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Toxicity Testing of Aromatic Hydrocarbons Utilizing a Measure of Their Impact on the Membrane Integrity of the Green Alga *Selenastrum capricornutum*

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Previously, we have revealed a strong linear relationship between toxicity and bioconcentration of volatile aromatic hydrocarbons by algal cells (Herman et al. 1991). Non-ionized organic pollutants are thought to disrupt membrane integrity through a non-specific narcotic-type mode of action (Boyles 1980; Hutchinson et al. 1980). The impact of organic pollutants on membrane integrity has previously been measured by monitoring the release of cellular manganese and potassium from algal cells exposed to pure aromatic hydrocarbons (benzene, naphthalene) and aqueous crude oil extracts (Kauss and Hutchinson 1978; Hutchinson et al. 1981). The release of ^{14}C-labeled photosynthates from algal cells exposed to various petroleum hydrocarbons (Hutchinson et al. 1979) and acetone (Stratton 1989) has also indicated an impact of organic pollutants on membrane integrity.

In the present study, the impact on membrane integrity of the aromatic hydrocarbons, benzene, toluene, ethylbenzene and three xylene isomers (para, meta, and ortho), collectively referred to as BTEX, was investigated using the green alga, Selenastrum capricornutum. BTEX are water-soluble components of crude oil and gasoline which are known to be toxic to aquatic life. Their impact on membrane integrity was determined by monitoring the release of ^{14}C-labeled photosynthates from algal cells exposed to benzene and o-xylene. An alternative method utilized a fluorescent dye, uranin (fluorescein sodium salt), which is commonly used as a vital stain (Stadelmann and Kinzel 1972). The purpose of the present study was to evaluate the usefulness and sensitivity of uranin staining as an indicator of the impact of organic pollutants on membrane integrity. The sensitivity of the uranin staining technique was compared to the concentration of BTEX required to inhibit algal growth.

MATERIALS AND METHODS

Stock cultures of the test alga, Selenastrum capricornutum, were maintained within 500-mL Erlenmeyer flasks, containing 200 mL of Bristol medium, and

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incubated under the conditions described by Herman et al. (1990). Direct counts of cell number were made using a 2-mm deep Fuchs-Rosenthal hemocytometer.

An inoculum of algal cells (1 x 10^5 cells/mL) was grown for 12 d within 2.5 L of Bristol medium to which either 100 μCi or 200 μCi (depending on the experiment) of ^{14}C-labeled bicarbonate ([^{14}C]-$NaHCO_3$, 56.6 mCi/mmole, ICN, California) had been added. The cells were harvested by sterile transfer into 250-mL centrifuge bottles, washed in Bristol medium and concentrated to approximately 4 x 10^8 cells/mL. A volume of the concentrated algal stock solution (1 mL) was transferred into 10-mL hypo-vials (Pierce, Rockford, Illinois) followed by 8 mL of aromatic hydrocarbons dissolved in Bristol medium to give a final concentration of 0, 40, 80, 160, 320 or 640 mg/L benzene or 0, 4, 8, 16, 32 or 64 mg/L o-xylene. The hypo-vials were sealed with "tuf-bond" septa and crimp-cap seals (Pierce, Rockford, Illinois.). The hypo-vials were placed on a gyratory shaker, and triplicate hypo-vials of each treatment were sampled after 2-, 4- and 8-d incubation periods. The hypo-vials were centrifuged at 1000 x g for 10 min, and a 2-mL volume of the supernatant was removed for liquid scintillation analysis. The samples were acidified using 1N HCl and, after 1 h, 15 mL of scintillation fluor (PCS II, Amersham, Illinois) was added. Radioactivity was measured using a LKB Wallac 1209 Rackbeta liquid scintillation counter. The supernatant was then completely removed from the hypo-vials, and the algal cells were washed in half-strength Bristol medium (pH adjusted to 4.5 using 1 N HCl), stained in uranin (J.C. Baker, Phillipsburg, New Jersey, 0.03% in half-strength Bristol medium) for 1 h, then washed three times in half-strength Bristol medium. Algal cells from replicate hypo-vials were combined, and a one-tenth dilution was used to fill a hemocytometer. The total number of cells was determined using white light; the number stained with uranin was determined using epifluorescence under blue light (Nikon B2 filter). A minimum of 300 cells from duplicate samples were counted.

To perform the uranin staining bioassay, exponential growth phase cells were concentrated to a density of 9 x 10^6 cells/mL in Bristol medium, and a 1-mL volume was transferred into 10-mL hypo-vials (Pierce, Rockford, Illinois), followed by 8 mL of a BTEX mixture containing benzene (52%), toluene (28%), ethylbenzene (5%) and p-, m-, o-xylene (each at 5%) dissolved in Bristol medium. These proportions were similar to those found within the water soluble fraction (WSF) of a standard gasoline (PS-6) provided by the American Petroleum Institute. Algal cells were exposed to BTEX mixtures of approximately 0, 12, 24 and 46 mg/L total aromatic hydrocarbons. The hypo-vials were sealed and incubated as previously described. After 1-, 4-, and 8-d exposure periods, triplicate hypo-vials from each treatment were centrifuged at 1000 x g for 10 min, and the algal cells stained with uranin as previously described. A 5-mL sample of each hypo-vial was used for fluorometric measurements. Non-stained cells (1 x 10^6 cells/mL) were used

as a blank. Uranin staining of algal cells was quantified using a Sequoia-Turner fluorometer (model 450) fitted with a narrow-band excitation filter (Sequoia-Turner 450-107) which produced blue light that matched the absorption peak of uranin (490 nm). An emission filter (Sequoia-Turner 450-108) with peak transmittance through a narrow (20 nm) window centered at 520 nm was used to separate uranin fluorescence from chlorophyll fluorescence (which has a peak at approximately 680 nm).

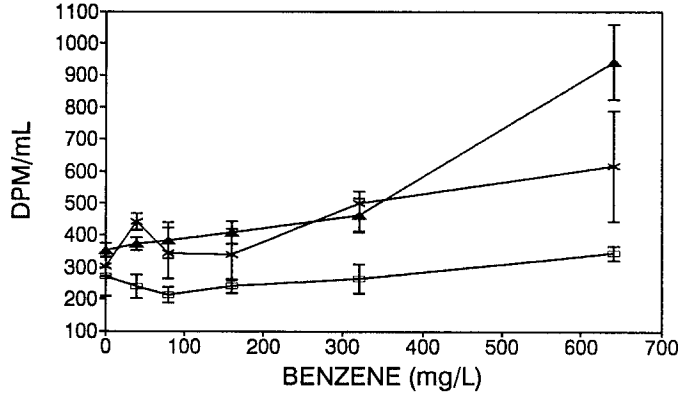
BTEX inhibition of algal growth was determined using the procedure described by Herman et al. (1990). Algal cells (1 x 10^5 cells/mL) were transferred to air-tight flasks and exposed, in triplicate, to 0, 12, 24 and 46 mg/L of the BTEX mixture described above. After 2, 4, 6, and 8 d, cell number was determined by direct microscopic counts. The ability of algal cells to recover following an 8-d exposure to BTEX was also determined. Algal cells were concentrated by centrifugation and used to inoculate fresh Bristol medium to an initial density of 1 x 10^5 cells/mL. The air-tight seals on the flasks were replaced by foam stoppers to allow the evaporative loss of any residual aromatic hydrocarbons.

RESULTS AND DISCUSSION

The release of labeled photosynthates was used to demonstrate the impact of aromatic hydrocarbons on membrane integrity. The ability of algal cells to retain ^{14}C-labeled photosynthates was diminished when the cells were exposed to aromatic hydrocarbons (Fig. 1), but the release of ^{14}C-labeled photosynthates occurred at concentration levels much greater than those required to reduce algal growth. Herman et al. (1990) found that the EC_{50} values (the concentration required to reduce growth by 50%) of benzene and o-xylene were 41.0 and 4.2 mg/L, respectively. The concentrations required for the release of ^{14}C-labeled photosynthates were an order of magnitude greater than the EC_{50} values.

In contrast to the release of labeled-photosynthates, uranin staining of algal cells demonstrated a dramatic response to aromatic hydrocarbon exposure. The proportion of total cell number capable of retaining the stain was greatly diminished at concentrations approximately twice the EC_{50} level of each aromatic hydrocarbon (Fig. 2).

The fluorometric bioassay was developed as an alternative to direct microscopic counts to provide a more expeditious and accurate means of quantifying uranin staining of algal cells. Cells were exposed to BTEX within sealed flasks for up to 8 d. Direct microscopic enumeration revealed no change in total cell number between treatment levels or between sample times. With a constant cell density, fluorometric readings could be compared directly between treatments and would reflect the degree to which the cells were stained, not the number of cells present in the sample. Fluorometric



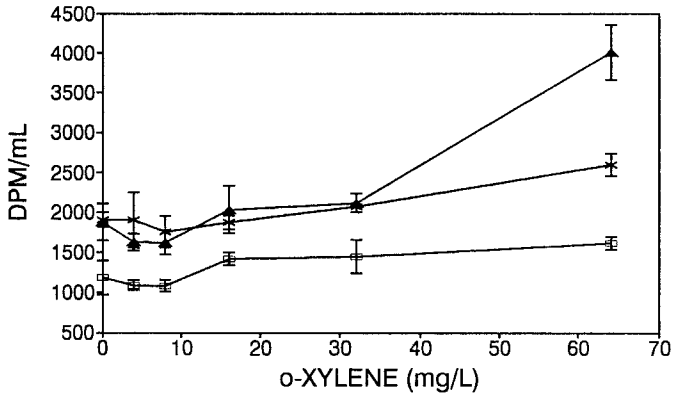
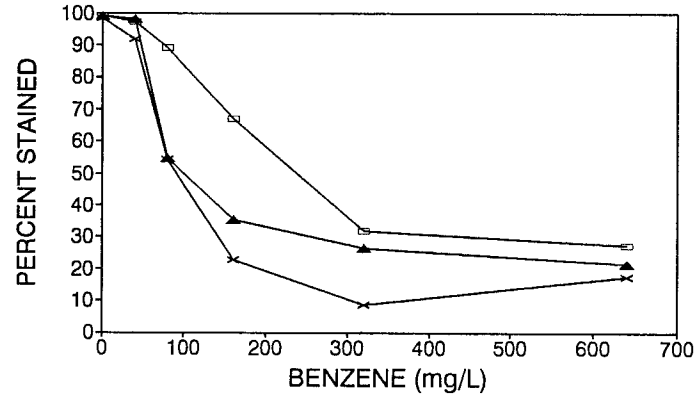


Figure 1. Release of ^{14}C-labeled photosynthates from algal cells exposed to benzene and o-xylene for 2 (□), 4 (×) and 8 (▲) days. The bars represent 1 standard deviation from the mean (n=3).



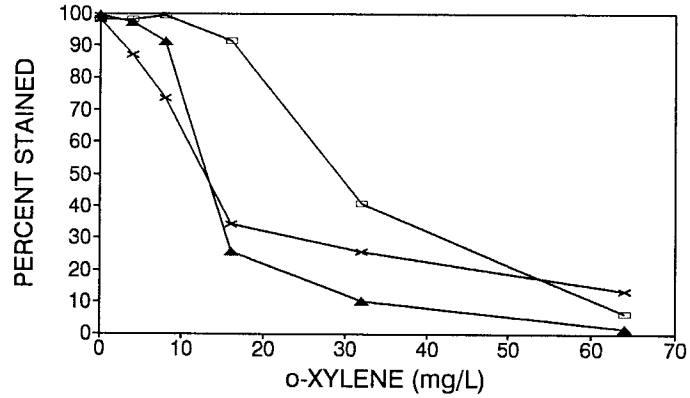


Figure 2. Percent of total cell number stained with uranin after exposure to benzene and o-xylene for 2 (□), 4 (×) and 8 (▲) days.

readings clearly showed a reduction in uranin staining of algal cells with exposure to BTEX (Fig. 3). The reduction in uranin staining with increased BTEX concentration was significant, as indicated by linear regression analysis ($p < 0.01$, $n = 12$, $r^2 = 0.74$ to 0.89).

Uranin staining of healthy algal cells is dependent on a pH gradient between an acidic staining solution and a more neutral cell cytoplasm (Stadelmann and Kinzel 1972). Under acidic conditions, uranin is lipophilic and can penetrate the cell membrane. The neutral cytoplasmic pH can convert the lipophilic molecule into a water-soluble compound that is trapped within the cell. There are two possible mechanisms by which a reduction in uranin staining can indicate an impact on membrane integrity. Damage to membrane integrity, resulting from exposure to aromatic hydrocarbons, may allow the cytoplasm, along with the water-soluble stain, to leak from the algal cells. It is also possible that membrane damage may prevent the cell from maintaining a pH gradient between the acidic staining solution and the neutral cytoplasm, thus reducing the ability of the cell to effectively trap uranin.

Increasing concentrations of BTEX were found to reduce algal growth, but recovery of algal growth was evident when the cells were removed from BTEX exposure (Fig. 4), indicating that BTEX was inhibitory but not algicidal (Payne and Hall 1979). When comparing growth inhibition with the uranin staining bioassay, it is evident that the uranin staining bioassay was sensitive within the range of BTEX concentrations which have an inhibitory effect on cell growth. Other staining procedures have indicated that cell death was required in order to reveal the toxic effects of environmental pollutants (Walsh 1983). The uranin staining bioassay did not require algicidal concentrations of BTEX to reveal the potential toxicity of aromatic hydrocarbons.

The sensitivity of the uranin staining bioassay can be further evaluated by a comparison between the BTEX concentrations required to inhibit algal growth after an 8-d exposure period and the reduction in uranin staining of algal cells exposed to the same BTEX mixture for 1 d (Table 1). The EC_{50} value for uranin staining was 19.0 mg/L compared with 22.7 mg/L for growth inhibition. These results demonstrated that uranin staining of algal cells exposed to BTEX for a relatively short time period provided an accurate prediction of the growth inhibition evident after an 8-d exposure period.

Uranin staining offers an alternative to the more traditional growth-inhibition bioassay as a means of determining the toxicity of aromatic hydrocarbons. Highly volatile organic pollutants, such as BTEX, require the use of sealed, air-tight flasks for toxicity testing, but the restriction of gas exchange can affect algal growth (Herman et al. 1990). The uranin staining bioassays were performed within sealed, air-tight flasks and did not require algal growth in order to demonstrate the potential toxicity of aromatic hydrocarbons.

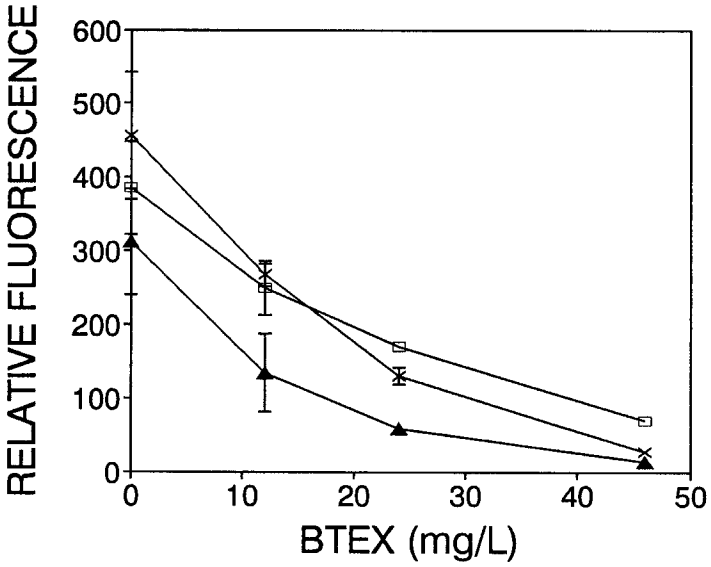


Figure 3. Fluorometric quantification of uranin staining of algal cells which had been exposed to a BTEX mixture for 1 (□), 4 (×) and 8 (▲) days. The bars represent 1 standard deviation from the mean (n=3).

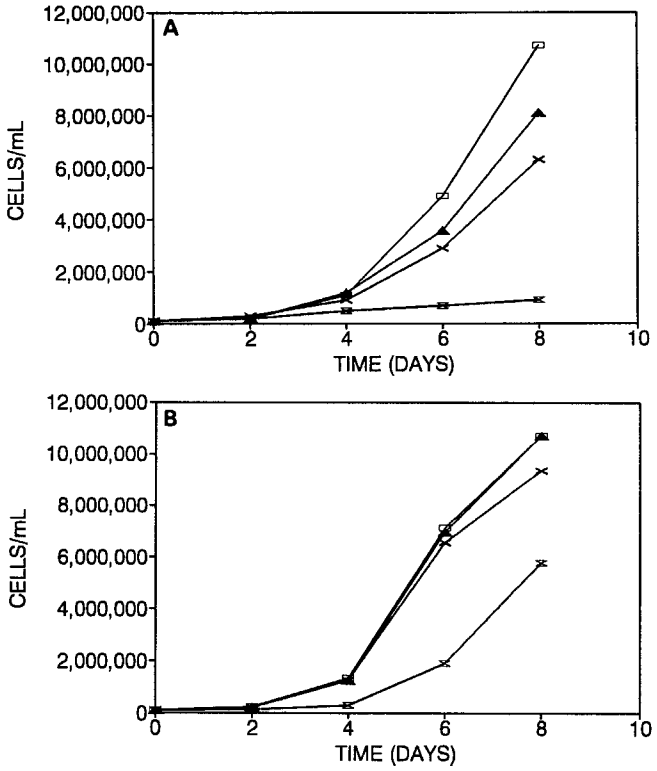


Figure 4. Impact of a BTEX mixture on algal growth (A), and recovery (B) of algal cells following the 8-day exposure period. Algal cells were exposed to 0 (□), 12 (▲), 24 (×) and 46 (⧗) mg/L total aromatic hydrocarbons.

Table 1. A comparison between the reduction in uranin staining of algal cells exposed to BTEX for 1 d and the reduction in algal cell number following an 8-d exposure period.

Compound	Uranin staining (relative fluorescence)			Cell growth (cells x 10^6/mL)		
BTEX (mg/L)	mean	SD[a]	%control	mean	SD	%control
0	385	63	100	10.7	1.3	100
12	249	36	65	8.1	0.5	76
24	170	9	44	6.4	1.0	60
46	71	3	18	0.9	0.2	8

[a] Standard deviation (n=3)

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