

## Lethal and Sublethal Effects of Azulene and Longifolene to Microtox<sup>®</sup>, *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas*

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The data from this study were needed to investigate the aquatic toxicity of the natural compounds azulene and longifolene, which were representatives of nearly 500 compounds of 19 chemical classes tentatively identified in Laurentian Great Lakes fish (Hesselberg and Seelye 1982; Passino and Smith 1987). There are growing concerns over the potential toxic and ecological effects of these hydro- and superhydro- phobic contaminants, with special regard to their accumulation and transport (Skoglund and Swackhamer 1994). To support the concentration-response evaluations and to estimate exposure, quantitative structure-activity relationships were used as predictive tools of toxicity.

The purpose of this study was to determine the acute and chronic toxicity of azulene (polynuclear aromatic hydrocarbon) and longifolene (cyclic hydrocarbon), to Microtox<sup>®</sup> bacteria (*Vibrio fischeri*), cladocerans (*Ceriodaphnia dubia* and *Daphnia magna*, and fathead minnow (*Pimephales promelas*). These test organisms have been utilized extensively for the evaluation of specific compounds and effluents, and in establishing water quality criteria. These natural chemicals are frequently present (spatially and temporally) but often neglected, and may require special attention.

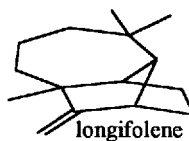
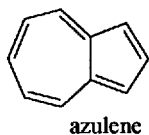
### MATERIALS AND METHODS

Standardized test procedures and methods were used in carrying out range finding and definitive toxicity tests with the bacteria, cladocerans, and fish in this evaluation process. Microtox<sup>®</sup> Model 500 test procedures (15±1°C) and methods were utilized according to “100% Test” protocols (Microbics Corporation 1992). All aspects of the testing, from culturing the cladoceran and fish test organisms (25 ±1°C), to their static-renewal exposure (25±1°C), were specifically conducted according to U.S. EPA methods (1989 and 1990).

Briefly, for the definitive acute toxicity tests with cladocerans and fish, up to 6 concentrations of each toxicant were prepared along with a diluent and vehicle control. Three replicate chambers, each containing ten organisms were used for each test concentration. Endpoints observed included inhibition of bioluminescence for the bacterial reagent, immobility for the cladocerans, and mortality for the fish. For the definitive chronic toxicity tests with cladoceran and fish, 5 concentrations of each toxicant were utilized along with a diluent and vehicle control. Three replicate chambers for fish

(each containing ten test organisms), and ten replicate chambers for the cladoceran species (each containing one test organism), were employed. Endpoints observed included survival and reproduction for the cladoceran species, and survival and growth for the fathead minnow.

For testing, azulene (CAS# 275514) was obtained from Aldrich Chemical Co. with 99% purity. The tricyclic sesquiterpene longifolene (CAS# 475207) was purchased from Fluka Chemical Co. with >99% purity.



The relative insolubility in water of azulene (solubility estimated at 0.02 g/L, from information downloaded from QSAR Database, U.S. EPA's Environmental Research Laboratory -- Duluth, MN) and longifolene (solubility estimated at  $4.3 \times 10^{-5}$  g/L, from QSAR Database, U.S. EPA) necessitated the use of the polar protic solvent acetone in preparing test solutions. Respective stock solutions were prepared with reconstituted moderately hard dilution water (U.S. EPA 1989 and 1990) in a laminar flow hood, with acetone as a carrier solvent, and were stored in amber bottles at 4°C in the dark. Azulene and longifolene exposure concentrations, expressed in mg/L, were prepared from the initial stock solutions using volumetric glassware and moderately hard dilution water. The test concentrations are presented as nominally defined. Water quality parameters such as alkalinity (60 -70 mg/L CaCO<sub>3</sub>L), hardness (80-100 mg/L CaCO<sub>3</sub>), pH (7.8-8.2), dissolved oxygen (7.6 - 8.0), and specific conductance (290 -320 umhos/cm) were measured to check for consistencies between batches and to provide general water quality control. These water quality parameters are known to contribute to changes in toxicity and bioavailability. Controls with acetone (maximum 1 mL acetone/L) were conducted for quality control and no adverse effects were observed on the test species,

The reference toxicant for Microtox<sup>®</sup> was zinc sulfate, and for cladocerans and fish sodium chloride was employed (results not shown). The following reagents were obtained from Microbics Corporation for the Microtox<sup>®</sup> test reagent (lyophilized bacteria in vials), osmotic adjusting solution (22% sodium chloride solution), and reagent diluent (2% sodium chloride solution). The marine luminescent gram-negative bacterium Vibrio fischeri (formerly Photobacterium phosphoreum) is a pure culture strain provided by Microbics Corporation. No culturing was necessary as the reagent was reconstituted from a lyophilized state.

The U.S. EPA Probit computer program (Version 1.5, U.S. EPA, Cincinnati, Ohio) was used to analyze the cladoceran and fish acute data and for calculating E(L)C50 values for azulene and longifolene. Probit requires at least two observations of partial kills, and assumes that the organism tolerances have a lognormal distribution with a typical sigmoidal dose response curve. Concentration versus acute percent response for azulene and longifolene was plotted for each test organism, and the Weibull distribution function (Elandt-Johnson and Johnson 1980) was used to statistically fit the data and yield the resulting shape of the curve.

The Microtox® “100% Test” protocol data were analyzed utilizing the Microtox® Version 7.0 software package employing the gamma function routine to calculate EC50 values (Microbics Corporation 1992). The gamma function routine involves linear plots of log light lost/light remaining, corrected for the control after a given time, versus log concentration.

Statistical methods for the chronic tests (survival, reproduction, and weight increase data from the 7-d tests) were analyzed using hypothesis testing. The data were tested for normality and homogeneity of variance. Dunnett’s test (Version 1.5, U.S. EPA, Cincinnati, Ohio) was employed to compare the treatment mean with the control mean to determine “no observed effects concentrations” (NOECs) and “lowest observed effects concentrations” (LOECs) for each endpoint (U. S. EPA. 1989) If data failed tests for normality and homogeneity, Steels Many One Rank Test was used with the chronic C.dubia tests (U.S. EPA 1989).

Hickey and Passino-Reader (1991) have applied the Linear Solvation Energy Relationship (LSER) paradigm to develop QSARS for toxicity of chemicals to the cladocerans Daphnia magna and Daphnia pulex, the fathead minnow Pimephales promelas, and the Microtox® reagent Vibrio fischeri (equations contained in, Hickey et al. 1990). The results of these predictive nonpolar narcosis models (Hickey et al. 1990) for the specified organisms are found in Table 1. These structure activity toxicity estimates are useful as screening tools over a particular range of conditions (such as nonpolar narcosis mode of action) (Nabholz et al. 1993); yet they are limited by design, as are all models of natural systems.

## RESULTS AND DISCUSSION

The toxicity evaluations for the compounds azulene and longifolene were initiated in March 1994 and completed in October 1994. The procedures employed over this period were consistent with U.S. EPA guidelines (U. S. EPA 1989). The resulting data generated from replicate acute and chronic tests (Sweet 1994) were analyzed utilizing the aforementioned statistical procedures and are reported in Tables 1 and 2.

**Table 1. Summary of Acute Toxicity Evaluation of Azulene and Longifolene.**

Toxicant Test Organism	Mean E(L)C50 (mg/L)	C.V. %	QSAR estimated E(L)C50, C.I. <sup>a</sup> (mg/L)
<b>Azulene</b>			
<u>V.fischeri</u> (n=12)	1.3 <sup>b</sup> (s.d. 0.57)	43	10.4 (2.2 - 51) (5 min @ 15°C)
<u>D.magna</u> (n=5)	11.5 (s.d. 2.65)	23	181 (62 - 536) (24 hr @ 22°C)
<u>C.dubia</u> (n=3)	8.7 (s.d. 1.63)	19	Not Available
<u>P.promelas</u> (n=3)	9.0 (s.d. 4.07)	45	11.4 (4.1 - 33) (96 hr @ 25°C)
<b>Longifolene</b>			
<u>V.fischeri</u> (n=3)	> 36	N.A	8.8E <sup>-5</sup> (9.9E <sup>-6</sup> -7.7E <sup>-4</sup> ) (5 min @ 15°C)
<u>D.magna</u> (n=3)	0.44 (s.d. 0.04)	9	0.36 (0.05 - 2.6) (24 hr @ 22°C)
<u>C.dubia</u> (n=3)	0.41 (s.d. 0.05)	13	Not Available
<u>P.promelas</u> (n=2)	10.2 (s.d. 0.33)	3	1.7E <sup>-3</sup> (3E <sup>-4</sup> - 9.5E <sup>-3</sup> ) (96 hr @ 25°C)

<sup>a</sup>C.I. = 95% confidence interval

<sup>b</sup>15 minute exposure value

Analysis of the acute data on the two selected polycyclic hydrocarbons shows that azulene is less toxic, with mean E(L)C50 values ranging from 1.3 to 11.5 mg/L among the test species (Table 1). By comparison, longifolene is more toxic with calculable mean E(L)C50 values ranging from 0.41 to 10.2 mg/L (Table 1). The Microtox® bacteria was most susceptible to azulene and least sensitive to longifolene. Coefficients of variation (C.V.s) calculated for E(L)C50 values are presented (Table 1) as indications of the variability of a calculated endpoint. The relatively high variability of the acute azulene results for the bacteria and fish are likely attributable to this compound's volatility in water (Henry's Constant estimated at  $1.99\text{E}^{-3}$  atm-m<sup>3</sup>/mole by QSAR Database, U.S.EPA).

The results of Microtox® proved to be a good estimate of the maximum toxicity of azulene (Table 1) for the test organisms. The Microtox® bacteria, however, were not sensitive to the highest test concentration of longifolene (36 mg/L). Furthermore, the stimulation of luminescence observed when the Microtox® bacteria were exposed to the highest test concentration of the longifolene toxicant may be an example of hormesis; in that the intensity of luminescence as compared to controls was greater than 10%.

Plots of concentration versus percent response (Figure 1) for C. dubia portray a relatively steep slope for longifolene (Weibull distribution function,  $F(x)=1-e^{-(x/0.435)^{4.9}}$ ) indicating low cladoceran variability in sensitivity, versus the more shallow slope for azulene ( $F(x)=1-e^{-(x/10.9)^{2.45}}$ ). The greatest "margin of safety" was observed with the lower molecular weight hydrocarbon azulene, and test organism D. magna, as indicated by the shallowest slope ( $F(x)=1-e^{-(x/16.78)^{1.77}}$ ) and spread of acute toxicity over 60 mg/L (versus a 1 mg/L spread for longifolene). Interestingly, these data plots of azulene and longifolene, fitted by the Weibull distribution function, suggest a sigmoidal relationship for all test organisms.

The results for longifolene tend to confirm the hypothesis that cladocerans are a sensitive test organism (Mayer and Ellersieck 1988). For azulene, the fathead minnow results suggest a comparable sensitivity to the cladocerans, with the bacterial reagent V. fischeri exhibiting the most sensitivity. Based on their physiology, it is reasonable that lyophilized bacteria show an increased sensitivity to some toxicants, as these may readily cross cell envelopes (Bitton and Dutton 1988). The residues in an organism at the receptor site(s), as the net result of many internal processes (diffusion, absorption, distribution, and binding), form a critical link between exposure and elicited bioresponse.

In order to validate the utility of the QSAR (general narcosis model) predictions in this research, a comparison was made between the observed and QSAR predicted acute toxicities of the chemicals by calculating their excess toxicity (Te) according to the following equation:  $Te = LC50_{pred}/LC50_{obs}$ , where  $LC50_{pred}$  and  $LC50_{obs}$  are the QSAR-predicted (baseline narcosis) and bioassay observed toxicities, respectively (Lipnick 1988). A validation ratio of 1.0 would demonstrate perfect agreement with a nonpolar narcosis model, for azulene and longifolene. For V. fischeri, D. magna, and P. promelas exposed to azulene, the resulting Te using averaged toxicity values (Table 1) equal 8, 15.7, and 1.27 respectively. For D. magna and P. promelas exposed to longifolene, the resulting Te utilizing averaged toxicity values equal 0.82 and 0.0002, respectively. A Te could not be calculated for V. fischeri for longifolene due to an observed stimulatory effect, as compared to controls. Thus, in this series of toxicity tests, QSARs proved to both underpredict (azulene and longifolene, Table 1) and overpredict (longifolene, Table 1) the

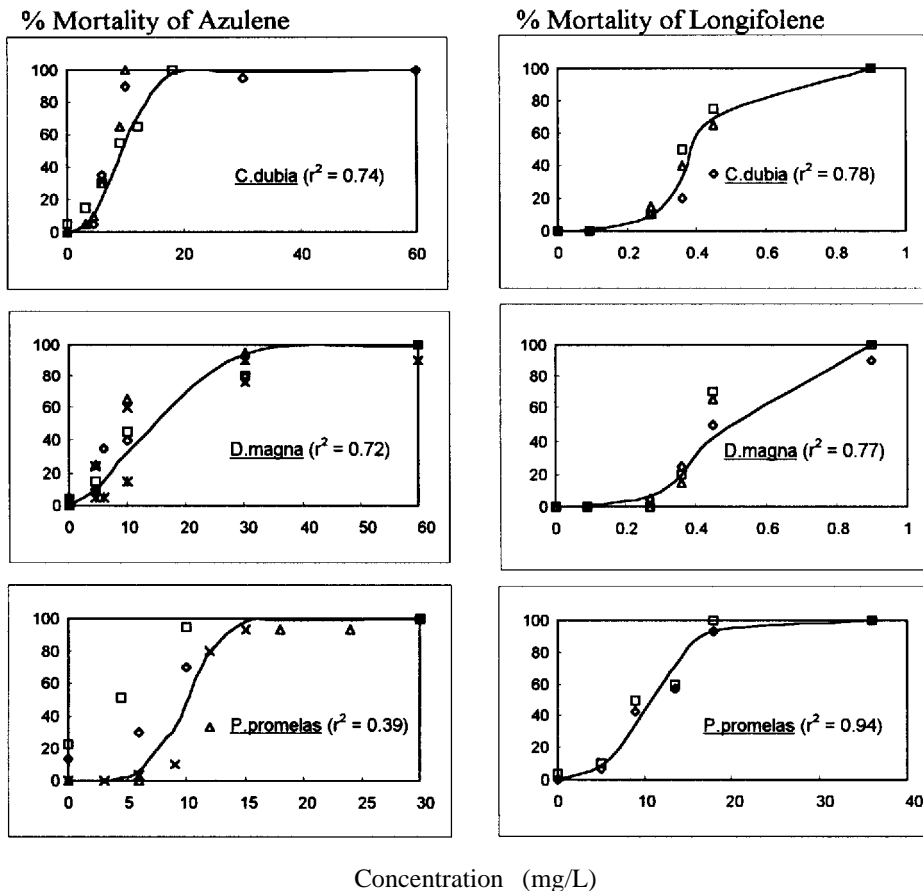


Figure 1. Weibull fit concentration - response curves with mortality/immobility as the test endpoints, from replicate bioassays with azulene and longifolene. Concentrations shown are nominal.

laboratory observed toxicity. Perhaps due to the estimated superhydrophobicity of longifolene ( $\text{Log}K_{ow}=6.1$ , versus 3.3 for azulene), the QSAR toxicological model failed to express some aspect of this hydrocarbon's functional toxicity. However, the solvatochromatic property values (Hickey and Passino-Reader, 1991) for azulene and longifolene were within the ranges used to develop the LSERs.

The 7-d chronic test results for *C. dubia* and *P. promelas* showed that azulene was significantly less toxic than was longifolene (Table 2), as determined by Tukey's method of multiple comparisons ( $p=0.05$ ). Azulene at the two highest test concentrations (3 and 6 mg/L) reduced both cladoceran survival and reproduction over the 7-d testing period. Also, for azulene, the acute-to-chronic ratio ( $\text{ACR} = L(E)/C50/\text{NOEC}$ ) for both *C. dubia* and *P. promelas* was approximately 9. Cladoceran reproduction proved to be the more sensitive endpoint with regard to the intermediate test concentration of longifolene (0.36 mg/L) (Table 2). For longifolene, the ACRs for *C. dubia* and *P. promelas* were approximately 1.5 and 23, respectively. The range of ACR values (from 1.5 to 23) for the

cladoceran and fish test species, in this research, support the investigation by Kenaga (1982) in which 86% of the chemicals tested displayed ACR values less than 100 for invertebrate and vertebrate species tested.

Table 2 . Summary of Azulene and Longifolene Chronic Toxicity Results

Toxicant Test Organism	NOEC <sup>a</sup> (mg/L)	LOEC <sup>b</sup> (mg/L)
Azulene		
<u>C.dubia</u> survival (n=3)	1.0	3.0
<u>C.dubia</u> reproduction (n=3)	1.0	3.0
<u>P.promelas</u> survival (n=3)	1.0- 3.0 <sup>d</sup>	3.0- 6.0 <sup>d</sup>
<u>P.promelas</u> growth (n=3)	1.0	3.0
QSAR “no effect” 30 d chronic level estimated: 0.57 mg/L <sup>c</sup>		
Longifolene		
<u>C.dubia</u> survival (n=2)	0.45	0.90
<u>C.dubia</u> reproduction (n=2)	0.27	0.36
<u>P.promelas</u> survival (n=2)	0.90	>0.9
<u>P.promelas</u> growth (n=2)	0.45	0.90
QSAR: none available		

<sup>a</sup>NOEC: No Observed Effect Concentration

<sup>b</sup>LOEC: Lowest Observed Effect Concentration

<sup>c</sup>QSAR data obtained from ASTER Database, U.S. EPA in Duluth

<sup>d</sup>Ranges overlap due to higher NOEC and LOEC survival results with one of the three replicate tests.

The principal advantages of this study’s trophic level approach to toxicity testing are the ease in standardization, replicability, low cost, and applicability to the ecological risk assessment of the Great Lakes. The traditional biotests with bacteria, cladocerans, and fish continue to provide accurate information on select toxic endpoints (such as immobility, diminished fecundity, and lethality), although they essentially provide little information on the more subtle effects such as immunotoxicity.

This battery of biotests did prove to be sensitive to the relatively low concentrations of azulene and longifolene, as representatives of naturally occurring and little investigated hydrocarbon species, that have been detected in the tissues of Laurentian Great Lakes fish. The combined acute and chronic results of this study indicate that polycyclic hydrocarbons such as azulene and longifolene have a likelihood of environmental hazard to the aquatic community. The results of the toxicity tests can be useful extrapolations to expected effects on natural populations. Each test component possesses unique attributes that make it a useful detector of toxic components. The useful features of the QSAR models include simplicity of calculation and applicability to cover a range of compounds and organisms; efficiency in time, energy, and cost; and provision of another element to the assessment of chemical risk to representative aquatic biota.

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