Measurement of Short- and Long-Term Toxicity of Polycyclic Aromatic Hydrocarbons Using Luminescent Bacteria

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Growing concern over environmental contamination has stimulated rigorous efforts to establish reliable biological monitoring assays. Methodology was developed for measuring photoinduced short- and long-term toxicity of an important group of contaminants, polycyclic aromatic hydrocarbons (PAHs), using the luminescent bacterium \textit{Vibrio fischeri}. The toxicity of most PAHs can be greatly enhanced on exposure of a living organism and/or the chemicals to ultraviolet (UV) radiation. There are two major mechanisms involved in photoinduced toxicity of PAHs: photosensitization and photomodification. In the former, production of singlet oxygen leads to cellular damage. In the latter, photooxidation of PAHs results in new compounds (usually oxygenated PAHs) that are often more toxic than their parent PAHs. Microbial toxicity assays were developed to measure short- and long-term photoinduced toxicity of PAHs. The bioassays were based on inhibition of luminescence and growth of \textit{V. fischeri}. The short-term assay should detect toxicity of chemicals that are taken up rapidly and/or whose photosensitization activity is immediate. The long-term assay should identify chemicals where the rate of assimilation is slow and/or time is required for photoinduced effects to be realized. The assays were tested with 12 different PAHs. The short-term assay did not reveal photoinduced toxicity for any of the test chemicals. However, photoinduced toxicity was apparent in the long-term assay, indicating that short-term assays may be opaque to this key mechanism of PAH toxicity.

Key Words: polycyclic aromatic hydrocarbons; \textit{Vibrio fischeri}; photoinduced toxicity; photomodification; photosensitization; ultraviolet radiation.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) constitute a class of hazardous organic chemicals made up of two or more fused benzene rings. They have low solubility in water, which contributes to their environmental persistence, especially in sediments and soils (Cerniglia, 1992; Edwards, 1983). Their lipophilicity, environmental persistence, and genotoxicity increase with molecular size (Jacob et al., 1986). Major sources of PAH contamination in the environment are the incomplete combustion of organic fuels including wood, coal, and petroleum (Edwards, 1983; Jacob et al., 1986). As well, the coking process to produce steel is a major source of PAHs (Edwards, 1983; Jacob et al., 1986).

Simulated solar radiation and natural sunlight can enhance the toxicity of PAHs to aquatic organisms such as fish, plants, and bacteria (Newsted and Giesy, 1987; Huang et al., 1993; Duxbury et al., 1997; Ren et al., 1994; McConkey et al., 1997; Mallakin et al., 1998). Photoinduced toxicity of PAHs is derived from two photochemical processes: photosensitization and photomodification (Newsted and Giesy 1987; Greenberg et al., 1993; Krylov et al., 1997; McConkey et al., 1997). During a photosensitization reaction, intracellular singlet-state oxygen (\(^{1}\text{O}_2\)) and other active oxygen species (ROS) are generated, which can cause oxidative damage in biological systems (Girotti, 1983; Foote, 1987). In the case of photomodification, PAHs are structurally altered to a variety of compounds most of which are oxygenation products (oxyPAHs). Many of these photoproducts are more toxic than their parent PAH (Huang et al., 1993, 1995; McConkey et al., 1997).

Several whole-organism assays tests have been developed to assess the toxicity of PAHs in aquatic environments. These include fish, macrophytes, protozoa, algae, and bacteria (Maciorowski et al., 1980). Because bacteria are simple to culture and grow rapidly, they have been used to evaluate various toxicological concerns in aquatic systems. Both growth and physiological measurements of bacteria have been employed (Taylor, 1936; Johnson et al., 1942; Sie et al., 1966; Gradbow et al., 1980; Mayfield et al., 1980). Among the different species of bacteria, luminescent bacteria have been found to be particularly useful in evaluating toxicant impacts. One of the first practical applications using luminescent bacteria was reported by Sie et al. (1966). In that case \textit{Photobacterium phosphoreum} was employed to detect toxic fumes within spacecraft. Bulich (1979) described
the first commercial toxicity test using luminescent bacteria. This experimental system was unique in that the test organisms were freeze-dried preparations that were hydrated immediately prior to use. The endpoint was luminescence from the bacteria following short (< 30 min) exposures to a toxicant. This bacterial test, sold under the trade name Microtox, used a selected strain of Photobacterium fischeri (formerly known as Photobacterium phosphoreum NRRL B-11177), later renamed to Vibrio fischeri (Bulich, 1979, 1986).

Because of the large amount of available data, and the simplicity and robustness of the test, it has been of great interest to compare the Microtox test with other bioassays, particularly other aquatic species (Curtis et al., 1982; Sanchez et al., 1988; Kaiser and Palabrica, 1991; Kaiser and Devillers, 1994; Kaiser et al., 1994). In general it has been found that toxicity of numerous chemicals to V. fischeri agrees well with toxicity to other aquatic organisms (Curtis et al., 1982; Sanchez et al., 1988; Kaiser and Palabrica, 1991; Fort, 1992; Kaiser and Devillers, 1994; Kaiser et al., 1994). The comparisons are especially good when one is interested in the relative toxicity of a large group of contaminants.

While the short duration of the Microtox test has obvious advantages for toxicant screening, there are major drawbacks. The test is sensitive only to fast-acting molecules. Further, the compounds must interfere with a process required for luminescence, and the chemical must be rapidly assimilated. The assay is opaque to toxic by-products of the applied chemicals that might form more than 30 min after the test begins. In the case of PAHs all of these are real concerns. Thus, a new bioassay was developed that employed both short-term and long-term endpoints. The short-term assay is based on measuring luminescence after 15 min exposure to a test chemical. In the long-term assay, V. fischeri cells are grown for 18 h in the presence of a toxicant. The methods are simple and rapid, and would permit a large number of samples to be tested quickly. Importantly, it was developed as a coupled short-term and long-term assay; that is, after the data from the 15-min assay are collected the bacteria are returned to the incubator for the 18-h assay. The assay procedure is quite different from the Microtox assay even though the same luminescent marine bacteria are employed. The assay is performed with bacteria in log-phase growth, instead of freeze-dried organisms. The assay is run in complex medium instead of a saline solution. Finally, the experiment can be performed in darkness and simulated solar radiation (SSR) to test for phototoxicity.

To evaluate these new assays, selected PAHs were tested for toxicity. They were aceanthrenequinone (AAQ), acenaphthene (ACE), acenaphthylene (ACY), acenaphthenequinone (ACQ), anthracene (ANT), anthraquinone (ATQ), 1,2-dihydroxyanthraquinone (1,2-dhATQ), naphthalene (NAP), 5-nitroacenaphthene (5-NACE), phenanthrene (PHE), phenanthrenequinone (PHQ), and pyrene (PYR). This group contains intact, oxy- and nitro-PAHs. It was found that while several of the compounds were directly toxic in the short-term assay, the impacts were not altered by SSR. Conversely, the long-term assay was useful for evaluating both direct and photoinduced toxicity of PAHs.

**MATERIALS AND METHODS**

**Bacterial Strain and Growth Conditions**

The strain of luminescent bacteria used for these experiments was Vibrio fischeri NRRL B-11177 (formerly known as Photobacterium phosphoreum). It was obtained from the Midwest Area National Center for Agricultural Utilization Research, Peoria, Illinois. Aseptic techniques were employed throughout. Vibrio fischeri were grown in a complex liquid medium that contained the following: KH₂PO₄ (18.4 mM), NaCl (0.5 M), MgSO₄·7H₂O (4.1 mM), glycerol (54.3 mM), yeast extract (1 mg L⁻¹), peptone (5 mg L⁻¹), and Bactopeptamin (1 mg L⁻¹). When solid medium was made, agar was used at 1.5% (w/v). These components were added to reverse osmosis-purified water (RO water) and brought to a volume of 1 L. After thorough mixing, the final pH of the medium was adjusted to 7.2 ± 0.1 with 10 M NaOH. The medium was distributed into 250-mL flasks (100 mL per flask), covered with cotton plugs, and autoclaved at 121°C for 30 min. Peptone and yeast extract were obtained from BDH Inc., Toronto, Ontario, and Bactopeptamin was obtained from Difco Laboratories, Detroit, Michigan. Stock cultures of V. fischeri were maintained on agar plates and used for primary inoculation of liquid cultures.

**Bacterial Growth and Luminescence Emission at Room Temperature**

Cells of V. fischeri were cultured in 100 mL of complex medium in 250-mL flasks at room temperature (20°C ± 1) with shaking for 20 h. Cultures were harvested when they reached log-phase growth as determined by absorbance at 650 nm using a spectrophotometer (Perkin-Elmer, Mississauga, Ontario, Canada). At this time, luminescence intensity was optimal for toxicity testing.

A 20-mL aliquot of the log-phase cells was diluted with complex medium to yield an absorbance of 0.40 at 650 nm. Several 500-μL aliquots from the diluted liquid culture were mixed with 500-μL aliquots of complex medium in the wells of a 48-well cell culture plate (Costar Safety Products, VWR Scientific Ltd, Toronto, Ontario, Canada). They were grown in the dark or SSR with shaking at room temperature for up to 48 h. SSR was generated with an artificial lighting system that mimics the spectral quality of natural sunlight (Fig. 1) (Huang et al., 1993; Greenberg et al., 1995). The SSR source contained eight daylight fluorescent lamps, two 350-nm UV lamps, and four 300-UV lamps (UV lamps were obtained...
FIG. 1. Spectral distribution of sunlight and a simulated solar radiation (SSR) source. (Top) Spectral output of natural sunlight at noon on a cloudless summer day in Waterloo, Ontario, Canada (43°N). (Bottom) Emission spectrum of an SSR source used in the toxicity tests with *V. fischeri*. The spectrum provided is from a source with a total fluence rate of 40 μmol m⁻² s⁻¹. Both spectra were collected with a photodiode array spectroradiometer.

from the Southern New England Ultraviolet Co., Branford Ontario, Canada). The light was filtered through two layers of cheesecloth and the polystyrene culture plate top. The latter absorbed all of UV-C (200–290 nm) in the light source. The fluence rate and spectral quality of the SSR source were measured using a photodiode array spectroradiometer (Oriel Instruments, Stratford, CT). The spectral output of the SSR source had a visible light:UV-A:UV-B ratio of 100:10:1 based on photon fluence rate, and the total fluence rate was 40 μmol m⁻² s⁻¹ (Fig. 1).

**Short-Term and Long-Term Toxicity Assays**

The short-term toxicity tests were based on inhibition of light production by the bacteria. The long-term toxicity tests were based on inhibition of both light production and growth of the bacteria. Cell densities of the cultures were measured with a spectrophotometer (Perkin-Elmer, Mississauga, Ontario, Canada) at 650 nm to exclude interference from the luminescence. The luminescence intensity of the *V. fischeri* was measured in a Cytofluor 2350 automated multiwell plate fluorescence measurement system (Millipore Ltd., Mississauga, Ontario, Canada) with an excitation light source switched off to eliminate any background fluorescence.

**Short-term assay.** Prior to toxicity testing, cultures of *V. fischeri* were grown at 20°C ± 1 for 20 h. Cultures were harvested when they reached a log phase and diluted to an absorbance of 0.4 as described above. Twenty-four 500-μL aliquots of cells were placed into the wells of a 48-well cell culture plate (Costar Safety Products, VWR Scientific Ltd, Toronto, Ontario, Canada). After 5 min acclimation in the 48-well cell culture plates, luminescence was measured. After the initial measurement of bacterial luminescence, the twenty-four 500-μL aliquots of bacteria were dosed with equal volumes of complex medium containing the test chemicals at twice the concentration required for the toxicity testing. The PAHs used in this study were purchased in high purity from Sigma Chemical Company (St. Louis, MO). Their structures are given in Fig. 2. A dilution series of each chemical plus a control were added to the wells in triplicate, providing three replicates of each concentration per individual assay. Seven concentrations of the PAHs in a geometric series were used (nominal concentrations from 0.065 to 4 mg/L). For toxicity testing of each chemical, stock solutions were prepared in dimethyl sulfoxide (DMSO) and diluted into culture medium to give the desired concentration. Because the aqueous solubilities of PAHs are low, it was necessary to employ a carrier solvent so that concentrations high enough for full dose–response curves could be achieved. Via this method, all PAHs (except ANT, AAQ, ATQ, and PYR) were soluble in the growth medium at the highest concentration tested. In the cases of ANT, AAQ, ATQ, and PYR, they form emulsified solutions above their solubility and have been shown to be bioavailable in this form (Huang et al., 1993). Note, benzo[a]pyrene is even less water soluble that PYR, and was therefore not used in this study. The concentration of DMSO was ≤ 0.1% (v/v) in each of the final mixtures. The carrier itself was not toxic at the concentrations used (McConkey et al., 1997). After dosing, the cells were incubated in darkness or SSR at room temperature for 15 min and the luminescence intensity was remeasured with the Cytofluor 2350 fluorescence measurement system (Millipore Ltd., Mississauga, Ontario, Canada).

**Long-term assay.** The long-term toxicity assay was simply a continuation of the short-term assay. After the above luminescence measurement, the 48-well culture plate was
FIG. 2. Structures of the 12 PAHs used in this study and their abbreviations.

Naphthalene NAP

Acenaphthene ACE

Acenaphthylene ACY

5-Nitroacenaphthene 5-NACE

Acenanthrenequinone AAQ

Acenanthrenequinone ACQ

Pyrene PYR

Phenanthrene PHE

Anthracene ANT

Phenanthrenequinone PHQ

Anthraquinone ATQ

1,2-dihydroxyanthraquinone 1,2-dhATQ

% light inhibition = 100/(1 + e^{-β(lnx - lnμ)}), \hspace{1cm} (2)

where x is the concentration of the test chemical, μ is the EC_{50}, and β is a measure of the slope of the concentration–response curve. Inhibition of growth was calculated for each chemical concentration as follows. Growth was determined as cell density according to

CD_t = CD_0 \times 2^n, \hspace{1cm} (3)

where CD_0 and CD_t are the cell density in absorbance units at time (zero) and time t (in hours) and n is the number of times the cells have doubled. Thus, n is given by

n = \log(CD_t/CD_0)/\log 2. \hspace{1cm} (4)

The growth rate (GR) is the number of cell doublings per hour given by

GR = n/t, \hspace{1cm} (5)

where t is the length of the assay in hours. Inhibition of growth was calculated for each concentration as follows:

% inhibition of GR = 100(1 - (GR(treated)/(GR(control)))). \hspace{1cm} (6)

A graph of GR versus concentration was plotted, and the concentration causing 50% inhibition of growth rate was determined by fitting Eq. (2) to the data as above.

RESULTS

Bacterial Growth and Luminescence Emission

The optimum conditions for growth of the V. fischeri were determined prior to the toxicity assays. The growth and luminescence of V. fischeri were determined in complex medium in darkness and SSR as a function of time (Fig. 3). When V. fischeri cells in log-phase growth are diluted into fresh medium, log-phase growth continues for about 30 h, at which points the cells reach stationary phase. In log-phase growth the doubling time was approximately 120 min, which is a good rate of growth for V. fischeri (Takahide and Nakamura, 1980). There was essentially no difference in V. fischeri growth in darkness or SSR (Fig. 3).
FIG. 3. Cell density and luminescence emission of \textit{V. fischeri} as a function of time. Cells were grown in a 48-well cell culture plate in darkness and SSR at room temperature for 48 h. Cells were grown in complex medium. Luminescence and cell density were determined as described under Materials and Methods.

Bacterial luminescence remained approximately constant for 8 h after the cells were diluted into fresh medium (Fig. 3). Following that luminescence rose with cell growth until it reached a peak at about 30 h. Luminescence then dropped as the cells went into stationary phase. Again there was no difference between SSR and darkness. From these data, it was concluded that a long-term assay of toxicity with \textit{V. fischeri} should last 15 to 20 h. In this way, both cell density and luminescence can be used as end points.

Short-Term Toxicity

\textit{Vibrio fischeri} were incubated in darkness and SSR with each of the 12 PAHs to determine their relative toxicity. The compounds tested were ACE, ACY, AAQ, ACQ, 5-NACE, PHE, ANT, ANQ, NAP, 1,2-dhANQ, PHQ, and PYR. The results of short-term toxicity of PAHs to \textit{V. fischeri} are given in Fig. 4 and Table 1. Full concentration–response curves are shown for the short-term toxicity of ACE, ACQ, ACY, and 5-NACE (Fig. 4). All four compounds caused increasing inhibition of luminescence with increasing chemical concentration. Effects increased approximately linearly with chemical concentration once the lowest observable effect concentration (LOEC) was reached for the four chemicals.

FIG. 4. Dose response of \textit{V. fischeri} to ACE, ACY, ACQ, and 5-NACE. \textit{Vibrio fischeri} were exposed to a concentration series of these PAHs for 15 min (short-term toxicity) in either SSR or darkness. Bacterial response was measured as percentage inhibition of luminescence and plotted versus chemical concentration (mg L$^{-1}$). Error bars are 95% confidence intervals.
### TABLE 1

Calculated EC$_{50}$ Values for Short-Term Toxicity of Selected PAHs to *Vibrio fischeri*

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>Abbreviation</th>
<th>Dark</th>
<th>SSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aceanthrenequinone</td>
<td>AAQ</td>
<td>24.39</td>
<td>23.70</td>
</tr>
<tr>
<td>Aceanthrene</td>
<td>ACE</td>
<td>2.41</td>
<td>2.52</td>
</tr>
<tr>
<td>Aceanaphthene</td>
<td>ACY</td>
<td>0.80</td>
<td>0.77</td>
</tr>
<tr>
<td>Aceanaphthenequinone</td>
<td>ACQ</td>
<td>2.67</td>
<td>2.65</td>
</tr>
<tr>
<td>Anthracene</td>
<td>ANT</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>Anthraquinone</td>
<td>ANQ</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>1,2-Dihydroxyanthraquinone</td>
<td>1,2-dhANQ</td>
<td>7.11</td>
<td>7.24</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>NAP</td>
<td>2.36</td>
<td>2.33</td>
</tr>
<tr>
<td>5-Nitroacenaphthene</td>
<td>5-NACE</td>
<td>2.29</td>
<td>2.16</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>PHE</td>
<td>7.33</td>
<td>6.89</td>
</tr>
<tr>
<td>Phenanthrenequinone</td>
<td>PHQ</td>
<td>0.92</td>
<td>0.81</td>
</tr>
<tr>
<td>Pyrene</td>
<td>PYR</td>
<td>N/D</td>
<td>N/D</td>
</tr>
</tbody>
</table>

*The assays were carried out under darkness or SSR. EC$_{50}$ values are based on percentage inhibition of luminescence after a 15-min incubation. Each experiment is the average of nine replicates and errors are the 95% confidence intervals.

N/D, no detectable effect at the highest concentration.

ACY was the most toxic to *V. fischeri*. There was no apparent difference in toxicity in SSR and darkness for any of the four chemicals.

Using concentration–response tests similar to those shown in Fig. 4, the EC$_{50}$ values were determined for all 12 chemicals (Table 1). Three of the compounds assayed (ANT, ANQ, and PYR) were nontoxic to *V. fischeri* at the maximum concentration tested. ACY and PHQ were found to be the most toxic in darkness and SSR. AAQ was the least toxic of the compounds that showed a measurable effect. Interestingly, in both PHQ and 1,2-dhANQ were more toxic than their parent PAHs (Table 1).

To determine if the toxicity of the 12 PAHs could be enhanced by light, *V. fischeri* were incubated in SSR with chemicals (Fig. 3, Table 1). The calculated EC$_{50}$ values for each chemical were not statistically different in darkness or SSR (Table 1). It is possible that the assay was not long enough for photomodification and/or photosensitization reactions to be effective.

**Long-Term Toxicity**

Because none of the PAHs were phototoxic in the short-term assay, the long-term assay was employed to test for photoinduced toxicity of PAHs to bacteria. *V. fischeri* were incubated in darkness and SSR for 18 h to determine the relative toxic potency of each chemical (Fig. 5, Table 2). The incubation was carried out in complex medium to allow bacterial growth. Two toxicity endpoints were employed. The first was inhibition of luminescence, and the second was inhibition of growth. Both endpoints gave similar results for each of the chemicals tested (Fig. 5, Table 2).

Full concentration–response curves for ACE, ACQ, ACY, and 5-NACE in darkness and SSR were determined for the long-term assay (Fig. 5). ACE and 5-NACE showed no toxicity to *V. fischeri* in darkness; however, both were toxic in SSR. In fact, 5-NACE became the most toxic of the four compounds in SSR. ACQ shows the same level of toxicity to *V. fischeri* in darkness and SSR. ACY was toxic to *V. fischeri* in darkness, and its toxicity was enhanced by exposure to SSR.

By use of concentration–response experiments, the EC$_{50}$ values of luminescence and growth were determined for all 12 chemicals in this study (Table 2). AAQ, ANT, ANQ, NAP, and PYR were not toxic to *V. fischeri* in darkness and SSR. The most toxic compound was PHQ. However, its toxicity was not enhanced by SSR. Two other compounds did not have lower EC$_{50}$ values in SSR compared with darkness (ACQ and 1,2-dhATQ). However, four compounds showed enhanced toxicity in SSR versus darkness: ACE, ACY, 5-NACE, and PHE.

In comparing the short-term and long-term toxicity test results two important conclusions emerge. First, the levels...
of toxicity of the compounds are quite different in the two assays. For instance ACE and ACY are less toxic in the long-term assay versus the short-term assay. Conversely for several compounds, the EC<sub>50</sub> values were similar in both assays (e.g., ACQ, 1,2-dhAQ, and PHQ). Second, photoinduced toxicity is evident only in the long-term assay.

### DISCUSSION

#### Growth and Luminescence Emission

The manners in which <i>V. fischeri</i> are cultured and the purpose of culture media vary widely. The medium used here was modified from other published media. The medium formulated here promotes strong growth and results in a high yield of luminescence. In previous studies, maximum luminescence was obtained when <i>V. fischeri</i> were grown in media containing glycerol as one of the carbon sources (Hill and Shoup, 1929; Kemper and Hanson, 1968; Takahide and Nakamura, 1980). As well, growth at pH 7.2 is optimal for luminescence from this bacterium (Johnson et al., 1942; Kemper and Hanson, 1968). The best temperatures for growth and luminescence of <i>V. fischeri</i> have been found to be between 20 and 25°C (Harvey, 1952; Kemper and Hanson, 1968; Takahide and Nakamura, 1980). Bacterial bio-luminescence is dependent on active respiration and, thus, requires oxygen. Vigorous shaking of cultures grown in flasks has been found to be adequate to achieve maximum luminescence (Takahide and Nakamura, 1980). Therefore, the <i>V. fischeri</i> in this study were cultured in a medium containing glycerol, yeast extract, peptone, and Bactopeptamin at pH 7.2 ± 0.1. Growth was at room temperature (20°C) with vigorous shaking. This achieved rapid growth and strong luminescence emission. It was found that after 15 to 20 h, the cells were in mid-log-phase growth and luminescence increased in parallel with growth. Therefore, the cells were grown for 18 h in the long-term toxicity assay.

#### Short-Term Toxicity

The calculated short-term toxicity for all PAHs showed the chemicals had the same impact on <i>V. fischeri</i> in the dark and SSR. The calculated EC<sub>50</sub> values of ACQ, ACE, ACY, NAP, PHE, and PHEQ were compared with previously published toxicity data (Jacobs et al., 1993; Kaiser et al., 1994; McConkey et al., 1997) (Fig. 6). When one compares the EC<sub>50</sub> values reported here with the above published data, one observes similar levels of toxicity (Fig. 6). A good example is PHQ where the toxicity to <i>V. fischeri</i> in the short-term assay agrees well with that published by McConkey et al. (1997). However, the EC<sub>50</sub> values reported here were consistently higher than those in the literature. For instance the calculated EC<sub>50</sub> of PHE is 7.33 mg/L, which is higher than that obtained by McConkey et al. (1997) (0.53 mg/L). Thus, the data from this study do not correlate strongly with other published data (Fig. 6). This is probably due to sugar and other forms of reduced carbon in the complex medium used here. They would probably bind to the PAHs, restricting their bioavailability. This is one reason DOC is thought to ameliorate PAH toxicity (Gensemer et al., 1996). In contrast, the Microtox assay is performed in a saline solution, allowing greater bioavailability of the chemicals. Thus, if a toxicity test is more relevant to

### TABLE 2

Calculated EC<sub>50</sub> Values for Long-Term Toxicity of PAHs to <i>Vibrio fischeri</i>

<table>
<thead>
<tr>
<th>Toxicant Abbreviation</th>
<th>Inhibition of luminescence</th>
<th>Inhibition of growth</th>
</tr>
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<tbody>
<tr>
<td>Dark</td>
<td>SSR</td>
<td>Dark</td>
</tr>
<tr>
<td>Acetanthenequinone</td>
<td>AAQ 0.11</td>
<td>0.62</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>ACE N/D</td>
<td>5.21 ± 0.26</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>ACY 6.57 ± 0.42</td>
<td>1.47 ± 0.24</td>
</tr>
<tr>
<td>Acenaphthenequinone</td>
<td>ACQ 1.88 ± 0.11</td>
<td>1.52 ± 0.18</td>
</tr>
<tr>
<td>Anthracene</td>
<td>ANT 0.42</td>
<td>1.47 ± 0.18</td>
</tr>
<tr>
<td>Anthraquinone</td>
<td>ANQ N/D</td>
<td>0.62 ± 0.19</td>
</tr>
<tr>
<td>1,2-Dihydroxyanthraquinone</td>
<td>1,2-dhANQ 5.80 ± 0.30</td>
<td>5.61 ± 0.20</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>NAP N/D</td>
<td>0.62 ± 0.19</td>
</tr>
<tr>
<td>5-Nitroacenaphthene</td>
<td>5-NACE N/D</td>
<td>0.62 ± 0.19</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>PHE 8.09 ± 0.33</td>
<td>7.15 ± 0.37</td>
</tr>
<tr>
<td>Phenanthrenequinone</td>
<td>PHQ 0.66 ± 0.11</td>
<td>0.69 ± 0.10</td>
</tr>
<tr>
<td>Pyrene</td>
<td>PYR N/D</td>
<td>0.62 ± 0.09</td>
</tr>
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*Bacteria were grown under SSR and darkness for 18 h. Inhibition of luminescence and inhibition of growth were determined for each chemical from which the EC<sub>50</sub> values were derived. All data are averages of nine replicates. The 95% confidence intervals are provided.

N/D, no detectable effect at the highest concentration tested.
autotrophic conditions, a minimal medium or saline solution may be more relevant. However, if the study is more relevant to oligotrophic or eutrophic conditions, a complex medium may be more relevant.

In the short-term assay none of the PAHs tested exhibited a large increase in toxicity in SSR compared with darkness. This indicates that photostimulation of PAHs was not a factor in these assays. This could be due to the short exposure to SSR. In 15 min there would be little or no photodestruction of PAHs. Also, there would be little time for cumulative damage from reactive oxygen species. That is, via photosensitization processes PAHs promote production of reactive oxygen species such as singlet O$_2$ and superoxide (Girotti, 1983; Krylov et al., 1997). This should be toxic to the bacteria via membrane, protein, and DNA damage. However, it may take more than 15 min for enough biological damage to accumulate to impede bacterial luminescence. The observed toxicity could thus be attributed solely to direct cytotoxicity of the compounds, and not to any photochemical processes that might arise from PAH exposure. This reveals an important limitation of short-term bacterial assays in detecting the effects of chemicals such as PAHs that may require an activation step.

**Long-Term Toxicity**

To examine the photoinduced toxicity of the PAHs, a longer-term assay of toxicity was attempted. Two endpoints of toxicity were used: growth and luminescence. This might help to assess if the chemicals had an impact on the luminescence mechanism (i.e., *V. fischeri* specific), or if the toxicity is more general in nature by affecting growth more than luminescence. It is striking that the EC$_{50}$ values were nearly the same for luminescence and growth, indicating the presence of broad effects on the bacteria from PAHs and modified PAHs.

Of the 12 chemicals tested, about half showed a toxic effect. Five of the chemicals, AAQ, ANT, ATQ, NAP, and PYR, showed no toxicity under darkness. ACE and 5-NACE were not toxic under darkness, but had toxicity under SSR. In addition the EC$_{50}$ for ACY dropped about fivefold in SSR relative to darkness to SSR. In the long-term assay there would have been time for photodestruction to occur and for photosensitized damage to accumulate. This showed that photochemical toxicity of PAHs can be observed with the long-term assay. Furthermore, of the four toxic chemicals that did not show an increase in toxicity in SSR, three were quinones (i.e., photooxidized PAHs). It is known that oxyPAHs do not require light to reveal toxicity (McConkey et al., 1997; Mallakin et al., 1998). The toxicity of PAHs is known to be greatly enhanced on exposure to sunlight or SSR (Huang et al., 1993; McConkey et al., 1997; Ren et al., 1994; Mallakin et al., 1998). Thus, it was somewhat surprising that two of the PAHs tested (ANT and PYR), both of which known to be phototoxic, did not exhibit toxicity under SSR in the long-term assay. It has been shown previously that many intact PAHs are rapidly photomodified to a mixture of photoproducts that are more toxic than the intact PAHs (Huang et al., 1993; McConkey et al., 1997; Ren et al., 1994; Mallakin et al., 1998). One possibility why ANT and PYR were not toxic is that the photoproducts did not have enough time to form in an 18-h assay. It is also possible that their photooxidation would be impeded in complex medium. This is because the reduced carbon compounds in the medium could act as antioxidants. Under such a scenario, early in exposure before photoinduced toxicity becomes a factor, the bacteria could outcompete the chemical by growth, and effectively dilute the chemical. Further, if the chemicals are not bioavailable (i.e., the sugar in the medium might lower bioavailability), the bacterium might grow faster than the rate of assimilation. In this regard, it is interesting to note that ANT and PYR are the least water-soluble compounds we tested. Another possibility is that the sugar in the medium quenches PAH excited states, thus preventing photooxidation of the chemicals. The other compounds found to be nontoxic in the long-term assay were AAQ, ATQ, and NAP. NAP is generally not a highly toxic compound in aqueous assays, probably due to its high volatility (Ren et al., 1994). In the cases of AAQ and ANQ, these PAH quinones are highly insoluble (Edwards, 1983). Thus, bacterial growth probably outpaces assimilation of these compounds.

It should be noted that ANT and ATQ have been found to be very phototoxic PAHs in the other systems (Huang et al., 1998).
et al., 1996). Perhaps their phototoxic potential would be realized if assimilation of the chemicals were allowed to occur. This might be achieved if there was a preincubation period in minimal medium. Therefore, modifications of our procedures have been investigated so that a wider range of photochemical effects can be observed (El-Alawi et al., 2001). It would also be useful to adapt the \textit{V. fischeri} assays for use in natural sunlight, to allow toxicity testing under fluence rates much higher than those that can be achieved with SSR. Indeed, a suit of assays for \textit{V. fischeri} would be valuable to allow an investigator to customize the assay to the properties of the test material and the question being asked.

CONCLUSIONS

Based on our work with the luminescent bacteria, a simple, rapid, reliable, and sensitive method for monitoring the toxicity of aquatic chemicals has been developed. The assays allowed the assessment of the toxicity of photomodified PAHs and intact PAHs. Importantly, using both assays, we are able to compare the contributions of direct effects and photoinduced effects to toxicity. Toxicity was greatly enhanced by light with the long-term assay. However, it was striking that the highly phototoxic ANT was not toxic in SSR in this study. It is possible that the low toxicity of some PAHs observed in this study can be attributed to two factors: first, the low bioavailability of PAHs to the bacteria; second, rapid growth of the bacteria, which can outstrip the effects of the chemicals.

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REFERENCES


