



TOXICITY OF CRUDE OIL TO THE MAYFLY, *Hexagenia bilineata* (EPHEMEROPTERA: EPHEMERIDAE)

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Abstract

Effects of crude oil on survival and behavior of the mayfly *Hexagenia bilineata* were evaluated in laboratory studies. Mayfly nymphs were exposed to the water soluble and oil residue fractions of crude oil. Mayfly survival was not reduced by a 96-h exposure to either the water soluble fraction or the oil residue mixed with sediment. However, significant mortality did result from a 21-day exposure to oil residue mixed with sediment at concentrations as low as 500 $\mu\text{g g}^{-1}$. Survival was also reduced after a 21-day exposure to oil-contaminated sediments (1905 $\mu\text{g g}^{-1}$) collected 6 weeks after a crude oil spill in the Chariton River, Missouri. In a behavioral test measuring habitat exclusion, organisms did not avoid contact with sediment containing oil residue (50–800 $\mu\text{g g}^{-1}$). Collectively, results from these studies indicate that exposure to oil residue in sediment will reduce survival of *H. bilineata* in the laboratory and may reduce survival in the environment for 6 weeks or more after an oil spill.

INTRODUCTION

During 1988, large inland oil spills in the Ohio River, Pennsylvania and the Gasconade River, Missouri focused attention on the effects of oil in freshwater ecosystems. Such spills result in deposition of oil in backwater areas that provide critical habitat for larval fishes and benthic invertebrates. Information available on the response of invertebrates to oil in freshwater ecosystems is limited (Green & Trett, 1989); however, several field studies suggest that crude oil spills in rivers have severe and persistent impacts on organisms in the benthic macroinvertebrate community (Crunkilton & Duchrow, 1990; Finger *et al.*, 1991).

When oil is released into the aquatic environment, it partitions into volatile, water soluble, and insoluble fractions. Volatile constituents evaporate quickly from the surface, while the water-soluble fraction (WSF) and

the oil residue represent a more persistent source of exposure to aquatic organisms (Giddings & Franco, 1986). Toxic responses to oil have been attributed to the rapidly degraded constituents of the WSF (Moore & Dwyer, 1974), but oil residue in sediment may pose long-term hazards to benthic communities (Giddings & Franco, 1986).

Several field studies have documented absence or reduction in numbers of mayflies (Ephemeroptera) after exposure to oil. For example, following a crude oil spill into Asher Creek, Missouri, Ephemeroptera were absent at one site for 9 months (Crunkilton & Duchrow, 1990). Similarly, a spill of No. 2 heating oil into the headwaters of the Makotuku River, New Zealand, resulted in disappearance of Ephemeroptera for 10 months (Michaelis, 1983). Rosenberg *et al.* (1980) found reduced colonization by Ephemeroptera nymphs on crude oil soaked substrates compared with unoiled substrates after 279 days in the Trail River, Northwest Territories. Experimental pond studies also showed decreased abundance of Ephemeroptera immediately after application of oil (Cushman & Goyert, 1984; Giddings *et al.*, 1984).

Collectively, the literature suggests that oil represents a hazard to *Hexagenia bilineata*, an ecologically important food species for fish. This species is abundant in a variety of aquatic environments and is universally recognized as a species representative of healthy aquatic ecosystems (Fremling, 1964). Habitat requirements including contact with and ingestion of sediment (Fremling, 1960) qualify *H. bilineata* as an appropriate organism for testing effects of oil. This study provides information about the concentration and duration of crude oil exposure that causes adverse effects on *H. bilineata*. Six toxicity studies were completed: two 96-h exposures with the water soluble fraction (WSF) from two different crude oils, one 24-h behavioral test with oil-contaminated sediment, one 96-h range-finding test and one 21-days exposure with oil-contaminated sediment, and one 21-days exposure with sediment contaminated during a recent oil spill in the Chariton River, Missouri. We also examined the relation between nominal additions and measured values of total petroleum hydrocarbons in sediments.

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MATERIALS AND METHODS

Test organisms

Hexagenia bilineata nymphs were obtained from a laboratory culture maintained at the National Fisheries Contaminant Research Center (NFCRC) field station in La Crosse, Wisconsin. Test organisms were 141-days-old at the start of testing (7 December 1991). A certified reference sediment (Ingersoll & Nelson, 1990) served as the substrate in the culture tank and in all tests with oil residue. This sediment is classified by the US Geological Survey as silt loam (1% total organic carbon, <0.01% inorganic carbon 8% sand, 66% silt, and 26% clay). Contaminant-free well water from NFCRC was used in culture, preparations, and tests (pH = 7.8, total hardness = 283 mg liter⁻¹ as CaCO₃, total alkalinity = 255 mg liter⁻¹ as CaCO₃).

Water soluble fraction (WSF) tests

Two different crude oils were used in these 96-h tests. Oil obtained from Shell Pipeline Corporation's Patoka Terminal in Patoka, Illinois, was used in one exposure. This intermediate sweet crude was similar to oil spilled into the Gasconade River in 1988. A second test conducted with Rothville crude No. 2, an intermediate weight oil that was spilled into the Chariton River, Missouri (5 November 1990), was obtained from Amoco's La Plata Terminal in La Plata, Missouri.

Methods for preparation of the WSF were adapted from Giddings and Franco (1986). A WSF stock solution was prepared by layering 2 liters of oil above 16 liters of well water and stirring gently in a sealed 20-liter glass jar for 48 h. All toxicant solutions were prepared at 22°C and under low light conditions (three 25-W yellow bulbs on a dimmer) to minimize photochemical changes. The WSF was removed and filtered through a Whatman No. 42 filter (2.5 µm cellulose). All preparations were stored at 4°C in the dark. Test waters were aerated 1 h prior to testing.

Artificial burrows constructed with a graded series of glass tubes (3–7 mm internal diameter), rather than sediment, provided the substrate during tests with the WSF (Henry *et al.*, 1986). Two 96-h acute WSF tests were performed with 10 organisms per replicate in each treatment. Treatments included 6.25, 12.5, 25, 50, 75, 100% WSF, and a well water control. Organisms were fed a light dusting of powdered trout chow after 24 h. Three 25-W yellow lights on a dimmer constantly illuminated the laboratory during all tests.

Oil residue tests

Oil residue was prepared with Rothville crude No. 2 by layering 50.4 ml of oil over 36 liters of well water in a glass aquarium for 7 days before skimming the residue from the water surface (Giddings & Franco, 1986). Of this material, 40 g was dissolved in 2 liters of methylene chloride to make an oil residue stock solution (20 000 mg liter⁻¹). Calculated amounts of stock solution were mixed with 500 g of reference sediment to prepare each oil residue treatment. Hereafter, micrograms of oil

residue per gram dry weight of sediment will be denoted as µg g⁻¹. All test chambers remained under a hood for 1 day to allow evaporation of the solvent carrier. Overlying water was then added to each chamber to attain a total volume of 2.4 liters. Suspended particles were allowed to settle for 24 h prior to adding test organisms. All experiments with oil residue mixed with sediment included a control and a solvent control.

The 21-day test exposing organisms to oil residue mixed with sediment had concentrations of 100, 200, 500, 800, and 1400 µg g⁻¹. Three 3.0-liter test chambers containing 20 organisms per chamber were prepared for each treatment level. Surviving organisms were enumerated on Days 7, 14, and 21 by sifting through the sediment in a test chamber from each treatment. Organisms were not fed during sediment tests. Total petroleum hydrocarbon (TPH) levels were measured at the start of this test using EPA method 418.1 (USEPA, 1979).

Organisms were also exposed for 21 days to oil-contaminated and upstream sediments collected from the Chariton River (Macon County, Missouri) 43 days after the 5 November 1990 oil spill. Sediments from backwater areas were collected and handled as described by ASTM (1990). Oil-contaminated sediment was collected 56 km below the spill site. Uncontaminated sediment was collected 10 km above the spill site. Sediments were processed and stored at 4°C in the dark for 2 weeks before testing (ASTM, 1990). The amount of sediment added to each test chamber was equivalent to 500 g dry weight. Chambers were filled to a volume of 2.4 liters with overlying water, allowed to settle for 1 day, and then aerated 1 h prior to testing.

A 24-h exposure with oil residue was conducted to assess habitat exclusion from the sediment. Sediments were prepared in the same manner as the 21-day tests and included concentrations of 50, 100, 200, 400, and 800 µg g⁻¹. Six organisms were placed in each chamber. Observations began 5 min after exposure and were repeated at intervals of 15 min, 30 min, 1 h, 2 h, 4 h, 12 h, and 24 h. If an organism was less than 50% buried it was considered out of the sediment.

Testing procedures

Established procedures were followed for conducting acute and chronic tests (EPA, 1989). Static tests were conducted in 2.8-liter glass aquaria filled to a volume of 2.4 liter and aerated. Temperature and dissolved oxygen were recorded daily during tests. Total ammonia, pH, alkalinity, hardness, and conductivity were recorded at the beginning and end of each 96-h test, every 96 h during the 21-day test, and at the beginning of the 24-h behavioral test. At the conclusion of all sediment tests, sediment was sieved to recover organisms and determine survival. Nymphs that were floating or lying in an abnormal position, or had ceased gill movement were placed in fresh water to see if they would recover. Death was defined as failure to respond to gentle prodding (Henry *et al.*, 1986).

Total petroleum hydrocarbons (TPH) measured during the 21-day sediment toxicity test were highly

variable and consistently lower (by at least 30) than nominal values. In response to this outcome, we examined the relation between nominal TPH and measured TPH. Samples for this study were prepared in the same manner as sediments used in the oil residue tests, except each nominal concentration was prepared by adding oil residue stock solution to 2500 g of reference sediment. Samples were prepared at nominal concentrations of 100, 200, 500, 800, 1400, 2800, and 5600 $\mu\text{g g}^{-1}$. Five subsamples were taken from each concentration for TPH analysis.

Statistical methodology

The Chariton River sediment test was analyzed using Fisher's exact test (on site treatment or control) versus survival (dead or alive) 2×2 contingency tables. The one-way alternate hypothesis [H_1 : mortality in contaminated sediment is greater than that in control (or upstream) sediment] was tested. Both 96-h and 21-day toxicity tests were analyzed using simple linear regression to relate survival to log of oil residue concentration. Regression models were fit by maximum likelihood methodology assuming a binomial error distribution (McCullagh & Nelder, 1989). Measured TPH concentrations were compared to nominal concentrations using a series of 2-sided one sample *t*-tests.

RESULTS

Water quality

Ranges for water quality data during all toxicity tests were within expected ranges except for total ammonia (0.04–2.72 mg liter⁻¹). Unionized ammonia (NH₃), determined from measurements of total ammonia (NH₄-N), did not exceed 0.35 mg liter⁻¹. Decreased survival caused by unionized ammonia is unlikely because the lowest 96-h LC50 recorded for 4 mayfly and 2 stonefly taxa is 1.80 mg liter⁻¹ (Thurston *et al.*, 1984), five times higher than concentrations observed during our tests.

Toxicity tests

Water soluble fractions prepared from Shell and Amoco oils did not affect survival after 96 h. Survival was 90% or greater in these tests. Similarly, a 96-h exposure to oil residue mixed with sediment at concentrations up to 3200 $\mu\text{g g}^{-1}$ did not affect survival.

In the 21-day test exposing organisms to oil residue mixed with sediment, a dose response relationship was evident at the conclusion of the test. Although little mortality was observed during Week 1 and 2, after 3 weeks, survival was <55% in the 3 highest treatments (Table 1). The relation between mortality and oil residue concentration was significant in Week 3 ($p = 0.001$), nearly significant in Week 2 ($p = 0.068$), and not significant in Week 1 ($p = 0.117$) (Fig. 1).

Similarly, significant mortality did not occur until Week 3 of the 21-day test with Chariton River sediment (Table 1). After 3 weeks, mortality in oil-contaminated Chariton River sediment (35%) was significantly

Table 1. Number of organisms surviving during 21-day oil residue test using Rothville No. 2 crude oil and oil-contaminated sediment from the Chariton River ($n = 20$ /chamber)

Treatment	Week 1	Week 2	Week 3
<i>Rothville No. 2</i>			
Control	16	19	19
Solvent control	19	19	18
100 $\mu\text{g g}^{-1}$	18	20	17
200 $\mu\text{g g}^{-1}$	19	17	16
500 $\mu\text{g g}^{-1}$	20	15	10
800 $\mu\text{g g}^{-1}$	19	17	11
1400 $\mu\text{g g}^{-1}$	20	17	8
<i>Chariton River</i>			
Upstream	19	19	20
Oil-contaminated sediment (1905 $\mu\text{g g}^{-1}$)	20	19	13

higher than mortality in the upstream sediment (0%, $p = 0.004$) or the control (5%, $p = 0.022$). Mortality in oil-contaminated sediment was not significantly higher than mortality in the upstream sediments or control after Weeks 1 and 2 (all p -values > 0.7). These results confirm that after 6 weeks of weathering in the river, sediment collected below the spill site remained toxic.

Habitat exclusion from oil residue was not apparent during the 24-h oil residue behavior test. The mean number of organisms ($n = 6$) out of sediment with oil residue (10–19%) was greater than in the controls (4–8%), but not statistically different ($p > 0.05$, $df = 37$). Organisms that were out of the sediment during this test generally remained on the sediment and did not exhibit increased swimming activity.

Total petroleum hydrocarbon (TPH) concentrations

Measured TPH of sediments in exposure chambers from the 21-day oil-contaminated sediment test was about 60% of nominal levels at 100 and 200 $\mu\text{g g}^{-1}$ treatments and between 19 and 40% of nominal levels

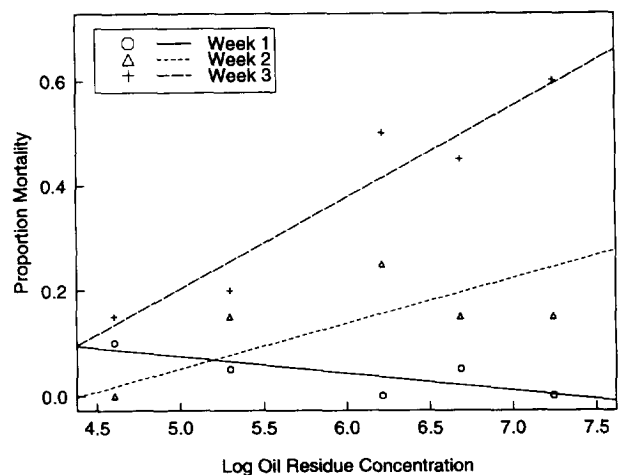


Fig. 1. Relation between mortality of *Hexagenia* and log-transformed concentrations of oil residue ($\mu\text{g g}^{-1}$) for Weeks 1, 2, and 3. The relation was significant for Week 3 ($p = 0.0004$), nearly significant for Week 2 ($p = 0.0678$) and not significant for Week 1 ($p = 0.1170$).

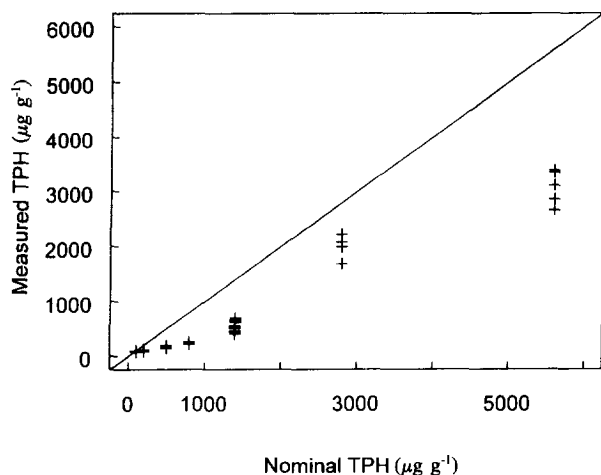


Fig. 2. Relation between measured TPH ($\mu\text{g g}^{-1}$) and nominal TPH ($\mu\text{g g}^{-1}$). Measures of TPH were highly variable underestimates of nominal concentrations.

at the higher concentrations. To identify whether this variability resulted from the analytical technique or from sediment preparation, we prepared additional samples and found similar results. Analysis of five replicate samples from each of seven concentrations revealed that measured TPH levels were 52 to 94% of nominal values for low concentrations and 30 to 39 within the high range (Fig. 2).

Overall, measures of total petroleum hydrocarbon were highly variable underestimates of actual concentrations (Fig. 2). Concentrations of 100 and 200 $\mu\text{g g}^{-1}$ were not significantly different ($p = 0.03$) from each other or from a blank. In addition, variance of the measurement increased with increases in oil-residue concentration. Caution should be used when evaluating TPH values from the literature determined using the analytical method employed in this study. We recommend reporting detailed information about preparation of oil residue, composition of test sediments, and exposure systems in addition to nominal TPH concentrations to facilitate interpretation of reported results.

DISCUSSION

The WSF was not acutely toxic to *H. bilineata* over a broad range of concentrations. However, laboratory exposure conditions do not parallel conditions during an actual oil spill. In the laboratory, the WSF was prepared in a sealed container that restricts evaporation of volatile components. Consequently, hydrocarbon levels in test water at the beginning of the WSF tests may be higher than during an oil spill. As the test progresses, evaporation will reduce WSF hydrocarbon levels. Conversely, a physical barrier formed by floating oil after a spill may restrict evaporation of volatile hydrocarbons from the water and prolong exposure to WSF components. These factors underscore the importance of field studies to complement laboratory findings.

Decreases in survival of *H. bilineata* occurred after the third week of exposure to laboratory-prepared,

oil-contaminated sediments. Similarly, survival was reduced after 3 weeks in oil-contaminated sediment from the Chariton River. Survival of organisms in these river sediments was significantly less than survival in sediments collected upstream of the spill site. Residues of oil remained high (1905 $\mu\text{g g}^{-1}$) in these river sediments. Weathering for 6 weeks in the river prior to testing had not eliminated the toxicity.

Determining specific toxic constituents in the oil residue is problematic (Tissot & Welte, 1984). Polycyclic aromatic hydrocarbons (PAHs) vary among crude oils and may be readily adsorbed to particulate matter and deposited in the sediment (Neff, 1979; Tissot & Welte, 1984). The persistence of crude oil is dependent on the composition of the oil mixture and on the characteristics of the environment into which it is released, particularly the temperature, oxygen concentration, and microbial nutrient availability. The toxicity of many PAH compounds to aquatic invertebrates (Anderson *et al.*, 1974; Finger *et al.*, 1985; Ott *et al.*, 1978; Rossi & Neff, 1978; Swartz *et al.*, 1990; Young, 1977) suggests that PAHs remaining in weathered oil residue are toxic.

Direct contact with and ingestion of oil residue from sediment are primary routes of exposure for nymphs because they generally remain in sediment burrows and feed on the substrate. Subsequent bioconcentration of PAHs by the organism may be an important factor contributing to the toxicity of oil residue to specific organisms and the bioaccumulation and retention of the toxic constituents in other components of the ecosystem. Our results demonstrated no acute toxicity after 96 h for mayflies, but suggested that extended exposure, allowing time for bioaccumulation of contaminants, would reduce survival. Stehly *et al.* (1990) reported substantial bioconcentration of two PAHs by a similar species (*Hexagenia limbata*) via water and assert that bioconcentration may also occur via sediment.

Avoidance of sediments contaminated with oil residues was not observed at levels below those causing mortality. Such contaminant-induced changes in behavior often occur rapidly as a response to dramatic changes in habitat quality (Little *et al.*, 1985). Habitat avoidance could increase the probability of survival by eliminating direct contact of the nymph with the contaminated substrate. Conversely, habitat exclusion could decrease *H. bilineata*'s ability to feed on substrate, while increasing susceptibility to predation and exposure to current. Lack of a strong avoidance response to oil residue mixed with sediment would allow these organisms to remain in the vicinity of a spill and bioaccumulate contaminants associated with the oil. Mortality similar to that demonstrated after extended exposure to oil-contaminated sediment in the laboratory may account for the consistent absence of nymphs from backwater areas recorded after oil spills in several field studies.

Community-level response to an oil spill will differ based on geographic locations due to variation in species composition, relative abundance of species, and

tolerance of individual species to PAHs (Resh *et al.*, 1988). Still, findings from this laboratory study and another by Giddings and Franco (1986), which detected increased mortality of the benthic organism *Chironomus tentans* after exposure to crude oil-contaminated sediment, suggest that residual oil in sediment will negatively alter the benthic macroinvertebrate community after an oil spill. Furthermore, an investigation into the effects of a massive crude oil spill in the Gasconade River, Missouri, documented substantial reductions in total numbers of benthic macroinvertebrates as well as adverse effects on specific functional feeding groups below the spill site (Finger *et al.*, 1991). After 18 months, functional groups of shredders and scrapers were either eliminated or reduced at sites below the spill, thus altering the functional capacity of that community. A similar study of a crude oil spill into Asher Creek, Missouri, reported that benthic organisms at a site below the spill were reduced to 0.1% of expected numbers 25 days after the spill and that substantial improvement in species diversity and abundance did not occur for 266 days; some taxa were adversely affected for at least 336 days (Crunkilton & Duchrow, 1990). In each study, the length of time that organisms were absent or reduced in number supports the contention that persistent oil residues affect the structure and organization of benthic invertebrate communities after an oil spill. These lengthy recovery times may be a consequence of mortality induced by initial exposure to oil, continuing toxicity of residual oil, or reduction in the rate of recolonization on oil-contaminated substrates. Knowledge of the persistence effects of freshwater oil spills should be valuable in determining the length of time required to assess the extent of environmental injury following a spill.

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