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Sublethal Effects of Phenanthrene, Nicotine, and Pinane on *Daphnia pulex*[1]

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Nearly 500 compounds were detected in the tissues of Great Lakes fish as compared to 8 in tissues of hatchery-reared fish (Hesselberg and Seelye 1982). Lethal concentrations for many representative compounds were determined by testing their acute toxicity (48-hr EC50) to Daphnia pulex (Passino and Smith 1987; Perry and Smith 1988; Smith et al. 1988). However, the population growth and survival of aquatic organisms over longer time intervals are usually affected at concentrations much lower than the EC50 for a specific chemical. To develop a general relationship between acute and chronic concentrations for representative compounds detected in Great Lakes fish, we initiated full-life-cycle testing on D. pulex with phenanthrene, nicotine, and pinane. Growth and fecundity of daphnids was measured in 16-d tests in the laboratory.

Phenanthrene and nicotine were highly toxic and pinane was moderately toxic to D. pulex in acute studies (Smith et al. 1988). For phenanthrene, a compound of the polycyclic aromatic hydrocarbons (PAHs) that has been associated with incomplete combustion of organic matter (Hallett and Brecher 1984), the EC50 was 0.35 mg/L (Smith et al. 1988). For nicotine [(S)-3-(1-methyl -2-pyrrolidinyl) pyridine], a compound in the heterocyclic nitrogen class of chemicals that has been used as an insecticide (Konar 1977), the EC50 was 0.24 mg/L (Perry and Smith 1988). Cyclic alkanes, many of which are constituents of crude oil (Brassell et al. 1978), were represented by pinane [(1S,2S)-2,6,6- trimethylbicyclo [3.1.1] heptane] for which the EC50 was 3.35 mg/L (Smith et al. 1988).

MATERIALS AND METHODS

We cultured and tested D. pulex in reconstituted hard water at 20° C, following standard procedures of the American Society for

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Testing and Materials (1980) and the US Environmental Protection Agency (1982). Dissolved oxygen (range 8-9 mg/L), total hardness (range 160-200 mg/L as $CaCO_3$), and alkalinity (range 100-125 mg/L as $CaCO_3$) were monitored by following ASTM procedures to ensure that the reconstituted hard water remained within a narrow range of values. Daphnid cultures were maintained as in Smith et al. (1988). Test chambers, which contained the same daphnid food (Chlorella pyrenoidosa, C. vulgaris, Chlamydomonus reinhardii, and Ankistrodesmus falcatus) as that used in daphnid cultures by Smith et al. (1988), averaged 9900 algal cells/mL.

Static-renewal, chronic bioassays were conducted for 16 d to test the effect of phenanthrene, nicotine, or pinane on growth and reproduction of D. pulex. Chemicals, obtained commercially, had a minimum purity of 97%. Stocks of phenanthrene and pinane were dissolved in acetone, and nicotine was dissolved in water. Each test consisted of a water or solvent control (0.5 mL/L), depending on compound tested, and five concentrations of each chemical.

Test chambers were similar to those used by Gersich (1984); they consisted of 1-L beakers containing five uniquely labeled, glass test tubes 3 cm in diameter. Nylon netting (0.29-mm mesh) was glued with a small bead of silicone aquarium sealant to the bottom of each tube to prevent Daphnia adults and neonates from escaping. Three replicate chambers, each containing five D. pulex in individual test tubes in 700 mL of water, were used for each test concentration.

At the start of the test, one Daphnia neonate was placed in each test tube in each chamber (15 per treatment, 90 per test). New culture media were provided for each treatment three times a week (Monday, Wednesday, Friday) during the 16-d study. To ensure consistency of the medium among replicates, we prepared one 2.1-L batch of medium, containing reconstituted hard water and daphnid food for each concentration. Aliquots of solvent and chemical stock (1 mL total) were added to each batch to obtain appropriate concentrations. The medium was then divided among three test chambers. Original daphnids were transferred with pipets to corresponding test tubes and test chambers and mortality was recorded. Neonates produced by the original daphnids were rinsed into petri dishes, counted, and discarded. The cumulative number of neonates produced by daphnids over 16 d was used to measure fecundity. On day 16 of the study (day 21 in phenanthrene tests), the original daphnids were placed in labeled test tubes containing ethanol for later determination of standard length (±0.01 mm). Tests were considered valid if mortality in controls was <20% by day 16.

Separate sets of chambers were used to determine changes in chemical concentrations under test conditions. One set contained chemicals in water only, to determine losses due to physical characteristics such as volatility or adhesion to glass. A

second set contained chemicals in the test medium that included algae and daphnids to determine additional losses from uptake by organisms. Chemical concentrations were analyzed at 1 hr, 48 hr, and 72 hr after preparation to simulate exposure in test media at the beginning and end of the renewal cycle (2-3 d).

Chemicals were extracted first from 100-mL aliquots of test solution into 10 mL of chloroform by liquid-liquid extraction in a 250-mL separatory funnel, and then with 5 mL of chloroform. The two chloroform extracts were combined and placed in 15-mL graduated centrifuge tubes and reduced to 2 mL on an evaporator (Meyer N-Evap Analytical Evaporator 111, Shrewsbury, Massachusetts) with a gentle flow of air. We quantified chemical concentrations with a Varian 3700 gas chromatograph (Walnut Creek, California) equipped with a photo ionization detector. Compounds were resolved on a 30-m, DB-17 wide-bore capillary column (0.53 mm id); helium was the carrier gas (5 mL/min) and nitrogen was the make-up gas for the detector (25 mL/min). Detector temperature was maintained at 230°C for all analyses, but the injector and column temperature were set differently for each compound (Table 1). An internal standard (Table 1) added to each sample before extraction was used for chemical quantification. A pure chloroform injection showed no peaks, indicating chemical purity of the solvent. A blank of reconstituted hard water provided estimates of background levels for each run. To determine recoverability of a chemical from the extraction and reduction process (Table 1), we spiked a 100-mL aliquot of reconstituted hard water with each chemical, processed it, and compared it to standards in chloroform.

Table 1. Gas chromatograph parameters for each chemical tested.

Chemical	Temperature (°C)		Recovered (%)	Internal Standard (10 mg/L chloroform)
	Injector	Column		
Phenanthrene	190	170	86-92	fluorene
Nicotine	130	170	95-98	phenanthrene
Pinane	100	80	88-95	naphthalene

We used analysis of variance (SAS Institute Inc. 1982), in which a set of orthoganol contrasts was used to compare control with treatments, to determine the first concentration that produced significant differences ($P \leq 0.05$) in fecundity or growth, designated as the lowest-observable-effect-concentration (LOEC).

RESULTS AND DISCUSSION

Recovery rates from the gas chromatography analysis at 1 hr after preparation in water only was highest for phenanthrene and relatively low (about 50% of expected) for nicotine and pinane (Table 2). At 48 and 72 hr after preparation, concentrations had

dropped substantially (<25% of expected) for all chemicals. Uptake by algae and daphnids from the test medium did not add to chemical loss 1 hr after preparation but further reduced phenanthrene and nicotine to negligible concentrations (<5% of expected) after 48 hr. The estimated LOECs were based on nominal concentrations instead of actual concentrations and therefore indicate upper limits. The actual concentrations of phenanthrene, nicotine, and pinane were much lower than the nominal concentrations, possibly due to adsorption to glassware, the volatility of these contaminants, and uptake by organisms.

Table 2. Recovery rates (mean % of nominal concentrations, standard error in parentheses) of chemicals through time after preparation in water only and in test media.

Chemical	% Recovery in Water Only			% Recovery in Test Media		
	1 hr	48 hr	72 hr	1 hr	48 hr	72 hr
Phenanthrene	84(1.3)	13(0.9)	10(0.4)	83(1.8)	0(0)	0(0)
Nicotine	57(3.0)	24(2.0)	9(4.5)	89(6.0)	3(3.0)	0(0)
Pinane	44(5.4)	3(0.9)	1(0.4)	41(5.4)	3(0.4)	1(0.9)

Mortality of the original daphnids was generally less than 50% by day 16, except in the highest concentration tested for each chemical (Table 3). This study now focuses on the sublethal effects of changes in fecundity and growth of the daphnids remaining in the lower concentrations tested.

Table 3. Mortality (%, mean and range) of Daphnia pulex by day 16 for bioassays with phenanthrene, nicotine, and pinane. Nominal concentrations (mg/L) of each chemical presented. Three replicates with phenanthrene and two each replicates for pinane and nicotine at each concentration except where noted (na).

Phenanthrene			Nicotine			Pinane		
mg/L	Mortality		mg/L	Mortality		mg/L	Mortality	
0.00	10	7-13	0.00	10	0-20	0.00	0	0-0
0.06	40	27-53	0.02	6	0-13	0.05	6	(na)
0.08	26	13-40	0.07	4	0-7	0.10	3	0-6
0.09	26	13-40	0.12	10	0-20	0.20	3	0-6
0.11	26	0-53	0.18	20	13-27	0.35	20	0-40
0.13	53	33-73	0.24	66	33-100	0.50	100	(na)

Fecundity and growth of daphnids decreased as the nominal concentration of phenanthrene increased from 0.06 to 0.13 mg/L (Figure 1). The LOEC (0.06 mg/L) was about 16% of the 48-hr EC50

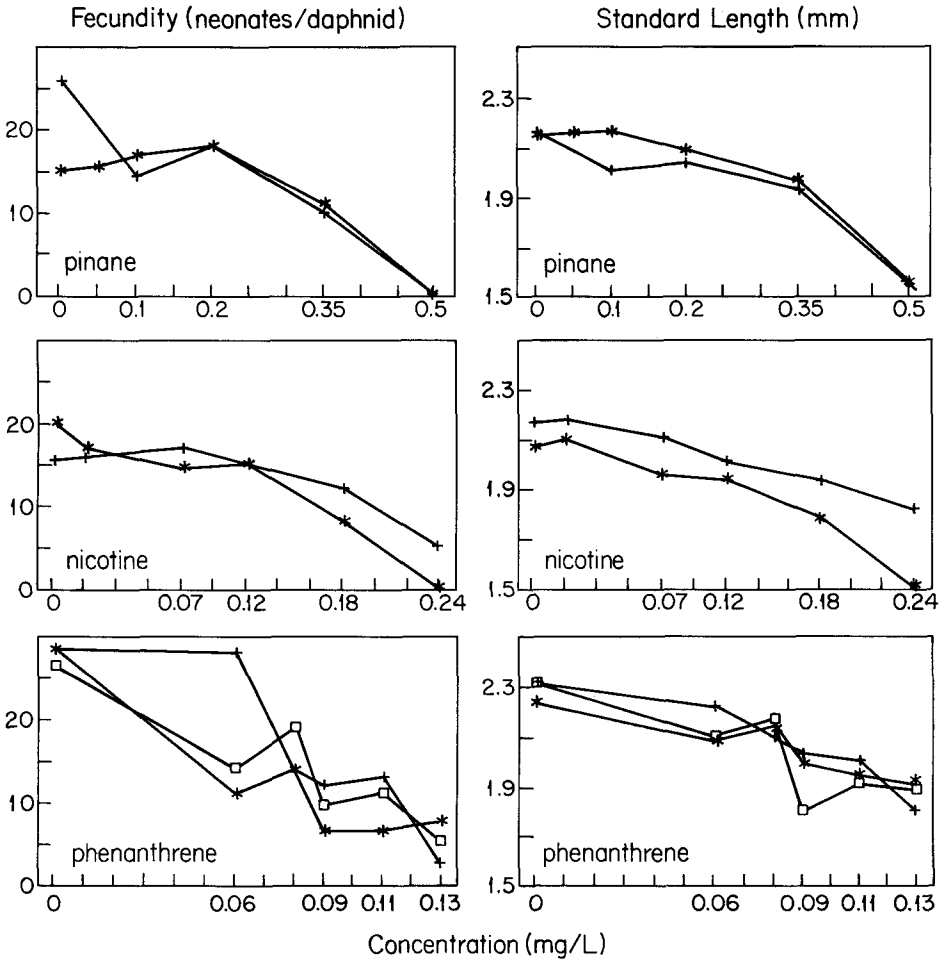

Figure 1. Change in mean growth (standard length) and fecundity of Daphnia pulex by day 16 (day 21 for growth in phenanthrene) for replicate bioassays with phenanthrene, nicotine, and pinane. Concentrations shown are nominal.

for phenanthrene (Smith et al. 1988). Even though an overall significant difference was detected at 0.06 mg/L in the three tests, one of the replicates produced no significant difference in length or fecundity when compared with the control. At 0.13 mg/L of phenanthrene, mortality was high (about 60%; Table 3) and fecundity approached zero. Therefore, over a relatively small range of phenenathrene concentrations, we encountered few or no

sublethal effects at 0.06 mg/L but substantial sublethal and lethal effects at 0.13 mg/L.

Nicotine also significantly reduced growth and fecundity of daphnids at nominal concentrations from 0.02 to 0.24 mg/L (Figure 1). The LOEC for length was 0.07 mg/L, 29% of the 48-hr EC50 for nicotine (Perry and Smith 1988). The LOEC for fecundity was 0.18 mg/L, 75% of the nicotine 48-hr EC50, and approached zero at 0.24 mg/L. Mortality also increased at this higher concentration (about 70%; Table 3).

Increasing concentrations of pinane significantly reduced growth and fecundity (Figure 1). The LOEC for both growth and fecundity was nominally 0.1 mg/L, i.e., 3% of the 48-hr EC50 (Smith et al. 1988). This LOEC also appeared to be at a threshold, since growth and fecundity were not affected at this concentration in one replicate. No neonates were observed at 0.50 mg/L. Hence, sublethal effects of pinane increased greatly from 0.1 to 0.5 mg/L, and mortality was complete at 0.70 mg/L (Table 3).

Chronic studies of D. pulex exposed to different concentrations of phenanthrene, nicotine, and pinane produced consistent sublethal effects among replicates and concentrations. The LOEC's for growth and fecundity with each chemical tested were 3 to 30% of the 48-hr EC50's. Growth decreased as concentration increased for each chemical tested, and fecundity approached zero at 2 to 5 times the LOEC for each chemical. In this study chemicals representing PAHs, heterocyclic nitrogen compounds, and cyclic alkanes, produced detectable sublethal effects in daphnids at less than 0.1 ppm in water. These chronic studies, in conjunction with the more extensive acute toxicity testing (Passino and Smith 1987; Perry and Smith 1988; Smith et al. 1988), provided a relatively quick but thorough toxicological assessment of a large array of chemicals and demonstrated the relative importance of different classes of compounds in changing growth and survival trends in given populations of native organisms. Classic toxicity tests continue to provide a reliable backdrop of results with which the effects of new chemicals or mixtures can be compared.

Inasmuch as aquatic organisms such as D. pulex are sensitive to low concentrations of dissolved chemicals that have been detected in the tissues of Great Lakes fish, the method of exposure and mode of transfer of these chemicals to aquatic biota must be verified. Organisms not only take up the chemicals in water, but are exposed to them through contact with contaminated sediment or through ingestion of contaminated food. After the most likely type of chemical exposure is verified, the consequences of this exposure to chemicals detected in the Great Lakes on populations of the native biota can be determined.

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