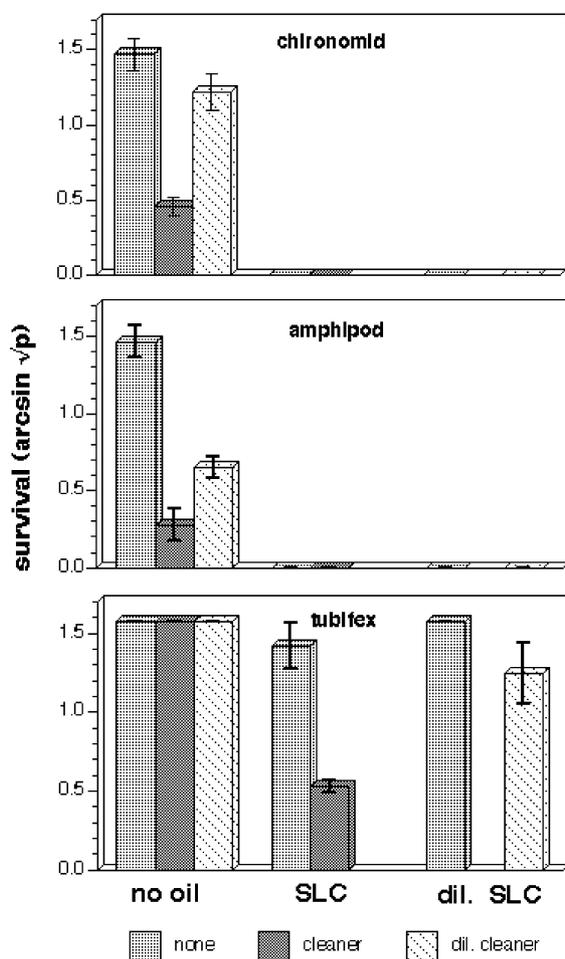


Fig. 4. Survival (least square mean \pm S.E., $n=4$) for each of the three benthic species in the test treatments of the side project (control, cleaner at two different levels, SLC at two different levels, and SLC + cleaner at two different levels). Results are shown separately for each of the test species. An arcsine \sqrt{p} value of 1.57 corresponds to 100% survival.

did not detect an increased loss of hydrocarbon from the microcosms in the presence of the cleaner or dispersant (unpublished results).

The toxicities of SLC and diesel diminished significantly over time in the daphnid and medaka bioassays, and SLC by itself was non-toxic by 6 months. In the chironomid bioassay, however, there was only a slight change in the toxicity of SLC and no change at all in the toxicity of diesel, even by 6 months. Both the cleaner and dispersant lost their toxicity over time, becoming virtually non-toxic to the daphnids, medaka, and chironomids by day 186. Similarly, for microcosms with both oil and chemical additives present, toxicities decreased over time. Improvements in the presence of the cleaner were more pronounced than those in dispersant-treated microcosms, while some improvement was observed for SLC, but not for diesel. This difference in toxicity reduction between the two oils could have been due to a difference in the rate of volatilization of the toxic com-

ponents of the two oils, or a difference in their rate of biodegradation. It has been shown that these rates are dependent on the composition of the oil (Green and Trett, 1989), and a study accompanying the present one showed that the temporal decline in hydrocarbon levels in the microcosms was faster for the SLC-containing ones than for those containing diesel (unpublished results). It has been shown that diesel added to mesocosms inhibits the oil-degrading bacterial communities during the first few weeks (Delille et al., 1998).

Mortalities in our experiments were especially high for the chironomid bioassay, with 100% mortalities in several treatments occurring over the entire 6-month period. The results are consistent with a field observation on the long term toxicity of oils and dispersants in sediments, where chironomids were totally eliminated even more than two years after an oil spill (Harrel, 1985). This long-term toxicity probably indicates that toxic components of oils and chemical additives persist in the sediments for quite a long time, and that rates of volatilization of the hydrocarbons from the soils are very slow. Previous studies have indicated that the sinking of oil into river and marsh sediments rapidly turns the sediment surface anoxic, resulting in anaerobic degradation of crude oil components by nitrate-reducing and methanogenic bacteria (Atlas, 1981). However, this anaerobic degradation is unlikely to remove significant quantities of oil from anoxic sediments. So oil may persist in the sediment for a long time once it becomes trapped in anoxic sediments (Green and Trett, 1989). In our experiment, the reduction in toxicity of the oil treatments was more pronounced for the water-column species than for the benthic species, indicating a quicker loss of the water-soluble fractions than the other fractions.

The results of the experiments with the chironomid (*C. tentans*) brought up the question whether chironomids were especially sensitive, or if the results indicate that hydrocarbon components other than the water-soluble fraction had a large contribution to toxicity (as the two water-column species were exposed to the microcosm water only while the chironomid bioassay used a complete microcosm subsample). The comparison of the sensitivities of the three benthic invertebrate species revealed that the chironomids are not especially sensitive. The sensitivity of the chironomids was intermediate to that of the amphipods and tubificid worms. These findings are consistent with the fact that oligochaetes, especially tubificid worms, are very tolerant benthic invertebrates (Chapman et al., 1979); and oligochaetes usually dominate the freshwater benthic invertebrate community immediately after an oil spill (Harrel, 1985). That benthic amphipods as a group are sensitive to oil pollution has been demonstrated repeatedly (Sanders et al., 1980; Elmgren and Frithsen, 1982; Elmgren et al., 1983). Our results are also consistent with other studies which showed that *Hyallolella azteca* was more sensitive to PAH-contaminated sediments

than *C. tentans* (Ankley et al., 1994) and *C. riparius* (Côté et al., 1998). So these results indicate that chironomids are not overly sensitive, and therefore probably a good indicator for overall effects of oil toxicity for the benthos.

In conclusion, this study revealed that the two oils in freshwater marsh microcosms did seriously affect the test species, and that this toxicity was especially pronounced for the benthic species. Diesel was more toxic than SLC, and the toxicity of both oils was enhanced on addition of the cleaner or dispersant. This enhancement persisted over time, indicating that these chemical additives may not be well suited for cleaning up oil spills in or near freshwater marshes. While applicability of the results is limited by the scope and design of the experiments, they shed light on an area in which little research has been done and improve our understanding of the effects of oil spills and chemical responses to oil spills in freshwater marshes.

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