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ROLE OF ARTIFICIAL BURROWS IN *HEXAGENIA*
TOXICITY TESTS: RECOMMENDATIONS FOR
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(Received 25 June 1985; Accepted 27 November 1985)

Abstract—*Hexagenia* are an important component of fish and waterfowl diets, provide an ecological link in the conversion of detritus to usable nutrients and are useful test organisms for monitoring trends in aquatic contaminants. Consequently, *Hexagenia bilineata* were used in toxicity tests to determine their sensitivity to leachate from spent shale oil and to evaluate the influence of including artificial glass burrows in exposure chambers. Gill beat frequency and mortality were significantly higher ($p = 0.01$) in nymphs that were not afforded an opportunity to burrow than in those exposed to the toxicant but that had access to artificial burrows. Molting frequency was depressed in *Hexagenia* lacking burrows, and it was further decreased as the amount of toxicant increased. Thus, thigmotactic stress accentuated by the presence of shale oil leachate was relieved by including artificial burrows in the exposure chambers. The resulting toxicity data are more ecologically meaningful because the burrowing life history characteristic of the *Hexagenia* was addressed and incorporated into the test protocol.

Keywords—Toxicity Mayfly Shale oil *Hexagenia*

INTRODUCTION

Throughout North America, burrowing mayfly nymphs of the genus *Hexagenia* are an important component of fish and waterfowl diets. They also provide an ecological link in the conversion of organic detritus and its associated microorganisms into a readily usable food source [1].

Where sediment consistency and the dissolved oxygen concentration at the sediment-water interface permit, *Hexagenia* nymphs live in U-shaped burrows. Since mayfly nymphs burrow into the sediment, which is often considered a sink for contaminants in aquatic ecosystems, they can be extensively exposed to contaminants and thus are useful monitoring organisms. In a laboratory testing scheme, Fremling and Mauck [2] developed a series of graded-size glass tube burrows (Fig. 1) to accommodate the intrinsic thigmotactic (need to be in contact with a substrate) requirement of the burrowing mayfly. Use of the burrowing mayfly in laboratory toxicity tests requires that a substrate

be provided that is chemically inert (thereby not affecting the bioavailability of the toxicant) and, concomitantly, that satisfies the thigmotactic requirement of the organism. However, actual toxicity tests using these artificial burrows have not been compared with tests run without them.

The purposes of our study were to (a) determine the sensitivity of nymphs of *Hexagenia bilineata* (Say) to leachate from spent shale oil, (b) determine whether the presence of glass tube burrows affected the organisms' sensitivity to the toxicant and (c) evaluate the relevance of including glass burrows in toxicity tests. Leachate from spent shale oil was selected as the test toxicant because there is a need for information on the potential impact of shale oil development on aquatic organisms, including burrowing mayflies.

MATERIALS AND METHODS

Eggs of the mayfly *Hexagenia bilineata* were collected in late July 1983 from female imagoes emerging from Lake Onalaska in LaCrosse, Wisconsin. The eggs were transported in aerated tanks of well water to Columbia, Missouri, and cultured in a 0.1-ha culture pond at the Columbia National Fisheries Research Laboratory.

Nymphs were retrieved for testing from the

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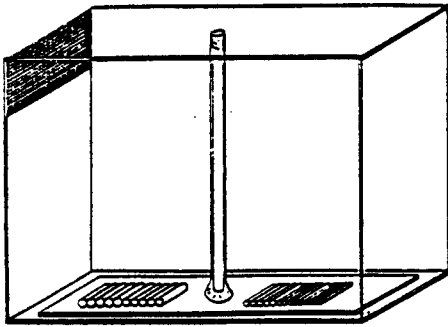


Fig. 1. Mayfly test chamber with artificial glass burrows.

pond 3 months after initial stocking. Nymphs were acclimated to test water for 24 h before they were placed in test chambers. One-half of the mayflies were also acclimated in glass tube burrows and the other half were held in tanks without burrows. Burrows were constructed using a series of graded-size glass tubes (3 to 7 mm i.d.). A range of tube sizes was incorporated into the chamber design so that as the mayflies grew they could select burrows of appropriate size. Five 5-cm sections of tube were cut, the ends flamed and then glued in size sequence to a 6 × 23 cm glass plate. The glass plate with burrows attached was then placed in a glass chamber (7 × 18 × 24 cm).

Test chambers with and without burrows were utilized in the study. Most often, mayfly nymphs crawled completely into the tubes, leaving neither head nor tail sticking out. Once inside, they oriented themselves ventral side down or lay on their sides. Gill movements, grooming behavior and feeding could be observed in both cases. The nymphs would leave the burrows to forage but would return to them readily. The animals usually left their burrows before they died.

Two replicate chambers holding 10 animals each were exposed to each treatment concentration under each of the two test conditions, with and without burrows. The test was conducted for 14 d. On days 0 and 13, nymphs were photographed for growth determination in a gridded, glass box that contained just enough water to cover them without producing optical distortions. Measurements were made on photographically enlarged images of the mayflies, and a correction factor, based on direct measurement of the grid, was used to compute actual lengths [3].

The toxicant and test water were delivered by a modified proportional diluter system [4]. A spe-

cial toxicant cell was added to the system to initially deliver the required amount of leachate (475 ml of 100% leachate). This volume was diluted and subsequently delivered to each test chamber every 30 min, replacing the total volume of each chamber four times a day. The high concentration consisted of 25% full-strength leachate (based on preliminary acute toxicity tests) and 75% well water. This concentration was diluted to create four additional concentrations, each one nominally 50% of the one preceding it. One-hundred percent well water served as the control. Daily maintenance of the diluter included the checking of diluter operation and of toxicant pump delivery, and the replenishing of the stock tank of shale leachate.

Well water (mean hardness, 302 mg/L as CaCO₃; mean alkalinity, 256 mg/L as CaCO₃; mean pH, 8.0) was used for all storage, culture, acclimation and testing. Hardness, alkalinity and pH were determined weekly for the water in each test chamber. Water temperature and dissolved oxygen, checked daily, averaged 17.7 ± 0.5°C and 7.58 ± 0.84 mg/L, respectively.

Mayfly nymphs were exposed in 2.8-liter glass chambers equipped with screens on one end that served as overflow drains (Fig. 1). Mortality and molting were observed daily, and gill beats and activity weekly. Because nymphs in their glass burrows were generally inactive it was often necessary to gently prod them to determine if they were still alive. The number of animals that had molted was determined by counting the number of exuviae in each chamber. Values were then adjusted to represent percent molts per number of surviving individuals. Activity and gill beats (amount of time active and total number of beats during a 3-min observation) were monitored weekly. Activity was defined as any movement other than gill beats, i.e., swimming, crawling, grooming and feeding. An individual gill beat was identified as a single undulation along the entire length of the abdominal row of gills. Gill beats resulting from passive undulation during swimming were not included in the frequency counts. For each behavioral observation, each animal in the selected chambers was watched continuously for 3 min. This period allowed adequate observation of individuals yet was not so long as to be impractical. The behavior of nymphs was monitored in one of the replicate chambers receiving each treatment level. The same chambers were observed each week, producing "repeated measures data" over time.

The nymphs in each chamber received 1 ml

daily of a mixture of yeast and powdered grass (Cerophyl®) in well water. A 16-h light:8-h dark photoperiod was simulated. Temperature was controlled using small aquarium heaters in the water-bath surrounding the test chambers.

Leachate was collected from lysimeters at Anvil Point, Colorado, which contains Paraho retorted shale. Colorado State University, under contract with the U.S. Environmental Protection Agency, designed and constructed these lysimeters in 1977 to simulate a canyon fill disposal site [5]. The lysimeters were constructed to facilitate collection of leachate water after it had percolated through spent shale tailings. Our leachate sample was a composite of samples taken from 22 April to 1 May 1983, from the high-elevation disposal lysimeter that had either no soil overburden or a 20-cm soil overburden. Leachate was mixed weekly and stored in two polyolefin food-grade tanks. Subsamples were taken and transported in 800-liter carboys to Columbia, Missouri, for immediate use in this study.

The chemical composition of the leachate was determined by Bartelle Pacific Northwest Laboratories (Table 1) [6]. Samples for inorganic analyses were taken and stored in plastic bottles; organic samples were stored in glass vials with Teflon-lined caps. Selected anions (F, Cl, NO₃, PO and SO₄) were quantified using a Dionex Model 16 ion chromatograph equipped with an AGI guard and ASI anion separator column, suppressor and conductivity detector. Samples were eluted with NaHCO₃ and Na₂CO₃. Nitrite was analyzed colorimetrically using the automated diazotizing method for nitrate but with the reduction step eliminated [7]. Quantitation was performed using a Model CFA-200 continuous-flow analyzer and selected metals were analyzed using a Jarrell Ash Model 975 (Fisher Scientific) inductivity-coupled plasma emission spectrometer.

Organic carbon was determined using a Dohrman DC-80 carbon analyzer equipped with a UV-enhanced persulfate oxidation system and an NDIR CO₂ detector (Horiba PIR2000). Nitrogen-containing organics were determined from methylene chloride extracts and analyzed on a Hewlett-Packard 5880 gas chromatograph equipped with flame ionization (FI) and nitrogen/phosphorous (N/P) detectors. Separation was achieved using a 50-m quartz capillary column programmed from 65 to 250°C. Aromatic hydrocarbons were quantitated using the FI detector and N/P detector. The leachate contained primarily sodium, magnesium and sulfate, and had total dissolved solids of about

Table 1. Comparison of the most prevalent cations and anions detected in three concentrations of spent leachate (% leachate: % dilution water) administered to *Hexagenia bifineata* in a 14-d study

Shale leachate: dilution water	Cation or anion concentration* (µg/ml ± sd)												
	B	Ca	K	Li	Mg	Mo	Na	Sr	F	Cl	NO ₃	SO ₄	
0:100 (control)	0.16 (0.0)	36.0 (0.9)	5.8 (0.3)	0.05 (0.0)	21.0 (0.4)	ND	49.0 (3.2)	0.24 (0.01)	0.61 (0.04)	22.0 (0.4)	ND	48.0 (0.7)	
6:94	0.19 (0.0)	50.0 (1.4)	35.0 (0.8)	0.47 (0.01)	142.0 (5.0)	0.32 (0.01)	164.0 (5.0)	0.57 (0.01)	0.96 (0.04)	40.0 (0.9)	4.1 (0.88)	793.0 (15.0)	
25:75	0.29 (0.01)	93.0 (5.6)	111.0 (7.6)	1.69 (0.09)	487.0 (28.0)	1.26 (0.04)	493.0 (15.0)	1.57 (0.04)	1.93 (0.07)	95.0 (1.7)	20.0 (0.9)	3,086.0 (51.0)	

ND, not detectable at 0.01 mg/ml.

*Mean ± sd in parentheses.

6,000 mg/L. Only background levels of toxic organic components were detected.

A YSI model 35 conductance meter equipped with a $K = 0.1$ probe was used to determine conductivity in the test chambers as a reflection of concentration. Total dissolved solids were determined weekly for total filterable residues [7]. This determination provided an additional monitoring tool for evaluating toxicant concentrations in the test chambers.

The experiment followed a "split-plot in time" design. Analysis of variance F tests were conducted to determine the significance of substrate, concentration, time and their interactions on activity, gill beats and molting. The same tests were also conducted on rank-transformed data for activity and gill beats as an alternative procedure that would be more robust in its distributional assumptions [8,9]. Because both procedures gave the same trends, only the results of the tests run on unranked data are presented. LC50 values and confidence intervals were calculated using methods outlined by Litchfield and Wilcoxon [10].

RESULTS AND DISCUSSION

Elevated shale leachate concentrations produced significant changes in mortality and activity levels, whereas the presence of burrows caused significant changes in all four biological indices (mortality, molting, activity, gill beats). Obviously, the presence of substrate was important to *Hexagenia*. Growth was not affected.

Mortality increased as shale leachate concentration increased under both testing regimes (Fig. 2). However, the cumulative mortality induced in chambers without glass tube burrows was 2 to 3 times higher from day 8 through day 14 than in chambers containing glass tubes (Table 2). This elevated mortality (20% even in control chambers without burrows) was attributed to thigmotactic stress associated with the lack of burrows combined with toxicity of the leachate. There were no mortalities observed in control chambers containing burrows.

In previous studies, *Hexagenia limbata* were exposed to hydrogen sulfide in chambers with mud and in chambers with mud overlaid with screens that prevented animals from burrowing [11]. The organisms were more susceptible to the hydrogen sulfide when the substrate was present but inaccessible. Although thigmotactic stress combined with toxicant exposure appeared to be responsible in that study for the elevated mortalities, as compared with similar exposures in which mayflies

Table 2. LC50s (% leachate) for mayflies exposed to five nominal concentrations of spent shale leachate for 14 d

Length of exposure (d)	LC50		Significant difference between values
	With substrate	Without substrate	
4	50.0 (27.2-91.8)	50.0 (18.6-134.1)	NS
6	50.0 (27.2-91.8)	25.0 (14.7-42.6)	NS
8	47.0 (23.2-95.1)	16.0 (8.6-29.9)	$p = 0.01$
10	42.0 (21.5-82.1)	15.0 (7.9-28.6)	$p = 0.001$
14	40.5 (17.2-95.6)	7.6 (4.1-14.1)	$p = 0.001$

95% confidence intervals in parentheses. NS, not significant, as determined by Student's t test.

could burrow, the exposures may not have been controlled enough to allow this comparison. The animals in the sediment could have received a lower contaminant exposure because of adsorption of toxicant to the sediment particles. The use of glass burrows in our study more clearly separated the influence of toxicant from that of thigmotactic stress. The results of our study indicate that burrowing requirements need to be met in *Hexagenia* toxicity tests and that contaminant exposures should be constant. The thigmotactic stress induced in tests without substrates may enhance toxicity but disallow the ecologically realistic predictive value of the data.

Thigmotactic stress was additionally evident in three other areas monitored during this study: type of activity performed, gill beat frequency and molting frequency. According to our definition of activity, mayfly nymphs were less active ($p = 0.001$) as shale leachate concentration increased (Fig. 2). Total activity was significantly ($p = 0.01$) higher in chambers with glass tubes than in those without (Fig. 2). At first glance, this would seem to contradict the idea that the absence of substrate results in thigmotactic stress as evidenced by increased respiration and activity. However, activity was described as the sum of the frequencies of several behaviors (swimming, crawling, grooming and feeding). Animals in the tubes appeared to allocate effort to grooming and feeding, while animals without substrates spent most of their active time crawling, spastically swimming and attempting to burrow either into the slight amount of food

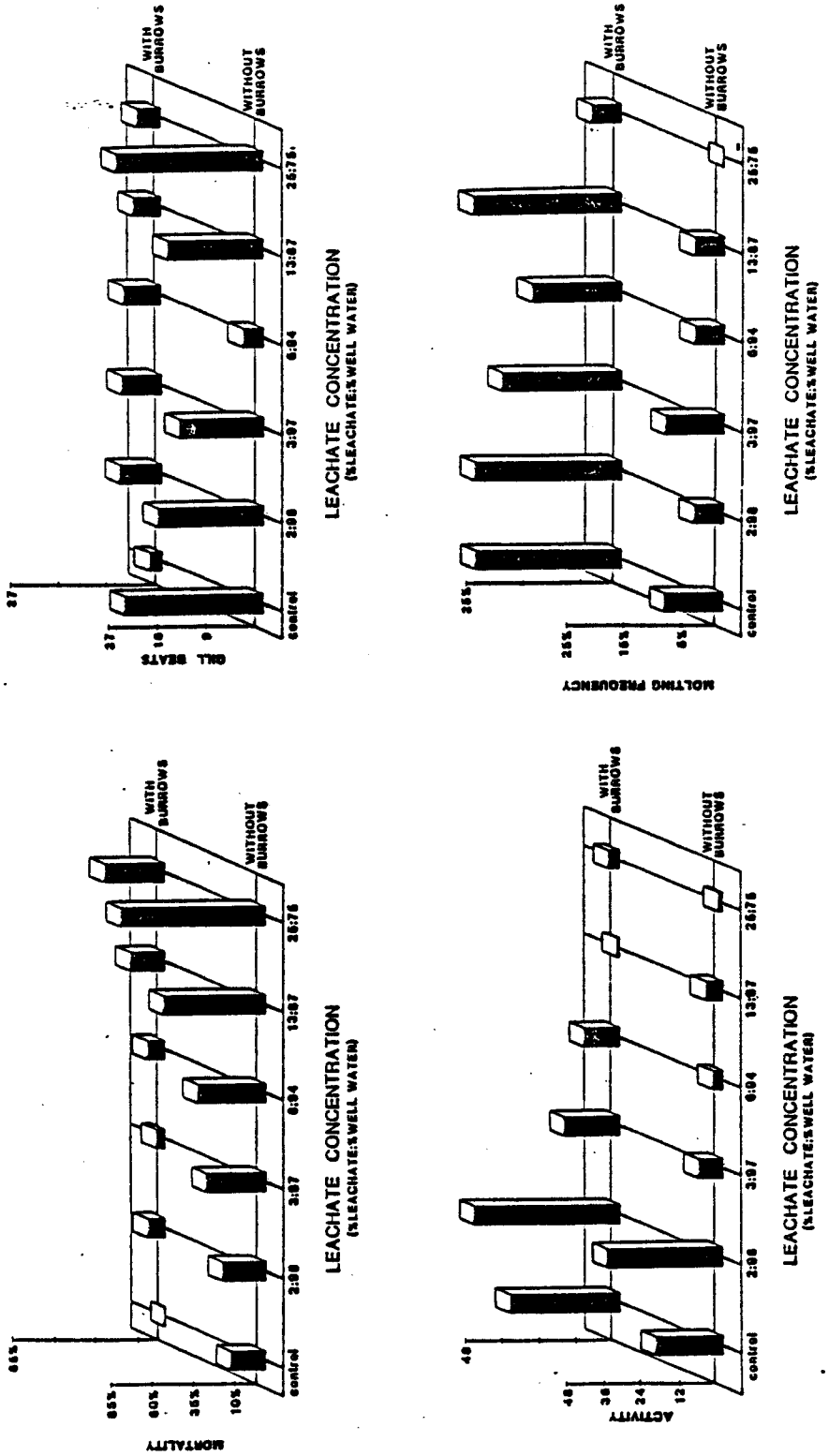


Fig. 2. Cumulative mortality and molting, and mean activity and gill beat frequency in mayflies exposed to shale oil leachate under two test conditions.

residue accumulated on the bottom of the test chambers or into the glass surface of the chambers themselves. These wide fluctuations in types of activity performed under each test condition were obscured when mean values were computed. In future studies, each of these four behaviors should be scored separately to permit quantitative confirmation of this division in activity patterns. The absence of substrate may cause fundamental changes in the time-energy budgets of mayflies, thus influencing toxicity results.

Shale leachate significantly influenced activity regardless of substrate. Animals under both test conditions were more active in control and in low-concentration chambers (Fig. 2) than in the three higher-concentration chambers. Many toxicants stimulate activity at low concentrations but depress activity at higher exposure levels [12]. Perhaps the toxic components in shale leachate influence mayflies similarly.

Gill beats were monitored during our study as a measure of respiration rate. When burrows were not present, the number of gill beats was significantly higher ($p = 0.05$) than when burrows were present (Fig. 2). There was no statistically significant difference in gill beats related to toxicant concentration or length of exposure. During the study, dissolved oxygen levels ranged from 70 to 100% saturation; thus they were never low enough to induce respiratory stress.

In other studies, higher respiration rates were found for *H. limbata* in test chambers without substrate than in those with substrate [13]. Furthermore, oxygen consumption varies with substrate type; in empty bottles in another study, oxygen consumption was highest, while the lowest consumption occurred in sediments of optimal particle size [14]. The evidence suggests that mayflies regulate respiration by altering the number of gill beats per unit time [13]. Increasing the number of gill beats increases the amount of water passing over gill and body surfaces. *Hexagenia* can regulate respiration rates to adjust to dissolved oxygen concentrations as low as 0.80 mg/L by increasing gill beat frequency. Smaller animals beat their gills more often than larger animals to regulate to changes in dissolved oxygen. This was measured by Ericksen [13] in a respirometer. Increased gill beat frequency therefore seems to indicate respiratory stress.

Despite the fact that *Hexagenia* can regulate respiration at very low dissolved oxygen concentrations, the combination of decreased oxygen and

the presence of a contaminant could result in increased mortality. The absence of substrate in this study obviously increased gill beat frequency, which seems to indicate increased respiratory stress.

Molting, monitored as a sublethal indication of animal health, was expressed as a cumulative percentage of the total number of animals present in each chamber (Fig. 2). Molting also seems to be a sensitive indicator of sublethal stress. Overall, more animals molted in chambers with tube burrows than in those without. In addition, the high concentration of leachate had a pronounced effect ($p = 0.001$) on molting frequency, reducing it below control rates and the rates observed at other concentrations under either test condition. In every treatment, the frequency of molting was significantly depressed in animals in chambers without burrows compared with those exposed to the same concentration in chambers with burrows.

Cadmium has been found to reduce the incidence of molting in three species of aquatic insects (mayflies, caddisflies and stoneflies) [15]. In addition, Henry [16] found that crayfish separately exposed to pentachlorophenol, Aroclor 1254 and triphenylphosphate showed the greatest susceptibility to these contaminants during molting. It was difficult to observe the exact time of molting in mayflies exposed to the shale leachate during our study. In future work, the relationship between molting and exact time of death should be examined. However, molting alone provides an indication of the developmental progress and overall health of the animal and, in our study, it was reduced both by toxicant and by lack of substrate.

In nature, *Hexagenia* require a fine-textured, rich, organic sediment for burrowing so that the integrity of the burrow is maintained (in contrast, sand collapses) [17]. Although toxicity testing with mayflies in sediment may provide an opportunity to evaluate the sorption kinetics or biological availability of a given contaminant, it obscures toxicological comparisons (because of the presence of humic acids and other organic materials) for fish and other nonburrowing invertebrates. Comparisons of classical toxicity test results (water exposures) for various test organisms require that the physical-chemical test conditions be as similar as possible while still accommodating the life history needs of each test species. For *Hexagenia*, these conditions can be satisfied using glass burrows.

Hexagenia also are excellent for monitoring long-lived, sediment-associated pollutants. In most

cases, the burrowing mayfly spends about 99% of its total life cycle in association with aquatic sediments before it emerges as a nonfeeding adult. Consequently, the contaminant body burden present in adult mayflies is the direct result of exposure in the aquatic environment at the mud-water interface [2]. This life history makes the mayfly nymph suitable for monitoring contaminants, and mayflies have been used successfully to monitor levels of polychlorinated biphenyls in the upper Mississippi River [18,19].

In our study, inclusion of glass tube substrates in toxicity tests with *Hexagenia* improved survival and presumably reduced thigmotactic stress. The resulting toxicity data are presumed to be more predictive of impacts that might occur under natural conditions and will provide more sound information for water quality standards because thigmotactic stress was reduced. Of the several types of substrates that have been devised [2], the glass tube burrows used in this study are, in our opinion, the least expensive, most inert and easiest to construct, and, because of the graduated sizes, they allow animals to choose burrows that will accommodate them as they grow larger. *H. bilineata* nymphs are an excellent alternative or complement to fish for use in studies evaluating toxicants because of their association with sediment, wide geographical distribution, importance in fish and water fowl diets, relatively sedentary habits, long life span (as compared with most insects) and ease of handling in the laboratory.

Acknowledgments—The authors thank Dan Woodward and Jim Coyle for assistance with leachate collection and transport, Ed Henry for technical assistance, Mark Kaiser for statistical help and Pat Hudson, Peter Landrum, Mike Mac and Don Palawski for their helpful reviews of the manuscript.

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