SHORT- AND LONG-TERM PHOTOINDUCED TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS TO LUMINESCENT BACTERIA

by

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ABSTRACT

Toxicity of most polycyclic aromatic hydrocarbons (PAHs) to aquatic organisms can be greatly enhanced upon exposure of the target organism and/or the chemicals to the ultraviolet (UV) radiation present in sunlight. There are two major mechanisms involved in the 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. In an effort to examine the photomodification and photosensitization processes of PAHs, microbial toxicity assays were developed to measure short- and long-term photoinduced toxicity. The test organism was the luminescent bacterium *Vibrio fischeri* (strain NRRL B-11177). Two physiological characteristics of this test organism that make it attractive for toxicity testing are a short division cycle and an inducible luciferase pathway. The bioassay methods were based on inhibition of luminescence and growth of *Vibrio fischeri*. The short-term assay developed was based on inhibition of luminescence after a 15 minute incubation with a test chemical. The long-term assay involved returning the cells to the incubator after the short-term endpoint was measured and growing them for 18 hours with the test chemical. The sensitivities of the assays were found to correlate well with other bioassays and they were effective at screening a large number of compounds. Both assays could be preformed in darkness or simulated solar radiation (SSR) to examine the effects of light on PAH toxicity. The short-term and long-term assays were tested with representative intact PAHs and modified PAHs. With the short-term assay the toxicity of all the chemicals was the same in SSR or darkness. This means photoinduced toxicity is not apparent in a short-term exposure. However, with the long-term assay, SSR did enhance PAH toxicity. Thus, photoinduced toxicity could be observed under appropriate conditions. Strikingly, ANT, one of the most phototoxic PAHs, was not toxic in SSR or darkness. It was thought that the reduced carbon in the medium might be limiting ANT bioavailability. The long-term assay was thus modified by incorporating an 8 hour pre-incubation period in minimal medium. This was found to be effective, as the photoinduced toxicity of ANT and other PAHs was readily observed if a pre-incubation in minimal medium was employed. Having developed the *V.*

fischeri assay, a quantitative structure-activity relationship (QSAR) previously developed for the aquatic plant *Lemna gibba* was applied to the bacteria. Summing two factors, one for photosensitization and one for photomodification resulted in predictive values that showed strong correlation to the *V. fischeri* toxicity data. Thus, a QSAR model derived for plants accurately described the toxicity of PAHs to a bacterial species. This indicates that the bipartite mechanism of PAH photoinduced toxicity is broadly applicable. The *V. fischeri* short- and long-term assays were finally applied to assessment of PAH-contaminated sediments. The sediments were collected from Hamilton Harbor, ON, and Mohawk Lake, Brantford, ON. They were fractionated and found to contain PAHs and oxyPAHs. Strikingly, the oxyPAH fractions were observed to be the most toxic samples. Thus, oxyPAHs in the environment have a hazard potential.

ACKNOWLEDGMENTS

No words can adequately express my gratitude, respect and appreciation to my supervisor Prof. Bruce M. Greenberg. I am very grateful for your imperturbable support and enthusiasm throughout my study. Sir your generosity and understanding of my eastern background are invaluable commodities. You always provided me with great ideas to help me to solve any problems with my research, and taught me the value of a relaxed and open laboratory atmosphere for bringing out the full potential of each member in a research group. You provided me with many memorable milestones and continue to be a great inspiration, both personally and professionally. I will always remember your words of wisdom to me: "Think...Think ". I am also very grateful and would like to give special thanks to Profs. Niels Bols and George Dixon for serving on my committee, and for their guidance and encouragement throughout my academic career at the University of Waterloo.

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DEDICATION

I would like to dedicate this thesis to my wife, children, parents, and the rest of my family in appreciation for all their support and encouragement.

Y. EL-ALAWI

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LIST OF ABBREVIATIONS USED IN THIS THESIS

ANT	anthracene
ACE	acenaphthene
ACY	acenaphthylene
BBF	benzo(b)fluorene
BAA	benzo(a)anthracene
BBA	benzo(b)anthracene
BAP	benzo(a)pyrene
BEP	benzo(e)pyrene
BGP	benzo(g,h,i)perylene
CHR	chrysene
COR	coronene
DAA	dibenzo(a,h)anthracene
DAP	dibenzo(a,i)pyrene
DMSO	dimethyl sulphoxide
EC50	concentration at which 50% of population was affected
ESS	excited singlet state
FLA	fluoranthene
FLU	fluorene
GSS	ground singlet state
$^1O_2$	singlet state oxygen
3Cs	triplet state PAH in solution
$^3O_2$	ground (triplet) state oxygen
PHE	phenanthrene
PHQ	phenanthrenequinone
NAP	naphthalene
PYR	pyrene
TRI	triphenylene
PAHs	polycyclic Aromatic Hydrocarbons
SSR	simulate Solar Radiation

UV	ultraviolet
UV_A	ultraviolet at A region [320-400 nm]
UV_B	ultraviolet at B region [280-320 nm]
UV_C	ultraviolet at C region [200-280 nm]
h	hour
$h\nu$	photon
QSAR(s)	quantitative structure activity relationship(s)
ppb	part per billion
ppm	Part per million
min	minute
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
nm	nanometer
PSF	photosensitization factor
PMF	photomodification factor

CHAPTER 1

GENERAL INTRODUCTION

For several decades there has been growing concern about pollution of the environment by xenobiotic chemicals (National Research Council, 1981; Ballantyn *et al.*, 1995). Chemical contaminants may result from industrial effluents, consumer or commercial products, mine wastes, petroleum products and natural gas (Truhaut, 1977; Ballantyn *et al.*, 1995). Some chemicals, such as pesticides, may be specifically designed to alter the assembly of biota present in natural or agricultural ecosystems. Xenobiotics may be organic, inorganic, metallic, or radioactive in nature (National Research Council, 1981; Moriarity, 1988; Manahan, 1994). Many are present naturally, but usually at much lower concentrations than have resulted from human activity. Many of these chemicals cause negative effects in biota, including humans, if applied in sufficiently high concentration. They may therefore be designated as toxic substances (Zakrzewski, 1991; Ballantyn *et al.*, 1995; Moriarity, 1988; Manahan, 1994).

Toxicology deals with the adverse effects of chemical agents on biological systems. A more encompassing definition may relate to the occurrence, physical and chemical properties, effects, and detection of xenobiotics (Moriarity, 1988; Manahan, 1994). Toxicology has three branches; clinical, forensic (legal), and environmental as described by Zakrzewski (1991). However, Ballantyne *et al.* (1995) suggested eight more branches in toxicology including veterinary, occupational and regulatory. Based on these definitions, environmental toxicology, is concerned with the nature, properties, effects, and detection of toxic substances in the environmental and in any environmentally exposed species (Zakrzewski, 1991; Ballantyn *et al.*, 1995). In general there are two main groups of toxicity screening tests in environment toxicology: health effects tests and ecological effects tests. Health effects toxicity tests are based on the use of sub-cellular components (e.g., enzymes, DNA, RNA), isolated cells (e.g., cell cultures, red blood cells), tissue sections, or whole organisms. These tests consist of the determination of organismal growth, cell viability (vital staining, plating efficiency, colony formation), cell reproduction, physiological integrity or macromolecular biosynthesis (Malcolm and

Pringle, 1975; Christian *et al.*, 1977). Ecological effect tests are conducted to measure the toxicity of chemicals to organisms representing various trophic levels of the food chain in natural and man-made ecosystems. In aquatic systems these organisms include bacteria, algae, zooplankton, protozoa, macrophytes, benthic invertebrates, fish, and amphibians (Patrick, 1973; Becking, 1979).

Ecotoxicology, which is environmental toxicology from an ecological perspective, is concerned primarily with the release of toxic substances into the environment, and their fate and distribution in the food chain. Qualitative and quantitative measurements of toxic responses in ecosystems and ecosystem components are especially important (Truhaut, 1977; Moriarity, 1988; Moriarity; Manahan, 1994). Ecotoxicology deals in an integrated way with microorganisms, plants, and animals, in relation to their environment and ecosystems (Truhaut, 1977; National Research Council, 1981; Moriarity, 1988). An ecosystem represents a community of organisms interacting with each other and their environment. An ecosystem can receive numerous manmade chemicals from industrial waste, domestic sewage, deposition from the atmosphere, and runoff from roads, parking lots, and farms. These contaminants in an ecosystem can cause harm to the environment and loss of habitat (Truhaut, 1977, National Research Council, 1981; Moriarity, 1988; Zakrzewski, 1991; Manahan, 1994).

Many environmental factors can enhance the toxicity of contaminants. A key factor is light. Phototoxicity, which can be induced by UV or visible radiation, plays major role in the environment (Oris and Giesy, 1986; Newsted and Giesy, 1987; Huang *et al.*, 1992; Huang *et al.*, 1993; Ren *et al.*, 1994). Light is involved in a large number of reactions in the atmosphere, natural waters, soil and living organisms (Tchan *et al.*, 1975). These reactions contribute to the synthesis and degradation of many organic substances (Tchan *et al.*, 1975; Wild *et al.*, 1991). Photochemical reactions are a way of eliminating some organic substances in the atmosphere, and they can play a significant role in the degradation of slightly biodegradable compounds in aquatic systems (Kochevar, 1982; Mueller *et al.*, 1989). Special attention is focused on the fate of organic pollutants such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls and pesticides, which are distributed in the environment. Many of these compounds are subject to photochemical modification and degradation (Dahl, 1992). The reactions

involved in photochemical transformations may result from direct excitation of substances absorbing sunlight. They may also be induced by various UV-absorbing species present in the environment, which turn in produce reactive intermediates such as hydrated electrons, singlet oxygen, superoxide, and hydroxy radicals (Larson and Berenbaum, 1988). In some cases, photosensitization by energy transfer may occur (Leifer, 1988). The objective of this thesis is to improve our ability to measure the effects of photoenhanced toxicity of PAHs. The luminescent bacteria, *Vibrio fischeri*, were used in short-term and long-term toxicity assays to carry out this research.

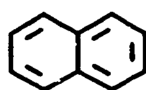
1.1 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) have been studied for well over a century. They are a large and structurally diverse class of organic molecules (NRC, 1983; Eisler, 1987; Harvey, 1997). PAHs constitute an important class of mutagenic and carcinogenic environmental contaminants that are produced during the processing of steel and the combustion of coal, gasoline, and diesel fuels (Gibson and Subramanian, 1984). PAHs consist of two or more fused carbons rings, which can have substituted groups attached to the rings (Eisler, 1987). The structures of PAHs range from naphthalene ($C_{10}H_8$, two rings), to coronene ($C_{24}H_{12}$, seven rings), (Figure 1.1). PAHs can be modified to oxygen and nitrogen-containing compounds (Figure 1.1) (Neff, 1979; Varanasi, 1989)

PAHs are relatively inert and nearly insoluble in aqueous solution (Eisler, 1987). They are thermodynamically stable due to the resonance energy of their extensive π-orbital systems. Nonetheless, in living cells these molecules can be metabolized to a variety of oxygenated products which are more water-soluble than the parent hydrophobic aromatic compounds (Jones and Leber, 1979; Cook *et al.*, 1983; Cooke and Dennis, 1983). These metabolites are more easily excreted by the organism (Geacintov, 1988; Harvey, 1997). However, they also become more toxic and mutagenic after oxidation (Payne *et al.*, 1987; Livingstone *et al.*, 1989; Timbrell 1991).

Because PAHs have low aqueous solubility they tend to be associated with particle surfaces in the environment (Environment Canada, 1994). This binding can

Figure 1.1 Structures and common names of polycyclic aromatic hydrocarbons. Included are the intact and oxygenated PAHs used in this study.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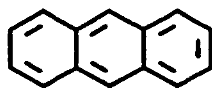
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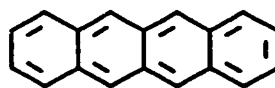
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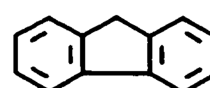
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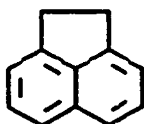
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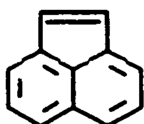
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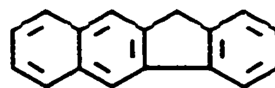
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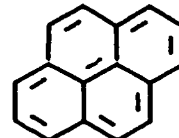
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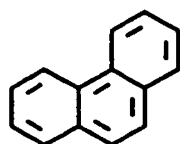
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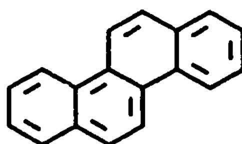
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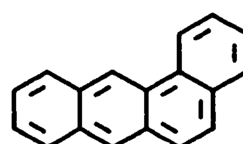
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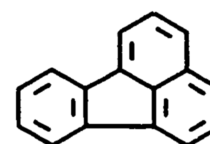
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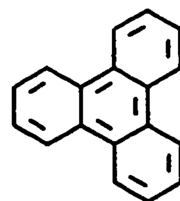
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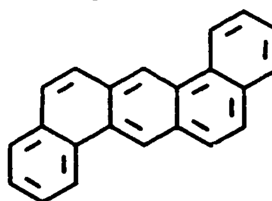
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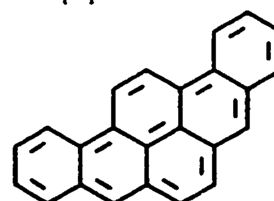
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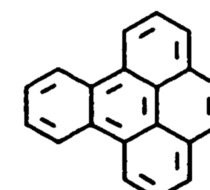
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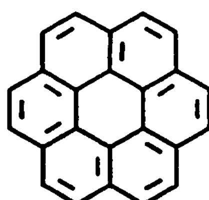
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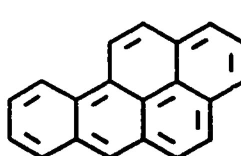
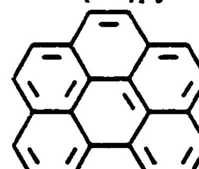

Benzo[a]pyrene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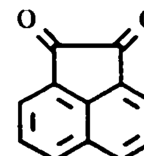
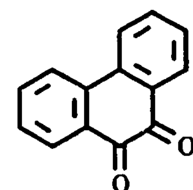
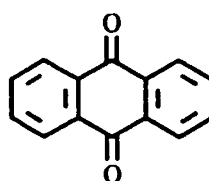


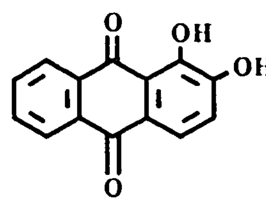
Acenaphthenequinone



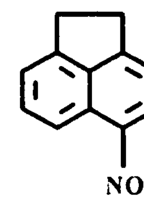
Phenanthrenequinone



Anthraquinone



1,2-dihydroxyanthraquinone



5-Nitroacenaphthene

interfere with volatilization, photolysis, and biodegradation (Cooke and Dennis 1983; Eisler, 1987). As a result, they are generally persistent in many aquatic environments. Their persistence, coupled with their potential carcinogenicity and toxicity, makes PAHs problematic and hazardous environmental contaminants (Eisler, 1987; Environment Canada, 1994). Because PAHs are hydrophobic, not only do they adsorb to particulate matter in water and sediments, but they are also readily assimilated into biological membranes (Environment Canada, 1994). Many PAHs are both cytotoxic and mutagenic. The mutagenicity results from the biotic or abiotic oxidation of PAHs to reactive electrophilic compounds that bind covalently to nucleophilic groups in DNA and proteins (Eisler, 1987).

1.2 Environmental sources of PAHs

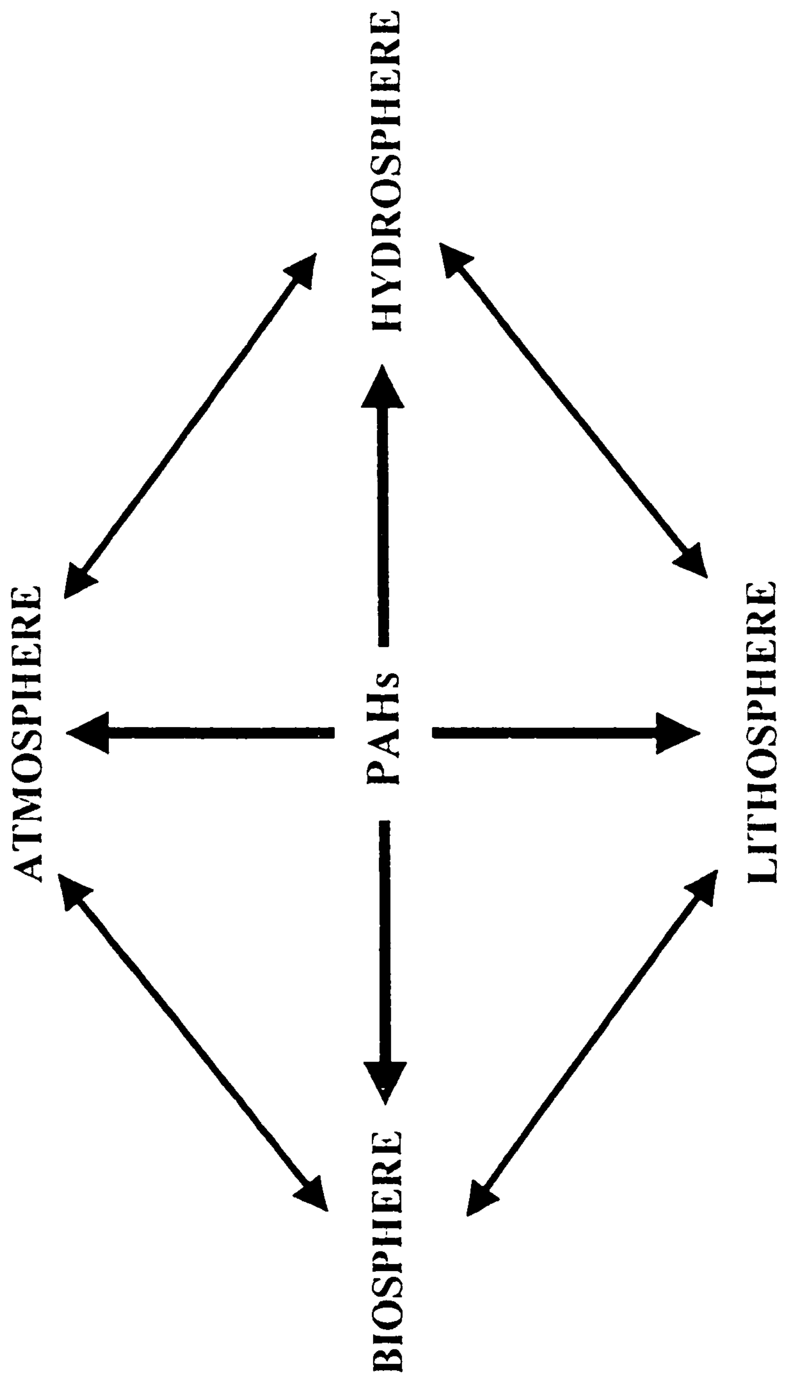
PAHs are released into the environment from biosynthetic, geochemical, and anthropogenic sources (Neff, 1979; Lee *et al.*, 1981; Cook and Dennis, 1983; Andersson *et al.*, 1983; Freeman and Cattell, 1990). Anthropogenic release is the most important source and includes two general types. One is the result of accidental spillage and intentional dumping of materials such as creosote, coal tar, and petroleum products. The other is from the incomplete combustion of organic material such as wood, municipal waste, coal, petroleum, and ore (Neff, 1979; lee *et al.*, 1981; Cook and Dennis, 1983; National Research Council, 1983). Natural sources of PAHs in the environment are volcanic activity, forest fires and biosynthesis by bacteria, plants, and fungi (Hopper, 1978; Neff, 1979; Lee *et al.*, 1981; Cook and Dennis, 1983). Recently, contamination of soils, sediments, and groundwater by PAHs has originated from four primary waste sources: creosote, coal tar, petroleum, and industrial effluents and gases. Overall, the main sources of PAHs in rivers, lakes, and oceans are urban runoffs, effluents from industrial processes, atmospheric depositions, and exhaust gases from ships (Neff, 1979; Lee *et al.*, 1981; Cook and Dennis, 1983; Mueller *et al*, 1989; Wild *et al*, 1991).

1.3 Environmental distribution of PAHs

Polycyclic aromatic hydrocarbons are widely distributed in the environment (Lee *et al.*, 1981). There are several routes for altering the location and/or structure of PAHs in the environment including volatilization, transport on atmospheric particles and aquatic particulate matter, photooxidation, chemical oxidation and biological oxidation (Cooke and Dennis, 1983, Hallet and Brecher, 1984, Varanasi, 1989). The physical environment where PAHs are distributed can be divided into four compartments (Figure 1.2): the atmosphere (air), hydrosphere (water), lithosphere (soil), and biosphere (living organisms or biota). Environmental sites contaminated by PAHs are very common. The areas are often small, but the PAH concentration can be high, and are usually co-contaminated with other organic compounds (e.g. PCBs) and heavy metals (Cooke and Dennis, 1983; Edwards, 1983). The highest concentrations of PAHs in the environment are generally close to the sources of contaminate release, and their concentrations generally decrease logarithmically with distance from the source (Lunde and Bjorseth, 1977; Jones and leber, 1979; Cooke and Dennis, 1983; Edwards, 1983).

PAHs are released to the atmosphere by combustion and volatilization. Virtually, any combustion process using organic fuel will generate PAH compounds (Varanasi, 1989). PAH emissions are usually higher in winter because of the increased use of heating systems. As well, the highest concentration of PAHs are in urban and industrialized areas (Neff, 1979; Cook *et al.*, 1983). The sources of PAHs in surface soils can be both natural and anthropogenic, depending on the location. For example, in forests a substantial amount of PAHs are transferred to the soil via leaf litter, which absorb PAHs from air (Varanasi, 1989). The distribution of PAH in surface soils is generally heterogenous, with some of the material in a oily phase on soil surfaces and some existing as free products in droplets lodged in the interstices of the soil (Varanasi, 1989). Their eventual distribution and transport will depend on local environmental conditions (Neff, 1979; Cook *et al.*, 1983). These include contaminant volume, viscosity, temperature, land contour, plant cover, and soil composition (Cook *et al.*, 1983). Vertical movement occurs as multiphase flows that will be controlled by soil chemistry, soil structure, pore size, and water content (Morgan and Watkinson, 1989). However, because

Figure 1.2 Distribution and dynamics of PAHs in the environment.


HYDROSPHERE
ATMOSPHERE
PAHs
LITHOSPHERE
BIOSPHERE

PAHs are hydrophobic they tend to remain at or near the soil surface, bound to hydrophobic particulate matter (Bossert and Bartha, 1986).

PAHs are more concentrated in aquatic systems than in any other environmental compartment. They are also more persistent in water than in air (Fox and Olive, 1977; Lunde and Bjorseth, 1977; Lee *et al.*, 1981). PAHs enter the aquatic environment via two routes. First, from the atmosphere and subsequently deposited on the surface of the water or soil. Second, direct and indirect releases of material into surface and ground water (Morgan and Watkinson, 1989). Since PAHs are hydrophobic, once they enter a water column, they adsorb to particulate matter and partition into sediments (Morgan and Watkinson, 1989). Humic substances in the sediment may enhance PAH mobility through emulsification and sorption to particle surfaces (Madsen *et al.*, 1992). This may account for the observation that significant concentrations of PAHs often occur in the water column bound to particulate matter (Madsen *et al.*, 1992). Thus, PAH concentrations in surface water are often higher than ground water because of the presence of suspended particles on which PAHs are adsorbed (Madsen *et al.*, 1992). Ground water is naturally filtered as it flows through the soil, and PAHs will partition onto the soil particles (Morgan and Watkinson, 1989).

PAH contamination in aquatic sediments has resulted primarily from atmospheric inputs, surface runoff, spills, and effluents from industrial areas (Bates *et al.*, 1984; LaFlamme and Hites, 1984). As PAHs settle through the water column, they are generally stripped of the low molecular weight PAHs through dissolution and volatilization. Thus, concentrations of high molecular weight PAHs in sediment can be very high ranging from 100 μg/g to greater than 100,000 μg/g in urban estuaries (Morgan and Watkinson 1989).

1.4 Physical and chemical properties of PAHs

Classically, PAHs are pure hydrocarbons containing two or more fused benzene rings (Neff, 1979; Lee *et al.*, 1981; Cook *et al.*, 1983). However, there are many PAHs which also contain heteroatoms such as nitrogen, sulfur, and oxygen (Mackay & Shiu, 1977; Neff, 1979). PAHs are non-polar and planar compounds (Mackay & Shiu, 1977;

Neff, 1979). In general, PAHs are crystalline solids having low solubility in water, high melting and boiling points, and low vapor pressure (Mackay and Shiu, 1977; Neff, 1979). With increasing molecular weight, solubility and vapor pressure decrease, and melting and boiling points increase (Neff, 1985). PAHs have melting points above 100 °C and boiling points above 300 °C. PAHs usually have vapor pressures in the range of 10^{-5} to 10^{-13} KPa at 25 °C and water solubilities in the range of < 1 $\mu g\ L^{-1}$ to 1 $mg\ L^{-1}$. Because they are very hydrophobic, they have high affinities for particle surfaces (Lee *et al.*, 1981; Hallet and Brecher, 1984).

A key parameter that indicates PAH behavior in the environment is the octanol-water partition coefficient (log K_{ow}). The log K_{ow} is a measure of the extent to which the chemical partitions between octonol and water at equilibrium. It is related to the lipophilicity of a compound, and the ability to partition into biological membranes (Government of Canada, 1994). PAHs present in an oxidizing environment may react to form more water-soluble compounds. This usually involves oxygenation of the compounds. The water solubility of PAHs increases following oxidation (Lee *et al.*, 1981; Hallet and Brecher, 1984).

PAHs have reactivity characteristics of aromatic molecules these include electrophilic substitutions, 1,2- and 1,4-additions, and addition-eliminations (Tipson, 1964; USNAS 1972). The physical and spectroscopic properties of PAHs are dominated by their conjugated π-electron systems, which makes PAH compounds photochemically active (Nakhimovsky, 1989). This structural feature also makes them relatively stable in the absence of strong oxidants (Nakhimovsky, 1989). PAH are more reactive than benzene. Their reactivity to methyl radicals increases with an increase in the number of alternating single and double bonds (U.S. EPA, 1987). In general, increased conjugation leads to greater stability, but also greater reactivity toward free radicals (U.S. EPA, 1987).

1.5 Photochemistry of PAHs

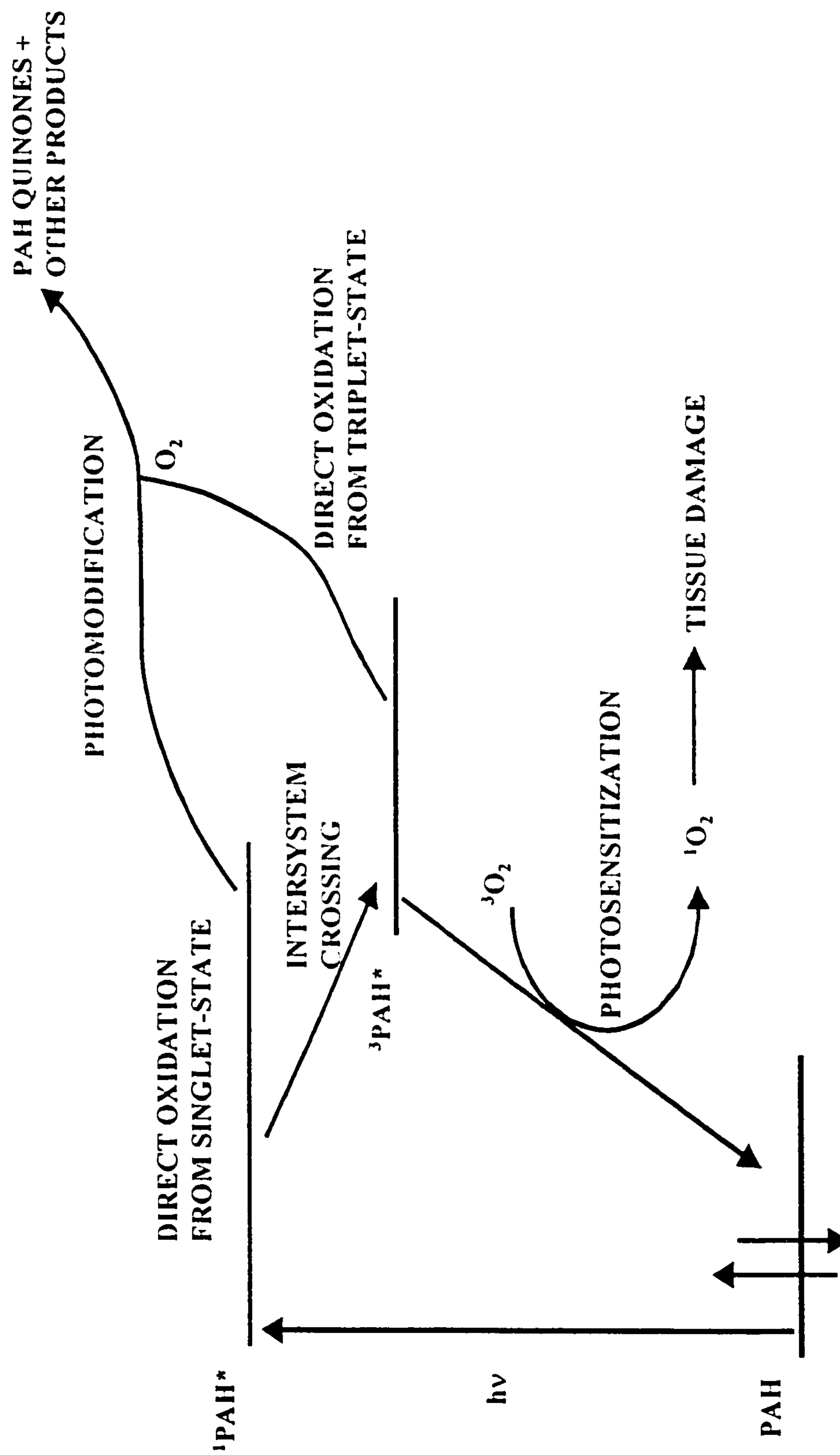
The photochemistry of PAHs has been studied in water and on solids in both environmental and laboratory settings (Fox and Olive, 1979). A wide variety of

photoproducts have been identified (Fox and Olive, 1979; Huang *et al.*, 1992; Huang *et al.*, 1993; Ren *et al.*, 1994; McConkey *et al.*, 1994; Mallakin *et al*, 1999). Most PAHs strongly absorb radiation in the wavelength range of 200 to 400 nm (Pitts 1979; Eisenberg *et al.*, 1983). In the presence of light and oxygen, polycyclic aromatic hydrocarbons readily undergo photooxidation (Pitts, 1979; Eisenberg *et al.*, 1983; Huang *et al.*, 1992; Huang *et al.*, 1993; Ren *et al.*, 1994; McConkey *et al.*, 1997; Mallakin *et al*, 1999).

When a PAH molecule absorbs a photon it is excited from the singlet-ground state to a singlet-excited state (Figure 1.3). From the singlet-excited state the PAH molecule may: 1) Return to the ground-state with the absorbed energy emitted as fluorescence. 2) Return to the ground-state with the energy dissipated as heat (thermal decay). 3) The excited-state may converted to the triplet-excited state by intersystem crossing (Lee *et al.*, 1981; Girotti, 1983; Foote, 1987). From the triplet-excited state, the PAH molecule can return to ground state by phosphorescence or heat dissipation (Girotti, 1983; Foote; 1987; Newsted and Giesy, 1987; Greenberg *et al.*, 1993). The conversion rate of PAH molecule to triplet-excited state is both rapid (rang from 10 to 100 ns), and efficient (quantum yields of 50 to 80%) (Kochevar, 1982; Nakhimovsky, 1989; Krylov *et al.*, 1995).

PAHs in singlet and triplet excited states can react by three routes (Figure 1.3) (Kochevar, 1982; Sinha and Chignell, 1983; Eisenberg *et al.*, 1983; Foote, 1987). First, PAHs are good photosensitizers, and in this process the excited molecule passes energy to another available molecule usually oxygen (O_2), generating highly reactive species such as singlet-state oxygen ($^1O_2$) (Girotti, 1983; Foote, 1987). Second, PAHs can be photooxidized through the direct reaction of excited molecules with oxygen forming PAH endoperoxides and other oxidized PAH products (McConkey *et al.*, 1997). Third, the dimerization of the excited state molecules in the absence of oxygen can occur (Lee *et al.*, 1981). In all cases, if oxygen is present, photooxidation and photosensitization are the most prevalent reactions. Importantly, when a PAH is in a triplet-excited state it can react with ground-state molecular oxygen to produce excited-singlet molecular oxygen ($^1O_2$), which can in turn react with a ground-state PAH molecule to yield oxygenated products (USNAS, 1972; McConkey *et al.*, 1994). Thus, PAHs are rapidly and efficiently converted to quinones and diols in the presence of light and O_2 (Cavalieri *et al.*, 1990;

Figure 1.3 Jablonski diagram representing ground and excited states of a PAH. Absorbance of a photon (*hv*) elevates an electron from the ground singlet-state (GSS) to an excited-singlet state (ESS). From there it can react directly, forming a photomodified compound. Alternatively, the excited molecule can intersystem cross to the excited triplet-state (ETS). The triplet-state molecule can also react directly, resulting in modification of the excited molecule, or it can react with ground state triplet oxygen ($^3O_2$), forming excited singlet oxygen ($^1O_2$) and returning the ETS molecule to the GSS.


PAH QUINONES +
OTHER PRODUCTS
PHOTOMODIFICATION
O_2
DIRECT OXIDATION
FROM TRIPLET-STATE
DIRECT OXIDATION
FROM SINGLET-STATE
INTERSYSTEM
CROSSING
$^3PAH^*$
$^3O_2$
PHOTOSENSITIZATION
$^1O_2$
TISSUE DAMAGE
$^1PAH^*$
hν
PAH

Huang *et al.*, 1993; Mallakin *et al*, 1999).

1.6 Photoinduced toxicity of PAHs

Phototoxicity presents an interesting twist in the measurement of toxicity (Suzuki *et al.*, 1982; Oris and Giesy, 1986). Certain chemicals can appear relatively nontoxic under normal indoor lighting conditions, yet exert a pronounced effect in sunlight (Oris and Giesy, 1986). When PAHs are exposued to actinic radiation, especially ultraviolet radiation, there are two processes that result in photoinduced toxicity: photosensitization and photooxidation (Oris and Giesy, 1986; Newsted and Giesy, 1987). Photosensitization occurs via the production of singlet oxygen (Figure 1.3), which has been shown to have a negative impact on fish, microorganisms, and plants (Oris and Giesy, 1986; Newsted and Giesy, 1987; Krylov *et al.*, 1995). PAH photooxidation products generally include diols, ketones and quinones, are highly toxic, often more toxic than the intact PAHs (Suzuki *et al.*, 1982; Huang *et al.*, 1992; Greenberg *et al.*, 1993; Ren *et al.*, 1994). This interesting phenomenon, is readily observed in fish, plants, microbes and aquatic invertebrates exposed to μM levels of various PAHs (Allred and Giesy, 1984; Oris *et al.*, 1984; Pengerud *et al.*, 1984; Ren *et al.*, 1994; Huang *et al.*, 1995; McConkey *et al.*, 1997). The immediate effect on fish is inhibited respiration as the compounds photosensitize gill tissue (Bowling *et al.*, 1983; Oris and Giesy, 1986; Newsted and Giesy, 1987; Schirmer *et al.*, 1998).

The photooxidiation of PAH is also very important in the environment (Oris and Giesy, 1986; Newsted and Giesy, 1987; Ren *et al.*, 1994; Huang *et al.*, 1995; McConkey *et al.*, 1997). PAHs are rapidly photooxidized in the presence of sunlight to a variety of products, including endoperoxides, hydroxy quinones and phenols. Many oxidized PAHs, including photooxidized PAHs, are known to be more hazardous to living organisms than intact PAHs (Young *et al.*, 1976; Cavalieri *et al.*, 1990; Huang *et al.*, 1993; Ren *et al.*, 1994; McConkey *et al.*, 1994). In this a microbial test will be developed to evaluate the short- and long-term photoinduced toxicity of PAHs.

1.7 Quantitative Structure-Activity Relationships of PAHs in the Environment

In aquatic toxicology, quantitative structure-activity relationships (QSARs) have been applied extensively to the study of acute toxicity of various classes of chemicals (Veith and Konasewich, 1975; Konemann and Musch, 1981). QSARs can provide correlations between the toxicity of a chemical and the physical properties of the chemical (Konemann and Musch, 1981; Krylov *et al.*, 1997). Because QSARs correlate the physicochemical properties of molecules to observable biological responses, they are useful for understanding the mechanisms of action of groups of related chemicals and for predicting the environmental risk associated with those chemicals (Blankley, 1983; Greenberg *et al.*, 1993; Krylov *et al.*, 1997). Many QSAR models have been developed to predict the potential hazards of untested compounds. They depend on physical properties, chemical properties and environmental factors for the contaminants (Blankley, 1983; Greenberg *et al.*, 1993; Krylov *et al.*, 1997). They can greatly augment empirical toxicity data by allowing estimations of toxicity to an organism to be based on readily measured or calculated chemical characteristics. At the same time, QSARs are used to predict properties and reactivity of related molecules (Blankley, 1983; Hermens and Opperhuizen, 1990). QSARs have the advantage of being able to predict toxicity of new chemicals without lab-intensive toxicity assessment. While, QSARs will never fully replace biological testing, they could reduce the number of such experiments required to confidently predict environmental risks (Blankley, 1983; Hermens and Opperhuizen, 1990).

Molecular properties used in QSARs can represent the whole molecule or can refer to selected functional groups. The types of physical properties used are hydrophobicity, electron density and steric hindrance. Many studies have demonstrated the validity of the QSARs approach for predicting the toxic effects of relatively homogenous classes of organic chemicals (Morgan *et al.*, 1977; Govers *et al.*, 1984; Newsted and Giesy, 1987; Ankley *et al.*, 1994). PAHs, being a large group of homogeneous contaminants have been used extensively in QSAR modeling (Blankley, 1983; Hermens and Opperhuizen, 1990). Attempts to develop QSARs models for PAH phototoxicity have taken into consideration the relationships between photoinduced

toxicity/carcinogenicity and the singlet, triplet and singlet-triplet splitting energy of PAHs as well as their phosphorescence lifetimes (Morgan *et al.*, 1977; Newsted and Giesy, 1987; Ankley *et al.*, 1994; Greenberg *et al.*, 1993; Krylov *et al.*, 1997). These models met with limited success.

The most recent QSAR model describing the photoinduced toxicity of PAHs use both the photosensitization and photomodification processes to describe toxicity (Greenberg *et al.*, 1993, Krylov *et al.*, 1997). Therefore, there are two physicochemical processes that can be used in QSAR modeling to describe the photoinduced toxicity of PAHs (Greenberg *et al.*, 1993; Krylov *et al.*, 1997). This was done using the aquatic higher plant *Lemna gibba* to describe the photoinduced toxicity of PAHs (Greenberg *et al.*, 1993; Huang *et al.*, 1995). For 16 PAHs, the two factors were shown to play approximately equal roles in toxicity. In this thesis, this QSAR model will be applied to the luminescent marine bacteria *Vibrio fischeri*.

1.8 Toxicity Mechanism of PAHs

PAHs have been shown to have numerous sites of action in living cells. When intact and photomodified PAHs penetrate into a living organism, a variety of reactions can occur (Cooney, 1982). Toxicity will depend on PAH structure, environmental conditions, and the organism being studied (Cooney, 1982; Josephy, 1997). There are many proposed mechanisms by which PAHs inhibit and eventually kill organisms (Davila *et al.*, 1995; Schirmer *et al.*, 1998). Therefore, various bioassays have been developed, including impaired growth to analyze various sites of action inhibition of enzymatic activities, changes in metabolism, inhibition of mitochondrial electron transport, and inhibition of photosynthesis (Gensemer *et al.*, 1996; Huang *et al.*, 1996; Huang *et al.*, 1997; Schirmer *et al.*, 1998). PAHs may cause damage to DNA or may induce protein denaturation (Josephy, 1997). PAHs are lipophilic molecules which interact with lipoprotein in membranes, affecting both membrane structure and function (Josephy, 1997; Schirmer *et al.*, 1998). Disruption of cell membranes results in leakage of macromolecules and metabolites from the cell (Willianmson, 1981; Josephy, 1997; Schirmer *et al.*, 1998).

Light can alter the toxicity of PAHs by two routes: photosensitization and photomodification (Oris and Giesy 1985; Larson and Berenbaum 1988; Greenberg *et al.*, 1993; Krylov *et al.*, 1995). Photomodification of PAHs, usually via oxidation, results in the formation of new compounds usually oxygenated PAHs (or oxyPAHs) (Larson and Berenbaum 1988; Huang *et al.*, 1993, Mallakin *et al.*, 1998). It has been found that oxyPAHs partition into photosynthetic membranes and inhibit the photosynthetic electron transport chain (Gensemer *et al.*, 1996, Huang *et al.*, 1996; Huang *et al.*, 1997; Duxbury *et al.*, 1997). For example, photooxidation products of anthracene were found to be more toxic than intact anthracene, and to inhibit photosynthetic electron transport at the cytochrome b/f complex (Gensemer *et al.*, 1996; Huang *et al.*, 1996, 1997). This is homologous to the cytochrome b/c complex in mitochondria and oxyPAHs have been found it inhibit respiration (Tripuranthakam *et al.*, 1999). The mechanism of photosensitization is well investigated as of route of photoinduced toxicity. Photosensitization results when a PAH molecule absorbs a photon, then undergoes an intersystem crossing and then reacts with $^3O_2$ to form $^1O_2$. $^1O_2$ is a highly reactive oxygen species and can cause significant damage to biomolecules such as lipid bilayers, proteins, and DNA (Landrum, 1986; Krylov *et al.* 1997).

Since enzymes drive all the metabolic reactions in microbial, plant, and animal cells, their inhibition could be the underlying cause of PAH toxicity to cells (Segal, 1968). It is believed that one of the most important effects of the toxic action of PAHs on bacteria is on enzyme activity (Iverson and Brinckman; 1978 Cooney, 1982; Josephy, 1997). Many chemicals alter the activity of an enzyme by altering the binding of the substrate and/or the enzyme turnover number (Segal, 1968; Josephy, 1997). PAHs are also carcinogenic as a result of their structure (Segal, 1968; Josephy, 1997). The flat, hydrophobic shape of PAHs makes them difficult to excrete. In addition, this shape allows a PAH to insert itself into the structure of DNA where it interferes with the proper functioning of the DNA and can lead to mutations and cancer (Morgan *et al.*, 1977; Lesko and Lorentzen 1985; Timbrell 1991). Carcinogenicity is not the only hazard which has been associated with chronic PAH exposure (Edwards, 1983). PAHs have also been shown to have adverse effects on the immune system (Edwards, 1983, Davila *et al.*, 1995). An important long-term toxicity concern in fish is carcinogenesis (Clements *et al.*,

1994). Liver tumours are the specific neoplasms, which are most strongly associated with exposure to PAHs (Clements *et al.*, 1994). The immunotoxicity of an individual PAH is dependent upon its structure and is not necessarily related to its carcinogenic potenial (Edwards, 1983; Davila *et al.*, 1995).

1.9 Phototoxicity assessment using the Luminescence Bacteria

Different metabolic functions of bacteria can be measured to assess the viability and/or the activity of the cell after exposure to a toxic substance (Alsop *et al.*, 1980; Chang *et al.*, 1981; Dutka and Kwan, 1981; Bossert and Bartha, 1986). Bacterial assays for chemical toxicity in aquatic environments can be placed in five categories based on the parameter being measured. They are bacterial growth, respiration (O_2, NO_3, organic molecules), cellular energy, consumption of a substrate, and luminescence (Qureshi 1981; Dutka and Kwan, 1981,1982; Serio and Pazzagli, 1982; Qureshi *et al.* 1984; Reteuna *et al.*, 1989; Zakrzewski, 1991). Two bacterial toxicity-screening tests were used to assess phototoxicity of PAHs in this thesis. They were inhibition of growth and bioluminescence.

1.9.1 Assays based on bacterial bioluminescence

Bioluminescence is the emission of light by living organisms (Hastings and Nealson, 1981; Hastings, 1986; Nealson, 1989). It has been exploited as analytical tool for measuring low levels of a variety of biologically important compounds (Deluca, 1978; Serio and Pazzagli, 1982; Krricka and Carter, 1982). Bioluminescence is observed in many diverse species in nature, such as bacteria, dinoflagellates, fungi, fish, insects, shrimp, and squid (Hastings and Nealson, 1981; Hastings, 1986). The luminescence produced by bacteria, in part because of its inherent beauty and ease of detection, has attracted scientific attention for more than 300 years (Hill and Shoup, 1929, Harvey 1952; Hastings *et al.*, 1985). In the 17th century the Irish chemist Robert Boyle observed the luminescent glow from the skin of dead rotting fish, presumably caused by luminescent marine bacteria growing in a saprophytic mode. By showing that the light

from dead fish was extinguished in a vacuum, the requirement for oxygen in bioluminescence was demonstrated for the first time (Hill and Shoup, 1929; Johnson 1947; Harvey, 1952). The mechanism of light emission is different among different organisms. Bioluminescent bacteria are mostly marine microorganisms, which usually live freely in ocean water (Sie *et al.*, 1966; Takahide and Nakamura, 1980). They can also occur as saprophytes, commensal symbionts, parasites of animals and specific light-organ symbionts (Hastings and Nealson, 1981; Dunlap and Greenberg, 1991). Presently, at least 11 species in four genera *(Vibrio, Photobacterium, Shewanella [Alteromonas]* and *Xenorhabdus)* have been described (Meighen, 1991). Although found predominantly in marine environments, luminescent bacteria are also present in freshwater and terrestrial habitats (Meighen, 1991). These bacteria are all gram-negative motile rods and can function as facultative anaerobes (Nealson *et al.*, 1979; Baumann *et al.*, 1983). The light-emitting bacteria that have been investigated in most detail are *Vibrio harveyi, V. fischeri, Photobacterium phosphoreum, P. leiognathi,* and *Xenorhabdus luminescens.* Other species of luminescent bacteria are *V. logei, V. splendidu,* and *V. cholerae* a freshwater species (Meighen, 1991).

The enzymes that catalyze the bioluminescence reaction leading to light emission in these organisms are called luciferases. The substrates are designated as luciferins, and have been shown to be similar in most prokaryotes (Hada *et al.*, 1985). The dramatic luminescence of cultures of luminescent bacteria is illustrated by *Vibrio fischeri* NRRL B-11177 on an agar plate and in a liquid culture (Figure 1.4). *V. fischeri* NRRL B-11177 (previously listed as *Photobacterium fischeri* and then as *Photobacterium phosphoreum*) has been developed into a commercial toxicity assay known as the Microtox assay (Azur Environmental, Carlsbad, CA). *V. fischeri* are a free-living marine bacteria which can become symbionts with squid and other marine fish, where they can colonize the corresponding light organs and produce bioluminescence (Breed *et al.*, 1957; Baumann and Baumann, 1980; ASTM, 1976; Haygood and Distel, 1993). *V. fischeri* produce yellow-orange colonies on marine agar containing 0.05% yeast extract and emit a blue-green color light when they are at high cell density. *V. fischeri* is a gram-negative rod

Figure 1.4 Light emission by *Vibrio fischeri* (strain NRRL B-11177) on an agar plate (A) and in liquid culture (B).

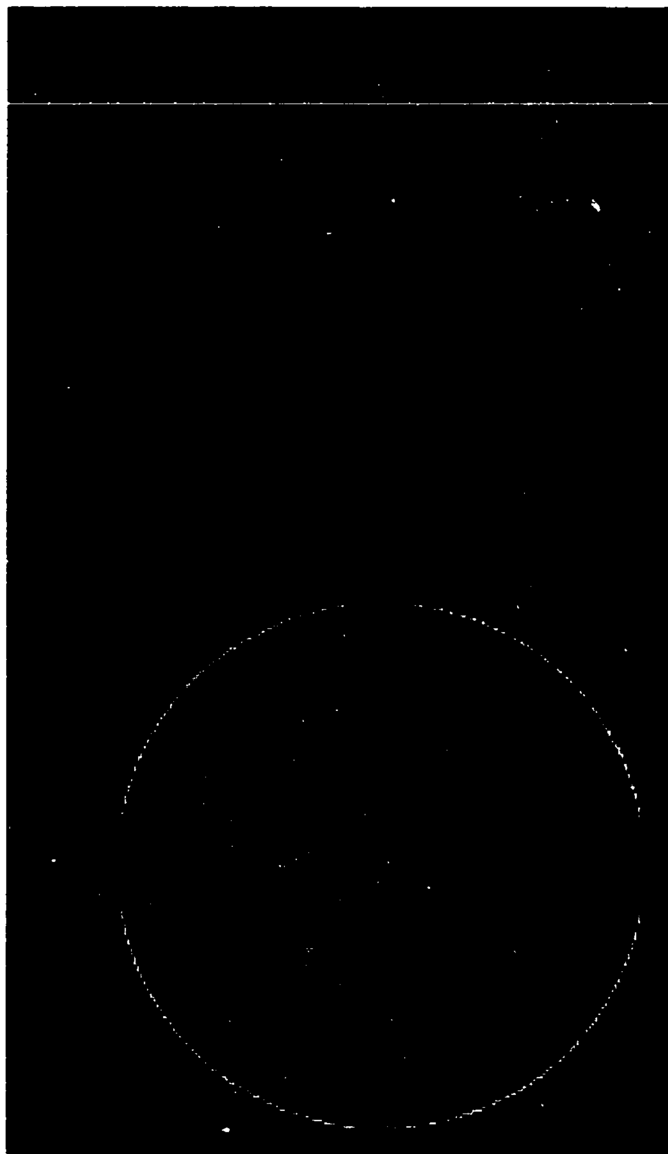
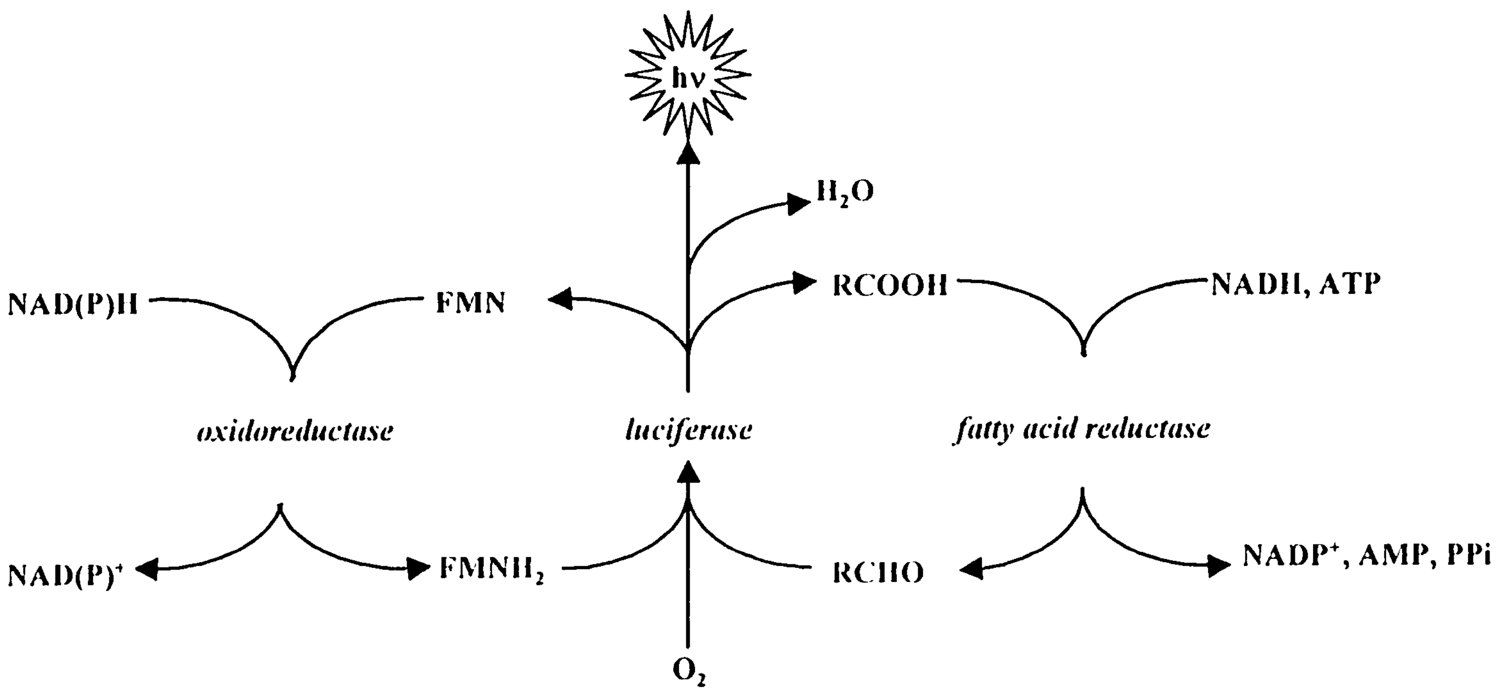


Figure 1.5 Mechanism of light emission from *V. fischeri*. This reaction is catalyzed by the enzyme luciferase. Oxygen is used to oxidize an aldehyde to a fatty acid. In the process, $FMNH_2$ is also oxidized, giving rise to an excited state form of FMN that quickly radiates away its extra energy as blue-green light 490 nm. Electrons are thus diverted from the respiratory electron transport chain, and light is produced at the expense of generation of ATP.

Luciferin (L) + Oxidant
luciferase (E)
Oxidized luciferin + light

RCHO + FMN2 + O2
luciferase (E)
RCOH + FMN + H2O + hν


hν
H2O
RCOOH
NADH, ATP
NAD(P)H
FMN
oxidoreductase
luciferase
fatty acid reductase
NAD(P)+
FMNH2
RCHO
NADP+, AMP, PPi
O2

bacteria, 0.5-0.8 μm in width, and 1.4-2.6 μm in length. They do not form endospores or microcysts. For growth *Vibrio fischeri* requires sodium chloride, temperatures in the range of 15-30 °C, and a pH of 6 to 8 (Nealson and Hasting, 1979; Takahide and Nakamura, 1980; Hastings *et al.*, 1985; Hastings, 1986).

Light emission in *V. fischeri*, NRRL-B-11177 occurs continuously as the result of a reaction shunt from the respiratory electronic transport chain (Figure 1.5) (Kurfurst *et al.*, 1984; Hastings *et al.*, 1985; Hastings, 1986). In the presence of oxygen (O_2) the reduced flavin mononucleotide ($FMNH_2$) bound to luciferase is oxidized to FMN, and FMN when formed in an excited state releases a photon of 490 nm when it relaxes to ground state. One oxygen atom from O_2 is reduced to form water. The second oxygen atom oxygenates a long-chain aliphatic aldehyde to form a fatty acid. In vivo both FMN and the fatty acid are rereduced by NAD(P)H to regenerate the substrates for the light emission reaction (Kurfurst *et al.*, 1984; Hastings *et al.*, 1985). Because of their ease of use scientists have exploited these bioluminescent bacteria as rapid assays of water quality and contamination. They are used for testing the quality of drinking water and for numerous other industrial applications (Bulich, 1979). As early at 1942, luminescent bacteria were used in toxicity assays. Johnson and Harryman (1942) showed that luminescent bacteria were sensitive to a wide range of heavy metal chlorides and narcotics. Sie and Jordan (1966) used *P. fischeri* in an agar plate culture to detect the effects of toxic materials in a spacecraft environment. Luminescent bacteria coupled with algae were used to detect herbicidal contamination (Tchan, *et al.*, 1975; Tchan and Chiou. 1977). There are many studies addressing the replicability and precision of these assays (Qureshi, 1981; Dutka and Kwan, 1981; Qureshi, *et al.*, 1984; Strosher, 1984; Bulich, 1986; Reteuna, *et al.*, 1989; Ribo, *et al.*, 1989; True and Hayward, 1990).

The luminescent bacterial assay also shows good correlation with results from other toxicity tests such as rainbow trout and *Daphina magna* (Munkittrick and Power, 1989; Munkittrick *et al.*, 1991). Linear regressions with correlation coefficients of approximately 0.7 to 0.97 were typically found with fish (Munkittrick and Power, 1989; Munkittrick *et al.*, 1991). The correlations were generally better for organic contaminants than metals. Further, the longer incubation times required for the fish assays tended produce lower correlation coefficients. Nonetheless when the toxicity tests were

compared based on toxic equivalency highly significant correlations were observed (Lebsack, *et al.*, 1981; Chang, *et al.*, 1981; Qureshi, *et al.*, 1982; Strosher, 1984; Bulich, 1986). Thus, toxicity assays based on bacteria luminescence play a key role in environmental toxicology and risk assessment.

1.9.2 Assays based on the measurement of bacterial growth inhibition

Use of bacterial growth inhibition, as a toxicological bioassay is not a new concept. It has been used to assess the sensitivity of microorganisms to antibiotics (Prier *et al.*, 1973) and environmental contaminants (Bowder and Krieg, 1974; Dutka and Switzer, 1978; Williamson and Johnson, 1981). Under optimal conditions, bacteria are characterized by rapid growth. Thus, the action of a toxic substance on growth can be obtained, then a dose-effect relationship can be measured. To measure growth in the effects of environmental toxicants, it is necessary to recognize that growth requires the ability to initiate and complete cell division. Furthermore, cell growth is dependent upon incubation temperature, pH, trace element concentrations, macronutrient concentrations, and ionic strength of the growth medium, oxygen content (Alsop *et al.*, 1980; Dutka and Kwan, 1982; Trevors *et al.*, 1982).

Growth assays are usually used over a longer period than the above luminescence assays. They are also dependent on more cellular factors than luminescence assays. That is, short-term luminescence assays depend on inhibition of respiration, where as any impact on the cells that shows growth will be detected in long-term assays. The assay, which is used here, was based on the measurement of turbidity as an index of *V. fischeri* growth. Luminescent bacteria are mixed with a toxicant at different concentrations in a liquid nutritive medium. Growth of the bacteria is recorded after about 24 hours. Samples containing toxicants will give absorbance values lower than the controls. The assay protocol is described in Chapters 2 and 3.

1.9.3 Comparison between Short- and Long-term bacterial Assays and Microtox Assay

A variety of bioassays using fish and other aquatic organisms are presently applied to detect toxic chemicals in marine and freshwater ecosystems (Maciorowki *et al.*, 1980). The use of microorganisms, bacteria in particular, as an assay agents allows development of simple, inexpensive, rapid, and generally sensitive tests to determine and monitor the ecotoxicity of effluents and contaminants in aquatic ecosystems (Grabow *et al.*, 1980). The Ames test is a bacterial assay used to detect mutagens in wastewater, drinking water and surface water (Grabow *et al.*, 1980). Physiological measurements of bacteria to monitor toxic materials in water have been used since 1960s (Axt, 1973; Bring-man and Kuhn, 1975; Hertkorn-Obst and Frank., 1979). For instance, bacterial populations in chemostat cultures and in freshwater microcosms were studied to evaluate the effects of toxic materials, including metals and herbicides (Ferebee and Guthrie, 1973; Mayvfield *et al.*, 1980).

Recently, one of the most commonly used microbial assays is the Microtox test. It was developed in the late 1970s (Bulich, 1979, 1986; Kaiser and Palabrica, 1991; Qureshi *et al.*, 1982, 1984; Ribo *et al.*, 1989). Using the knowledge that activity of the respiratory electron transport chain is an indication of the metabolic state of the cell, the Microtox test is based on rehydrating freeze-dried luminescent marine bacteria *Vibrio fischeri* (strain NRRL-B-11177) and measuring the toxicant-induced inhibition of ATP/NADPH-dependent bioluminescence (Qureshi *et al.*, 1982, 1984; Ribo *et al.*, 1989). The results obtained using the microbial test systems needed to be correlated with those of other established bioassays employing a wide range of representative toxic substances (Munkittrick and Power, 1989). Some published reports of this type have evaluated the sensitivity of the Microtox system in determining the toxicity of pure compounds and complex effluents (Chang *et al.*, 1981). The sensitivity of this test is, in general, similar to that of acute lethality tests using fish (reviewed by Munkittrick and Power, 1989; Munkittrick *et al.*, 1991). Compared to lethality tests that used fathead minnows, trout, and *Daphnia*, Microtox was about as sensitive to pure organic compounds, municipal wastes, and the toxic industrial effluents, but was often less sensitive to inorganic toxicants and pesticides (Munkittrick and Power, 1989; Munkittrick *et al.*, 1991).

However, because of the short-term nature of Microtox test (< 30 min) slow acting chemicals often give false negatives. Therefore, a long-term assay with *V. fischeri* would be useful. Another problem with the Microtox test is that it uses freeze-dried bacteria that are rehydrated immediately prior to use. It might be better to use cells that are taken immediately from cultures in log-phase growth.

To date, *V. fischeri* has not been used extensively in long-term (~24 h) growth assays. However, for toxicity assessment of PAHs, such an assay would be important. This is because photoactivation is a major mechanism of PAH toxicity. This could be missed in short-term toxicity, and indeed light has not been used as a factor in the Microtox assay. In the case of photosensitization, time would be required for photoactivated $^1O_2$ to have an effect. In the case of photooxidation, time is required for modification reactions to proceed. In general, there are many contaminants that require longer incubation reactions to be toxic. They may be slowly assimilated by the organism, or may need to be biologically modified. Therefore, there is great need to develop a long-term assay for *V. fischeri* and compare it to the short-term Microtox-like assays.

1.10 Research objectives

A major goal of this thesis is to study the direct and photoinduced short- and long-term toxicity of PAHs to luminescent bacteria *V. fischeri*. Further, a Quantitative Structure Activity Relationship (QSAR) of photoinduced toxicity PAH to *L. gibba* will be applied to *V. fischeri*. Last, as an application of the short- and long-term *V. fischeri* assays, whole extraction and fractions isolated from different PAH contaminated sediments were tested.

The subject of chapter two was first to develop new methodologies for growth *V. fischeri* (strain NRRL B-11177) under darkness and simulated solar radiation (SSR) by using a specially formulated medium. The second objective was to develop in vivo assays for measuring short- and long-term toxicity of intact and photomodified PAHs. The assays were conveniently timed in that after the short-term data (15 min) were collected, the cells were allowed to grow for 18 h to give long-term toxicity data. This assay was tested with intact and modified PAHs. The subject of chapters three and four was to use a

pre-incubation technique to promote standardization of the assay by studying the effects a sugar–source (reduced carbon) in the growth media This was, to test how the medium affects the bioavailability of PAHs in the short- and long-term toxicity test. It was found that the medium had a lager impact on the toxicity of PAHs.

PAHs differ in their physico-chemical properties such as water solubility, volatility, lipophilicity (Mackay and Callcott, 1999) and susceptibility to photochemical degradation (Huang *et al.*, 1991, Huang *et al.*, 1997, Arey, 1999). The photoinduced toxicity of PAHs is derived through two mechanisms, photosensitization and photomodification. Huang *et al.*, (1997) established a QSAR model for photoinduced toxicity of PAHs to the aquatic plant *Lemna gibba*. The model was based on physico-chemical properties of PAHs. The subject of chapter Five was apply this QSAR to *V. fischeri* using data from the assays developed for this thesis. This was a test both of the *V. fischeri* toxicity assays and the generality of the mechanism of PAH photoinduced toxicity.

Sediments are an effective substrate for various hydrocarbon contaminants. In particular, PAHs in aquatic environments reside in sediments. As an application of the *V. fischeri* short- and long-term toxicity assays, PAHs contaminated and reference sediments were tested. It was found that the extracts from PAH contaminated sediments were toxic to *V. fischeri*, especially in the long-term assay. Further, when the sediment was fractionated, the most toxic fraction was the oxyPAH-containing fraction.

With this, a new bacterial assay was developed it allows short-term and long-term data to be collected in a single test. The long-term assay is able to detect photoinduced toxicity of PAHs. The data collected is consistent with photoinduced toxicity of PAHs to *L. gibba*. Finally, the *V. fischeri* toxicity test can be applied to the evaluation of environmental samples.

CHAPTER 2

MEASUREMENT OF PHOTOINDUCED SHORT- AND LONG-TERM TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS USING LUMINESCENT BACTERIA [(1)]

2.1 ABSTRACT

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 bacteria *Vibrio fischeri*. The toxicity of most PAHs can be greatly enhanced upon exposure of a living organism and 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 PAHs results in new compounds (usually oxygenated PAHs) that are often more toxic than their parent PAHs. In an effort to differentiate between the photomodification and photosensitization processes, microbial toxicity assays were developed to measure short- and long-term toxicity. The bioassays were based on inhibition of luminescence and growth of *Vibrio fischeri*. The short-term assay should detect toxicity of chemicals that are taken up rapidly and either are directly cytotoxic or whose photosensitization activity is immediate. The long-term assay identifies chemicals where the rate of assimilation is slow and/or time is required for photoinduced effects to be realized. Thus, the long-term assay is important for testing the toxicity of photo-labile toxicants over a larger portion of the modification process. The efficiency of the assays

[(1)]Submitted to Ecotoxicology and Environmental Safety. Co-authors are D. G. Dixon and B. M. Greenberg.

were tested with twelve different PAHs and modified PAHs (aceanthrenequinone, acenaphthene, acenaphthylene, acenaphthenequinone, anthracene, anthraquinone, naphthalene, phenanthrene, phenanthrenequinone, pyrene, 1,2-dihydroxyanthraquinone and 5-nitroacenaphthene). The sensitivity of the short- and long-term assays correlates well with other bioassays, especially when the importance of photosensitization and photomodification are considered. As well, the assays are applicable to non-photoactive chemicals, especially those where the rate of assimilation might be a factor.

2.2 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 as their molecular size increases (Jacob *et al.*, 1986). A major sources of PAH contamination in the environment is 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; Huang thesis, 1995; 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). During a photosensitization reaction, intracellular singlet-state oxygen ($^1O_2$) 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 the parent PAHs (Huang *et al.*, 1993; Huang *et al.*, 1995; McConkey *et al.*, 1997).

Several whole organism assays 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 (Beijerinck, 1889; Taylor, 1936; Johnoson *et al.*, 1942; Sie *et al.*, 1966; Grabow *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), *Photobacterium phosphoreum* was employed to detect toxic fumes within spacecraft. Bulich (1979) described the first commercial toxicity test using luminescent bacteria (Bulich 1979; 1986). This experimental system was unique in that the test organisms were freeze-dried preparations that were hydrated immediately prior to the assay. The end point is luminescence from the bacteria following short (<30 min) exposures to a toxicant. This bacterial test, sold under the trade name Microtox, utilized a selected strain of *Vibrio fischeri* (formerly known as *Photobacterium phosphoreum* NRRL B-11177) (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 (Lebsack *et al.*, 1981; Curtis *et al.*, 1988; Sanchez *et al.*, 1988; Kaiser and Palabrica 1991; Kaiser and Devillers 1994; Kaiser *et al.*, 1994). In general, it has been found that the toxicity of numerous chemicals to *V. fischeri* agrees well with toxicity to other aquatic organisms (Lebsack *et al.*, 1981; Curtis *et al.*, 1988; Sanchez *et al.*, 1988; Kaiser and Palabrica 1991; Fort 1992; Kaiser and Devillers 1994; Kaiser *et al.*, 1994). The comparisons of 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 only sensitive to fast acting molecules.

Further, the compounds must interfere with a process required for luminescence, and they must be rapidly assimilated. The assay is opaque to toxic by-products of the applied chemicals. In the case of PAHs all of these are real concerns. Thus, *V. fischeri* bioassay was developed that employed both short-term and long-term end-points. The short-term assay is based on measuring luminescence after 15 min exposure to a chemical. The long-term assay assesses the growth of *V. fischeri* after 18 h in the presence of a toxicant. The methods are simple, rapid and inexpensive, 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 15 min assay are collected, the bacteria can be 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. That is the assay is performed with bacteria in log-phase growth, instead of freeze-dried organisms. The assay is run in complex media instead of a saline solution. Finally, the experiment can be performed in the darkness and simulated solar radiation (SSR) to test for phototoxicity.

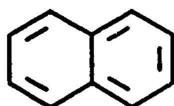
To evaluate this new assay, selected PAHs were tested for toxicity. They were aceanthrenequinone (AAQ), acenaphthene (ACE), acenaphthylene (ACY), acenaphthenequinone (ACQ), anthracene (ANT), anthraquinone (ATQ), 1,2-dihydroxyanthraquinone (1,2-dhATQ), naphthalene (NAP), 5-nitroacenaphthene (5-NACE), phenanthrene (PHE), phenanthrenequinone (PHQ), and pyrene (PYR) (Figure 2.1). This group contains intact-, oxy- and nitro-PAHs. It was found both the short-term and long-term *V. fischeri* assays were useful for evaluating direct and photoinduced toxicity of PAHs.

2.3 MATERIALS AND METHODS

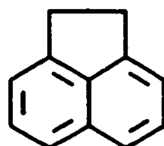
2.3.1 Bacterial Strain and Growth Conditions

The strain of luminescent bacteria used for these experiments was *Vibrio fischeri* NRRL B-11177 (formerly known as *Photobacterium phosphoreum*). It was obtained from

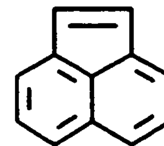
Figure 2.1 Structures of the twelve polycyclic aromatic hydrocarbons used in this study and their three-letter abbreviations.



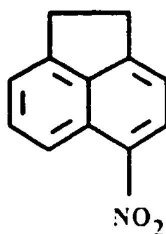
Naphthalene
NAP



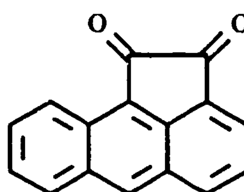
Acenaphthene
ACE



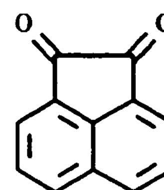
Acenaphthylene
ACY



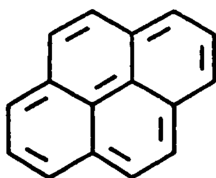
5-Nitroacenaphthene
5-NACE



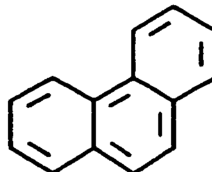
Aceanthrenequinone
AAQ



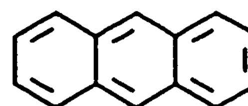
Acenaphthenequinone
ACQ



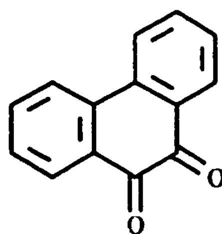
Pyrene
PYR



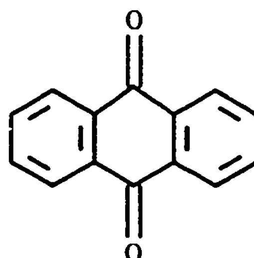
Phenanthrene
PHE



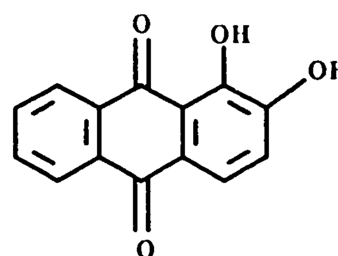
Anthracene
ANT



Phenanthrenequinone
PHQ



Anthraquinone
ATQ



1,2-dihydroxyanthraquinone
1,2-dhATQ

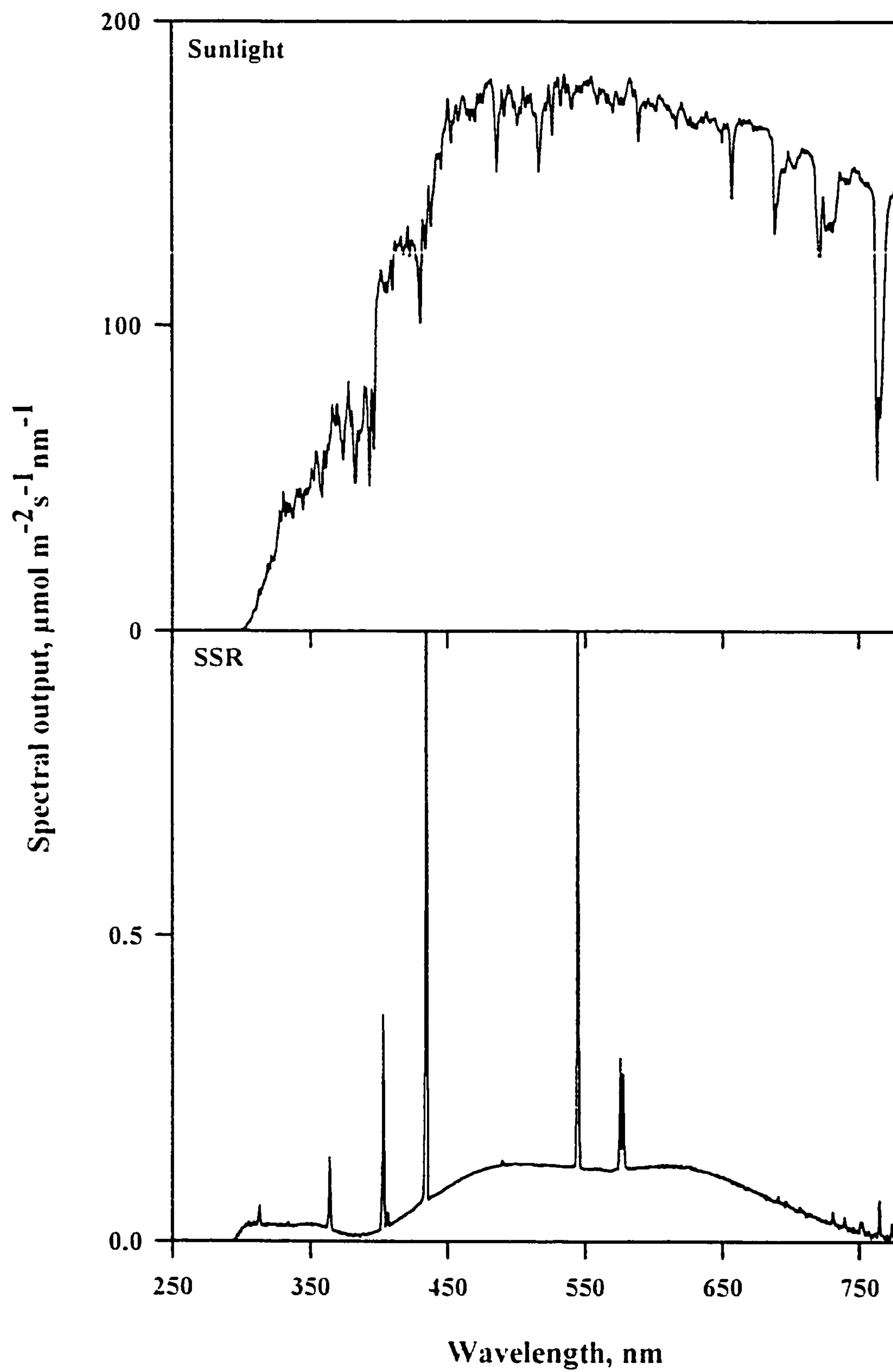
the Midwest Area National Center for Agricultural Utilization Research, Peoria, Illinois, USA. Aseptic techniques were employed throughout. *Vibrio fischeri* were grown in a complex liquid media which contained the following: KH_2PO_4 18.M, NaCl 0.5 M, $MgSO_4.7H_2O$ 4.1 mM, Glycerol 54.3 mM, Yeast extract (1 mg L^{-1}) Peptone (5 mg L^{-1}), and Bactopeptamin (1 mg L^{-1}). When solid media 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 media was adjusted to 7.2 ± 0.1 with 10 M NaOH. 1 L of complex media was distributed into 250 ml flasks (100 ml per flask) and covered with cotton plugs, and autoclaved at 121 °C for 30 minutes. Peptone and Yeast extract were obtained from BDH Inc., Toronto, ON, and Bactopeptamin was obtained from Difco Laboratories, Detroit, MI. Stock cultures of *V. fischeri* were grown on agar plates and used for primary inoculation of liquid culture.

2.3.2 Bacterial Growth and Luminescence emission at room temperature

Cells of *V. fischeri* were cultured in 100 ml of complex media in 250 ml flasks at room temperature (20°C ± 1) with shaking for 20 hours. 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.

An aliquot of 20 ml of cells was taken from the flask and diluted with complex media to yield an absorbance of 0.40 at 650 nm. Several aliquots of 500 µl from the diluted liquid culture were mixed with 500 µl of complex media and placed in a 48-well cell culture plate (Costar Safety Products, VWR Scientific Ltd, Toronto, Ontario, Canada). They were then grown in the dark or simulated solar radiation (SSR) with shaking at room temperature for up to 48 hours. SSR was generated with an artificial lighting system that mimics the spectral quality of natural sunlight Figure 2.2 (Huang *et al.*, 1993; Greenberg *et al.*, 1995). The SSR source contained eight daylight fluorescent lamps, two 350 nm UV lamps and four 300 nm UV lamps (UV lamps were obtained from Southern New England Ultraviolet Co., Brantford, Ontario, Canada). The light was

Figure 2.2 Spectral distribution of sunlight and a simulated solar radiation (SSR) source. **Panel A:** The spectral output of natural sunlight at noon on a cloudless summer day in Waterloo, Ontario, Canada (43°N). **Panel B:** Emission spectrum of an SSR source used in toxicity test with *Vibrio fischeri*. The spectrum provided are from a source with a total fluence rate of 40 µmol m^{-2} s^{-1}. Both spectra were collected with a photodiode array spectroradiometer.



Sunlight
SSR
200
100
0
0.5
0.0
250
350
450
550
650
750
Spectral output, µmol m-2s-1nm-1
Wavelength, nm

filtered through two layers of cheese cloth and the polystyrene culture plate top. The latter absorbed all 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, USA). 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 $m^{-2} s^{-1}$ (Figure 2.2).

2.3.3 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 multi-well plate fluorescence measurement system (Millipore Ltd., Mississauga, Ontario, Canada) with the excitation light source switched off to eliminate any background fluorescence.

2.3.3.1 Short-term assay

Prior to toxicity testing, cultures of *V. fischeri* were grown at 20°C ± 1 for 20 hours. 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, ON, Canada). After 5 minutes acclimatization in the 48-well cell culture plates, luminescence was measured. After the initial measurement of bacterial luminescence, the twenty-four of 500 µl bacteria cell culture dosed with equal volumes of complex media containing the test chemicals at twice the concentration required for the toxicity testing. 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 test chemical in a geometric series were used. For toxicity testing of each chemical, stock solutions were prepared in dimethyl sulphoxide (DMSO) and diluted with culture media 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. 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. After dosing, the cells were incubated in darkness or SSR at room temperature for 15 minutes and the luminescence intensity re-measured with the Cytofluor 2350 fluorescence measurement system (Millipore Ltd., Mississauga, Ontario, Canada).

2.3.3.2 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 returned to the incubator. The cultures were incubated for 18 hours in darkness or SSR, at room temperature with shaking. After 18 hours of incubation, luminescence intensity and cell density was measured for each sample.

2.3.4 Calculations of EC50s and data analysis

The procedure for calculating the EC50 is as follows. Inhibition of luminescence intensity was used to assess the EC50 in the short-term test. For the long-term test, both inhibition of light emission and growth were used. Percent inhibition of luminescence was determined according to equation (1).

$$\% \text{ Light Inhibition} = 100\,(1-(L_f \, . \, C_i / L_i \, . \, C_f)) \quad (1)$$

Where L_i is the initial luminescence of the bacteria prior to toxicant exposure, L_f is the luminescence of the bacteria following a 15 minute or 20 hour chemical exposure. C_i and C_f are the initial and final luminescence of the control (non-treated) bacteria.

Calculation of EC50s (the toxicant concentration effective at causing a 50% reduction in light output) were based on a logit function for continuous response data (McConkey *et al.*, 1997; Sananthanan, 1987). The data for % inhibition vs. chemical concentration were fit to equation (2).

$$\% \text{ Light Inhibition} = 100/\left(1 + e^{\beta(\ln x - \ln \mu)}\right) \quad (2)$$

Where x is the concentration of the test chemical, μ is the EC50, 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 equation (3)

$$CD_t = CD_0 \times 2^n \quad (3)$$

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

$$n = \log\left(CD_t / CD_0\right) / \log 2 \quad (4)$$

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

$$GR = n / t \quad (5)$$

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

$$\% \text{ Inhibition of GR} = 100\left(1 - \left(GR(\text{Treated}) / GR(\text{Control})\right)\right) \quad (6)$$

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

2.4. RESULTS

2.4.1 Bacterial Growth and Luminescence Emission

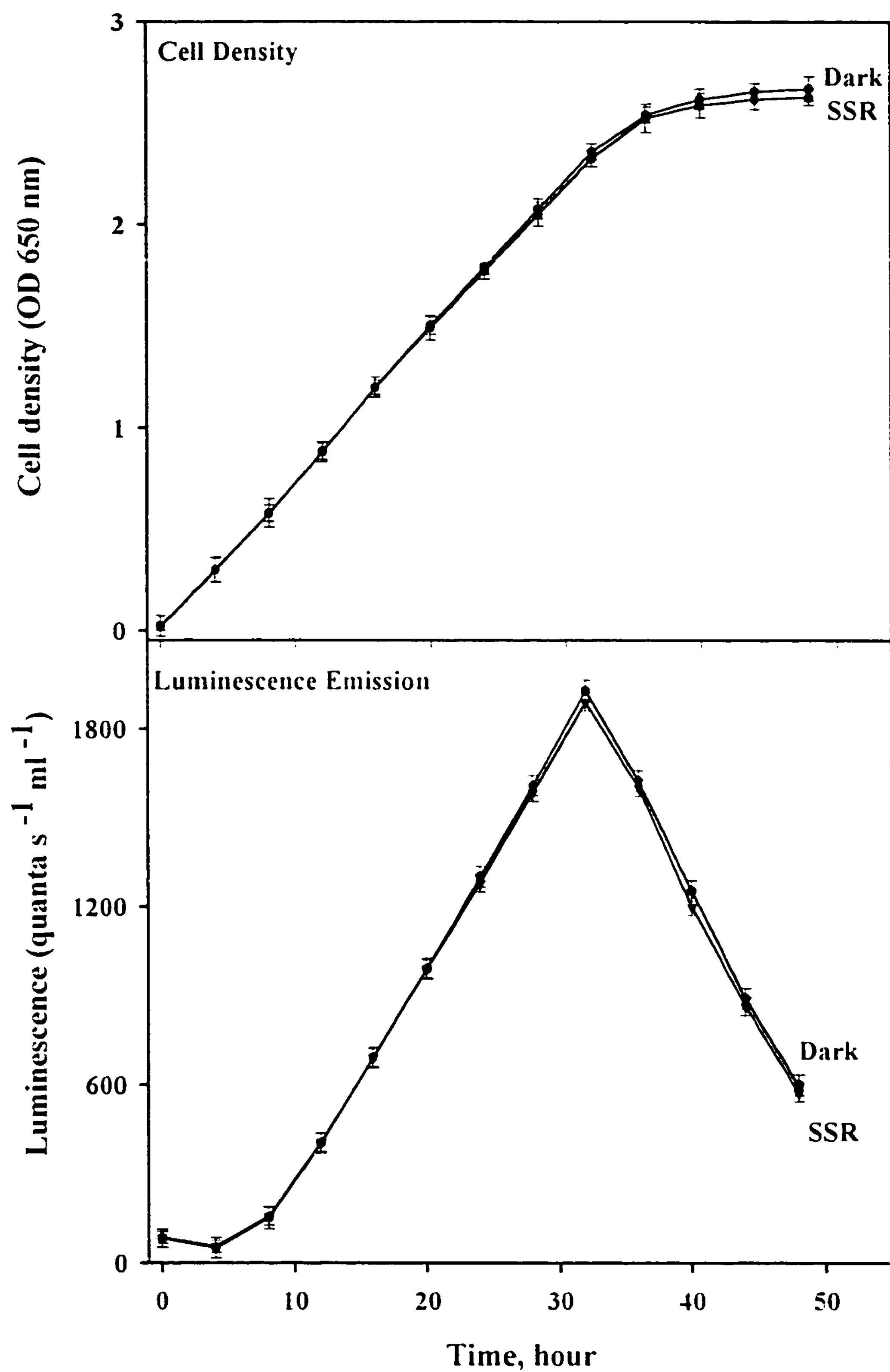
The optimum conditions for growth of the *Vibrio fischeri* was determined prior to the toxicity assays. The growth and luminescence of *Vibrio fischeri* were determined in complex media in darkness and SSR as a function of time (Figure 2.3). When *V. fischeri* cells in log-phase growth are diluted into fresh media, log-phase growth continues for about 30 hours, at which point cells shift into stationary phase. In log-phase growth the doubling time was approximate 120 min, which this is a good rate of growth of *V. fischeri* (Takahide, 1980). There was essentially no difference in *V. fischeri* growth in darkness or SSR (Figure 2.3).

Bacterial luminescence remained approximately constant for 8 hours after the cells were diluted into fresh media (Figure 2.3). Following that, luminescence rose with cell growth until it reached a peak at about 30 hours. Luminescence then dropped as the cells went into stationary phase. Again there was no difference between SSR and darkness. From this data, it was concluded that a long-term assay of toxicity with *V. fischeri* should last 15 to 20 hours. This way, both cell density and luminescence can be used as endpoints.

2.4.2 Short-term Toxicity

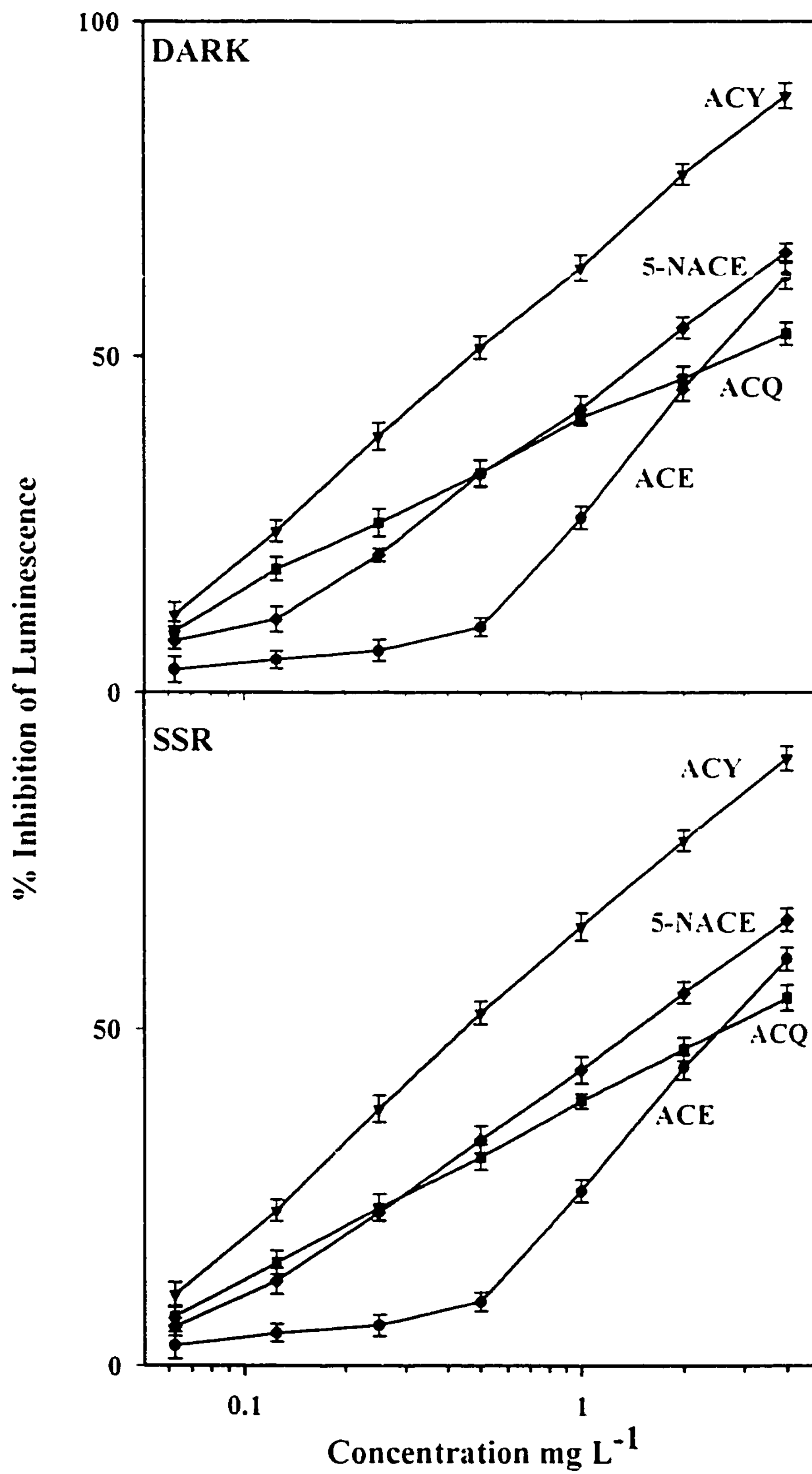
Vibrio fischeri was incubated in dark and SSR with each of the twelve PAHs to determine their relative toxicity. The compounds tested were ACE, ACY, AAQ, ACQ, 5-NACE, PHE, ANT, ANQ, NAP, 1,2-dhANQ, PHQ and PYR. The results of short-term toxicity of PAHs to *V. fischeri* are given in Figure 2.4 and Table 2.1. Full concentration-

Figure. 2.3 Cell density and luminescence emission of *Vibrio 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 hours. Cells were grown in complex media. Luminescence and cell density were determined as described in materials and methods.



Cell Density
Dark
SSR
Cell density (OD 650 nm)
3
2
1
0
Luminescence Emission
Luminescence (quanta s -1 ml -1)
1800
1200
600
0
Dark
SSR
0
10
20
30
40
50
Time, hour

Figure. 2.4 Dose-response of *V. fischeri* to ACE, ACY, ACQ, and 5-NACE. *Vibrio fischeri* was exposed to a concentration series of these PAHs for 15 minutes in either SSR or darkness. Bacterial response was measured as percent inhibition of luminescence and plotted versus chemical concentration (mgL^{-1}). Error bars are the 95% confidence intervals.



DARK
ACY
5-NACE
ACQ
ACE
SSR
ACY
5-NACE
ACQ
ACE
100
50
0
50
0
0.1
1
% Inhibition of Luminescence
Concentration mg L-1

Table 2.1 Calculated EC50s (mg/L) for short-term toxicity of selected PAHs to *Vibrio fischeri*. The assays were carried out under darkness or SSR. EC50s are based on % inhibition of luminescence after a 15 minute incubation. All experiments are the average of nine replicates and errors are the 95% confidence intervals.

		Short-term toxicity	
Toxicant	**Abbreviation**	**Dark**	**SSR**
Aceanthrenequinone	AAQ	24.39 ± 0.24	23.70 ± 0.41
Acenaphthene	ACE	2.41 ± 0.46	2.52 ± 0.48
Acenaphthylene	ACY	0.80 ± 0.08	0.77 ± 0.05
Acenaphthenequinone	ACQ	2.67 ± 0.24	2.65 ± 0.28
Anthracene	ANT	N/D	N/D
Anthraquinone	ANQ	N/D	N/D
1,2-dihydroxyanthraquinone	1,2-dhANQ	7.11 ± 0.16	7.24 ± 0.25
Naphthalene	NAP	2.36 ± 0.34	2.33 ± 0.25
5-Nitroacenaphthene	5-NACE	2.29 ± 0.29	2.16 ± 0.21
Phenanthrene	PHE	7.33 ± 0.41	6.89 ± 0.32
Phenanthrenequinone	PHQ	0.92 ± 0.04	0.81 ± 0.06
Pyrene	PYR	N/D	N/D

N/D: No detectable effect at the highest concentration.

response curves shown for short-term toxicity of ACE, ACQ, ACY, and 5-NACE. 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. ACY was the most toxic to *V. fischeri*. There was no apparent change in toxicity in SSR vs. darkness for any of the 4 chemicals.

Using concentration response tests similar to those shown in Figure 2.4, EC50s were determined for all 12 chemicals. The EC50s for the compounds are in Table 2.1. 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 an effect. Interestingly, in both SSR and darkness, PHQ and 1,2-dhANQ were more toxic than their parent PAHs (Table 2.1).

To determine if the toxicity of the twelve PAHs could be enhanced by light, *V. fischeri* was incubated in SSR with the chemicals (Figure 2.3). The calculated EC50s for each chemical were nearly the same in darkness or SSR (Table 2.1). It is possible that the time period of the assay was not long enough for photomodification and/or photosensitization reactions to be effective.

2.4.3 Long-term Toxicity

Because none of the PAHs were phototoxic in the short-term assay, a long-term assay was developed to test for photoinduced toxicity to bacteria. *Vibrio fischeri* was incubated in darkness and SSR for 18 hours to determine the relative toxic potency of each chemical (Figure 2.5 and Table 2.2). The incubation was carried out in complex media to allow bacterial growth. Two toxicity endpoints were employed. The first was inhibition of luminescence, and second was inhibition of growth. Both endpoints gave similar results for each of the chemicals tested (Figure 2.5 and Table 2.2).

Full concentration-response curves for ACE, ACQ, ACY, and 5-NACE in darkness and SSR were determined for the long-term assay (Figure 2.5). ACE and 5-

Figure. 2.5 Dose-response of *V. fischeri* to Acenaphthene, Acenaphthylene, Acenaphthenequinone and 5-Nitroacenaphthene. *Vibrio fischeri* was exposed to a concentration series of PAHs for 18 hours (long-term toxicity) in darkness or SSR. Bacterial response was measured as percent inhibition of luminescence and as percent inhibition of growth. The percent inhibition in both cases were plotted versus chemical concentration (mg L^{-1}). n = 9.

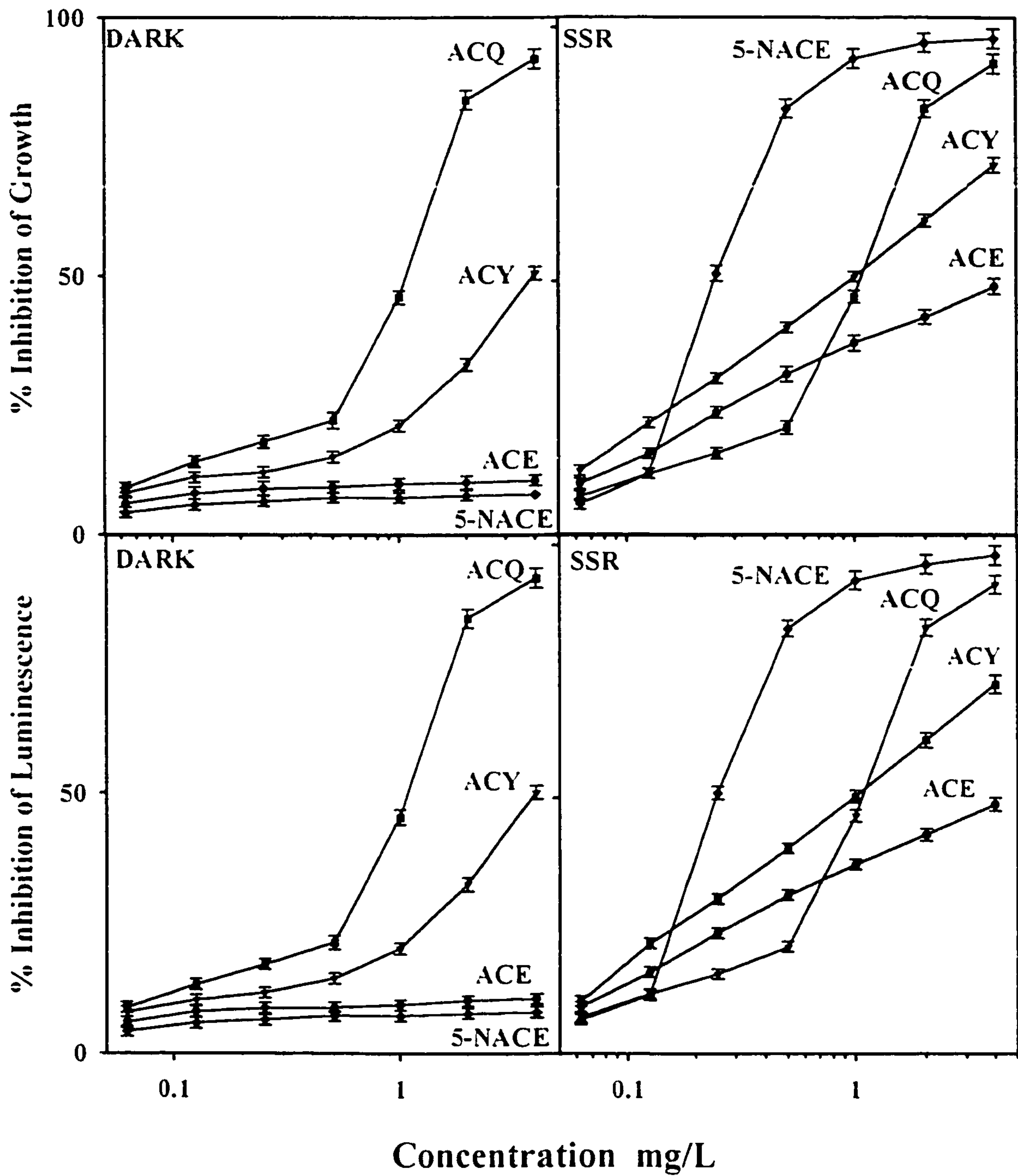

DARK
SSR
ACQ
ACY
ACE
5-NACE
100
50
0
% Inhibition of Growth
% Inhibition of Luminescence
0.1
1
Concentration mg/L

Table 2.2 Calculated EC50s (mg/L) for Long-term Toxicity of PAHs to *Vibrio fischeri*. Bacteria were grown under SSR and darkness for 18 hr. Inhibition of luminescence and Inhibition of growth were determined for each chemical from which the EC50s were derived all data are the average of nine replicates. The 95% confidence intervals are also provided.

		Long-term toxicity			
		Inhibition of Luminescence		Inhibition of Growth	
Toxicant	**Abbreviations**	**Dark**	**SSR**	**Dark**	**SSR**
Aceanthrenequinone	AAQ	N/D	N/D	N/D	N/D
Acenaphthene	ACE	N/D	5.21 ± 0.26	N/D	4.68 ± 0.30
Acenaphthylene	ACY	6.57 ± 0.42	1.47 ± 0.24	6.05 ± 0.29	1.39 ± 0.21
Acenaphthenequinone	ACQ	1.88 ± 0.11	1.52 ± 0.18	1.74 ± 0.10	1.41 ± 0.25
Anthracene	ANT	N/D	N/D	N/D	N/D
Anthraquinone	ANQ	N/D	N/D	N/D	N/D
1,2-dihydroxyanthraquinone	1,2-dhANQ	5.80 ± 0.30	5.61 ± 0.20	5.45 ± 0.35	5.28 ± 0.31
Naphthalene	NAP	N/D	N/D	N/D	N/D
5-Nitroacenaphthene	5-NACE	N/D	0.62 ± 0.19	N/D	0.58 ± 0.17
Phenanthrene	PHE	8.09 ± 0.33	7.15 ± 0.37	7.38 ± 0.41	6.72 ± 0.35
Phenanthrenequinone	PHQ	0.66 ± 0.11	0.69 ± 0.10	0.62 ± 0.09	0.65 ± 0.11
Pyrene	PYR	N/D	N/D	N/D	N/D

N/D: No detectable effect at the highest concentration tested.

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 4 compounds in SSR. ACQ shows same level of toxicity to *V. fischeri* in darkness and SSR. ACY was toxic to *V. fischeri* in darkness. However, toxicity was enhanced by exposure to SSR. Using concentration-response experiments, the EC50s for luminescence and growth were determined for all 12 chemicals in this study. For both end points the experiments were done in SSR and darkness (Table 2.2). AAQ, ANT, ANQ, NAP and PYR were nontoxic 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 EC50s in SSR relative to darkness (ACQ and 1,2-dhATQ). However, 4 compounds showed enhanced toxicity in SSR vs. darkness (ACE, ACY, 5-NACE and PHE).

Upon comparing the short-term and long-term toxicity test, two trends 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 vs. the short-term assay. Conversely, for several compounds, the EC50 were similar in both assays (ACQ, 1,2dhAQ, and PHQ). Second, photoinduced toxicity was only evident in the long-term assay.

2.5 DISCUSSION

2.5.1 Growth and Luminescent Emission

The manner in which *Vibrio fischeri* is cultured and the purpose of culture media vary widely. The media used here was modified from other published media. The media formulated promotes strong growth and results in a high yield of luminescence. In previous studies, maximum luminescence was obtained when *V. fischeri* was grown in media containing glycerol as a carbon source (Hill and Shoup, 1929; Kempner and Hanson, 1968; Takahide and Nakamura, 1980). As well, growth at pH 7.2 is optimal for luminescence from this bacterium (Johnson, 1947; Kempner and Hanson, 1968). The best temperatures for growth and luminescence of *V. fischeri* have been found to be between

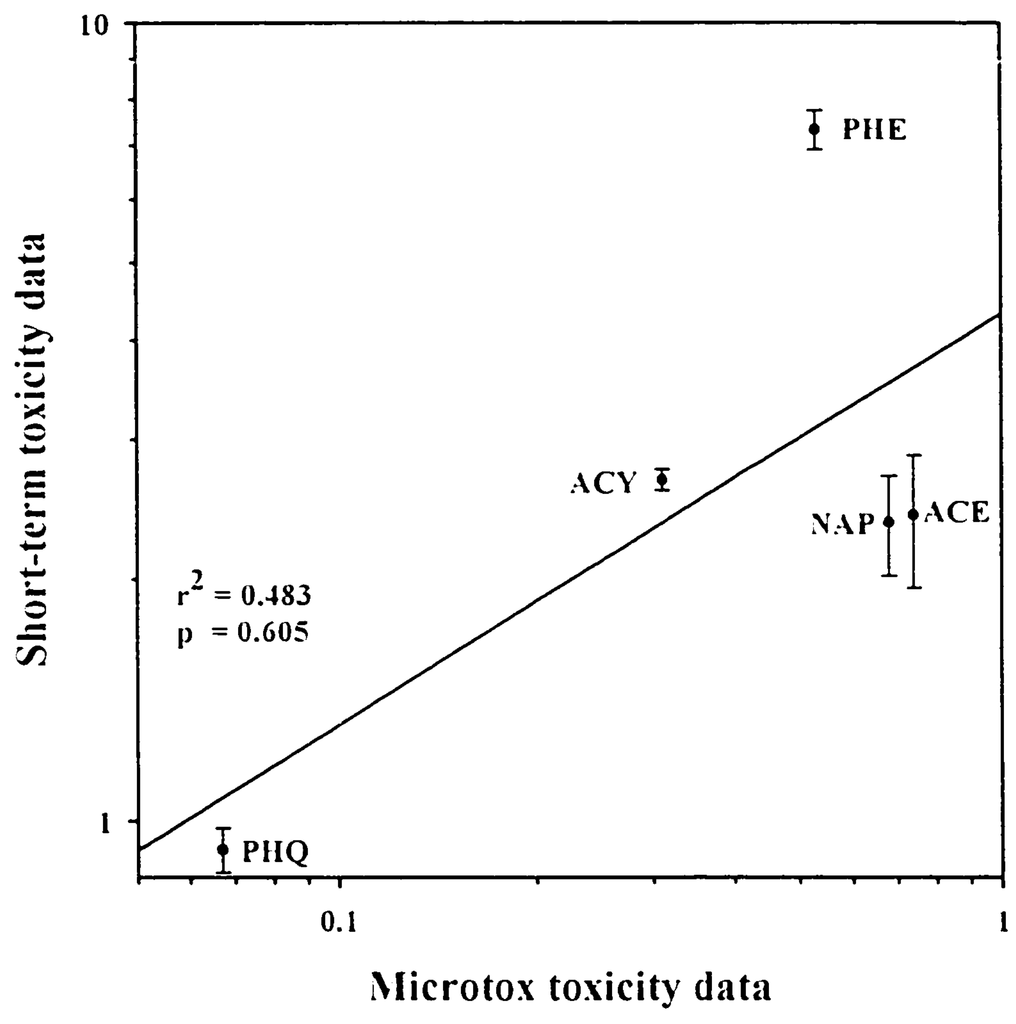
20 °C and 25 °C (Harvey, 1952; Kempner and Hanson, 1968; Takahide and Nakamura, 1980). Bacterial bioluminescence is dependent on active respiration, and thus requires oxygen. Vigorous shaking of cultures grown in flasks has been found to be adequate to achieve maximum luminescence (Nakamura and Matsuda 1971; Takahide and Nakamura, 1980). Therefore, *V. fischeri* in this study was cultured in a media containing glycerol, Yeast extract, Peptone and Bactopeptamin at pH 7.2 ± 0.1. Growth was at room temperature (23 °C) with vigorous shaking. This achieved rapid growth and strong luminescence emission. It was found that, after 15 to 20 hours, the cells were in mid log-phase growth and luminescence increased in parallel with growth. Therefore, the cells were grown for 18 hours in the long-term toxicity assay.

2.5.2 Short-term Toxicity

The calculated short-term toxicity for all PAHs showed that all the chemicals had the same impact on *V. fischeri* in darkness and SSR. The calculated EC50s of ACQ, ACE, ACY, NAP, and PHE were compared with toxicity published (Jacobs *et al.* 1993; Kaiser, 1994) (Figure 2.6). The calculated EC50 of PHE was 7.33 mg/L, which was higher than that obtained by McConkey *et al.*, (1997). However, PHQ toxicity to *V. fischeri* in the short-term toxicity agrees well with that published by McConkey *et al.*, (1997). When one compares the EC50s reported here to the above published data, one observes a similar level of toxicity (Figure 2.6). However, the EC50s reported here were consistently higher than those in the literature (Figure 2.6). This is probably due to the sugar and other forms of reduced carbon in the complex media. This would probably bind to the PAHs, restricting their bioavailibility. In contrast, the Microtox assay is performed in a saline solution.

There was little or no toxicity from ANT and PYR to *V. fischeri* in the short-term assay, similar to published results for these chemicals (Jacobs *et al.*1993; Kaiser 1994) One might attribute the above discrepancies between this data and the Microtox data to differences in media used. The complex media used in this study contains high levels of reduced organic carbon (sugar, amino acids, and proteins). Conversely, the Microtox

Figure 2.6 Correlation between short-term toxicity (From Table 2.1) and Microtox assay data (Jacob et al., 1993)



10
1
0.1
1
PHE
ACY
NAP
ACE
PHQ
r2 = 0.483
p = 0.605
Short-term toxicity data
Microtox toxicity data

assay is performed in saline solution. The high organic content of the complex media could impact on PAH bioavailability. This is one reason DOC is thought to ameliorate PAH toxicity (Gensemer *et al.*, 1996).

In the short-term assay none, of the PAHs tested exhibited a large increase in toxicity in SSR compared to darkness. This indicates that photoactivation of PAHs was not a factor in these assays. This could be due to the short exposure time to SSR. In 15 minutes there would be little or no photooxidation of PAHs. Also, there would be little time for cumulative damage from reactive oxygen species. 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 problem with short-term bacterial assays in detecting the effects of chemicals such as PAHs that may need time to be activated.

2.5.3 Long-term Toxicity

To allow for the measurement of the photoinduced toxicity of the PAHs, a longer term assay of toxicity was attempted. Two end points of toxicity were used: growth and luminescence. This might help to assess if the chemicals impacted on the luminescence mechanism (i.e. *V. fischeri* specific), or if toxicity is more general in nature by affecting growth more than luminescence. It is striking that the EC50s for both assays 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 or SSR. ACE and 5-NACE were not toxic under darkness, but had toxicity under SSR. In addition, the EC50 for ACY dropped about 5-fold, in going from darkness to SSR. This showed that photochemical toxicity of PAHs can be observed when a long term assay is used. Moreover, of the 4 toxic chemicals that did not 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.*, 1997).

The toxic response of PAHs is known to be greatly enhanced upon exposure to sunlight or SSR. This is especially true for ANT (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, PYR), both of which are known to be phototoxic, did not exhibit any 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.*, 1999). One possibility why ANT and PYR were not toxic is that the photoproducts did not have enough time to form in an 18 hour assay. It is also possible that their photooxidation would be impeded in complex media. This is because the reduced carbons in the media would act as antioxidants. Under such a scenario, early in exposure, before photoinduced toxicity sets in, the bacteria could out-compete the chemical and effectively dilute the chemical. Further, if the chemicals are not bioavailable (i.e., the sugar in the media might lower bioavailability), the bacteria might grow faster than the rate of assimilation. Another possibility is the sugar in the media quenches PAHs in excited state thus preventing photooxidation of the chemicals. The other compounds found to be non-toxic in the long-term assay were AAQ, ATQ and ANP. NAP is generally not a highly toxic compound in aqueous assays. This is probably due to its high volatility (Ren *et al.*, 1994). In the case of AAQ and ANQ, PAH quinones are often 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.*, 1997). Perhaps their phototoxic potential would be realized if assimilation of the chemicals was allowed to occur. This might be achieved if there was a pre-incubation in minimal media. Therefore, a modification of the procedure is warranted to be able to observe a wider range of photochemical effects. This is the subject of the next two chapters.

2.6 CONCLUSIONS

Based on our work with the luminescent bacteria, a simple, rapid, repeatable 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 to toxicity direct effects and photoinduced effects. In a few cases, toxicity was greatly enhanced by light. However, it was striking that the highly phototoxic ANT was not toxic in SSR in this study. It appeared that the low toxicity of some PAHs observed in this study can be attributed to two different reasons. First, the low bioavailability of PAHs to the bacteria. Second, rapid growth of the bacteria, which can outstrip the effects of the chemicals.

CHAPTER 3

EVALUATION OF PRE-INCUBATION FACTORS ON SHORT AND LONG TERM PHOTOINDUCED TOXICITY OF ANTHRACENE TO LUMINESCENT BACTRIA [1]

3.1 ABSTRACT

Anthracene toxicity increases significantly after exposure to simulated or natural sunlight. A simple, rapid method for monitoring the photoinduced short- and long–term toxicity of PAHs has been developed. The assay is based on changes in the luminescence intensity and growth rate of *Vibrio fischeri*. Under suitable conditions, *V. fischeri* emits a constant amount of light as a metabolic by-product. After exposure to a toxicant, the light intensity can be diminished by an amount, which is often proportional to the extent of toxicant impact. Generally, *V. fischeri* has been used in short-term (15-30 min) assays. However, this does not reveal the toxicity of all hazardous chemicals. In particular, we found some intact PAHs were not highly toxic in short-term assays. As well, the known phototoxicity of those compounds was not revealed in short-term assays. Further, in a long-term assay based on cell growth, the highly phototoxic PAH, ANT was inert. Therefore, a long-term assay was developed that employs a light or dark pre-incubation period. This was found to reveal the extreme photoinduced toxicity of PAHs.

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[1] Submitted to American Society for Testing and Materials. Co-authors are D. G. Dixon and B. M. Greenberg.

3.2 INTRODUCTION

Many intact and photomodified polycyclic aromatic hydrocarbons (PAHs) are present in aquatic environments (Huang, *et al.*, 1999). PAHs are ubiquitous in the environment primarily as a product of incomplete combustion of fossil fuels (Cook, *et al.* 1983; Edwards, 1983; Jacob, *et al.* 1986; Cook, *et al.* 1983). They have been identified in surface and drinking water, ambient air, exhaust emissions from internal combustion engines, tobacco smoke, smoked food, edible aquatic organisms, and in sediments (Edwards, 1983; Cook, *et al.* 1983; Jacob, *et al.* 1986). A large body of literature exists on the toxicity and carcinogenicity of PAHs (Jacob, *et al.* 1986, Cook, *et al.* 1983).

The phototoxic properties of PAHs are well studied in aquatic environments (Allred and Giesy, 1985; Kagan, *et al.*, 1990, Krylov, *et al.*, 1997). These studies strongly suggest that there is a real hazard to aquatic ecosystems from photoinduced toxicity of PAHs (Kochevar, *et al.*, 1982; Allred and Giesy, 1985, Kagan, *et al.*, 1990; Johnson, *et al.*, 1993; Huang, et al 1993, 1995, Mallakin, *et al.*, 1999). Previous work using ANT showed that toxicity is enhanced through via photosensitization and photomodification reactions (Ren *et al.*, 1994; Huang, *et al.*, 1993, 1995; Duxbury, *et al.*, 1997; Mallakin, *et al.*, 1999). In particular, several photoproducts are generated when ANT is exposed to simulated or natural solar radiation. The time frame for generation of photoproducts is on the order of hours. Many of the photoproducts have significant toxic activity (Huang, *et al.* 1997; Mallakin, *et al.*, 1999).

Several bioassays have been developed to test toxicity to aquatic organisms. These include changes in growth, survival, metabolism, inhibition of enzymatic activities, and inhibition of photosynthetic and mitochondrial electron transport (Bulich and Isenberg, 1981; Steinberg, *et al.*, 1995). Among these toxicity tests are bacterial luminescence assays. Bacterial luminescence assays are important tools in aquatic toxicology because they are rapid, simple and accurate (Bulich, 1979; Curtis, *et al.*, 1982; Bulich and Isenberg, 1981; Environment Canada, 1993). The luminescent bacteria *Vibrio fischeri* has been used extensively as a short-term assay of toxicity (Environment Canada, 1993; Steinberg, *et al.*, 1995). For instance, the Microtox assay is a 15 min test based on loss of luminescence (Environment Canada, 1993; Steinberg, *et al.*, 1995). However, this

assay returns false negatives for chemicals that act slowly or require time to be assimilated. To wit, ANT, which increases dramatically in toxicity following photomodification, has been found to have little impact in the Microtox test (Kaiser and Devillers, 1994). Furthermore, in the previous chapter a long-term assay was developed to probe for photoinduced toxicity. It was an 18 hour growth test. However, ANT was non-toxic in that assay, possibly because cell growth outpaced toxic effects. Thus, the *V. fischeri* long-term toxicity assay was modified to include a pre-incubation step in minimal media to allow ANT to be photomodified and assimilated before cell growth ensues. ANT was indeed found to have high toxicity when there was a pre-incubation period in simulated solar radiation (SSR).

3.3 MATERIALS AND METHODS

3.3.1 Chemicals and Bacterial Strain

Anthracene was purchased in high purity (Sigma Chemical Co., St. Louis, Mo). The strain of luminescent bacteria used for this study was *Vibrio fischeri* (strain NRRL B-11177). It was obtained from the Midwest Area National Center for Agricultural Utilization Research (Peoria, Illinois, USA).

3.3.2 Growth Media

V. fischeri was cultured in complex media of the following formulation: Monobasic Potassium Phosphate (KH_2PO_4), 2.5 g/L; Sodium Chloride (NaCl), 30 g/L; Magnesium Sulfate ($MgSO_4.7H_2O$), 1 g/L; Glycerol ($C_3H_8O_3$), 5 g/L; Yeast extract, 1 g/L; Peptone, 5 g/L; Bactopeptamin, 1 g/L; and agar when required, 15 g/L. The final pH of the media was adjusted to 7.2 ± 0.1 with 10 M NaOH. The mixture was autoclaved at 121°C for 30 minutes. Minimal media was the above without glycerol, yeast extract, peptone, and bactopeptamin. Peptone and Yeast extract were obtained from BDH Inc., Toronto, ON, and Bactopeptamin was obtained from Difco Laboratories, Detroit, MI. Stock cultures of *V. fischeri* were grown on agar plates and used for primary inoculation

of liquid cultures. A Spectrophotometer (Perkin-Elmer, Mississauga, Ontario, CA) and Cytofluor 2350 multi-well-plate fluorescence measurement system (Millipore Ltd., Mississauga, Ontario, Canada) were used to measure cell density and light output.

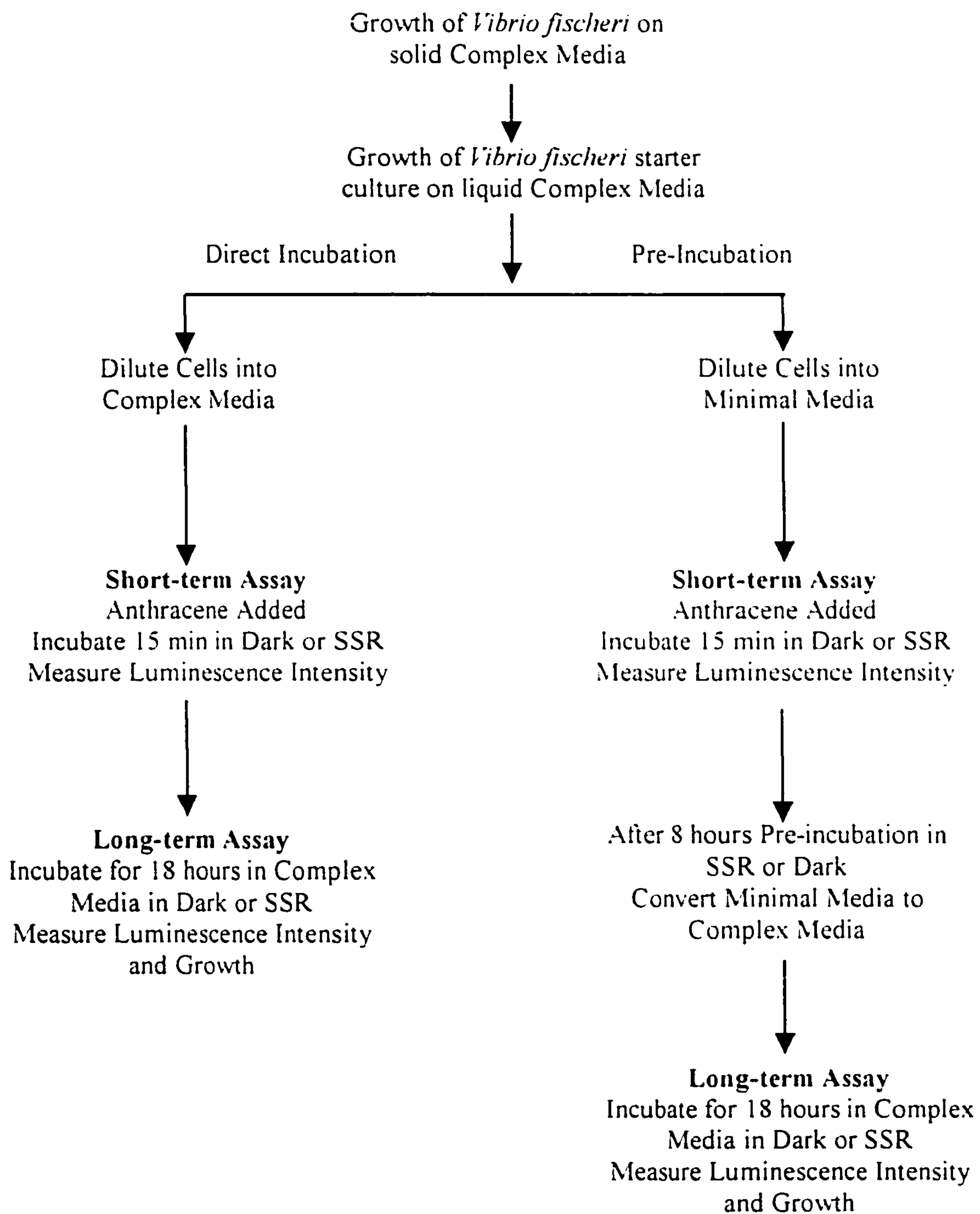
3.3.3 Growth Conditions

To generate a starter culture for toxicity tests, cells of *V. fischeri* were cultured in 100 ml of complex media with shaking at room temperature (20°C ± 1) for 24 hours. Two methods were used to follow *V. fischeri* growth (Figure 3.1). The first method involved diluting cells from the starter culture into complex media. The second method involved diluting cells into minimal media (complex media without a reduced carbon source) for a 4 or 8 hour pre-incubation period, at which point the media was made complex by addition of the above reduced carbon sources (Figure 3.1)

For the first method, 0.5 ml of actively growing starter cells in log-phase were harvested and put into 20 ml of complex media to give an approximate cell absorbance of 0.4 at 650 nm. Aliquots of 0.5 ml of this diluted bacterial suspension were added to 0.5 ml complex media in the wells of a 48-well culture plate (Costar Safety Products, VWR Scientific Ltd, Toronto, Ontario. Canada). The bacteria were then grown in the darkness or SSR with shaking at 20°C for up to 28 hours.

For the second method, 0.5 ml of *V. fischeri* cells from the starter culture were diluted into 20 ml of minimal media to an optical density (OD) of 0.4 at 650 nm. Aliquots (0.5 ml) of the diluted cells were added to 0.5 ml of minimal media in the wells of a 48-well culture plate. Cells were pre-incubated for 4 hr or 8 hr in minimal media in SSR or darkness. After the pre-incubation period, an equal volume of complex media with glycerol ($C_3H_8O_3$) at 10 g/L, yeast extract at 2 g/L, peptone at 10 g/L bactopeptamin at 2 g/L was added to each well in the culture plate. Cells were grown for up to 28 hours in the darkness or SSR. Cell density and luminescence intensity of the culture was measured every 4 hours. The cell density of the culture was measured with spectrophotometer. The luminescence intensity was measured with a Cytofluor 2350 multi-well plate reader.

Figure 3.1 Flow-chart for short and long-term toxicity tests in complex media and minimal media.



Growth of *Vibrio fischeri* on solid Complex Media
Growth of *Vibrio fischeri* starter culture on liquid Complex Media
Direct Incubation
Pre-Incubation
Dilute Cells into Complex Media
Dilute Cells into Minimal Media
Short-term Assay
Anthracene Added
Incubate 15 min in Dark or SSR
Measure Luminescence Intensity
Short-term Assay
Anthracene Added
Incubate 15 min in Dark or SSR
Measure Luminescence Intensity
Long-term Assay
Incubate for 18 hours in Complex Media in Dark or SSR
Measure Luminescence Intensity and Growth
After 8 hours Pre-incubation in SSR or Dark
Convert Minimal Media to Complex Media
Long-term Assay
Incubate for 18 hours in Complex Media in Dark or SSR
Measure Luminescence Intensity and Growth

3.3.4 Short and long-term assays of toxicity

The short-term toxicity test was based on inhibition of light production in luminescent bacteria incubated for 15 minutes under darkness or SSR. For the long-term toxicity test, inhibition of light production by the bacteria and inhibition of growth were used. In the latter case, toxicity was measured after the 4 or 8 hour pre-incubation period and 18 hours of growth of the bacteria (Figure 3.1).

3.3.4.1 Short-term assay

The starter culture was diluted with complex or minimal media and twenty-four 500 µl aliquots of cells were distributed in a 48-well cell culture plate as above. After 5 minutes acclimatization in the 48-well cell culture plates, the luminescence intensity was measured. After the initial measurement of bacterial luminescence, the bacteria were dosed with ANT. Aliquots of 500 µl of the cell culture were combined with equal volumes of complex or minimal culture media with the test chemical. ANT was used, in a geometric concentration series in triplicate. Stock solutions of ANT were prepared in dimethyl sulphoxide (DMSO) and added by 1000-fold dilution to the culture liquid media to give the specified concentration. The DMSO concentration of 0.1% does not affect luminescence or growth of *V. fischeri*. After dosing, the cells were incubated in darkness or SSR at room temperature for 15 minutes and the luminescence intensity was measured.

3.3.4.2 Long-term assay

After the short-term test was completed, the multi-well culture plate was returned to the growth chamber. If the assay was in complex media, the cells were incubated for 18 hours in SSR or darkness at 20°C growth and luminescence were then determined as above. If the assay was in minimal media, cells were pre-incubated with the chemical in darkness or SSR at 20°C for 4 or 8 hours. After this pre-incubation, the media was made complex as above. The cells were then incubated for an additional 18 hours in SSR or darkness at 20 °C. Toxicity was determined by inhibition of growth and luminescence.

3.4 RESULTS AND DISCUSSION

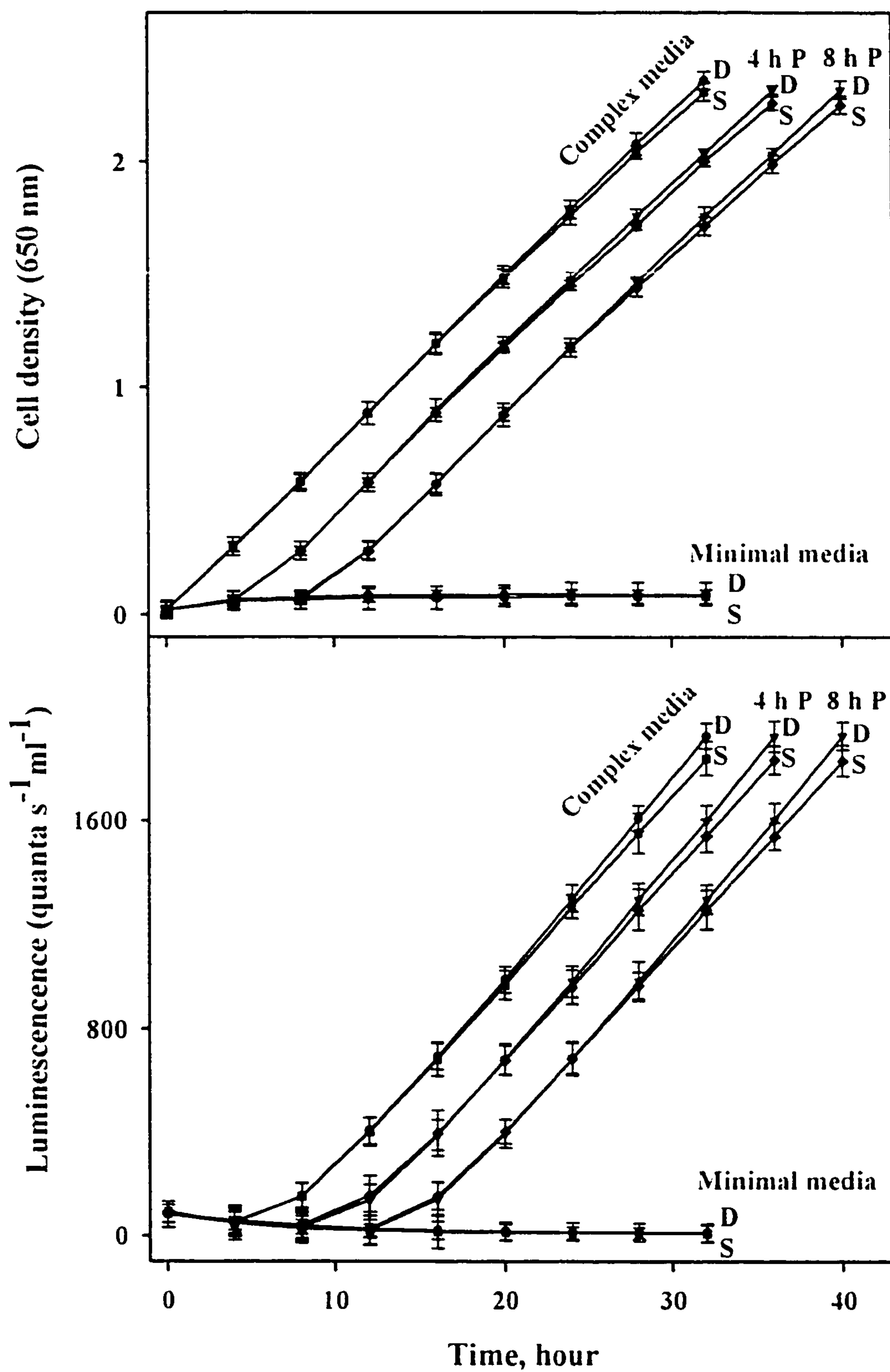
3.4.1 Growth and luminescence emission

When *V. fischeri* cells in log-phase growth were diluted into complex media, log-phase growth continued for more than 20 hours (Figure 3.2). For these cells, changes in luminescence showed a lag period of 8 hours, after which, increases in luminescence intensity mirrored cell growth. Relative to darkness, there was no effect of SSR on growth of the cells. When cells were placed in minimal media, little growth was observed and luminescence gradually dropped. After a 4 to 8 hours pre-incubation in minimal media, the media was made complex, and the cells shifted almost immediately into log-phase growth. The growth rate was identical to that of cells placed directly in complex media. Increases in luminescence again recovered after 4 or 8 hours lag period, and then mirrored growth. Once again, there was no effect of SSR relative to darkness. This demonstrates that a pre- incubation for 4 or 8 hours in minimal media in SSR or darkness does not harm the *V. fischeri*.

3.4.2 Short-term toxicity of PAHs on V. fischeri

In the short-term toxicity assay, V. fischeri was incubated with ANT in darkness and SSR in minimal or complex media (Figure 3.3). There was a modest inhibition of luminescence in minimal media at high ANT concentrations. The calculated EC50 of ANT in darkness in minimal media was 17.92 mg/L (Table 3.1), which agrees well with published EC50 obtained using the Microtox assay (Jacobs, et al. 1993). When the assay was performed in complex media no toxicity was observed (Figure 3.3, Table 3.1). The high reduced carbon content of the complex media could be limiting the bioavailability of ANT. This is consistent with previous studies where humic acids were shown to ameliorate toxicity of PAHs (Oris, et al., 1990; Gensemer, et al., 1997).

Figure 3.2 Optical density and luminescence intensity of *Vibrio fischeri* incubated in darkness or SSR. Log-phase cells were diluted into complex or minimal media as described in material and methods. For cells pre-incubated in minimal media for 4 or 8 hour (4 h P, 8 h P), the media was made complex as described in methods and materials. Bacteria were incubated in 4 ways: Complex media: bacteria were growth in complex media for 30 hours. 4 h P: bacteria were pre-incubated for 4 hour in the minimal media, the media was made complex and cells were grown for 30 hours. 8 h P: bacteria were pre-incubated for 8 hours in the minimal media, the media was made complex and cells were grown for 30 hours. Minimal media: bacteria were incubated in minimal media for 32 hours. All incubations were run in the darkness (D) and SSR (S).



Cell density (650 nm)
0
1
2
Complex media
D
S
4 h P
D
S
8 h P
D
S
Minimal media
D
S
Luminescencence (quanta s^{-1} ml^{-1})
0
800
1600
Complex media
D
S
4 h P
D
S
8 h P
D
S
Minimal media
D
S
0
10
20
30
40
Time, hour

Conversely, the complex media could allow for healthier cells, and thus tolerance to chemical stress.

To determine if short-term toxicity of ANT could be photoinduced, the assay was performed in SSR. No light effect was observed in complex or minimal media (Table 3.1). This is consistent with the mechanism of photoinduced toxicity of ANT, which depends in large part on photooxidation of the chemical to more toxic forms (Huang, *et al.*, 1997; Mallakin, *et al.*, 1998).

3.4.3 Long-term effects of ANT on V. fischeri

The results for long-term toxicity of ANT to *V. fischeri* are given in Table 3.2 and Figure 3.4. When the assay was carried out in complex media (i.e., with no pre-incubation) ANT had little effect in darkness or SSR (Table 3.2). The SSR result was surprising because ANT is known to be highly phototoxic (Huang, *et al.*, 1997, Mallakin, *et al.*, 1998). It is possible that, in complex media, the ANT is not sufficiently bioavailable and growth of the cells is rapid enough to escape toxic effects.

To probe whether cell growth allowed escape from toxicity, the cells were pre-incubated with ANT in minimal media (Table 3.2 and Figure 3.4). First, *V. fischeri* was pre-incubated in minimal media for 4 or 8 hours in SSR, to allow up-take and photooxidation of ANT Toxicity was then assessed following 18 hours growth in complex media under SSR. This resulted in very high toxicity (EC50 = 1.63 and 0.13 mg/L, for 4 and 8 h P respectively, Table 3.2 and Figure 3.4). Second, *V. fischeri* was pre-incubated in dark for 8 hours and then allowed to grow in darkness for 18 hours. This resulted in low toxicity (Table 3.2 and Figure 3.4). Third, *V. fischeri* was pre-incubated in minimal media for 8 hours in darkness followed by 18 hours growth in SSR in complex media or vice versa. Both of these treatments showed intermediate toxicity (Table 3.2 and Figure 3.4), with SSR pre-incubation followed by growth in darkness being the more toxic of the two types of treatments. This work shows that direct assays in complex media greatly limit ANT impacts on the bacteria. It implies growth can out-compete uptake and photomodification. Importantly, the highest levels of toxicity were observed when the pre-incubation was carried out in SSR. This indicates that photooxidation of ANT ($t_{1/2}$ =

Figure 3.3 Dose-response of *V. fischeri* to ANT in the short-term toxicity assay. *V. fischeri* was exposed to a concentration series of ANT in minimal and complex media as described method and materials. Bacterial response was measured as inhibition of luminescence and plotted versus chemical concentration. Each data point represents an average of 6 replicates. Error bars are 95% confidence intervals.

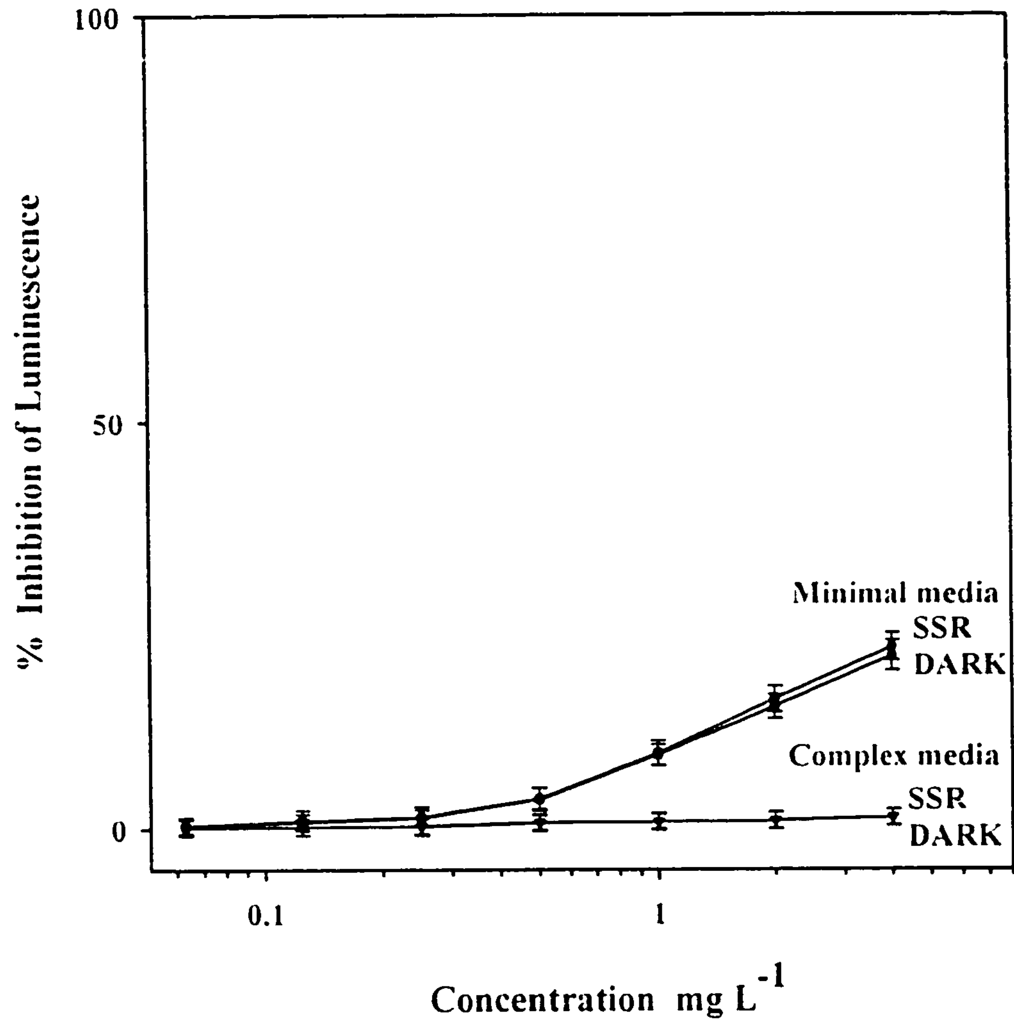

100
50
0
% Inhibition of Luminescence
Minimal media
SSR
DARK
Complex media
SSR
DARK
0.1
1
Concentration mg L-1

Table 3.1 Calculated EC50s for short-term toxicity of ANT to *V. fischeri*. Inhibition of luminescence in complex and minimal media was determined in the darkness and SSR

Media	EC50 mg L^{-1}	
	Dark	**SSR**
Complex Media	N/D	N/D
Minimal Media	17.92 ± 0.072	16.73 ± 0.088

Data are presented as EC50s in mg L^{-1} ± 95% confidence interval, n = 6

N/D: No detectable effect.

Table 3.2. Calculated EC50s (mg/L) for long-term toxicity of ANT to *V. fischeri* as inhibition of luminescence after pre-incubation in minimal media with different ways.

Pre-incubation	Incubation	Luminescence Inhibition		Growth Inhibition	
		4 h P	**8 h P**	**4 h P**	**8 h P**
NONE	DARK	N/D	N/D	N/D	N/D
NONE	SSR	N/D	N/D	N/D	N/D
DARK	DARK	85.78 ± 0.097	85.78 ± 0.097	84.88 ± 0.098	84.88 ± 0.097
DARK	SSR	11.53 ± 0.082	6.49 ± 0.052	10.75 ± 0.096	6.11 ± 0.098
SSR	DARK	4.99 ± 0.062	1.95 ± 0.042	4.79 ± 0.081	1.89 ± 0.062
SSR	SSR	1.63 ± 0.082	0.13 ± 0.052	1.53 ± 0.095	0.12 ± 0.068

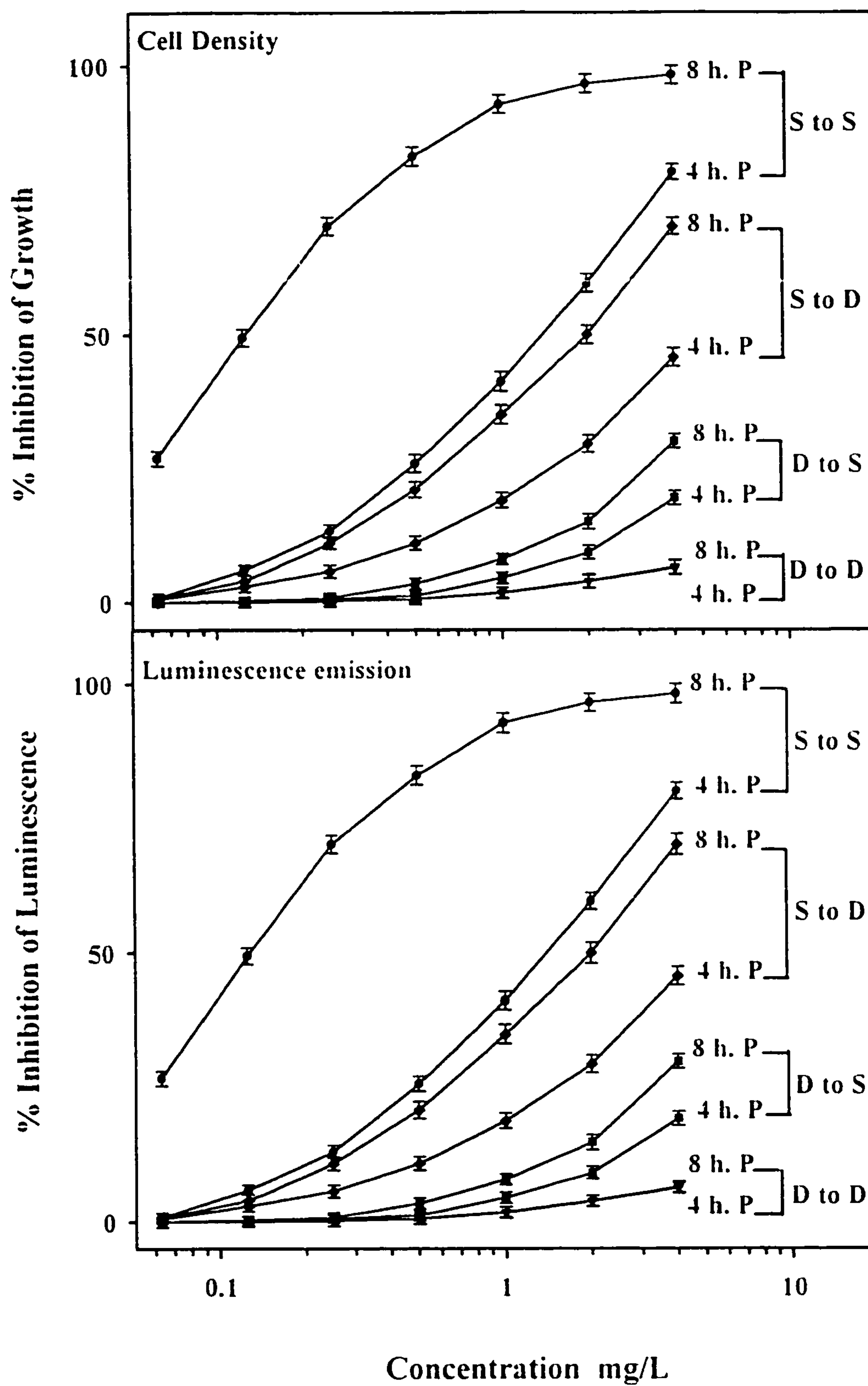
Data are presented as EC50s in mg L^{-1}, ± 95 % confidence interval, n = 6

4 h P: 4 h pre-incubation

8 h P: 8 h pre-incubation

N/D: No detectable effect

Figure 3.4 Dose-response of *V. fischeri* to ANT in the long-term toxicity assay. *V. fischeri* was exposed to a concentration series of PAHs as described in Table 3.2. Bacterial response was measured as inhibition of luminescence and inhibition of growth, and plotted versus chemical concentration. Each data point represents an average of six replicates. Error bars are the 95 % confidence intervals. 8 h P: 8 h pre-incubation in minimal media. 4 h P: 4 h pre-incubation in minimal media. S to S: Pre-incubation in SSR, growth in SSR; S to D: Pre-incubation in SSR to dark, growth in SSR to dark; D to S: Pre-incubation in dark to SSR, growth in dark to SSR; D to D: Pre-incubation in dark to dark, growth in dark to dark.


Cell Density
% Inhibition of Growth
100
50
0
8 h. P
4 h. P
S to S
8 h. P
4 h. P
S to D
8 h. P
4 h. P
D to S
8 h. P
4 h. P
D to D
Luminescence emission
% Inhibition of Luminescence
100
50
0
8 h. P
4 h. P
S to S
8 h. P
4 h. P
S to D
8 h. P
4 h. P
D to S
8 h. P
4 h. P
D to D
0.1
1
10
Concentration mg/L

2 hours in SSR) is important for bacterial toxicity. Importantly, this work shows how much experimental conditions can influence toxicity. Indeed the short-term assay and the assay without pre-incubation completely missed the extreme phototoxicity of ANT. Thus, one must consider the properties of a test chemical when designing a toxicity test.

3.5 CONCLUSIONS

This study suggests that the *V. fischeri* assay can be adapted to screen for phototoxicants and/or slow acting contaminants. With the assays we have developed, it will be possible to compare toxicity due to photomodification or photosensitization. From this work it is clear that ANT toxicity to bacteria requires time for photomodification. We note that our manipulations are environmentally relevant, as bacteria in natural conditions are often nutrient limited, and therefore exposure to toxicants under sub-optimal growth conditions will be common. Under these sub-optimal growth conditions there is time for contaminant modification and assimilation.

CHAPTER 4

APPLICATION OF PRE-INCUBATION TO SHORT- AND LONG-TERM PHOTOINDUCED TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS TO LUMINESCENT BACTRIA [1]

4.1 ABSTRACT

Irradiation of polycyclic aromatic hydrocarbons (PAHs) in aqueous solution with simulated solar radiation (a light source with a visible light: UV-A: UV-B ratio similar to that of sunlight) can greatly enhance their toxicity. Two microbial toxicity tests with *Vibrio fischeri* were used to investigate the effect of composition of the growth media and pre-incubation on photoinduced toxicity of PAHs. For the short-term toxicity assay, inhibition of bacterial luminescence was measured. For the long-term toxicity assay, both inhibition of bacterial luminescence and inhibition of growth were recorded. To broaden this test, *V. fischeri* was pre-incubated with PAHs in media without a sugar source (minimal media) to facilitate assimilation of the contaminants and prevent bacterial growth at the outset of the assay. *V. fischeri* was more sensitive in minimal media than in complex media in both short- and long-term toxicity assays. Moreover, in the long-term assay, SSR greatly increased toxicity. This indicated that both assimilation and photomodification of PAHs are important to their toxicity to *V. fischeri*.

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[1] Submitted to Environmental Toxicology. Co-authors are D. G. Dixon and B. M. Greenberg.

4.2 INTRODUCTION

Most environmental polycyclic aromatic hydrocarbons (PAHs) are anthropogenic, formed as a result of incomplete combustion of organic compounds (Edwards, 1983; Jacob *et al.*, 1986; Tuominen *et al* 1988). Because PAHs are hydrophobic, in aquatic environment they are generally found in sediments (Neff, 1979; Pearlman *et al.*, 1984). Previous work has shown that light dramatically enhances the toxicity of PAHs (Huang *et al.*, 1993; Ren *et al.*, 1996; McConkey *et al.*, 1996). Because PAHs have extensive π-orbital systems, they absorb strongly in the ultraviolet-B (UV-B; 290-320 nm) and ultraviolet-A (UV-B; 320-400 nm) regions of the solar spectrum (Greenberg *et al.*, 1995; Huang *et al.*, 1993). When PAHs are excited by actinic radiation they have photosensitizer activity, forming singlet-state oxygen ($^1O_2$) in high yield (Huang *et al.*, 1995) $^1O_2$ is highly damaging to biological tissue (Huang *et al.*, 1995). Additionally, exposure of PAHs to actinic radiation results in the photomodification of the compounds (Morgan *et al.*, 1977, Cook *et al.*, 1983; Nikolaou *et al.*, 1984; Huang *et al.*, 1993; Huang *et al.*, 1996). Photomodification generally is the result of oxidation of PAHs The rates of photomodification of PAHs are rapid enough for the oxidized compounds to contribute to toxicity. Given that photomodified PAHs are often more toxic than the parent PAHs, this is an environmentally important problem. As well, toxicity can be correlated to photomodification, with impacts increasing in parallel with the extent of photomodification (Zepp and Schlotzhauer, 1979; Schoeny *et al.*, 1988; Huang *et al.*, 1993; Ren *et al.*, 1995).

Another key process dictating toxicity of PAHs is assimilation of the compounds. Two related factors that dictate up-take of contaminants are bioavailability and hydrophobicity (Anderson *et al.*, 1987; Dickson *et al.*, 1994). Bioavailability of PAHs is a major concern in their toxicity because they are so hydrophobic (Belfroid *et al.*, 1996). The lower molecular weight PAHs prevail in the dissolved water phase, and they are often cytotoxic in short exposures due to rapid assimilation (Anderson *et al.*, 1987; Dickson *et al.*, 1994; Belfroid *et al.*, 1996). Prolonged exposure time often increases availability of higher molecular weight PAHs. The higher molecular weight PAHs have large octanol water partition coefficients and given enough time will accumulate in biological

membranes to high levels. Therefore, to fully understand PAH toxicity it is important to be able to temporally examine toxicity (Dickson *et al.*, 1994; Belfroid *et al.*, 1995).

The objective of this chapter only was to examine the impact of pre-exposure to PAHs in minimal media on the toxicity of PAHs to *V. fischeri*. In the previous chapters, ANT toxicity to *V. fischeri* was found to greatly increase in a long-term assay if there was a pre-incubation period in minimal media. This increase was attributed to photomodification and elevated bioavailability. In this chapter, this work was extended by examining if pre-incubation increased the toxicity of other PAHs.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strain and growth Media

The strain of marine gram-negative luminescent bacteria *Vibrio fischeri* NRRL B-11177, formerly known as *Photobacterium phosphoreum*, was used for the experiments presented here (see chapters 2 and 3). Luminescent bacteria were cultured in a complex media using the following formulation: monobasic potassium phosphate 18.4 mM; sodium chloride 0.5 M; magnesium sulfate 4.1 mM; glycerol 54.3 mM; yeast extract 1 mg/L; peptone 5 mg/L; bactopeptamin 1 mg/L; and agar when required 15 mg/L. The final pH of the media was adjusted to 7.2 ± 0.1 with 10 M NaOH. The media was distributed into 250 ml flasks to form a shallow layer of media. The mixture was autoclaved at 121 °C for 30 minutes. Peptone and Yeast extract were obtained from BDH Inc., Toronto, ON, and Bactopeptamin was obtained from Difco Laboratories, Detroit, MI. Stock cultures of *V. fischeri* were grown on agar plates used for primary inoculation.

4.3.2 Minimal media

Minimal media consists of 18.4 mM monobasic potassium phosphate, 0.5 M sodium chloride and 4.1 mM magnesium sulfate. The final pH of the media was adjusted to 7.2 ± 0.1 with 10 M NaOH. The mixture was autoclaved at 121 °C for 30 minutes.

4.3.3 Growth and luminescent emission

Bacteria were grown in darkness or simulated solar radiation (SSR). SSR was generated with a lighting system that mimics the spectral quality of natural sunlight. The light source consisted of eight daylight fluorescent lamps, two UV-A (350 nm) lamps and four UV-B (300 nm) lamps. The UV-B was filtered through two layers of cheese cloth. The light was filtered through a standard polystyrene cell culture plate top, which absorbed all UV-C (200-290 nm). The spectral output of the SSR sources had a visible light: UV-A: UV-B ratio of 100:10:1 based on photons, and the total fluence rate was 40 $\mu mol\ m^{-2}s^{-1}$ (Chapter 2).

To generate a starter culture for toxicity tests, cells of *V. fischeri* were cultured in 100 ml of complex media with shaking at room temperature (20°C ± 1) for 24 hours in darkness. Two methods were used to follow *V. fischeri* growth. The first type involved diluting cells from the starter culture into complex media. The second type involved diluting cells into minimal media (complex media without the reduced carbon sources) for 8 hours, at which point the media was made complex by addition of the reduced carbon sources found in the complex media. For the first type of test, 0.5 ml of actively growing starter cells in log-phase were harvested and put into 20 ml of complex media to give an approximate cell density of 0.4 at 650 nm. A 0.5 ml aliquot of this bacterial suspension was added to 0.5 ml complex media in the wells of a 48-well culture plate (Costar Safety Products, VWR Scientific Ltd, Toronto, Ontario. Canada) and grown in darkness or SSR with shaking at 20°C for up to 28 hours.

For the second type of experiment, 0.5 ml of *V. fischeri* cells from the starter culture were diluted into 20 ml of minimal media to an optical density (OD) of 0.4 at 650 nm. The diluted cells (0.5 ml) were added to 0.5 ml of minimal media in the wells of a 48-well culture plate. Cells were pre-incubated for 8 hour in minimal media in SSR or darkness. After the 8 hour pre-incubation period an equal volume of complex media with glycerol at 10 g/L, yeast extract at 2 g/L, peptone at 10 g/L and bactopeptamin at 2 g/L was added to each well in the culture plate. Cells were grown for up to 28 hours. Cell density and luminescence intensity of the culture was measured every 4 hours. The cell

density of the culture was measured with spectrophotometer. The luminescence emission of the *V. fischeri* was measured with a Cytofluor 2350 multi-well fluorescence plate reader.

4.3.4 Bacterial short- and long-term toxicity assays

Inhibition of light production in *V. fischeri* after incubation with the toxicant for 15 minutes in darkness or SSR was used to assess the short-term toxicity of PAHs. To assess long-term toxicity of PAHs on *V. fischeri*, both inhibition of light production and growth rate were used. In the long-term assay, toxicity was measured after 18 hours of growth of the bacteria in the presence of the toxicants.

4.3.4.1 Bacterial short-term assay

The starter culture was diluted as above with minimal media and twenty-four 500-µl aliquots of cells were added to a 48-well cell culture plate (Costar Safety Products, VWR Scientific Ltd, Toronto, Ontario, Canada). After 5 minutes acclimatization in the 48-well cell culture plates, the luminescence intensity was measured. After the initial measurement of bacterial luminescence, the bacteria were dosed with PAHs. The 500-µl aliquots of the cell culture were combined with equal volumes of the test chemical in complex or minimal culture media. A dilution series of each chemical (7 concentrations plus the control) were added in a geometric series to the wells in triplicate. Stock solutions of PAHs were prepared in dimethyl sulphoxide (DMSO) and added by 1000-fold dilution to the culture liquid media to give the specified concentration. The DMSO concentration of 0.1% does not affect luminescence or growth of *V. fischeri*. After dosing, the cells were incubated in darkness or SSR at room temperature for 15 minutes with shaking and the luminescence intensity was measured.

4.3.4.2 Bacterial long-term assay

After the short-term test was completed, the multi-well culture plate was returned to the growth chamber. The cells continued to be incubated with the toxicant in darkness or SSR at 20°C. If the experiment started in complex media, growth was allowed to proceed for 18 hours. If the experiment involved an 8 hour pre-incubation in minimal media, after this pre-incubation, the media was made complex as above. The cells were then incubated for an additional 18 hours in SSR or darkness at 20°C. Toxicity was determined by inhibition of growth and luminescence.

4.3.5 Calculations of EC_{50} and data analysis

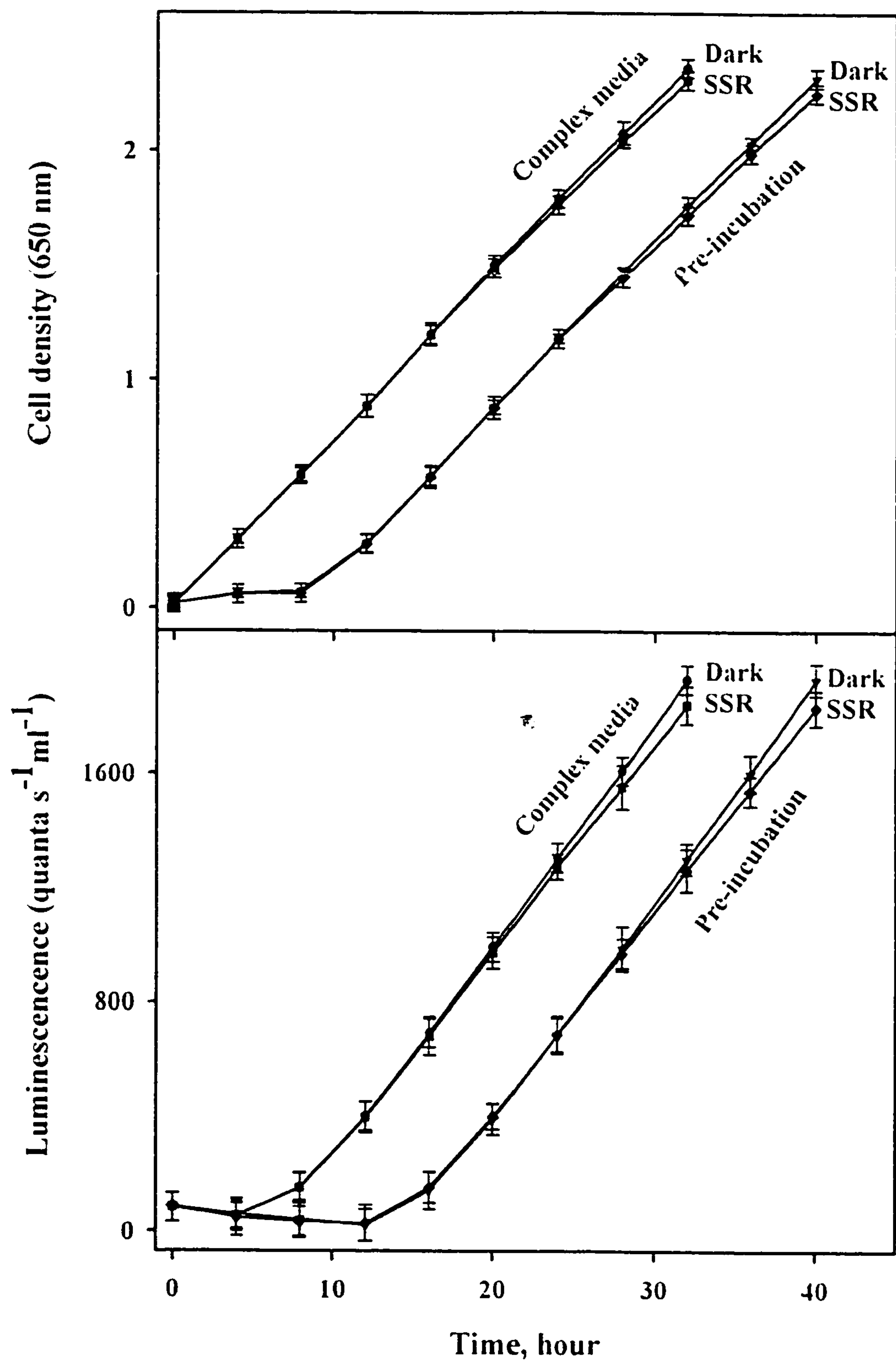
Toxicity is expressed as the concentration causing 50 % inhibition of luminescence (EC50). This was used for short-term and long-term assay. Inhibition of luminescence was plotted vs. concentration. These data were fit to a log it function as in chapter 2. From this regression, the EC50 was determined for the long-term toxicity assays the concentration that causing 50 % inhibition of growth was also calculated. The 95 % confidence intervals of the EC50 values were calculated. All calculations are based on nominal test concentrations (Chapter 2).

4.4 RESULTS

4.4.1 Growth rate and luminescent emission of V. fischeri

When *V. fischeri* cells in log-phase growth were diluted into complex media, log-phase growth continued for more than 20 hours (Figure 4.1). For these cells, luminescence remained constant for a lag period of 8 hours, after which increased light output mirrored cell growth. Relative to darkness, there was no effect of SSR on growth of the cells. When cells were placed in minimal media instead, no growth was observed and luminescence gradually declined. After a 8 hours pre-incubation in minimal media, the

Figure. 4.1 Cell density and luminescence intensity of *Vibrio fischeri*. Cultures of bacteria were grown in 48-well cell culture plates under darkness and SSR at room temperature. Bacteria growth in two different ways: First, the bacteria were grown continuously in complex media for 30 hours. Cell density and luminescence intensity were measured every 4 hours. Second, bacteria pre-incubated for 8 hours in minimal media and then grown for 30 hours in complex media as described in materials and methods. Cell density and luminescence intensity were measured every 4 hours.



Cell density (650 nm)
0
1
2
Complex media
Dark
SSR
Dark
SSR
Pre-incubation
Luminescence (quanta s-1 ml-1)
0
800
1600
Complex media
Dark
SSR
Dark
SSR
Pre-incubation
0
10
20
30
40
Time, hour

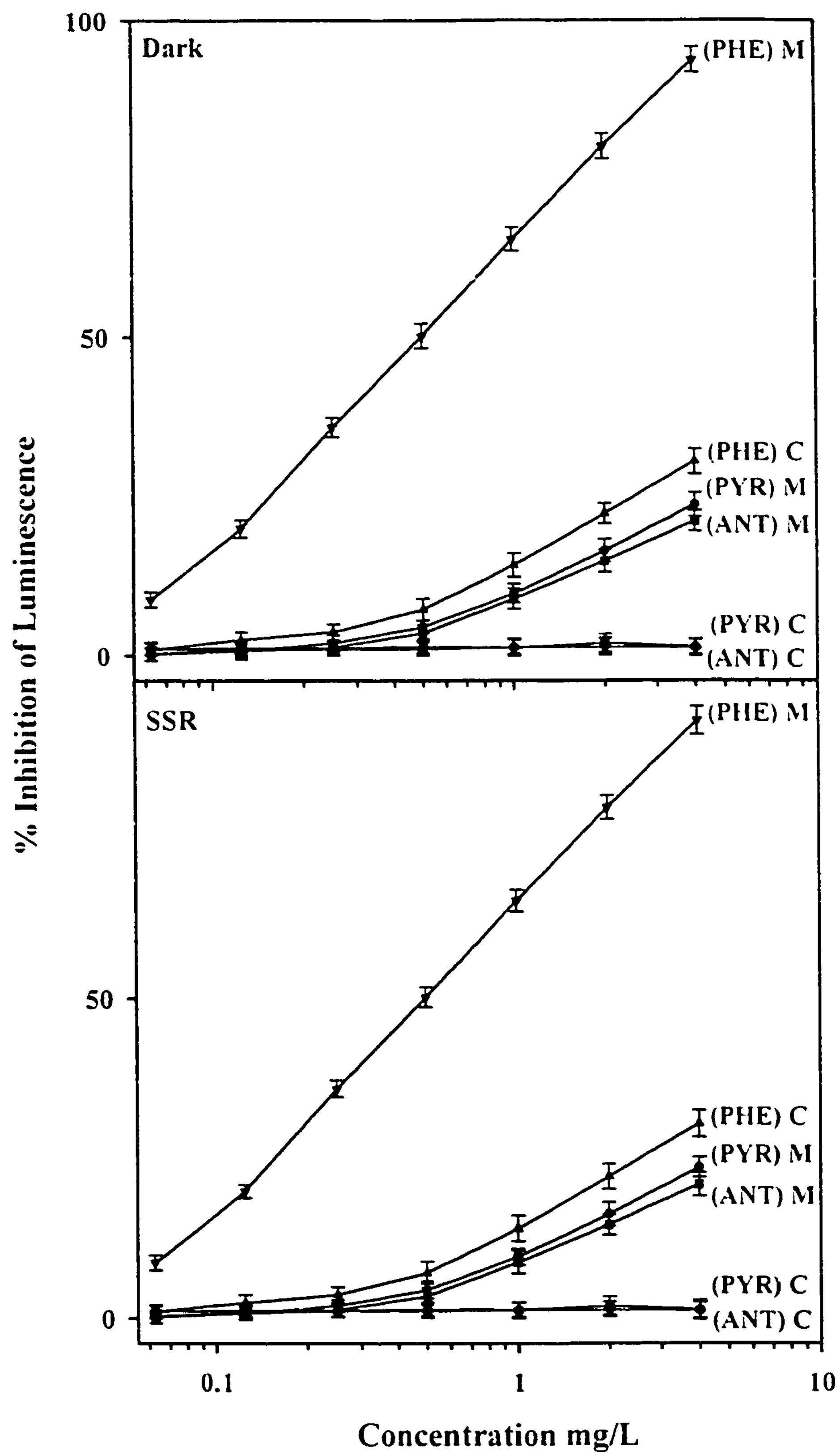
media was made complex and the cells shifted almost immediately into log-phase growth. The growth rate was identical to that of cells placed directly in complex media. Luminescence recovered after an 8 hour lag period and then mirrored growth. Once again there was no effect of SSR relative to darkness. This demonstrates that a pre-incubation for 8 hours in minimal media does not harm the *V. fischeri*.

4.4.2 Short-term toxicity of selected PAHs to V. fischeri

Vibrio fischeri was incubated for 15 min in darkness or SSR with 12 different PAHs to determine the toxic potency of each chemical (Fig 4.2 and Table 4.1). Full dose-response curves are shown for ANT, PHE and PYR as examples of the 12 PAHs used in this study. For ANT, in complex media there was no toxicity observed at any concentration tested. However, in minimal media ANT showed toxicity at concentrations ranging from 1 to 4 mg/L (Figure 4.2). The calculated EC50 of ANT in minimal media was 17.92 mg/L in darkness and 16.78 mg/L in the SSR (Table 4.1). These are in the same range as those reported for the Microtox test (Jacobs *et al.*, 1993). PHE was toxic to *V. fischeri* in complex media, (Figure 4.2 and Table 4.1) but the EC50 was much higher than that reported for the Microtox assay (Jacobs *et al.*, 1993). Interestingly, PHE toxicity in minimal media (EC50 = 0.51 mg/L) was similar to that in the Microtox assay (Jacobs *et al.*, 1993). Similarly the toxicity of PYR in the short-term assay was more pronounced in minimal media than in complex media (Figure 4.2). For all this chemicals there was no effect of SSR on toxicity (Figure 4.2).

These calculated EC50s of the twelve PAHs in short-term assay are presented in (Table 4.1). The lowest EC50 was obtained for PHQ (0.07 mg/L) in minimal media. This value agreed with EC50 for PHQ observed by McConkey *et al.* (1997). The toxic impact of the PHQ in complex media was different from that in minimal media. Toxicity was less pronounced in complex media (Table 4.1). Of the 12 PAHs tested the highest EC50 was obtained for ANQ. In minimal media the EC50 was 96 mg/L and there was no toxicity observed in complex media (Table 4.1).

Figure. 4.2 Dose-response of *Vibrio fischeri* to ANT, PHE and PYR in the short-term assay. The assay was run in complex (C) and minimal (M) media for 15 min in darkness and SSR. The toxicity affect was detected as inhibition of luminescence.



100
Dark
(PHE) M
50
(PHE) C
(PYR) M
(ANT) M
(PYR) C
(ANT) C
0
SSR
(PHE) M
50
(PHE) C
(PYR) M
(ANT) M
(PYR) C
(ANT) C
0
0.1
1
10
% Inhibition of Luminescence
Concentration mg/L

Table 4.1 Calculated EC50s (mg/L) for short-term toxicity of PAHs to *Vibrio fischeri* under different lighting conditions. EC50s are based on % Inhibition of luminescence. All experiments are the average of 6 replicates. ± 95 % confidence intervals.

		EC50 for Inhibition of Luminescence			
		Complex Media		Minimal Media	
Toxicant	**Abbreviation**	**Dark**	**SSR**	**Dark**	**SSR**
Aceanthrenequinone	**AAQ**	24.39 ± 0.24	23.70 ± 0.41	7.85 ± 0.59	7.05 ± 0.41
Acenaphthene	**ACE**	2.41 ± 0.46	2.52 ± 0.48	0.81 ± 0.10	0.83 ± 0.09
Acenaphthylene	**ACY**	0.80 ± 0.08	0.77 ± 0.05	0.33 ± 0.06	0.34 ± 0.04
Acenaphthenequinone	**ACQ**	2.67 ± 0.24	2.65 ± 0.28	1.27 ± 0.07	1.26 ± 0.05
Anthracene	**ANT**	N/D	N/D	17.92 ± 0.07	16.78 ± 0.09
Anthraquinone	**ANQ**	N/D	N/D	95.89 ± 0.88	96.09 ± 0.82
1,2-dihydroxyanthraquinone	**1,2-dhANQ**	7.11 ± 0.16	7.24 ± 0.25	2.03 ± 0.14	1.62 ± 0.11
Naphthalene	**NAP**	2.36 ± 0.34	2.33 ± 0.25	0.72 ± 0.12	0.70 ± 0.11
5-Nitroacenaphthene	**5-NACE**	2.29 ± 0.29	2.16 ± 0.21	1.18 ± 0.08	1.09 ± 0.03
Phenanthrene	**PHE**	7.33 ± 0.41	6.89 ± 0.32	0.51 ± 0.02	0.52 ± 0.03
Phenanthrenequinone	**PHQ**	0.92 ± 0.04	0.81 ± 0.06	0.07 ± 0.02	0.06 ± 0.01
Pyrene	**PYR**	N/D	N/D	15.07 ± 0.07	14.53 ± 0.08

*N/D: No toxicity observed at the highest concentration tested.

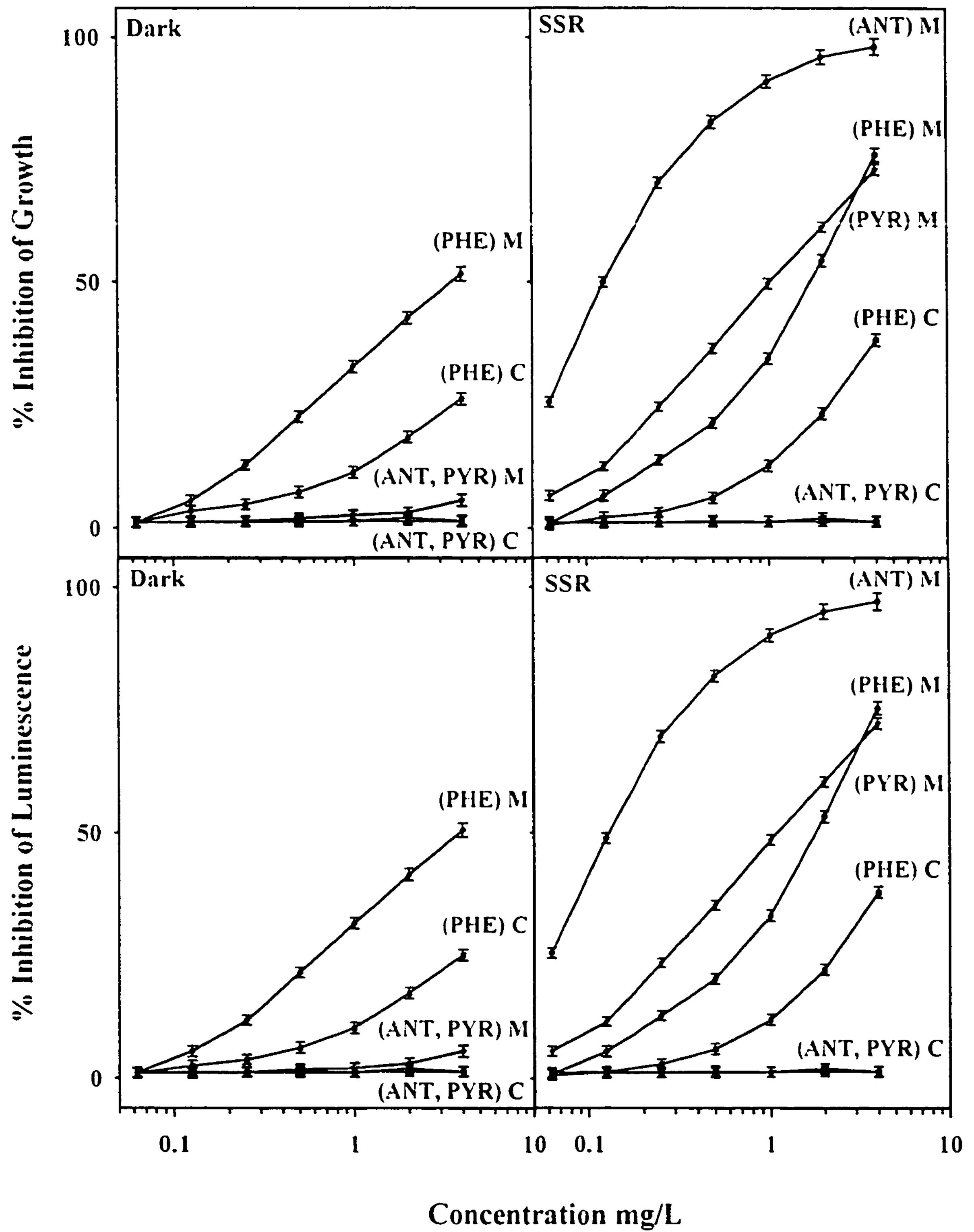
Except for ANT, the rest of the PAHs had EC50s ranging from 0.3 mg/L to 8 mg/L (Table 4.1). In all cases, the EC50 were greater in complex media than in minimal media. The EC50s in minimal media for ACE, ACY, ACQ, ANT, NAP, PHE, and PHQ agreed well with published EC50s obtained using Microtox assay (Jacobs *et al.*, 1993; Kaiser and Devillers, 1994). The calculated EC50s for all twelve PAHs in the darkness are nearly same in SSR except for 1,2-dhANQ. 1,2-dhANQ was slightly more toxic in the SSR than darkness (Table 4.1).

4.4.3 Long term assay of selected PAHs to V. fischeri

Toxicity of the 12 PAHs was assessed in long-term assays using growth and luminescence inhibition as end-points. Full concentration-response curves in darkness and SSR are provided for ANT, PHE and PYR (Figure 4.3). ANT was not toxic to *V. fischeri* in the dark or SSR in complex media. ANT had modest impacts in minimal media at high concentrations in darkness (Figure 4.3). However, the ANT was highly toxic to *V. fischeri* in SSR in minimal media (Figure 4.3). Similar results were achieved with PYR (Figure 4.3). Relative to complex media, the toxicity of PHE was enhanced when minimal media was used along with a pre-exposure. The toxicity of PHE was only slightly enhanced by SSR. Thus, for all three chemicals, toxicity was generally greater when minimal media was used for a pre-exposure period. Furthermore, when there is a pre-exposure in minimal media, the photoinduced toxicity of the PAHs became readily apparent.

Similar results were achieved with the other PAHs examined in this study (Table 4.2). No toxicity was observed with ANQ or NAP in the long-term assay when it was run with complex media, but both ANQ and NAP showed slight toxicity when there was a pre-incubation in minimal media (Table 4.2). The toxic effect of AAQ on *V. fischeri* in the long-term assay was different between complex media and minimal. AAQ in complex media had no toxic impact in darkness or SSR. However, when pre-incubated in minimal media AAQ was toxic in darkness and the impact was enhanced with SSR (The EC50 was an order of magnitude lower in SSR) (Table 4.2). ACE had no toxic

Figure. 4.3 Dose-response of *V. fischeri* to ANT, PHE, and PYR in the long-term assay. *V. fischeri* was exposed to concentration series of ANT, PHE, and PYR for 18 hours in darkness or SSR. Bacterial response was measured as percent inhibition of luminescence and the growth rate. Each data point represents an average of nine replicates of the given concentration. Two types of assays were performed. C: the exposures were in complex media for 18 h in darkness or SSR. M: the cells were pre-exposed to the chemicals in minimal media for 8 hours in darkness or SSR, and then allowed to grow with the chemicals in complex media in darkness or SSR.



Dark
SSR
(ANT) M
(PHE) M
(PYR) M
(PHE) C
(ANT, PYR) M
(ANT, PYR) C
% Inhibition of Growth
% Inhibition of Luminescence
100
50
0
0.1
1
10
Concentration mg/L

impact on the *V. fischeri* under darkness in complex media, but was slightly toxic following a pre-incubation in minimal media. Toxicity of ACE was greatly enhanced by SSR, with the EC50 dropping nearly 40-fold due to light exposure. 5-NACE did not have a toxic impact in darkness in complex media, but the toxicity was enhanced both by exposure to SSR and by a pre-incubation in minimal media. Similarly, ACY, ACQ, 1,2-dhANQ and PHQ all showed more impact on *V. fischeri* with a minimal media pre-exposure relative to complex media. In all cases their toxicity was enhanced by SSR (Table 4.2), although the light impact on PHQ was minimal. However, PHQ was so toxic in the dark that there was little change possible upon exposure to SSR (Table 4.2).

The order of toxicity of the twelve PAHs, tested was PHQ > ANT > AAQ > 5-NACE > ACQ > ACY >1,2-dhANQ > PYR > PHE > ACE > ANQ > NAP (Table 4.2). Note, both bioindicator end-points (inhibition of luminescence and inhibition of growth) gave very similar results for all the chemicals tested (Table 4.2). All of the chemicals except NAP and ANQ showed increased toxicity in SSR relative to darkness. Further, all the chemicals had elevated impacts with a minimal media pre-incubation period. Thus, photoinduced toxicity of PAHs to *V. fischeri* can be observed when appropriate conditions are used.

4.5 DISCUSSION

The results of these experiments show that the *V. fischeri* after incubation for 8 hours in minimal media can grow successfully and emit luminescence at a normal rate when shifted into complex media. This allows one to pre-incubate *V. fischeri* in minimal media with test chemicals to allow up-take and modification of the chemicals. The bacteria can then be challenged with growth in complex media to determine if the chemical is toxic. This greatly expands the well used short-term assay of toxicity using *V. fischeri* (Microtox). This pre-incubation is environmentally relevant, as bacteria are commonly nutrient limited in aquatic systems. Importantly, the long-term assay of *V. fischeri* involving an 8 hour pre-incubation in minimal media clearly revealed the prevalent mechanism of PAH photoinduced toxicity.

Table 4.2 Calculated EC50s (mg/L) for long-term toxicity of PAHs to *Vibrio fischeri* under darkness or SSR. All experiments are the average of 6 replicates. ± 95 % confidence intervals. The difference between the complex media and minimal media experiments are as in Figure 4.3.

	Inhibition of Luminescence				Inhibition of Growth			
	Complex Media		Minimal Media		Complex Media		Minimal Media	
Toxicant	**Dark**	**SSR**	**Dark**	**SSR**	**Dark**	**SSR**	**Dark**	**SSR**
AAQ	N/D	N/D	3.56 ± 0.08	0.21 ± 0.02	N/D	N/D	3.24 ± 0.05	0.20 ± 0.01
ACE	N/D	5.21 ± 0.07	88.45 ± 0.09	2.54 ± 0.07	N/D	4.68 ± 0.08	86.75 ± 0.09	2.39 ± 0.08
ACY	6.57 ± 0.08	1.47 ± 0.04	3.62 ± 0.09	0.53 ± 0.03	6.05 ± 0.11	1.39 ± 0.05	3.54 ± 0.08	0.52 ± 0.02
ACQ	1.88 ± 0.05	1.52 ± 0.05	0.52 ± 0.03	0.39 ± 0.02	1.74 ± 0.09	1.41 ± 0.04	0.51 ± 0.03	0.37 ± 0.01
ANT	N/D	N/D	85.78 ± 0.07	0.13 ± 0.01	N/D	N/D	84.88 ± 0.07	0.12 ± 0.01
ANQ	N/D	N/D	95.85 ± 0.09	96.78 ± 0.12	N/D	N/D	97.89 ± 0.11	98.80 ± 0.09
1,2-dhANQ	5.80 ± 0.11	5.60 ± 0.09	1.81 ± 0.05	0.59 ± 0.02	5.75 ± 0.09	5.61 ± 0.08	1.79 ± 0.06	0.58 ± 0.03
NAP	N/D	N/D	99.85 ± 0.11	98.95 ± 0.13	N/D	N/D	99.79 ± 0.12	99.90 ± 0.15
5-NACE	N/D	0.62 ± 0.02	9.44 ± 0.12	0.35 ± 0.02	N/D	0.58 ± 0.03	9.07 ± 0.08	0.33 ± 0.02
PHE	8.09 ± 0.11	7.15 ± 0.09	4.53 ± 0.04	1.93 ± 0.02	7.38 ± 0.08	6.72 ± 0.09	4.48 ± 0.04	1.92 ± 0.02
PHQ	0.66 ± 0.02	0.69 ± 0.03	0.08 ± 0.02	0.04 ± 0.01	0.64 ± 0.03	0.68 ± 0.03	0.07 ± 0.01	0.04 ± 0.01
PYR	N/D	N/D	97.51 ± 0.09	1.43 ± 0.03	N/D	N/D	97.21 ± 0.09	1.42 ± 0.02

*N/D: No toxicity observed at the highest concentration tested.

4.5.1 Short-term toxicity

PAHs are common contaminants of terrestrial and aquatic ecosystems. Traditional toxicological studies for defining the potential hazard of PAHs in the environment have been conducted in the absence of sunlight. However, recent work, particularly in aquatic toxicology, have shown that PAHs become substantially more toxic upon exposure to sunlight. Sunlight can rapidly oxidize PAHs in the environment to oxyPAHs, many of which more toxic than intact PAHs (Huang *et al.*, 1993; Ren *et al.*, 1994; McConkey *et al.*, 1997). In this study, the luminescent bacteria *V. fischeri* was used as bioindicator to evaluate photoinduced toxicity of PAHs in short-term and long-term assays.

The solubility properties of PAHs severely limit the amounts that can be utilized in experiments in aqueous media. Small amounts of DMSO thus were used to introduce the PAHs into the media to increase the solubility and the bioavailability. For toxicity testing of polycyclic aromatic compounds individually, DMSO was used for direct delivery of the PAHs to appropriate aqueous media by 1,000-fold dilution. With DMSO, supersaturated solutions of PAHs can be generated allowing for full concentration response experiments. A 0.1% (v/v) DMSO solution was used for the control experiments and this concentration showed no toxic effects on *V. fischeri* (Environment Canada, 1993).

The short-term assays with individual PAHs were preformed in complex media and in minimal media. Because the complex media data have been compared with published PAH toxicity data in Chapter 2 (Figure 4.3), in this discussion the minimal media data will be compared with published data. The data from the short-term assays in minimal media were in good agreement with data obtained from Microtox tests (Kaiser *et al.*, 1984; Bulich, 1986; Jacobs *et al.*, 1993; Kaiser and Devillers, 1994). This can be seen by the high correlation of the data for the PAHs performed here in minimal media and the 6 corresponding compounds found in the above reference (Figure 4.4). The EC50s of both PHE and PHQ (0.5 mg/L and 0.07 mg/L) agreed well with the published EC50 data from McConkey *et al.* (1997) and Jacobs *et al.* (1993). ANT, ANQ and PYR were not toxic to *V. fischeri* in complex media but they showed low toxicity in minimal media and the Microtox assay. This suggests that sugar and other reduced carbons sources in the

complex media effect the bioavailability of ANT, ANQ and PYR. The application of minimal media raised the sensitivity of the short-term assay to PAHs, with the most striking effect on ANT, ANQ, and PYR.

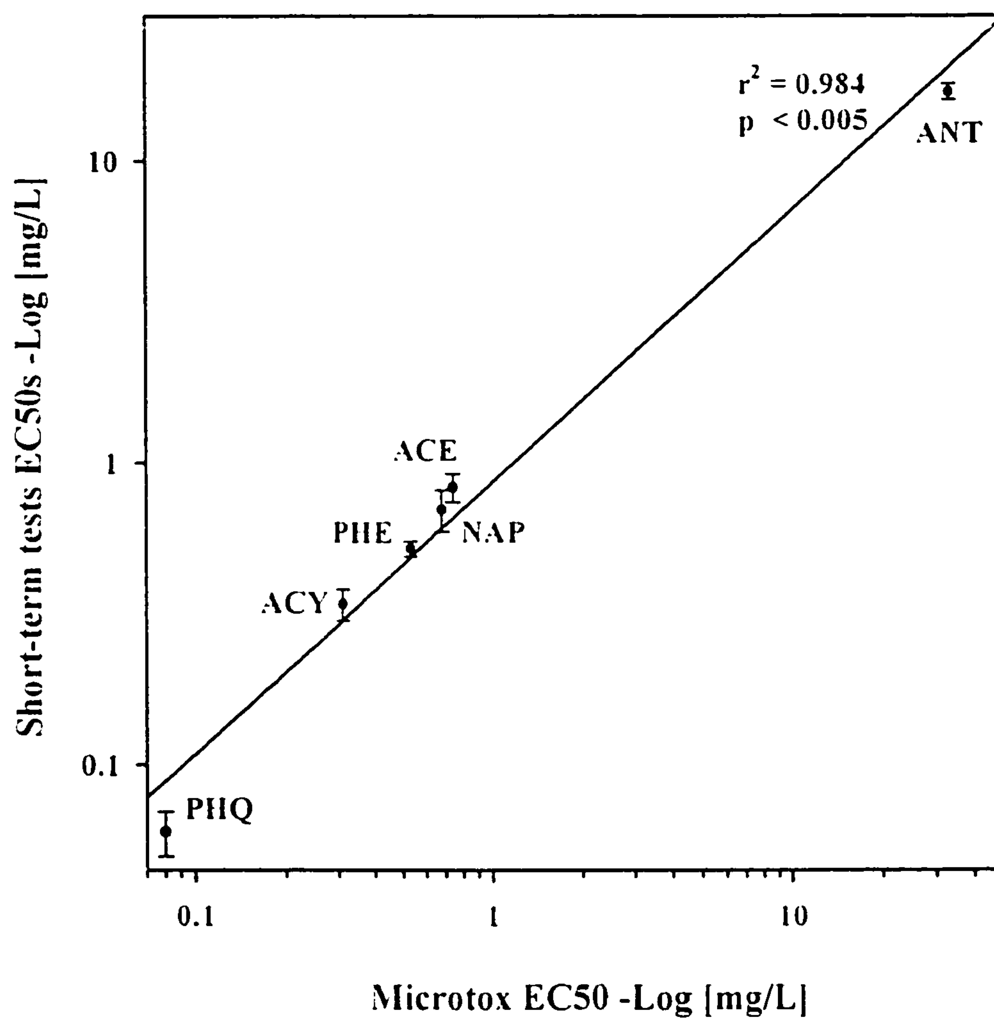
To determine if the toxicity of PAHs observed in SSR could be attributed to photosensitization or photomodification reactions, *V. fischeri* was incubated in darkness and SSR with the PAHs. The calculated EC50 for each PAHs in the darkness or SSR were not different in the short-term assay (Table 4.1). This indicated that photoactivation of PAHs was not a factor in the short-term toxicity assay. Because toxicity increased in minimal media relative to complex media, it can be concluded that bioavailability is the key factor in short-term toxicity of PAHs. This explains why log K_{ow} has been found to be a predictive factor in PAH toxicity in several studies.

4.5.2 Long-term toxicity

PAHs are rapidly photomodified to a mixture of photoproducts under SSR that are more toxic and water soluble than the intact compounds (Huang *et al.*, 1993; Ren *et al.*, 1994; McConkey *et al.*, 1997). The twelve PAHs used in this study generally showed more toxicity in the long-term assays than in the short-term assays. As well, toxicity was greatly increased by SSR in the long-term assays. ANT is phototoxic in a number of bioassays (Allred and Giesy, 1985; Kagan *et al.*, 1987; Holst and Giesy, 1989; Mallakin *et al.*, 1999). ANT exhibited the properties of a true phototoxicant (Table 4.2 and Figure 4.3) in that toxicity to *V. fischeri* was very low in darkness (EC50 = 85.7 mg/L) but was enhanced upon exposure to SSR (EC50 = 0.14 mg/L). Note, ANT was completely non-toxic in the short-term assay in darkness or SSR. This dramatic increase was seen for several of the PAHs tested.

NAP and ANQ had the lowest toxic impact to *V. fischeri* under darkness and their toxicity was not enhanced upon exposure to SSR. The toxicity of PYR and ACE were significantly enhanced by SSR. PHE and 5-NACE had low toxicity in darkness to *V. fischeri*, but they showed high toxic impacts upon exposure to SSR. The toxic responses of *V. fischeri* to AAQ and ACY were enhanced upon exposure to SSR, but relatively high

Figure 4.4 Regression analysis of the short-term assay for *Vibrio fischeri* versus Microtox assay. EC50s for (ANT, ACE, ACY, NAP, PHE and PHQ) for the short-term assay and the Microtox assay were plotted against each other. Liner regression was performed with Sigma Plot and the p value was determined by ANOVA using Systat.

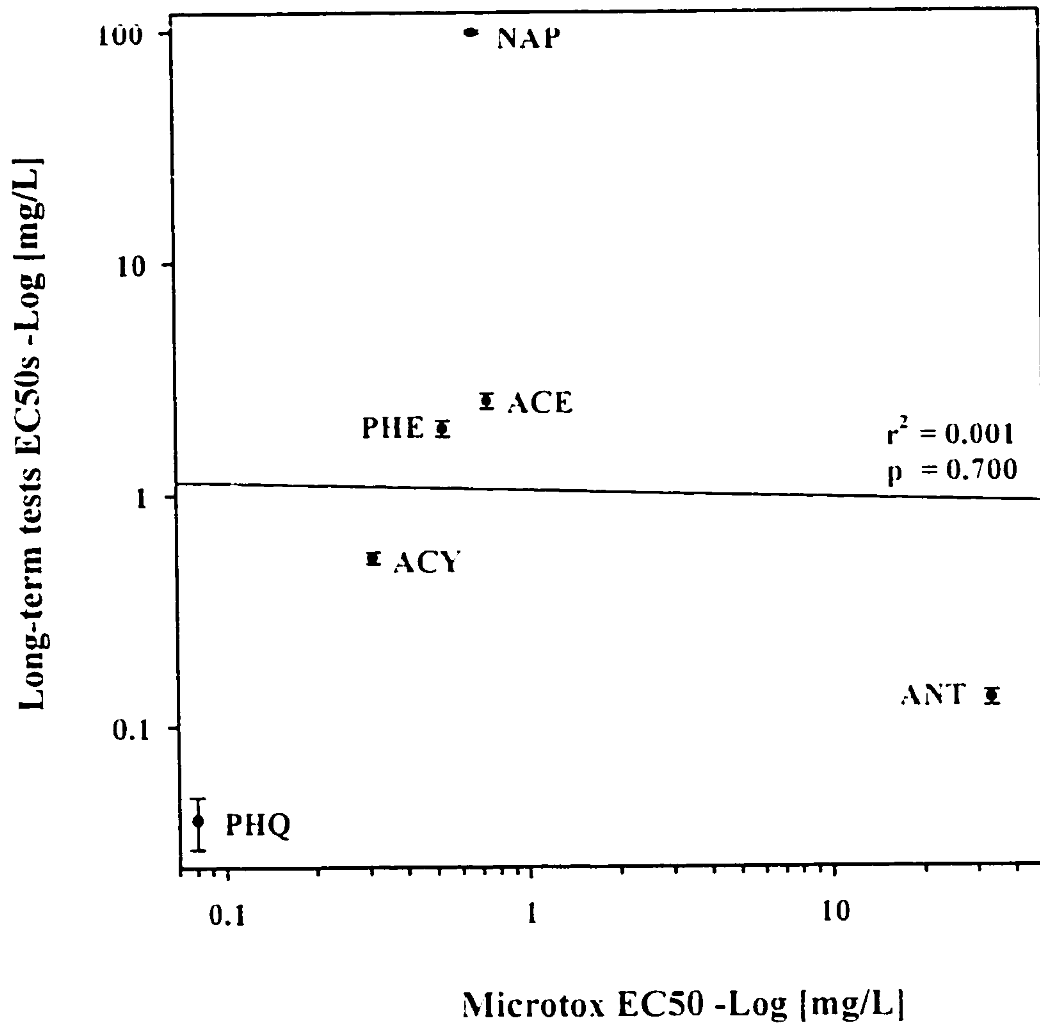

r² = 0.984
p < 0.005
ANT
ACE
PHE
NAP
ACY
PHQ
10
1
0.1
0.1
1
10
Short-term tests EC50s -Log [mg/L]
Microtox EC50 -Log [mg/L]

toxicity was observed in darkness. 1,2-dhANQ and PHQ were both toxic to *V. fischeri* darkness and SSR. Phototoxicity shows an apparent increase with increasing PAH/oxyPAH solubility. This may indicate one reason why oxyPAHs were more toxic than the intact PAHs. That is, they are more soluble, and thus, more bioavailable (Huang *et al.*, 1993; Kagan *et al.*, 1987; Kocchevar *et al.*, 1982). Previous work with PAHs has demonstrated that photomodified solutions of a given PAH exhibit greater toxicity than a solution of the parent PAH (Huang *et al.*, 1993; Ankley *et al.*, 1994; Ren *et al.*, 1994; Ren *et al.*, 1996; Huang *et al.*, 1995).

This study extends the previous observations of correlations between Microtox assay data, and short-term toxicity data under different assay procedures. The comparison of EC50 values for the short-term assays in darkness or SSR condition (Figure 4.4) strongly correlated with data from Microtox test. However, there was no correlation between the long-term assay versus the Microtox assay (r^2 = 0.00061) (Figure 4.5). This holds for both the SSR and dark data. Thus the *V. fischeri* test is highly sensitive to experimental conditions. In particular, the short-term assays are completely transparent to the highly prevalent phototoxicity of PAHs.

Exposure to SSR enhanced the toxicities of PAHs to *V. fischeri* in the long-term assay. There is likely relationship to the photosensitization and photomodification processes of PAHs. It was observed that both photosensitization and photomodification of PAHs are important factors in their phototoxicity (Huang *et al.*, 1997). However, to improve our understanding of these factors on photoinduced toxicity of PAHs, a QSAR model of photoinduced toxicity was developed by Huang *et al.* (1997) for plants. This model can now be applied to *V. fischeri* to determine if it works with other organisms. Thus, the aim of the next chapter (Chapter 5) was to utilize the plant QSAR model on *V. fischeri*.

Figure 4.5 Regression analysis of the long-term assay for *Vibrio fischeri* versus Microtox assay. EC50s for (ANT, ACE, ACY, NAP, PHE and PHQ) for the long-term assay and the Microtox assay were plotted against each other. Liner regression was performed with Sigma Plot and the p value was determined by ANOVA using Systat.



100
10
1
0.1
NAP
ACE
PHE
ACY
ANT
PHQ
r² = 0.001
p = 0.700
0.1
1
10
Long-term tests EC50s -Log [mg/L]
Microtox EC50 -Log [mg/L]

4.6 CONCLUSIONS

The results of this study indicate that *V. fischeri* is useful for the detection of short- and long-term chemical toxicity. The growth assay used here gave very similar results to luminescence in the long-term assay. *V. fischeri* was more sensitive to the toxicity of the chemicals in minimal media than in complex media. Thus, bioavailability of PAHs is key to their microbial toxicity. The short-term assay showed a good correlation with Microtox when it was run in minimal media. The pre-incubation of PAHs with bacteria in minimal media overcame the limited bioavailability PAHs. The photoinduced toxicity of PAHs is only observed in the long-term assay, and photoinduced toxicity is further enhanced if there is a pre-incubation period in minimal media.

CHAPTER 5

QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIP FOR THE PHOTOINDUCED TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS TO LUMINESCENT BACTRIA[1]

5.1 ABSTRACT

Many studies have shown that natural sunlight greatly enhances the toxicity of PAHs. Photosensitization reactions (e.g., generation of singlet-state oxygen) and photomodification reactions (e.g., photooxidation of PAHs to more toxic species) are both pathways of photoinduced toxicity of PAHs in aquatic environments. The principles of quantitative structure-activity relationships (QSARs) were applied to PAHs showing that a photosensitization factor (PSF) and photomodification factor (PMF) both can be additively combined to describe photoinduced toxicity. This QSAR model was based on the photoinduced toxicity of 16 PAHs to the duckweed *Lemna gibba* (Huang *et al.* 1997). The objective of this study was to apply the QSAR model developed for *L. gibba* to another organism. The organism chosen was the luminescent marine bacteria *Vibrio fischeri*. Toxicity data for the QSAR model was inhibition of luminescence and inhibition of growth to *V. fischeri* by sixteen intact PAHs. The PMFs for the PAHs from the *Lemna gibba* QSAR showed a moderate correlation to bacterial toxicity, where as the PSFs showed only a weak correlation to toxicity. As was the case for *L. gibba*, summing the PMF and the PSF resulted in a strong correlation to toxicity that had predictive value. Thus, a QSAR model derived for plants accurately described the toxicity of PAHs to a bacterial species. This indicates that the bipartite mechanism of PAH photoinduced toxicity should be broadly applicable.

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[1]Submitted to Environmental Toxicology and Chemistry. Co-authors X.-D. Huang, D. G. Dixon and B. M. Greenberg.

5.2 INTRODUCTION

The principles of quantitative structure activity relationships (QSARs) are based on the premise that properties of a chemical are implicit in its molecular structure. The idea is to link groups of related chemicals with a particular biological response, such as toxicity. One can hypothesize relevant physico-chemical parameters to establish QSARs to describe biological responses (Newsted and Giesy, 1987; Hermens, 1989). QSARs are useful for understanding the mechanisms of toxic action of chemicals, and predicting toxicity for risk assessment. They can predict the potential hazards of untested compounds, identify physical traits of chemicals that contribute to biological impacts, describe the routes of chemical interaction with an organism, and aid in elucidating mechanisms of toxicity (Newsted and Giesy, 1987; Hermens, 1989; Hermens and Opperhuizen, 1990).

In developing a QSAR model, it is essential to consider the attributes of the environmental compartment in which the contaminant of interest resides. This will dictate which physico-chemical properties of a molecule are likely to be most influential in toxicity (Schoeny *et al.*, 1988; Malkin 1992). For PAHs, to better understand the process of photoinduced toxicity, it is of interest to relate their hazards in the presence of actinic radiation to the physical and structural properties of the compounds. To generate a QSAR for the photoinduced toxicity of PAHs, the mechanisms of the photochemical reactions involved have to be considered as well as how they impact negatively on living organisms (Cooper and Herr, 1987; Huang *et al*, 1997; Krylov *et al*, 1997).

Photoactivation by sunlight is one of the most important routes of activation of PAHs in the environment (Larson and Berenbaum, 1988). There are two routes by which radiation can alter the toxicity of PAHs: photosensitization and photomodification (Foote, 1987; Huang *et al.*, 1993, Krylov *et al.*, 1997). Photosensitization reactions initiated by PAHs usually proceed via formation of singlet-state oxygen (Larson and Berenbaum, 1988; Foote, 1991). Photosensitization begins with the sensitizing molecule absorbing a photon, which elevates it to the excited singlet-state. Then the molecule can be transformed by intersystem crossing to the excited triplet-state, where it can react with ground triplet-state oxygen to form excited singlet-state oxygen ($^1O_2$). PAHs have high

quantum yields for triplet-state formation, and have triplet lifetimes that are sufficiently long to allow for reaction with triplet-state oxygen. Singlet-state oxygen formed within a biological organism is highly damaging and toxic (Morgan *et al.*, 1977; Newsted and Giesy, 1987; Larson and Berebaum, 1988). Photomodification of PAHs, usually via oxidation reactions, leads to formation of new compounds that have different bioactivities than the parent compounds (Larson and Berenbaum, 1988; Huang *et al.*, 1993; Ren *et al.*, 1994; McConkey *et al.*, 1997). Oxidized PAHs are often more toxic than intact PAHs. By understanding photosensitization and photomodification, QSARs for photoinduced toxicity of PAHs can be generated (Greenberg *et al.*, 1993; Huang *et al.*, 1997; Krylov *et al*, 1997).

Initially, QSAR models for photoinduced toxicity of PAHs were based on photosensitization reactions, because most photoinduced PAH toxicity data relied only on the intact compounds (Morgan *et al*, 1977; Newsted and Giesy, 1987; Kochavar, 1987). In these models, correlations between PAH bioactivity and the triplet-state lifetimes, and/or the degree of splitting between singlet, and triplet-state energy levels were correlated to toxicity. However, some of the chemicals did not fit the models (Newsted and Giesy, 1987). These early QSAR models did not consider photomodifed of PAHs, because the toxicity of photomodifed PAHs had not yet been established (Huang *et al*, 1997).

The rates of PAH photomodification in sunlight are relatively rapid and photomodification has been shown to be a major factor in environmental toxicity of PAHs (Greenberg *et al*, 1993; Huang *et al*, 1993). Because photomodified PAHs are highly toxic, it follows that a complete model for the photoreactions of PAHs and their resultant photoinduced toxicity must incorporate determinants for both photosensitization and photomodification. Krylov *et al.* (1997) demonstrated the merits of using both a photomodification and photosensitization to describe the toxicity of PAHs to the aquatic higher plant *Lemna gibba*. This QSAR model demonstrated the photochemical processes important in the toxicity of sixteen PAHs to the higher aquatic plant *L. gibba*. It was a detailed chemical kinetic model to describe the key photochemical reactions of PAHs within leaf tissue and the surrounding aqueous medium. The model showed that

photosensitization and photomodification contribute additively to toxicity (Krylov *et al.* 1997; Huang *et al.* 1997). The results of two series of experiments on the photoinduced toxicity of 16 PAHs to *L. gibba*, were used to solve for two complex constants in the model. The first series, which determined the toxicity of the intact PAHs, was used to address that component of toxicity due to photosensitization. This led to a photosensitization factor (PSF) for each PAH. The second series of experiments, which determined the toxicity of the PAHs after photomodification, was used to address that component of toxicity due to the photomodified PAHs. This lead to a photomodification factor (PMF) for each PAH. The PSF and PMF were then used to explain toxicity on the basis of both photosensitization reactions initiated by the intact chemicals and direct toxicity of the photomodification products (Huang *et al.* 1997, Krylov *et al.* 1997).

The aim of this chapter was to determine if the QSAR model for the photoinduced toxicity of PAHs to *L. gibba* could be applied to another organism. The main question was whether a good correlation between the *L. gibba* QSAR model and photoinduced toxicity data for the aquatic bacterium *V. fischeri* could be achieved. This was important as it would indicate that both aspects of PAH phototoxicity, photosensitization and photomodification, are broadly applicable. The luminescent bacteria *V. fischeri* was used to assess photoinduced short- and long-term toxicity for the 16 PAHs used in the *L. gibba* QSAR. It was found that short-term toxicity showed no correlation to *L. gibba* toxicity nor to the *L. gibba* QSAR for photoinduced toxicity. However, data from long-term toxicity assay correlated well to the *L. gibba* toxicity data and the QSAR for photoinduced toxicity to *L. gibba*.

5.3 MATERIALS AND METHODS

5.3.1 Growth medium and Growth Conditions

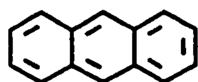
Vibrio fischeri (strain NRRL B-11177) was used as test organism. They were obtained from the Midwest Area National Center for Agricultural Utilization Research, Peoria, Illinois, USA. Aseptic techniques were employed to avoid contamination by

extraneous bacteria. *V. fischeri* growth medium was prepared using the following formulation: Monobasic Potassium Phosphate (KH_2PO_4), 18.4 mM; Sodium Chloride (NaCl), 0.5 M; Magnesium Sulfate ($MgSO_4.7H_2O$), 4.1 mM; Glycerol ($C_3H_8O_3$), 54.3 mM; Yeast extract, 1 mg/L; Peptone, 5 mg/L; Bactopeptamin, 1 mg/L. These components were added to distilled/deionized 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 Sodium Hydroxide (NaOH) and autoclaved at 121 °C for 30 minutes. A minimal medium was also used. It was as above without glycerol, yeast extract, peptone and bactopeptamin. Peptone and Yeast extract were obtained from BDH Inc., Toronto, Ontario, and Bactopeptamin was obtained from Difco Laboratories, Detroit, MI. Stock cultures of *V. fischeri* were grown on agar plates and used for primary inoculation of liquid cultures. A spectrophotometer (Perlsin-Elmer, Mississauga, Ontario, CA) and Cytofluor 2350 fluorescence measurement system (Millipore Ltd., Mississauga, Ontario, Canada) were used to measure cell density and light output. The chemicals used for toxicity testing were anthracene (ANT), benzo(a)anthracene (BAA), benzo(a)pyrene (BAP), benzo(b)anthracene (BBA), benzo(b)fluorene (BBF), benzo(e)pyrene (BEP), benzo(g,h,i)perylene (BGP), chrysene (CHR), coronene (COR), dibenzo(a,h)anthracene (DAA), dibenzo(a,i)pyren (DAP), fluoranthene (FLA), fluorene (FLU), phenanthrene (PHE), pyrene (PYR) and triphenylene (TRI). Their structures are given in Figure 5.1. Some of their physical properties are given in Table 5.1.

5.3.3 Bacterial short- and long-term toxicity assay

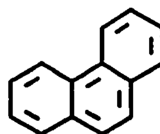
The short-term toxicity test was based on inhibition of light production in luminescent bacteria incubated for 15 minutes in darkness or SSR. Inhibition of light production and inhibition of growth were used as endpoints in long-term toxicity test. In the latter case, toxicity was measured after 18 hours of growth of the bacteria in dark or SSR.

Figure 5.1 Structures of the sixteen PAHs used in this study

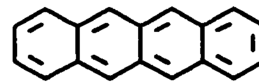


Anthracene
ANT

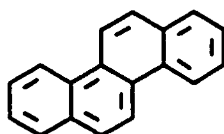
Fluorene
FLU



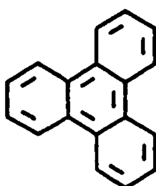
Phenanthrene
PHE



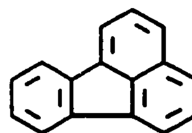
Benzo(b)anthracene
BBA



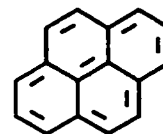
Chrysene
CHR



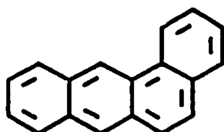
Triphenylene
TRI



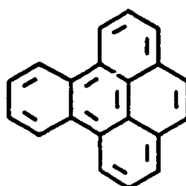
Fluoranthene
FLA



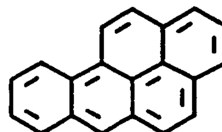
Pyrene
PYR



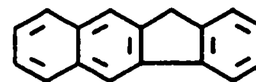
Benzo(a)anthracene
BAA



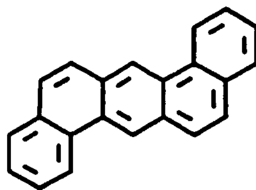
Benzo(e)pyrene
BEP



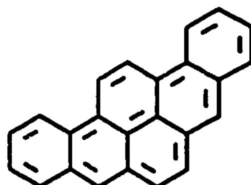
Benzo(a)pyrene
BAP



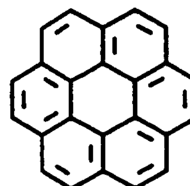
Benzo(b)fluorene
BBF



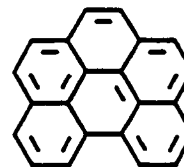
Dibenzo(a,h)anthracene
DAA



Dibenzo(a,i)pyrene
DAP



Coronene
COR



Benzo(g,h,i)perylene
BGP

Table 5.1 Physical-chemical parameters for the sixteen PAHs used in this study.

PAHs	ABB.	M. F	M. W	W. S (µmol/L)[1]	Log K_{ow} range[2]
Anthracene	ANT	$C_{14}H_{10}$	178.23	0.409	4.54 - 4.45
Benzo(a)anthracene	BAA	$C_{18}H_{12}$	228.29	0.040	5.49 - 5.91
Benzo(a)pyrene	BAP	$C_{20}H_{12}$	252.32	0.016	6.06 - 6.50
Benzo(b)anthracene	BBA	$C_{18}H_{12}$	228.28	0.048	5.66 - 6.02
Benzo(b)fluorene	BBF	$C_{17}H_{12}$	216.30	0.009	5.27 - 5.69
Benzo(e)pyrene	BEP	$C_{20}H_{12}$	252.30	0.025	6.06 - 6.50
Benzo(g,h,i)perylene	BGP	$C_{22}H_{12}$	276.34	0.002	6.51 - 7.10
Chrysene	CHR	$C_{18}H_{12}$	228.29	0.013	5.61 - 5.91
Coronene	COR	$C_{24}H_{12}$	300.36	0.001	5.40 - 8.00
Dibenzo(a,h)anthracene	DAA	$C_{22}H_{14}$	278.35	0.002	5.97 - 7.19
Dibenzo(a,i)pyrene	DAP	$C_{24}H_{14}$	302.40	0.001	6.44 - 7.40
Fluoranthene	FLA	$C_{16}H_{10}$	202.26	1.280	4.90 - 5.22
Fluorene	FLU	$C_{13}H_{10}$	166.22	12.000	4.18 - 4.47
Phenanthrene	PHE	$C_{14}H_{10}$	178.23	7.200	4.45 - 4.57
Pyrene	PYR	$C_{16}H_{10}$	202.30	0.720	4.90 - 5.18
Triphenylene	TRI	$C_{18}H_{12}$	228.29	0.180	5.61 - 5.91

ABB: Abbreviations

M. F: Molecular formula

M.W: Molecular weight

W. S: Water solubility (µmol/L)[1]

Log K_{ow}: n-octanol/water partition coefficient

[1]From Mackay *et al.*, 1992; Neff 1985

[2]From van Brummelen 1995

5.3.3.1 Short-term assay

A starter culture was diluted into minimal media, and twenty-four 500 µl aliquots of cells were distributed in a 48-well cell culture plate as in chapter 3. After 5 minutes acclimatization in the 48-well cell culture plates, the luminescence intensity was measured. After the initial measurement of bacterial luminescence, the bacteria were dosed with the PAHs. Aliquots of 500 µl of the cell culture were combined with equal volumes of the test chemical in minimal culture medium. A dilution series of each chemical (7 concentrations plus the control) were added in a geometric series to the wells in triplicate. Stock solutions of PAHs were prepared in dimethyl sulphoxide (DMSO) and added by 1000-fold dilution to the culture medium to give the specified concentration. The DMSO concentration of 0.1% does not affect luminescence or growth of *V. fischeri*. After dosing, the cells were incubated in darkness or SSR at room temperature for 15 minutes and the luminescence intensity was measured.

5.3.3.2 Long-term assay

After the short-term test was completed, the multi well culture plate was returned to the growth chamber. The cells were incubated in minimal media with the chemical in darkness or SSR at 20°C for 8 hours. After this pre-incubation, the medium was made complex as in chapter 3. The cells were then incubated for an additional 18 hours in SSR or darkness at 20°C. Toxicity was determined by inhibition of growth and luminescence.

5.3.3.3 Statistical analysis

For the short-term assay, the measured responses were used as a percent decrease in light production for *V. fischeri*. For the long-term assay percent decrease in light production and a percent decrease in growth rate for *V. fischeri* were used. Data analysis and curve fitting were performed using SYSTAT software (Wilkinson, 1994). This was used to calculate EC50s and confidence intervals the EC50 value for each replicate assay

was calculated, and then an average EC50 was obtained from the 6 replicates. Confidence intervals on the resulting mean EC50s were calculated according to Kuehl (1994). Average inhibition of luminescence and growth at a concentration 0.5 mg/L for each chemical were also calculated. The quality of fit of the regressions (r^2) and the significance of the regressions (p) were determined with SYSTAT. Linear and log-linear least squares regressions were performed to determine the quality of the correlation of a given parameter to toxic strength.

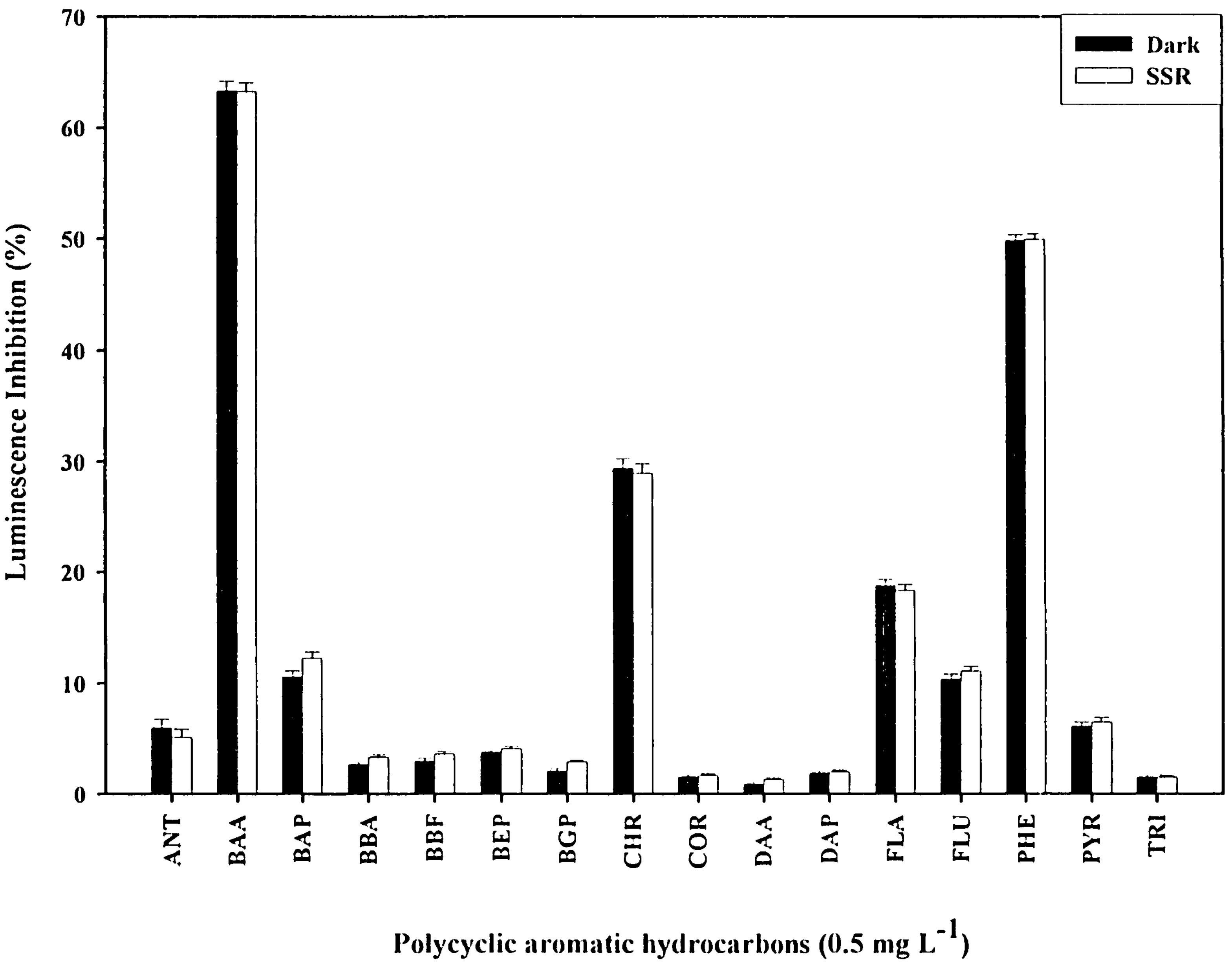
5.4 RESULTS AND DISCUSSION

5.4.1 Short-term Toxicity

The *Vibrio fischeri* short-term toxicity assay was performed individually with sixteen different intact PAHs in minimal medium. The luminescent bacteria were dosed with serial concentrations of PAHs. Inhibition of luminescence intensity was used to assess the toxicity after 15 minutes exposure in the darkness or SSR. The photoinduced short-term toxicity of PAHs are presented as EC50 and as inhibition of luminescence at 0.5 mg/L of a given PAH (Figure 5.2 and Table 5.2). In darkness, BAA (abbreviations of PAHs are given in Figure 5.1) had the highest toxicity of the sixteen PAHs tested. BAA had the same level of toxicity in SSR and darkness. The PAH with the next highest toxicity was PHE, and it also had the same level of effect in darkness and SSR. Three PAHs had moderate toxicity (10 to 30 % inhibition of luminescence) (CHR, FLA, and FLU). The remaining chemicals (ANT, BBA, BBF, BEP, BGP, COR, DAA, DAP and TRI) had low toxicity at 0.5 mg/L (<10 % inhibition of luminescence) (Figure 5.2 and Table 5.2).

For all the PAHs tested there was no difference in toxicity to *V. fischeri* in SSR or darkness. The short exposure time to SSR (15 min) precluded any measurable photooxidation or photomodification of the PAHs, ensuring that the observed toxicity could be attributed solely to intact PAHs. Further, the chemicals were apparently not causing appreciable photosensitized damage in this short period of time. This implies that

Figure 5.2 Short-term toxicity of PAHs to the luminescent bacteria *V. fischeri* in minimal medium. *Vibrio fischeri* were incubated in either darkness or SSR for 15 min in the presence of 0.5 mg/L of a given PAH. The toxicity was monitored by percent inhibition of luminescence relative to the controls. Error bars represent 95% confidence intervals (n = 6).



Dark
SSR
Luminescence Inhibition (%)
70
60
50
40
30
20
10
0
ANT
BAA
BAP
BBA
BBF
BEP
BGP
CHR
COR
DAA
DAP
FLA
FLU
PHE
PYR
TRI
Polycyclic aromatic hydrocarbons (0.5 mg L-1)

Table 5.2 Calculated EC50s and photoinduced short-term toxicity at 0.5 mg/L for the sixteen intact PAHs to *Vibrio fischeri* under darkness and SSR. Data are means ± 95% confidence intervals, n = 6.

	EC50s (mg/L)		% Inhibition of luminescence (0.5 mg/L)	
PAHs	**Dark**	**SSR**	**Dark**	**SSR**
Anthracene	17.07 ± 0.78	16.13 ± 0.69	6.06 ± 0.78	5.19 ± 0.77
Benzo(a)anthracene	0.30 ± 0.28	0.29 ± 0.16	63.33 ± 0.88	63.25 ± 0.82
Benzo(a)pyrene	8.04 ± 0.51	7.93 ± 0.48	10.60 ± 0.53	12.24 ± 0.55
Benzo(b)anthracene	90.63 ± 0.88	89.86 ± 0.80	2.69 ± 0.19	3.40 ± 0.18
Benzo(b)fluorene	16.69 ± 0.66	16.68 ± 0.68	3.03 ± 0.17	3.69 ± 0.16
Benzo(e)pyene	17.42 ± 0.29	17.67 ± 0.28	3.87 ± 0.13	4.15 ± 0.13
Benzo(g,h,i)perylene	89.38 ± 0.65	88.91 ± 0.69	2.13 ± 0.17	2.91 ± 0.17
Chrysene	1.43 ± 0.11	1.37 ± 0.10	29.44 ± 0.81	28.99 ± 0.81
Coronene	98.09 ± 0.75	98.01 ± 0.77	1.56 ± 0.18	1.69 ± 0.18
Dibenzo(a,h)anthracene	97.18 ± 0.65	96.55 ± 0.61	0.88 ± 0.17	1.30 ± 0.16
Dibenzo(a,i)pyrene	91.21 ± 0.35	90.81 ± 0.44	1.92 ± 0.14	2.01 ± 0.14
Fluoranthene	2.16 ± 0.18	2.12 ± 0.13	18.80 ± 0.52	18.34 ± 0.51
Fluorene	3.20 ± 0.37	3.13 ± 0.21	10.37 ± 0.44	11.07 ± 0.42
Phenanthrene	0.51 ± 0.09	0.51 ± 0.11	49.84 ± 0.51	49.91 ± 0.51
Pyrene	15.07 ± 0.69	14.53 ± 0.76	6.22 ± 0.37	6.59 ± 0.38
Triphenylene	96.99 ± 0.75	95.97 ± 0.88	1.53 ± 0.18	1.54 ± 0.19

if short-term bacterial toxicity will be compared to *L. gibba* toxicity there will not be a correlation, as PAH toxicity to *L. gibba* is highly dependent on actinic radiation (Huang et al, 1997). This also predicts that the QSAR for *L. gibba* will have no correlation to the *V. fischeri* short-term toxicity data.

5.4.2 Long-term Toxicity

Vibrio fischeri cells were incubated with the PAHs for 8 hours in darkness or SSR in minimal medium, and then for 18 hours in complex medium under the same lighting conditions to determine the relative toxic potency of each chemical (Figure 5.3). For all 16 chemicals tested, the results based on inhibition of luminescence were very similar to those based on growth inhibition. Also, the results based on EC50s and percent inhibition at 0.5 mg/L were similar. This was important because toxicity data at one concentration is better suited for QSAR modeling than EC50s. This is because the kinetic QSAR model for *L. gibba* mathematically relates better to inhibition of an effect than to EC50s (see below and Krylov *et al.*, 1997). As well, inhibition of luminescence at 0.5 mg/L is spread over only 2 orders of magnitude, while the EC50s are spread over 3 orders of magnitude.

The toxicity of ANT in darkness was very low, however, the toxicity was enhanced greatly upon exposure to SSR. Similarly, the toxicity of BAA and FLA were enhanced upon exposure to SSR compared to their toxicity in darkness. Indeed, for 15 of the 16 PAHs tested, toxicity was increased in SSR relative to darkness. Only for CHR was the toxicity same (and very low) in both SSR and darkness. Also for three chemicals, toxicity only increased about 2-fold due to SSR exposure.

All of the data collected to assess the photoinduced long-term toxicity of intact PAHs using inhibition of either luminescence or growth at 0.5 mg/L were within two orders of magnitude (Table 5.3). The results show the highest toxic effect in SSR was from ANT the followed by BAA and FLA. The least toxic chemicals were CHR, TRI and COR. Long-term assays for the PAHs in darkness revealed very little effect for all 16 compounds. However, long-term toxicity in SSR showed about half the PAHs to have moderate to high toxicity (>25 % inhibition of growth).

Figure 5.3 Long-term toxicity of PAHs to *V. fischeri*. *Vibrio fischeri* were incubated in either darkness or SSR in the presence of 0.5 mg/L of a given PAH. The assay involved an 8 h pre-incubation in minimal medium followed by an 18 h incubation in complex medium. Toxicity was monitored by percent inhibition of luminescence and growth relative to the controls. Error bars represent 95% confidence intervals (n = 6).

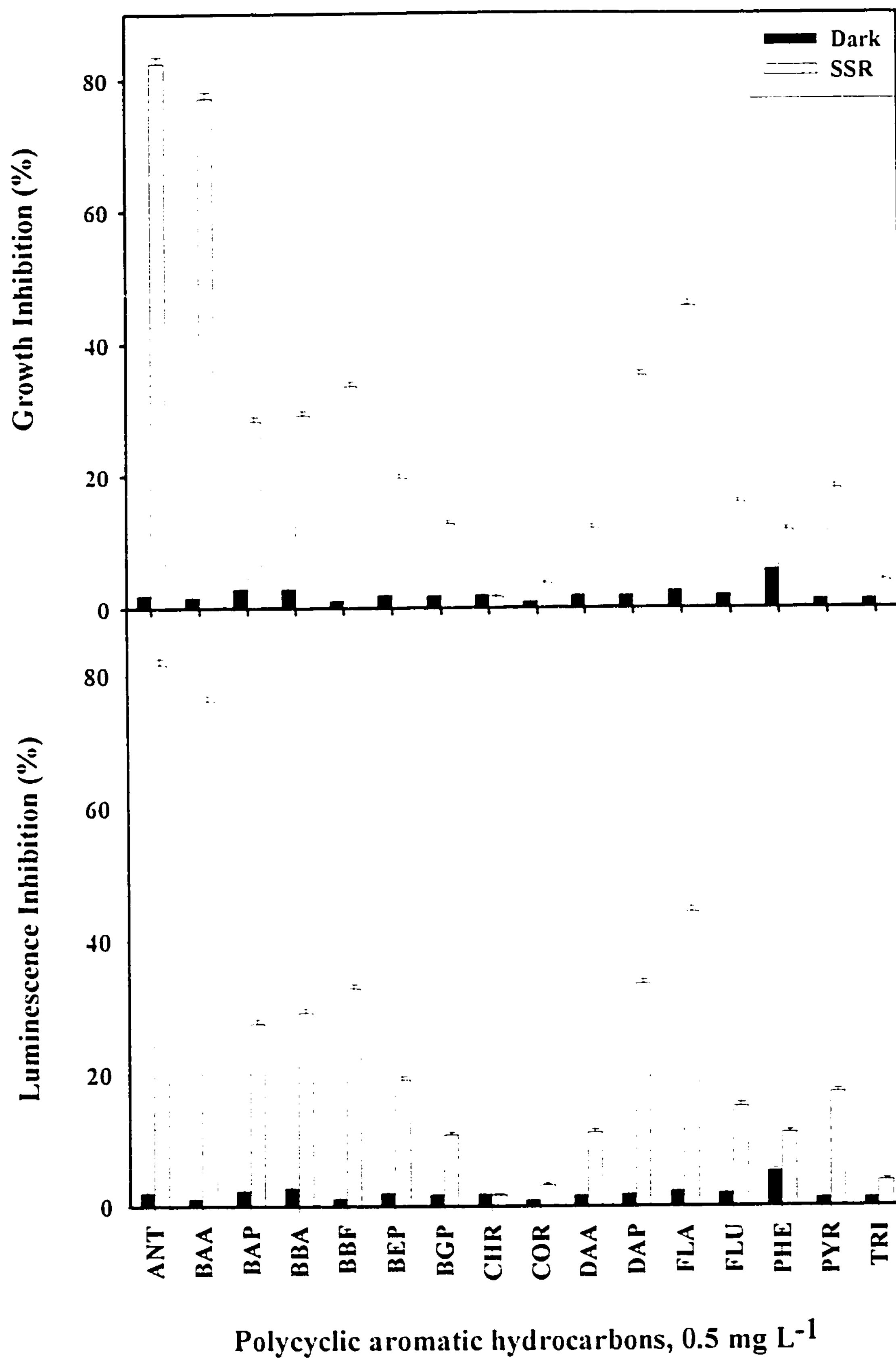

Dark
SSR
Growth Inhibition (%)
Luminescence Inhibition (%)
0
20
40
60
80
ANT
BAA
BAP
BBA
BBF
BEP
BGP
CHR
COR
DAA
DAP
FLA
FLU
PHE
PYR
TRI
Polycyclic aromatic hydrocarbons, 0.5 mg L-1

Table 5.3 Calculated EC50s and toxicity at 0.5 mg/L for the sixteen PAHs to *V. fischeri* under SSR or darkness. Data are means ± 95% confidence intervals (n = 6).

PAHs	Inhibition of Luminescence				Inhibition of Growth			
	EC50 mg/L		Toxicity at 0.5 mg/L*		EC50 mg/L		Toxicity at 0.5 mg/L*	
	Dark	SSR	Dark	SSR	Dark	SSR	Dark	SSR
ANT	85.78 ± 0.73	0.13 ± 0.13	1.98 ± 0.17	81.61 ± 0.91	84.88 ± 0.69	0.12 ± 0.11	2.01 ± 0.17	82.55 ± 0.91
BAA	89.81 ± 0. 85	0.23 ± 0.18	1.01 ± 0.16	76.04 ± 0.88	89.25 ± 0.69	0.22 ± 0.14	1.62 ± 0.11	77.22 ± 0.81
BAP	67.81 ± 0.78	0.81 ± 0.28	2.28 ± 0.25	27.56 ± 0.63	67.11 ± 0.86	0.80 ± 0.21	2.89 ± 0.12	28.23 ± 0.62
BBA	89.31 ± 0.75	0.72 ± 0.18	2.68 ± 0.28	29.18 ± 0.61	88.51 ± 0.69	0.71 ± 0.18	2.93 ± 0.13	29.96 ± 0.62
BBF	85.21 ± 0.85	0.95 ± 0.18	1.09 ± 0.14	32.77 ± 0.62	84.95 ± 0.77	0.93 ± 0.17	1.15 ± 0.11	33.62 ± 0.62
BEP	80.66 ± 0.75	1.50 ± 0.26	1.94 ± 0.18	19.09 ± 0.33	79.99 ± 0.88	1.49 ± 0.28	1.99 ± 0.15	19.62 ± 0.33
BGP	93.77 ± 0.55	18.49 ± 0.15	1.72 ± 0.16	10.78 ± 0.30	93.21 ± 0.75	17.75 ± 0.13	1.91 ± 0.12	12.71 ± 0.31
CHR	89.98 ± 0.85	84.75 ± 0.79	1.82 ± 0.19	1.62 ± 0.18	89.68 ± 0.65	84.94 ± 0.85	1.99 ± 0.13	1.64 ± 0.19
COR	99.86 ± 0.65	99.89 ± 0. 77	0.86 ± 0.12	3.21 ± 0.18	99.85 ± 0.80	99.84 ± 0.91	0.97 ± 0.11	3.66 ± 0.19
DAA	94.85 ± 0.69	1.35 ± 0.11	1.55 ± 0.13	11.15 ± 0.27	93.99 ± 0.83	1.34 ± 0.09	1.88 ± 0.12	11.95 ± 0.21
DAP	92.55 ± 0.65	1.54 ± 0.13	1.79 ± 0.14	33.47 ± 0.61	92.41 ± 0.77	1.52 ± 0.10	1.92 ± 0.11	35.07 ± 0.61
FLA	83.96 ± 0.65	0.70 ± 0.09	2.22 ± 0.17	44.34 ± 0.71	83.85 ± 0.55	0.70 ± 0.08	2.59 ± 0.16	45.63 ± 0.71
FLU	80.99 ± 0.72	1.04 ± 0.14	1.96 ± 0.17	15.13 ± 0.39	81.29 ± 0.75	1.04 ± 0.11	2.01 ± 0.13	15.83 ± 0.31
PHE	4.53 ± 0.35	1.93 ± 0.17	5.29 ± 0.31	11.12 ± 0.22	4.48 ± 0.39	1.92 ± 0.15	5.79 ± 0.30	11.61 ± 0.22
PYR	97.51 ± 0.85	1.43 ± 0.12	1.19 ± 0.12	17.25 ± 0.41	97.21 ± 0.88	1.42 ± 0.19	1.29 ± 0.15	17.95 ± 0.42
TRI	96.89 ± 0.81	91.20 ± 0.75	1.19 ± 0.13	3.80 ± 0.18	96.79 ± 0.86	91.20 ± 0.81	1.32 ± 0.10	4.02 ± 0.18

* Toxicity at 0.5 mg/L is % inhibition of luminescence or growth.

It is interesting that ANT and BAA showed the largest increase in toxicity in SSR compared to the other 14 PAHs. Both ANT and BAA are photomodified faster than the rest of the PAHs, and they increase greatly in toxicity following photomodification (Huang *et al*, 1997). Thus, the increased toxicity of PAHs after incubation in SSR is consistent with the previous studies which showed that PAHs photomodified in SSR are more toxic than intact PAHs (Huang *et al.*, 1993; Ren *et al.*, 1994; Huang *et al*, 1997). The dramatic increase in toxicity in SSR showed photoinduced toxicity to be important for PAH toxicity to bacteria. This indicates that it might be possible to relate the QSAR of PAH photoinduced toxicity for *L. gibba* to the *V. fischeri* long-term toxicity data.

5.4.3 Summary of the L. gibba QSAR model for the photoinduced toxicity of PAHs

To generate a QSAR model describing the photoinduced toxicity of PAHs, the mechanism of the primary photochemical reactions involving light-sensitive molecules and the impacts of those reactions on living organisms must be considered (Morgan *et al*, 1977; Newsted and Giesy, 1987; Foote, 1991). The phototoxicity of PAHs occurs by two routes: photosensitization reactions initiated by PAHs and photomodification of PAHs. Both photosensitization and photomodification can be used in toxicity modeling (Larson and Berenbaum, 1988; Foote, 1991, Krylov *et al.*, 1997; Huang *et al.*, 1997). Indeed, a QSAR model based on these two factors for the photoinduced toxicity of 16 PAHs to *L. gibba* was successfully generated (Krylov *et al.*, 1997; Huang *et al.*, 1997).

In that QSAR model, a relationship based on a sum of photosensitization and photomodification descriptors were used to predict and explain photoinduced toxicity. These descriptors were a photomodification factor (PMF) and a photosensitization factor (PSF). When the PMFs and PSFs for each PAH were summed, an excellent correlation of PAH toxicity to L. gibba was achieved (Huang *et al.*, 1997). Merging the PMF and PSF into a QSAR model requires incorporation of a large number of parameters that may have varying degrees of importance in toxicity. The number of parameters required was minimized using toxicity data for *L. gibba* (Krylov *et al*, 1997; Huang *et al*, 1997).

For the photomodification factors (PMF), a PAH photoproduct in aqueous solution is generated with first order rate constant k_m. The photoproduct will be assimilated by the plants with a rate of constant, k_p. P_L will cause growth inhibition of plants with a rate constant k_J. Each of these constants are given in Table 5.4.

This gives a PMF of:

$$PMF = f\{k_m, k_p, k_J\} \qquad (1)$$

Because the rate of assimilation, k_p, is much faster than photomodification process, k_m, (Duxbury *et al.*, 1996), the rate limiting process for generation of photoproducts in a test organism will be the photomodification rate, k_m. Thus, k_p can be excluded from equation 1, giving:

$$PMF = f\{k_m, k_J\} \qquad (2)$$

The PMF for each PAH is the product of normalized values for k_m and k_J (Huang *et al*, 1997) (Table 5.4).

The photosensitization Factor (PSF) for *L. gibba* was shown to be dependent on the concentration of the PAH in the leaf and the efficiency of formation of the PAH triplet state (Krylov *et al*, 1997). The former is dependent on the assimilation rate, k_c, of PAHs. The latter is dependent on three factors: total irradiation (energy) in the SSR source (E), quantum yield for formation of the triplet excited state (φ), and efficiency of photon absorbance by the PAH (J). Frequency of photon absorbance, J, is described by the integral of the overlap between the absorbance spectrum of a given PAH and the emission spectrum of the SSR source. The photosensitization process can then be described by a photosensitization factor (PSF):

$$PSF = f\{k_c, E, \varphi, J\} \qquad (3)$$

Table 5.4 Physical constants and toxicity data used for the calculation of PSFs and PMFs. Toxicity is inhibition of growth of *Lemna gibba* by the intact PAHs ± standard deviation, n = 9. $[C_L]$, in μmol/g fresh weight, represents plant uptake of PAHs. φ is the quantum yield for triplet-state formation. J represents absorption of SSR by the PAHs. The PSF is the product of $[C_L]^n$, φ^n, and J^n (The superscript n denotes that the respective values were normalized). $t_{1/2}$ is the half-life in the h of the PAH in SSR, and k_m (h^{-1}) is the exponential decay rate constant based on $t_{1/2}$. T^n_{pm} is the normalized value of the toxicity (inhibition of growth) of the fully photomodified PAHs to *L. gibba*. The PMF is the product of k^n_m and T^n_{pm}.

PAHs	Toxicity (I)	$[C_L]$	φ	J	$[C_L]^n$	φ^n	J^n	PSF	$t_{1/2}$	k_m	k^n_m	T^n_{pm}	PMF
ANT	1.000 ± 0.024	0.080	0.60	36.9	0.250	0.63	0.119	0.019	2	0.347	1.000	1.00	1.000
BAA	0.745 ± 0.014	0.012	0.80	58.7	0.038	0.84	0.190	0.006	5	0.139	0.461	1.00	0.401
BAP	0.255 ± 0.014	0.046	0.40	200.3	0.144	0.042	0.647	0.039	52	0.013	0.038	0.98	0.037
BBA	0.259 ± 0.024	0.035	0.65	37.0	0.109	0.68	0.120	0.009	27	0.026	0.075	0.97	0.073
BBF	0.354 ± 0.038	0.093	0.50	46.5	0.291	0.53	0.150	0.023	70	0.010	0.029	0.66	0.019
BEP	0.165 ± 0.061	0.051	0.70	78.8	0.159	0.74	0.255	0.030	75	0.009	0.026	0.78	0.020
BGP	0.139 ± 0.057	0.054	0.60	126.2	0.169	0.63	0.408	0.044	100	0.007	0.020	0.26	0.005
CHR	0.016 ± 0.043	0.039	0.67	34.8	0.122	0.70	0.112	0.010	56	0.012	0.035	0.74	0.026
COR	0.118 ± 0.026	0.037	0.80	102.0	0.116	0.84	0.330	0.032	100	0.007	0.020	0.55	0.011
DAA	0.085 ± 0.052	0.058	0.50	144.2	0.181	0.53	0.466	0.044	16	0.043	0.124	0.17	0.021
DAP	0.458 ± 0.038	0.045	0.50	309.5	0.141	0.53	1.000	0.074	40	0.017	0.049	0.64	0.031
FLA	0.580 ± 0.028	0.288	0.60	84.8	0.900	0.63	0.274	0.156	40	0.017	0.049	0.88	0.043
FLU	0.160 ± 0.061	0236	0.31	3.0	0.738	0.33	0.010	0.002	19	0.037	0.107	0.30	0.032
PHE	0.104 ± 0.071	0.320	0.80	8.9	1.000	0.84	0.029	0.024	14	0.050	0.144	0.27	0.039
PYR	0.170 ± 0.024	0.251	0.27	72.7	0.784	0.28	0.235	0.052	46	0.015	0.043	0.45	0.020
TRI	0.071 ± 0.043	0.094	0.95	8.9	0.294	1.00	0.029	0.008	65	0.011	0.032	0.58	0.018

Because the same SSR source was used for all toxicity test, total irradiation energy, E, can be excluded form the equation 3, giving:

$$PSF = f\{k_c, \varphi, J\} \quad (4)$$

The QSAR model by Huang *et al.*, (1997) showed the PSF to be a product of the three normalized factors. These constants and the PSF for each PAH are given in Table 5.4.

Because the photomodification and photosensitization processes function independently (Krylov *et al.*, 1995), the QSAR for toxicity to *L. gibba* was found to be a sum of the PSF and PMF (Huang *et al.*, 1997), where:

$$\text{Toxicity } \alpha \ f\{PMF + PSF\} = f\{k_m, k_d\} + f\{k_c, \varphi, J\} \quad (5)$$

These factors (Table 5.4) will be regressed against toxicity data for *V. fischeri*. However, to do this one must consider how to describe toxicity of the bacteria.

The QSAR model for *L. gibba* was mathematically related to a measure of toxicity. Leaf production of the plants was used for this. The number of leaves at a given time, *t*, was used to measure plant growth. When nutrients and space are not limiting (Greenberg *et al.*, 1992), the growth rate, dN/dt, is proportional to the number of leaves, N, present at any moment in a toxicity test, or:

$$dN/dt = k'N. \quad (6)$$

Toxicity of PAHs for the *L. gibba* QSAR model was described as growth inhibition, I. Since growth is proportional to N, the number of leaves in the treated samples (N_t) relative to the control sample (N_c) during a log phase of growth rate were used to generate inhibition of growth according to the following equation

$$I = (\ln N_c - \ln N_t)/\ln N_c, \quad (7)$$

However, since N is proportional to growth, Huang *et al.*, (1997) were able to relate I to the PMF and PSF by:

$$I \alpha f\{PSF + PMF\} = f\{k_m, k_d\} + f\{k_c, \varphi, J\}. \quad (8)$$

Thus, to relate the *V. fischeri* toxicity to the *L. gibba* QSAR model, the *V. fischeri* toxicity data to be used will be inhibition of growth or luminescence at one concentration. The concentration used was 0.5 mg/L (Table 5.2 and 5.5). To apply the *L. gibba* QSAR model to *V. fischeri*, the *V. fischeri* short-term and long-term data were correlated to the *L. gibba* toxicity data, the PMF, the PSF and the sum of PMF + PSF.

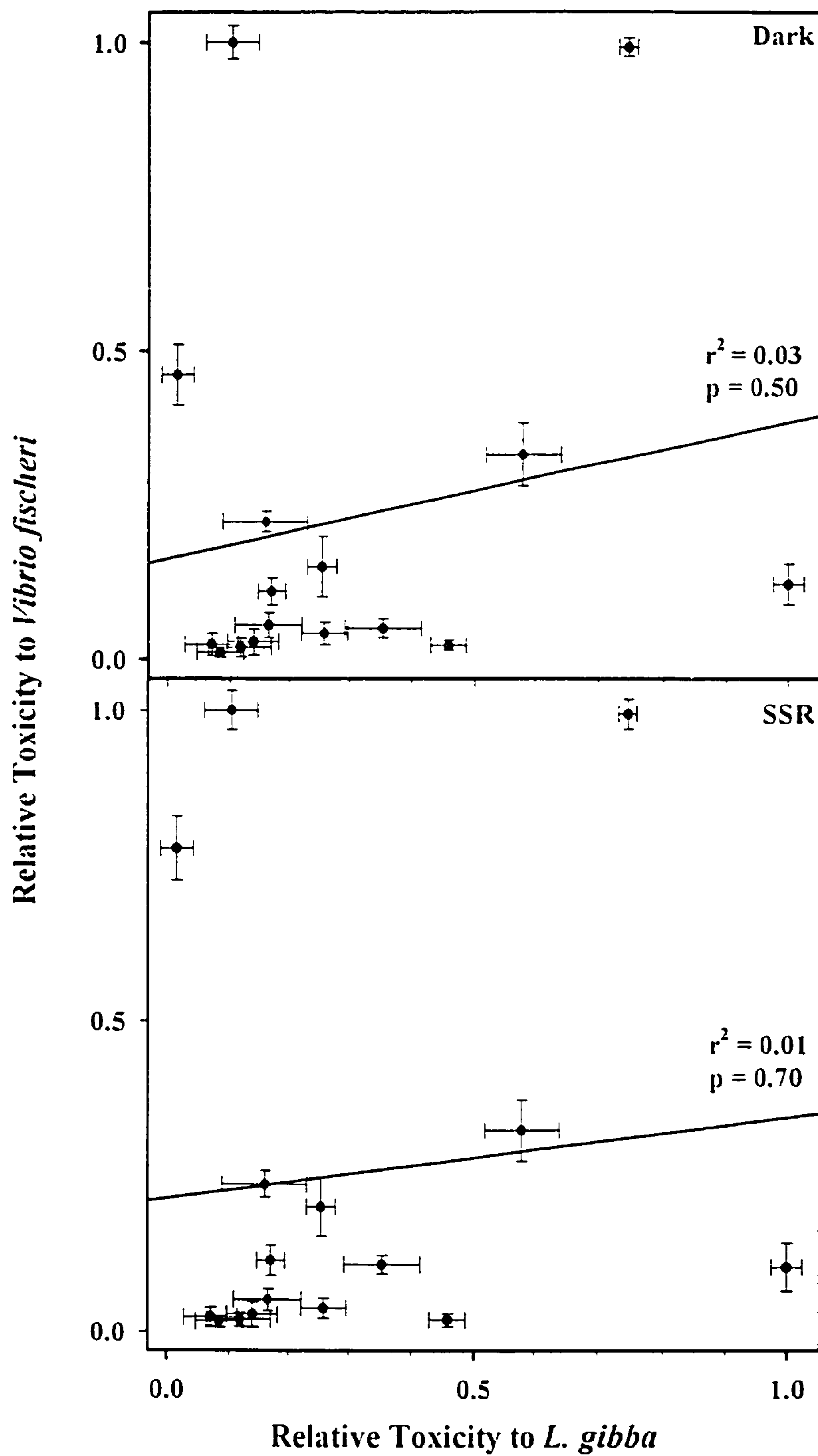
5.4.4 Correlation between short-term toxicity and L. gibba toxicity

The QSAR toxicity model developed by Huang *et al.*, (1997) was based on toxicity of PAHs to *L. gibba* (Table 5.5). Therefore, it was necessary to determine if the *L. gibba* data could be correlated with the toxicity of PAHs to *V. fischeri* (Table 5.5). The toxicity of intact PAHs to *V. fischeri* in the short-term assay was plotted versus the toxicity of PAHs to *L. gibba* (Figure 5.4). The inhibition of luminescence for PAHs to *V. fischeri* at 0.5 mg/L were normalized from 0 to 1 and then plotted against the toxicity data for *L. gibba* (Figure 5.4). No significant correlation to toxicity was observed in the dark or SSR. This result is not surprising because there was no evidence of photoinduced toxicity in the short term *V. fischeri* assay and PAH toxicity to *L. gibba* is highly dependent on light.

Table 5.5 Summary data for relative short-term toxicity of the sixteen PAHs to *V. fischeri* and *L. gibba*. PSF, PMF and PSF+PMF from the *L. gibba* QSAR are also given.

PAHs	Short-term toxicity		Toxicity to *L. gibba*	PSF	PMF	PSF + PMF
	Dark	SSR				
Anthracene	0.122 ± 0.033	0.105 ± 0.039	1.000 ± 0.024	0.019	1.000	0.019
Benzo(a)anthracene	0.992 ± 0.014	0.995 ± 0.024	0.745 ± 0.014	0.006	0.401	0.407
Benzo(a)pyrene	0.150 ± 0.049	0.162 ± 0.048	0.255 ± 0.014	0.039	0.037	0.076
Benzo(b)anthracene	0.042 ± 0.018	0.038 ± 0.016	0.259 ± 0.024	0.009	0.073	0.082
Benzo(b)fluorene	0.050 ± 0.014	0.061 ± 0.015	0.354 ± 0.038	0.023	0.019	0.042
Benzo(e)pyene	0.055 ± 0.020	0.052 ± 0.018	0.165 ± 0.061	0.030	0.020	0.050
Benzo(g,h,i)perylene	0.028 ± 0.021	0.028 ± 0.020	0.139 ± 0.057	0.044	0.005	0.049
Chrysene	0.461 ± 0.049	0.578 ± 0.051	0.016 ± 0.043	0.010	0.026	0.036
Coronene	0.019 ± 0.015	0.020 ± 0.011	0.118 ± 0.026	0.032	0.011	0.043
Dibenzo(a,h)anthracene	0.011 ± 0.005	0.017 ± 0.009	0.085 ± 0.052	0.044	0.021	0.065
Dibenzo(a,i)pyrene	0.023 ± 0.008	0.019 ± 0.010	0.458 ± 0.038	0.074	0.031	0.105
Fluoranthene	0.332 ± 0.051	0.325 ± 0.049	0.580 ± 0.028	0.156	0.043	0.199
Fluorene	0.223 ± 0.017	0.239 ± 0.021	0.160 ± 0.061	0.002	0.032	0.034
Phenanthrene	1.000 ± 0.027	1.000 ± 0.021	0.104 ± 0.071	0.024	0.039	0.064
Pyrene	0.110 ± 0.022	0.116 ± 0.025	0.170 ± 0.024	0.052	0.020	0.072
Triphenylene	0.024 ± 0.018	0.024 ± 0.015	0.071 ± 0.043	0.008	0.018	0.026

Figure 5.4 Short-term toxicity of intact polycyclic aromatic hydrocarbons (PAHs) to *V. fischeri* compared to the toxicity of PAHs to *L. gibba*. Toxicity data for intact PAHs on *V. fischeri* in (Table 5.3) normalized from 0 to 1 and then plotted against the toxicity data of *L. gibba* (Table 5.5).


Dark
$r^2 = 0.03$
$p = 0.50$
SSR
$r^2 = 0.01$
$p = 0.70$
1.0
0.5
0.0
Relative Toxicity to Vibrio fischeri
Relative Toxicity to L. gibba

5.4.5 Correlation of short-term toxicity to the PSF

In the short-term assay period (15 minuets), the only potential light-induced toxicity would be from photosensitization reactions. However as, stated above there was no different in toxicity to *V. fischeri* in darkness or SSR. Therefore, a correlation to either the PSF or PMF from the *L. gibba* QSAR was not anticipated. Nonetheless, to confirm this the *V. fischeri* toxicity data in darkness and SSR was plotted against the PSF (Table 5.4 and Figure 5.5). A plot of luminescence inhibition versus the PSF is shown in Figure 5.5. As anticipated, no relationship between the two data sets was observed.

5.4.6 Correlation of short-term V. fischeri toxicity to the PMF

The short-term toxicity data in darkness and SSR for *V. fischeri* normalized from 0 to 1 and then plotted against the PMF from the *L. gibba* QSAR (Table 5.5 and Figure 5.6). A plot of inhibition of luminescence versus PMF did not reveal a relationship. Again, because toxicity from the short-term assay was not light dependent and did not show any correlation to the *L. gibba* toxicity data, it is not surprising that there was no correlation to the PMF. Further, with the short-term assay, there was no time for photomodification, and thus a correlation was not expected.

5.4.7 Correlation of short-term toxicity to the sum of PSF and PMF

To be complete, the short-term *V. fischeri* toxicity data was plotted against the sum of the PMF and PSF from the *L. gibba* QSAR (Figure 5.7). This was done because the full QSAR was needed to describe photoinduced toxicity to *L. gibba*. Once again no correlation was observed. This is mostly attributable to the fact that there was no photoinduced toxicity in the *V. fischeri* short-term test. Nonetheless, it was important to confirm that a correlation was not observed for any of the *L. gibba* QSAR factors. Indeed, an important test of QSAR models is that they do not correlate to all toxicity data. This is a crucial test when a relationship is not expected, as is the case here.

Figure 5.5 Short-term toxicity of intact PAHs to *V. fischeri* compared to the PSF from the *L. gibba* QSAR. Toxicity data for intact PAHs on *V. fischeri* normalized from 0 to 1 and then plotted against the PSF (Table 5.5). No significant correlation between toxicity and the PSF was observed in the darkness or SSR.

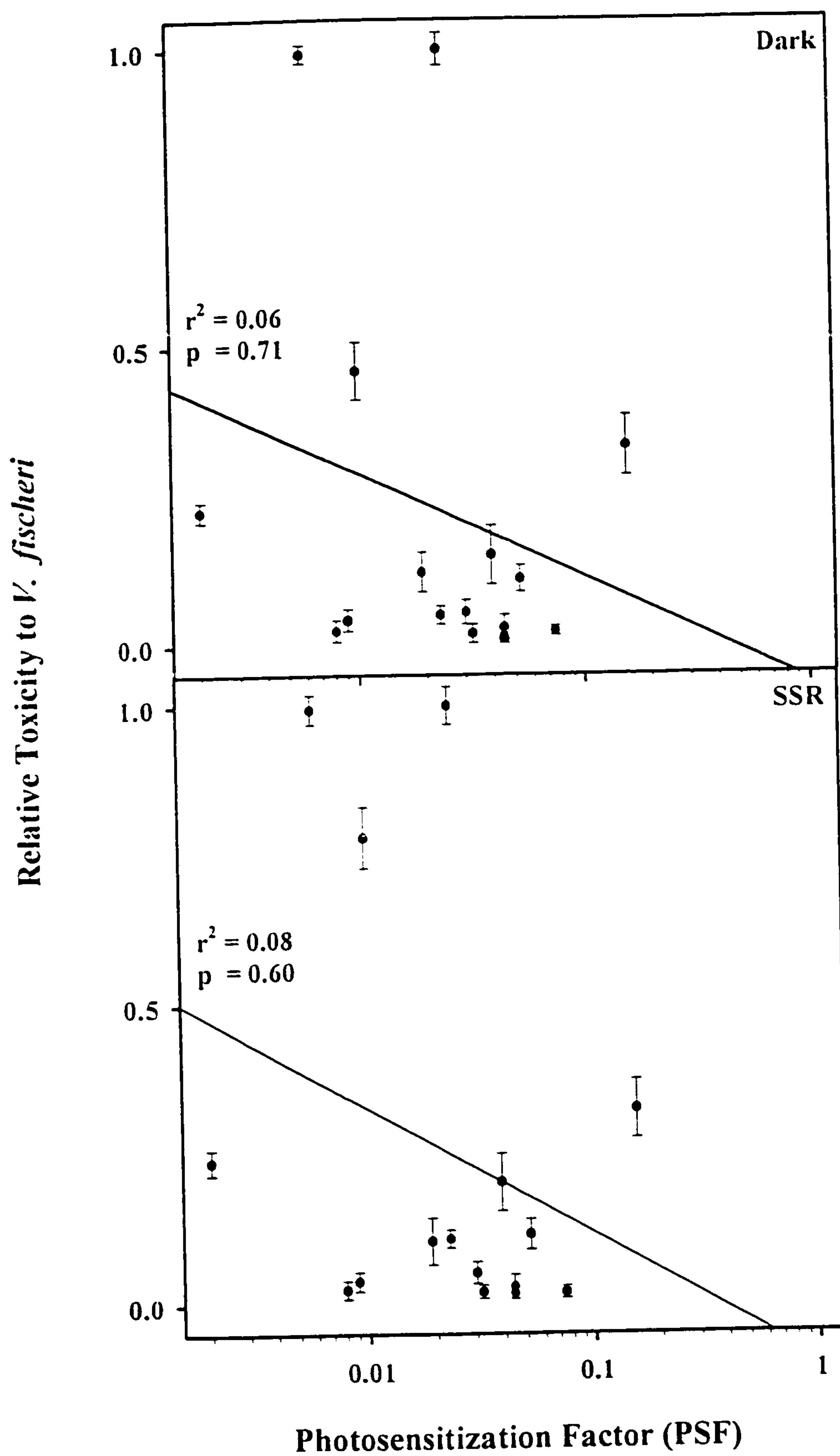
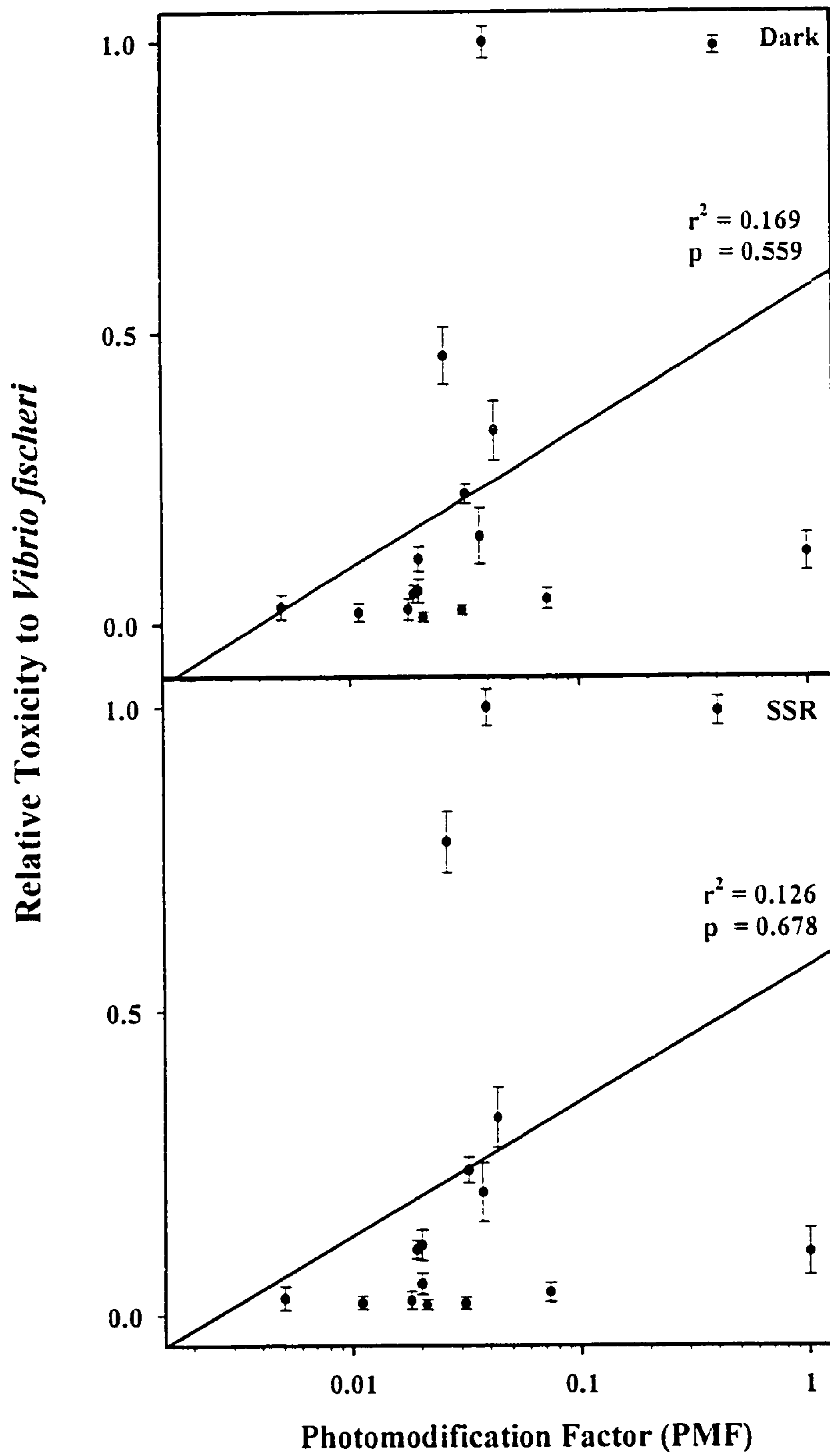
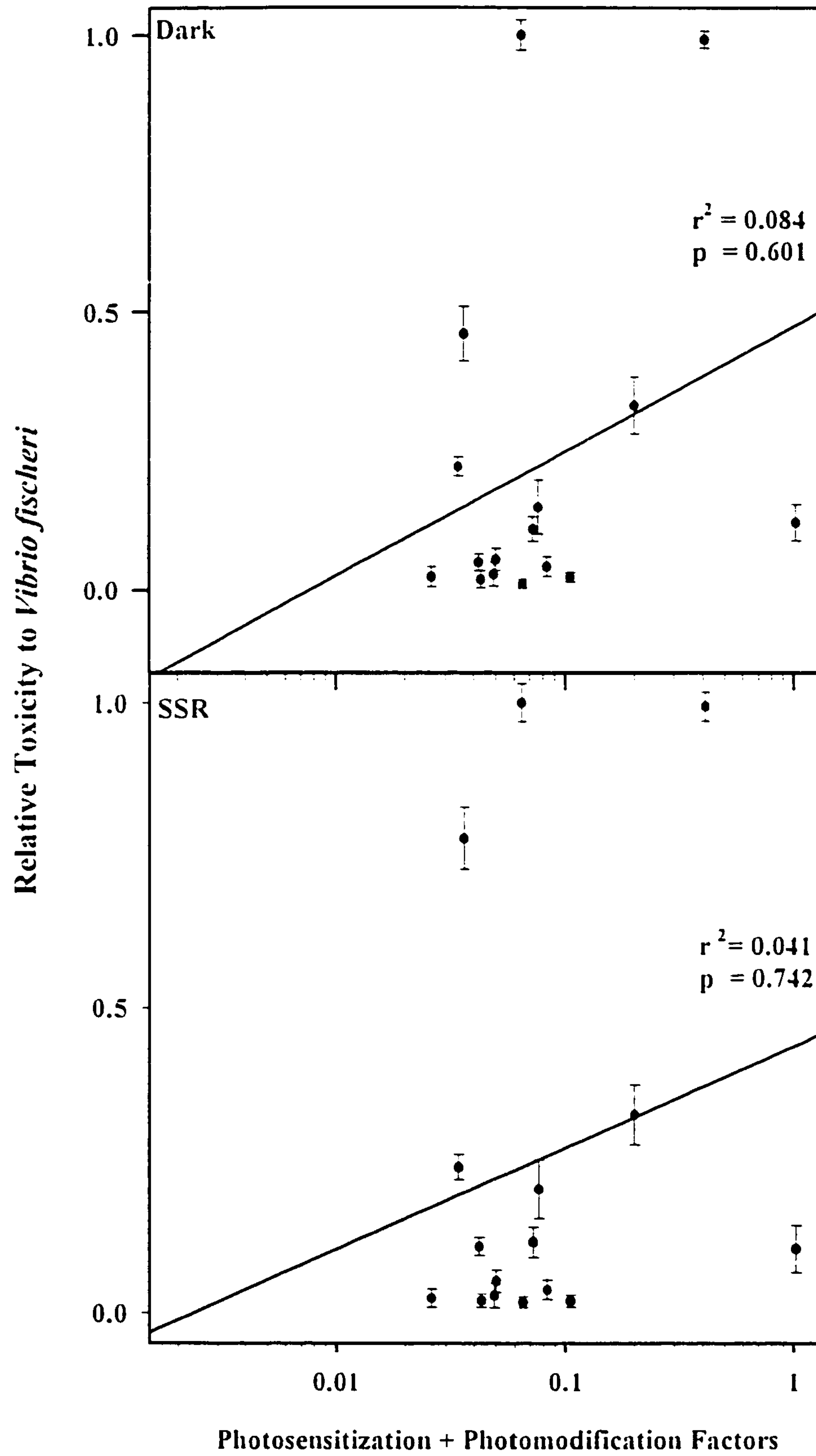

Dark
1.0
0.5
0.0
$r^2 = 0.06$
$p = 0.71$
SSR
1.0
0.5
0.0
$r^2 = 0.08$
$p = 0.60$
0.01
0.1
1
Relative Toxicity to *V. fischeri*
Photosensitization Factor (PSF)

Figure 5.6 Short-term toxicity of intact PAHs to *V. fischeri* compared to the PMF from the *L. gibba* QSAR. Toxicity data for intact PAHs on *V. fischeri* normalized from 0 to 1 and then plotted against the PMF (Table 5.5). No significant correlation between toxicity and the PMF was observed in the darkness or SSR.



Dark
$r^2 = 0.169$
$p = 0.559$
SSR
$r^2 = 0.126$
$p = 0.678$
1.0
0.5
0.0
0.01
0.1
1
Relative Toxicity to Vibrio fischeri
Photomodification Factor (PMF)

Figure 5.7 Short-term toxicity of intact PAHs to *V. fischeri* compared to the sum of the PSF and the PMF from the *L. gibba* QSAR. Toxicity data for intact PAHs on *V. fischeri* normalized from 0 to 1 and then plotted against the sum of PSF and PMF (Table 5.5). NO significant correlation to toxicity was observed in the darkness or SSR.


Dark
1.0
0.5
0.0
r² = 0.084
p = 0.601
SSR
1.0
0.5
0.0
r² = 0.041
p = 0.742
0.01
0.1
1
Relative Toxicity to Vibrio fischeri
Photosensitization + Photomodification Factors

5.4.8 Correlation between long-term toxicity and L. gibba toxicity

The long-term toxicity of PAHs to *V. fischeri* was light inducible (Table 5.6). Thus, it is possible that inhibition of growth and luminescence in caused by the PAHs in SSR would correlate with PAH toxicity to *L. gibba*. If this is observed, one might also expect that the QSAR for *L. gibba* would predict toxicity to *V. fischeri*. The *V. fischeri* long-term toxicity data in darkness and SSR were plotted against the *L. gibba* toxicity data (Figure 5.8). No correlation was observed for the toxicity of PAHs to *V. fischeri* in darkness compared to the *L. gibba* data. However, when the SSR toxicity data for *V. fischeri* was plotted against the *L. gibba* data, an extremely strong correlation was observed ($r^2 = 0.95$, $p < 0.005$) (Figure 5.8). This indicates the same phototoxicity effect that dictates impacts on *L. gibba* can be applied to *V. fischeri*. Therefore, the *L. gibba* QSAR should be applicable to the *V. fischeri* long-term SSR data.

5.4.9 Correlation of long-term toxicity to the PSF

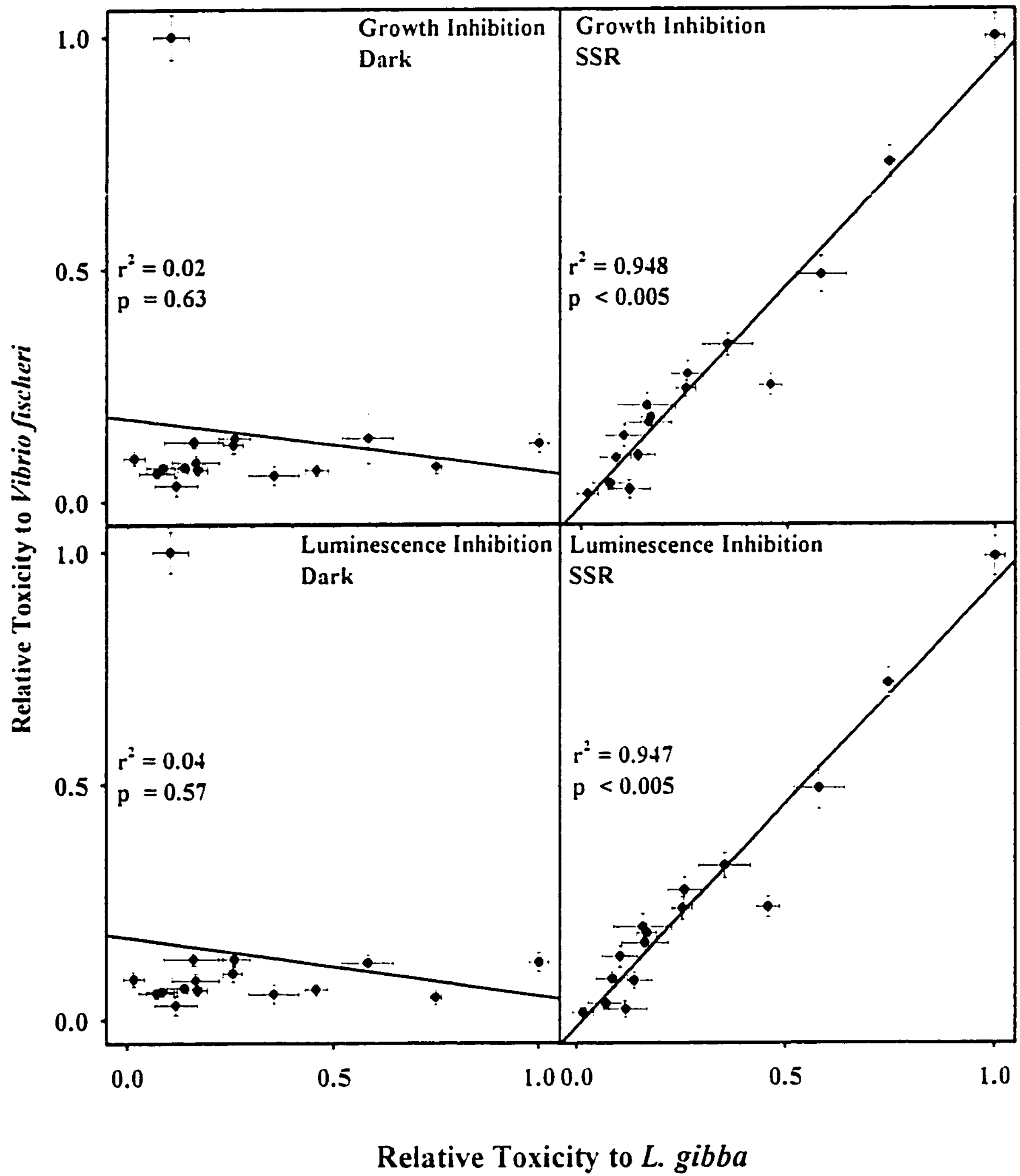
The QSAR model for PAH photoinduced toxicity developed for *L. gibba* was based on a sum of the PMF and PSF. To apply the model to another organism it was necessary to determine if either factor alone could be strongly correlated with the toxicity of PAHs. First, the PSF was compared to the toxicity of PAHs to *V. fischeri* in SSR. Because their should be no correlation between the toxicity of PAHs to *V. fischeri* in darkness, and this data did not correlate to the *L. gibba* toxicity data (Figure 5.8), these data were not used. The PSF was plotted versus inhibition of luminescence and growth of *V. fischeri* on log-linear scale (Figure 5.9). No relationship was readily apparent between PSF factor and the toxicity data. However, if 5 PAHs (ANT, BAA, BBA, BBF and FLU) were excluded from the regression, a good correlation is observed ($r^2 = 0.660$, $P < 0.005$).

It is interesting to note that four of the five chemicals (ANT, BAA, BBA and FLU) that were excluded from the above regression derive most of their toxicity from photomodification (Huang *et al.*, 1997). All four have low PSFs and high PMFs, and they are moderately to highly toxic. Therefore, their low PSFs will not adequately predict their

Table 5.6 Summary long-term toxicity at 0.5 mg/L for intact PAHs to *Vibrio fischeri* and *L. gibba*. Also relisted are the PSF and PMF. Toxicity is inhibition of luminescence and inhibition of growth of *V. fischeri* by intact PAHs ± 95 % confidence intervals, n = 6. This is from tables 5.3 and 5.5, and is only regenerated here to aid in the QSAR modeling that follows.

PAHs	Luminescence Inhibition		Growth Inhibition		Toxicity	PSF	PMF	PSF +
	Dark	SSR	Dark	SSR	to *L. gibba*			PMF
ANT	0.122 ± 0.010	1.000 ± 0.043	0.125 ± 0.011	1.000 ± 0.049	1.000 ± 0.024	0.019	1.000	1.019
BAA	0.048 ± 0.015	0.727 ± 0.032	0.075 ± 0.016	0.730 ± 0.034	0.745 ± 0.014	0.006	0.401	0.407
BAP	0.099 ± 0.018	0.239 ± 0.025	0.122 ± 0.018	0.242 ± 0.018	0.255 ± 0.024	0.039	0.037	0.076
BBA	0.128 ± 0.015	0.279 ± 0.028	0.136 ± 0.014	0.274 ± 0.026	0.259 ± 0.038	0.009	0.073	0.082
BBF	0.055 ± 0.010	0.331 ± 0.027	0.056 ± 0.011	0.336 ± 0.024	0.354 ± 0.061	0.023	0.019	0.042
BEP	0.084 ± 0.013	0.165 ± 0.019	0.084 ± 0.010	0.170 ± 0.018	0.165 ± 0.057	0.030	0.020	0.050
BGP	0.068 ± 0.009	0.085 ± 0.017	0.073 ± 0.008	0.099 ± 0.012	0.139 ± 0.043	0.044	0.005	0.049
CHR	0.087 ± 0.015	0.016 ± 0.010	0.093 ± 0.012	0.016 ± 0.013	0.016 ± 0.026	0.010	0.026	0.036
COR	0.031 ± 0.010	0.023 ± 0.018	0.034 ± 0.011	0.026 ± 0.020	0.118 ± 0.052	0.032	0.011	0.043
DAA	0.061 ± 0.010	0.088 ± 0.016	0.072 ± 0.011	0.093 ± 0.013	0.085 ± 0.038	0.044	0.021	0.065
DAP	0.065 ± 0.012	0.242 ± 0.022	0.067 ± 0.009	0.250 ± 0.023	0.458 ± 0.028	0.074	0.031	0.105
FLA	0.120 ± 0.018	0.479 ± 0.046	0.136 ± 0.015	0.487 ± 0.040	0.580 ± 0.061	0.156	0.043	0.199
FLU	0.129± 0.013	0.199 ± 0.029	0.128 ± 0.011	0.206 ± 0.027	0.160 ± 0.071	0.002	0.032	0.034
PHE	1.000 ± 0.035	0.136 ± 0.023	1.000 ± 0.039	0.141 ± 0.025	0.104 ± 0.043	0.024	0.039	0.064
PYR	0.064 ± 0.011	0.186 ± 0.019	0.068 ± 0.010	0.181 ± 0.020	0.170 ± 0.024	0.052	0.020	0.072
TRI	0.057 ± 0.010	0.036 ± 0.012	0.061 ± 0.009	0.038 ± 0.011	0.071 ± 0.043	0.008	0.018	0.026

Figure 5.8 Long-term toxicity of intact PAHs to *V. fischeri* compared to the toxicity of PAHs to *L. gibba*. Toxicity data for intact PAHs on *V. fischeri* were normalized from 0 to 1 and then plotted against the toxicity to *L. gibba* (see Table 5.6). A strong correlation to toxicity was observed for the *V. fischeri* SSR data, but not for the dark data.


Growth Inhibition
Dark
$r^2 = 0.02$
$p = 0.63$
Growth Inhibition
SSR
$r^2 = 0.948$
$p < 0.005$
Luminescence Inhibition
Dark
$r^2 = 0.04$
$p = 0.57$
Luminescence Inhibition
SSR
$r^2 = 0.947$
$p < 0.005$
Relative Toxicity to Vibrio fischeri
Relative Toxicity to L. gibba
0.0
0.5
1.0

toxicity. In the case of BBF, it has the third highest toxicity and it has a PSF and PMF that are nearly equal. It follows that to describe its phototoxicity both factors will be required. Also, BBF is the closest of the 5 chemicals to the regression for the chemicals that fit the PSF.

These results show that a factor other than photosensitization is required to predict the photoinduced toxicity of PAHs to luminescent bacteria. This other factor is likely to be the PMF. This is consistent with the *L. gibba* QSAR model, where the PSF correlated with toxicity for only some of the chemicals (Huang *et al.*, 1997).

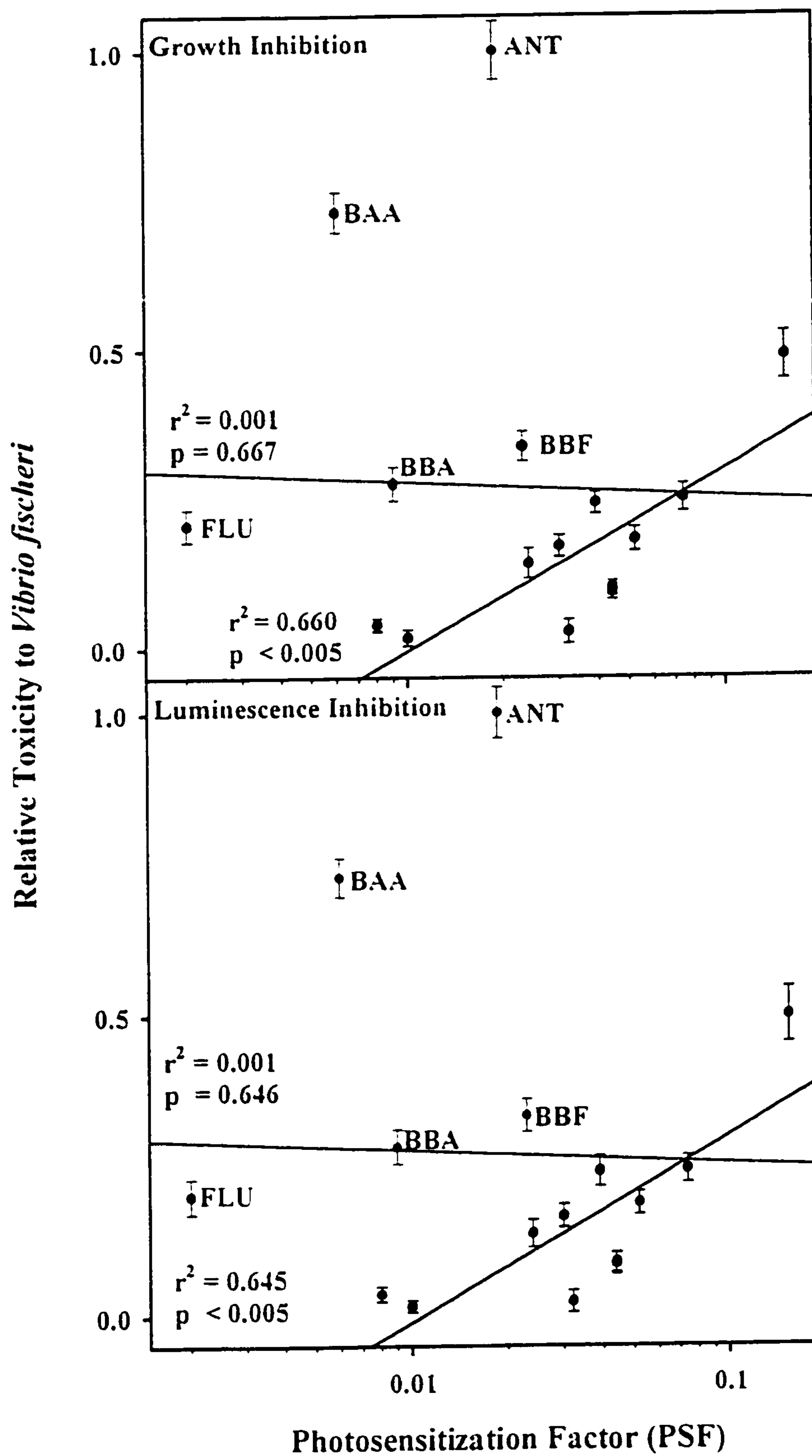
5.4.10 Correlation of long-term toxicity to the PMF

PAHs are more toxic after becoming photomodified by SSR (Huang *et al.*, 1997; McConkey *et al.*, 1997). Toxicity of the compounds in SSR when applied in the intact form might depend on how rapidly they are converted to the more toxic photomodified compounds. This was the case for photoinduced toxicity to *L. gibba*. The PMF is largely dependent on the rate of photomodification. Thus, the PMFs were plotted against toxicity of *V. fischeri* on a log-linear scale (Figure 5.10). A strong relation ($r^2 = 0.801$, $p > 0.005$) with high predictive capacity was apparent between relative toxicity of PAHs on *V. fischeri* and the photomodification factor. There were no apparent outliers in this regression. This result indicates that photoinduced toxicity is more dependent on the PMF than the PSF.

5.4.11 Correlation of long-term toxicity to the sum of PSF and PMF

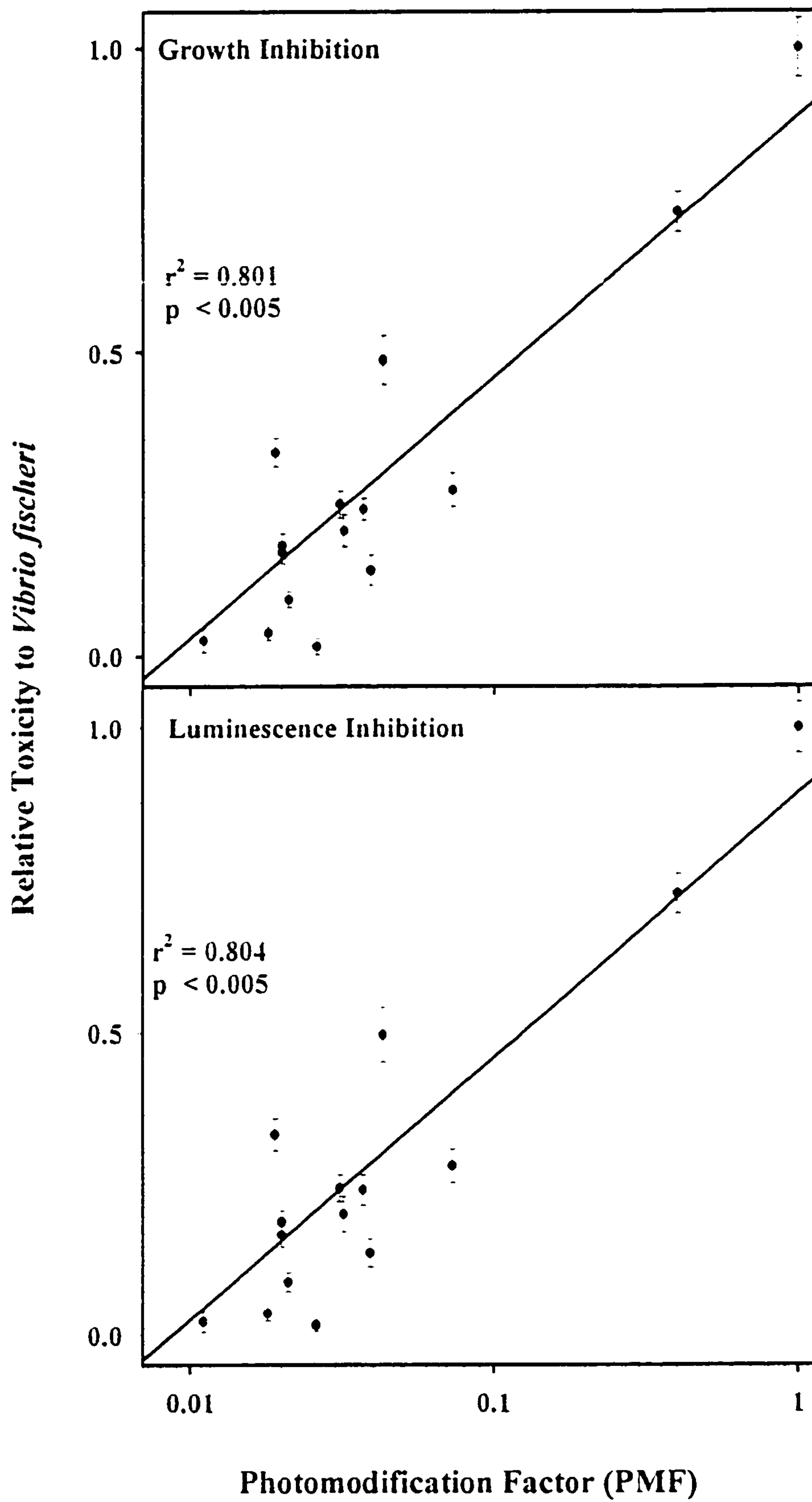
The PSF and the PMF were both able to describe photoinduced toxicity of PAHs to *V. fischeri*. Indeed the *L. gibba* QSAR used both the PSF and PMF as a sum to describe toxicity (Huang *et al.*, 1997; Krylov *et al.*, 1997). Thus, it was logical to combine the two factors into a single determinant to improve the QSAR model for toxicity of PAHs to *V. fischeri*. The PSF and the PMF were summed and plotted against the long-term toxicity to *V. fischeri* in SSR (Figure 5.9). A very good regression with high

Figure 5.9 Long-term toxicity of intact PAHs to *Vibrio fischeri* compared to the PSF. Toxicity data for intact PAHs on *V. fischeri* was normalized from 0 to 1 and then plotted against the PSF (Table 5.6). When all values are included in the analysis no significant correlation to toxicity was observed in the SSR. With 5 chemicals removed from the regression (ANT, BAA, BBA, BBF and FLU), a significant correlation was observed.



Growth Inhibition
ANT
BAA
$r^2 = 0.001$
$p = 0.667$
BBF
BBA
FLU
$r^2 = 0.660$
$p < 0.005$
Luminescence Inhibition
ANT
BAA
$r^2 = 0.001$
$p = 0.646$
BBF
BBA
FLU
$r^2 = 0.645$
$p < 0.005$
1.0
0.5
0.0
0.01
0.1
Relative Toxicity to *Vibrio fischeri*
Photosensitization Factor (PSF)

Figure 5.10 Long-term toxicity of intact PAHs to *Vibrio fischeri* compared to the PMFs of the PAHs. Toxicity data for intact PAHs on *V. fischeri* was normalized from 0 to 1 and then plotted against the PMF (Table 5.6). A strong correlation between toxicity and the PMF was observed.


Growth Inhibition
r² = 0.801
p < 0.005
Luminescence Inhibition
r² = 0.804
p < 0.005
1.0
0.5
0.0
1.0
0.5
0.0
0.01
0.1
1
Relative Toxicity to Vibrio fischeri
Photomodification Factor (PMF)

predictive value was achieved ($r^2 = 0.876$, $p < 0.005$). If three PAHs (BBF, FLU, TRI) that did not fit the regression quite as well were excluded from the regression, the correlation improves dramatically ($r^2 = 0.980$). Explanatory and theoretical models by Huang *et al.*, 1997; Krylov *et al.*, 1997 and *V. fischeri* data show that about half the PAHs have a PSF greater than the PMF. Additionally, for 10 of the 16 PAHs, the same factor dominates in the QSAR model.

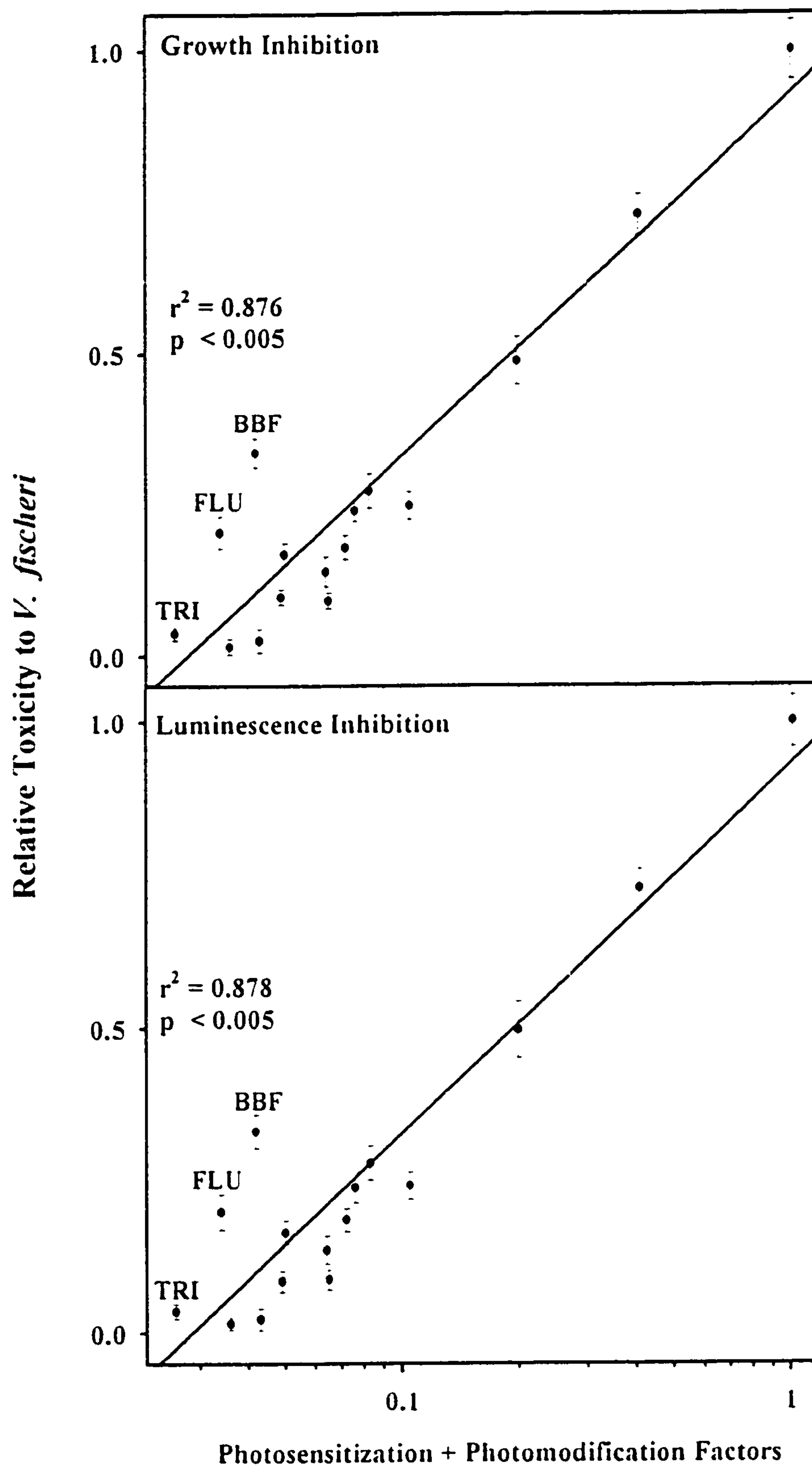
The data show that one factor alone (PSF or PMF) does not fully describe the photoinduced toxicity of PAHs to *V. fischeri*. Based on the dynamics of the photochemical reactions of PAHs in a two-compartment system (intracellular and extracellular space) it was possible to postulate that both photomodification and photosensitization are crucial for describing the toxicity of PAHs in the environment. The relation of the two factors should be additive, since they cannot operate concurrently; that is, intact PAHs will act as photosensitizers until they are photomodified. The toxicity that follows will depend on the mechanisms of action of the photomodified PAHs. Interestingly, it has recently been shown that photomodified PAHs inhibit bioenergetic electron transport (Tripuranthakam *et al.*, 1999, Huang *et al.*, 1997), possibly explaining why they are toxic to aerobic organisms like *V. fischeri*.

This study showed that the PMF and PSF describe photoinduced toxicity to *V. fischeri* as they did for *L. gibba*. Thus, the QSAR model for the photoinduced toxicity of PAHs can be applied to other organisms and may be broadly applicable to other photochemically active contaminants, especially those where photomodification of the parent compounds has a major impact on toxicity. Lastly, this research shows that photomodification of PAHs is a factor that must be considered in environmental risk assessment.

5.5 CONCLUSIONS

For the toxicity of PAHs to *V. fischeri*, it was possible that the phototoxic mechanism might be different from the mechanism in *L. gibba*. However, the QSAR model for *L. gibba* was readily applicable to PAH photoinduced toxicity to *V. fischeri*.

Figure 5.11 Long-term toxicity of intact PAHs to *Vibrio fischeri* compared to the sum of PSF and PMF from the *L. gibba* QSAR. Toxicity data for intact PAHs on *V. fischeri* normalized from 0 to 1 and then plotted against the sum of PSF and PMF (Table 5.6). A strong correlation to toxicity was observed for the *V. fischeri* SSR data with the sum of PSFs and PMFs.


Growth Inhibition
r^2 = 0.876
p < 0.005
BBF
FLU
TRI
Luminescence Inhibition
r^2 = 0.878
p < 0.005
BBF
FLU
TRI
1.0
0.5
0.0
1.0
0.5
0.0
0.1
1
Relative Toxicity to V. fischeri
Photosensitization + Photomodification Factors

Photosensitization and photomodification are the key factors presented in this model and both processes contribute together in toxicity to *V. fischeri*. This shows that toxicity to microbes occurs through Photomodification factors or photosensitization factors. One can conclude from this model that both factors will be broadly applicable across the biosphere. It also shows that the photomodification process is crucial to under standing the environmental toxicology of PAHs.

CHAPTER 6

ASSESSMENT OF THE PHOTOINDUCED TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS CONTAMINATED SEDIMENTS USING LUMINESCENT BACTRIA[1]

6.1 ABSTRACT

PAHs are of great concern at the present time because they are being released into environment at a rapid rate, and they are carcinogenic and toxic. The identification and detection of PAHs are very important goals. Because they are hydrophobic, PAHs partition into sediments in aquatic environments. The objective of this research was to assess the hazards PAH contaminated sediments from Hamilton Harbor, Ontario and Mohawk Lake, Brantford, Ontario using the *V. fischeri* short- and long-term toxicity tests. The sediments were analyzed for intact and modified PAHs and tested for toxicity. The sediments were collected from contaminated and reference sites in Hamilton Harbor and Mohawk Lake. They were extracted into organic solvent and fractionated by normal-phase high performance liquid chromatography (HPLC). Six fractions were collected and analyzed by reversed-phase HPLC. Three fractions contained intact PAHs, one contained a mixture of intact and modified PAHs and two fractions contained only modified PAHs. The fractions were tested for toxicity with *V. fischeri*. Most of the fractions were phototoxic to *V. fischeri*. One of the fractions that contained only modified PAHs was found to be the most toxic.

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[1]Submitted to: Environmental Toxicology and Chemistry. Co-authors are X.-D. Huang, M. A. Lampi, B. J. McConkey, D. G. Dixon, and B. M. Greenberg.

6.2 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants resulting from combustion and processing of fossil fuels, coal-burning power plants, and production of steel. They have been identified in a variety of environmental compartments including air, water, sediment and soil (Cook *et al.*, 1983; Edwards, 1983; Jacob *et al.*, 1986; Schmidt *et al.*, 1987; Fetzer, 1989; Cerniglia, 1992). PAHs are usually present in environmental samples as complex mixtures. These mixtures vary greatly in relative concentrations of the individual components, and thus in carcinogenic and/or mutagenic properties. As well, modified PAHs (e.g. nitro- and oxyPAHs) have been found in mixtures containing PAHs (Neff 1979, Bowling *et al.*, 1983, Nikolaou *et al.*, 1984; Smith 1987; Legzdins *et al.*, 1995; Enya *et al.*, 1997; Kosian *et al.*, 1998; Huang *et al.*, 1999). However, the extent and ubiquity of modified PAHs in aquatic sediments, and the toxicity of modified PAHs in environment remains largely unexplored. Nonetheless, given the ease of photomodification of PAHs, they should be in the environment in high enough concentrations to present a hazard.

Sediments are the major repository for contaminants introduced into surface waters. This is especially true for hydrophobic compounds, which can accumulate in sediments to several orders of magnitude higher in concentration than those in the surounding water (Lyman, 1984, Lee and Jones, 1984). Aquatic sediments may in fact constitute the most important reservoir or sink of pollutants in the environment. Contaminated sediments can be directly toxic to aquatic life or they can be a source of contaminants for accumulation in the food chain (Mackay *et al.*, 1992). The assessment of sediment quality has often involved only the acquisition of analytical chemistry data (Ankley *et al.*, 1994). However, quantifying contaminant concentrations alone often does not provide enough information to adequately assess their potential adverse effects. The bioavailability and toxicity of contaminants in sediments requires the use of bioassays to estimate biological damage that can be caused to aquatic organisms (Anderson *et al.*, 1987). Many biological testing methods used to assess the toxicity of contaminants in sediments rely on relatively short-term exposures. These will miss the toxicity of many of compounds in contaminated sediments. For instance, it was shown in earlier chapters of

this thesis that for PAHs, long-term exposures are required to observe phototoxicity. Thus, it was of interest to apply the short- and long-term *V. fischeri* toxicity tests to determine the hazards of sediments contaminated with PAHs and modified PAHs.

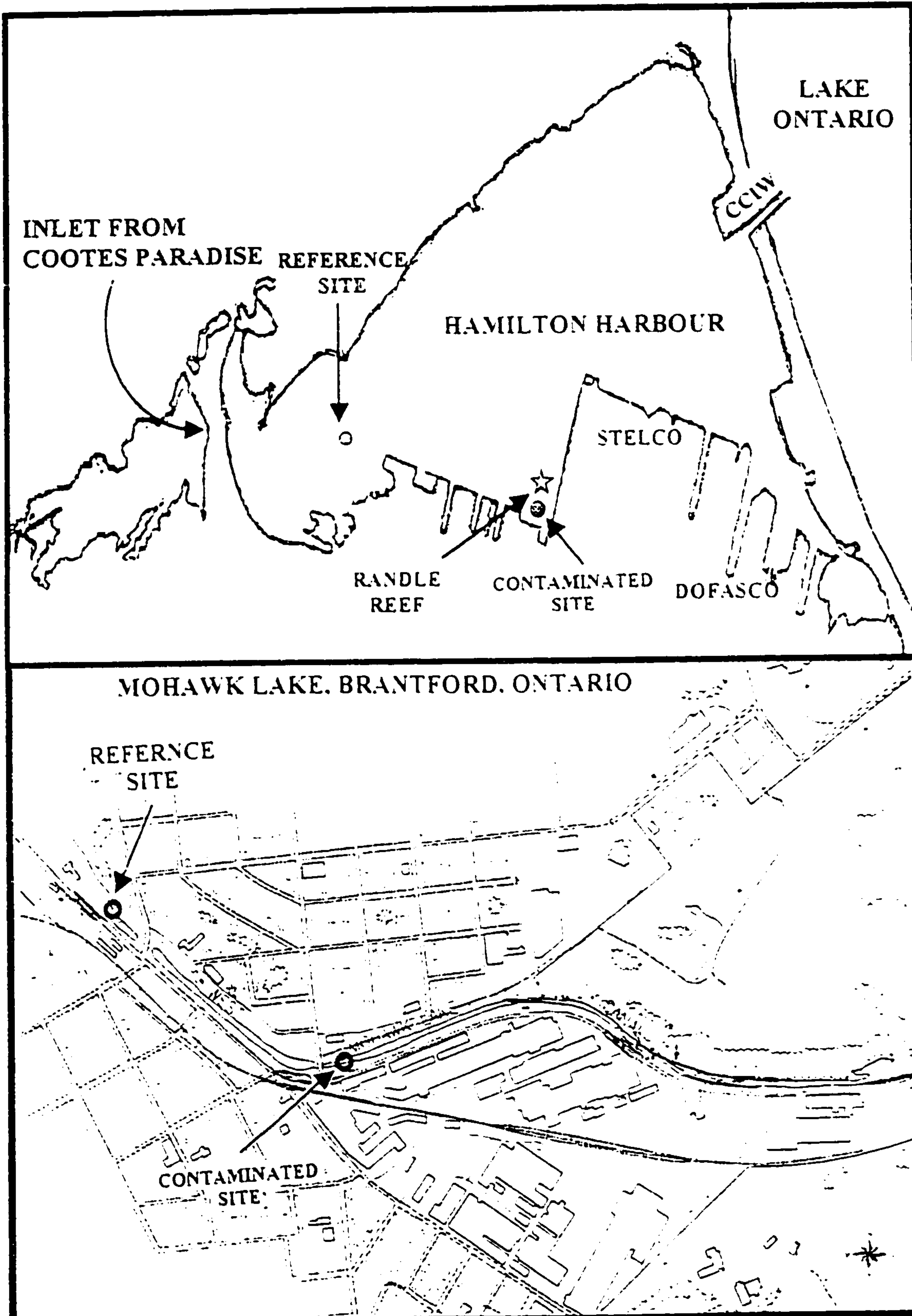
The objectives of this study were to extract PAH-contaminated sediments and analyze them for PAHs and oxyPAHs to determine if modified PAHs can be readily detected. The extracts were then tested for toxicity to determine if the modified PAHs presents account for some of the toxicity of the sediments. The sediments were collected from Hamilton Harbor, Ontario and Mohawk Lake, Brantford, Ontario (Figure 6.1). The extracts were separated by a two-dimensional HPLC technique. The compounds were divided into PAHs and modified PAH containing fractions. Using the short- and long-term *V. fischeri* assays the modified PAH fractions were found to be most toxic.

6.3 MATERIALS AND METHODS

6.3.1 Collection, extraction and fractionation of sediments

Sediments used in the toxicity tests were collected with a stainless steel grab from Hamilton Harbor, ON and Mohawk Lake, Brantford, ON (Figure 6.1). Sediment samples were taken from a contaminated site near the docking port at Randle Reef in Hamilton Harbor (Hamilton, Ontario), and a documented reference site in Hamilton Harbor. Sediment samples were also collected from a PAH contaminated site and a reference site in Mohawk Lake (Brantford, ON). The industries surrounding both lakes are known to have released PAHs that have partitioned to the sediments. All of the sediment samples were drained and air-dried at room temperature in darkness. The US EPA standard method was used to extract the sediments. Sediments samples (4 g) were dried at room temperature and suspended in 10 ml of hexane: acetone (1:1, v:v), and ultrasonicated for 20 minutes (500 W, Fisher Ultrasonicator) this step was repeated 3 times. The extracts were concentrated to 10 ml with a gentle stream of Nitrogen (N_2). The 10 ml extracts were divided in two. One 5 ml extract was dried completely with Nitrogen (N_2) and re-suspended in 2 ml of dimethyl sulfoxide (DMSO) to determine the whole sediment extract

Figure 6.1 Maps of Hamilton Harbor and Mohawk Lake. Contaminated sediment and reference sediment sampling sites are indicated.


LAKE ONTARIO
CCIW
INLET FROM COOTES PARADISE
REFERENCE SITE
HAMILTON HARBOUR
STELCO
RANDLE REEF
CONTAMINATED SITE
DOFASCO
MOHAWK LAKE, BRANTFORD, ONTARIO
REFERNCE SITE
CONTAMINATED SITE

toxicity.

The second 5 ml extract was fractionated using normal-phase high performance liquid chromatography (HPLC) (Lampi *et al.*, 2000). 250 µl was loaded onto a Whatman 5 µm Partisil PAC RAC column (Whatman, Clifton, NJ, 9.4 mm i.d. × 10 cm length) was used. Samples were eluted at a flow rate of 2 ml/min, with a step gradient of hexane:tetrahydrofuran (THF) (95:5, v/v, solvent A) and methanol:THF (80:20, v/v, solvent B). The gradient used was as follows (elapsed time): 0-5 min., 100% solvent A; 5-10 min., 90% solvent A; 10-25 min., 50% solvent A; 25-30 min., 100% solvent B. A Beckman system GOLD HPLC with a diode array detector was used. The method was designed to elute compounds with increasing polarity as retention time increased, thus allowing isolation of more polar compounds like modified PAHs, in the later fractions. Six fractions were collected for toxicity testing and further analysis by reverse phase HPLC. The following retention time intervals were used to collect fractions: 2-4 min., fraction 1; 4-7 min., fraction 2; 7-12 min., fraction 3; 12-17 min., fraction 4; 17-22 min., fraction 5; 22-30 min., fraction 6. For toxicity testing, the fractions were brought to dryness under a stream of N_2 and resuspended in 250 µl of DMSO (equal to the loading volume).

Reversed phase HPLC was used to identify some of the individual components of the fractions. Samples (250 µl) of the fractions were eluted through a 5 µm Supelco Supelcosil LC-18 analytical column (Sigma-Aldrich Canada Ltd., Oakville, Ontario., 4.6 mm i.d. × 25 cm length). A Shimadzu HPLC with diode array detector was used. A linear gradient beginning with 50:50 acetonitrile:water (v/v), and ramping to 100% acetonitrile over 40 min was used at a flow rate of 1.5 ml/min. A photodiode array detector, scanning from 210 nm to 400 nm, was used to collect absorbance spectra of individual components eluting off of the column. Spectra and retention times were then compared with authentic compounds in a library of PAHs and modified PAHs enabling identification of many of the components.

6.3.2 Toxicity of Sediment Extracts Using Luminescent bacteria

The above whole sediment extracts, and fractions 1 to 6 from the sediments were tested for toxicity. The marine luminescent bacteria *V. fischeri* was used as in previous chapters. Both the short-term and long-term assays were employed. In all cases, concentrations of the test sample are reported as mg equivalents of sediment per liter (mgEQS/L). That is, all concentrations are reported as the amount of sediment extracted.

6.3.2.1 Short-term Assay

Briefly a starter culture was diluted with minimal media as in chapter 3, and twenty-four 500 µl aliquots of the cells were added to a 48-well cell culture plate. After 5 minutes acclimatization in the 48-well cell culture plates, the luminescence intensity was measured. After the initial measurement of bacterial luminescence, the bacteria were dosed with one of the sediment whole extract samples or fractions. Aliquots of 500 µl of the cell culture were combined with equal volumes of the test material in minimal culture medium. A dilution series of each extract or fraction (7 concentrations plus the control) were added in a geometric series to the wells in triplicate. Stock solutions of the sediment extract and fractions were prepared in dimethyl sulphoxide (DMSO), and added by 1000-fold dilution to the culture liquid medium to give the specified concentration. A DMSO concentration of 0.1% does not affect luminescence or growth of *V. fischeri*. After dosing, the cells were incubated in darkness or SSR at room temperature for 15 minutes with shaking and the luminescence intensity was measured.

6.3.2.2 Long-term Assay

After the short-term test was completed, the multi-well culture plate was returned to the growth chamber. The samples were then pre-incubated for 8 h in minimal medium. After this pre-incubation, the medium was made complex as in chapter 3. The cells were

then incubated for an additional 18 hours in SSR or darkness at 20°C. Toxicity was determined by inhibition of growth and luminescence.

Toxicity was expressed as the concentrations causing 50 % inhibition of luminescence or growth (EC50). To find the EC50, inhibition of luminescence and growth were plotted vs. concentration (mgEQS/L), and fit to a log it function as in chapter 2. The 95 % confidence intervals of the EC50s values were calculated. All calculations are based on nominal test concentrations (Chapter 2).

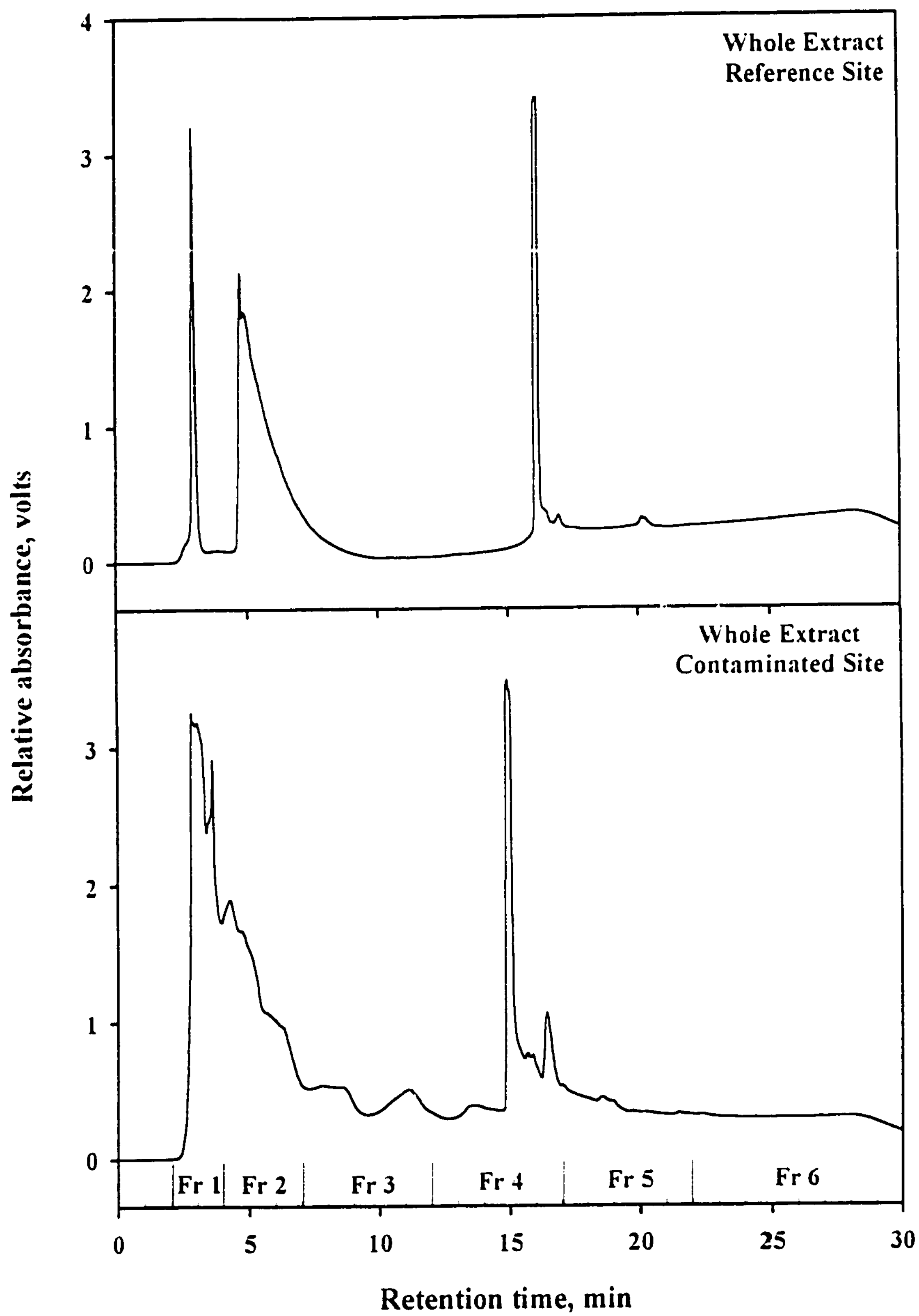
6.4 RESULTS

6.4.1 Sediments extraction and Fraction analysis

Sediments were collected from Hamilton Harbor, ON, and Mohawk Lake (Brantford, ON). Sediment samples were collected from PAH contaminated sites in shallow water close to shore and reference sites. The sediments were extracted and the analyzed by a 2D HPLC method developed by Lampi *et al.* (2000) as described in materials and methods. The sediment sample from Hamilton Harbor was heavily contaminated by the steel industry and various spills over many years. In the first dimension, a Partisil PAC-RAC column (normal phase) was used (Figure 6.2). The Partisil PAC-RAC column is effective for the preparatory fractionation of complex environmental mixtures. The normal-phase HPLC chromatogram of the contaminated sediment was quite different from the reference sediment (Figure 6.2). One can observe two major peaks with many minor peaks following each major peak. There were many more peaks in the contaminated sediment and they were larger than the peaks from the reference sediment.

The whole extracts were separated into 6 fractions. These fractions were used for 2nd dimension analysis. The fractions are indicated on Figure 6.2. The fractions contained compounds with less hydrophobicity as the retention time increased. All the 6 fractions were subjected to 2nd dimension HPLC analysis. A C-PAH (C-18) reversed-phase column was used. The compounds were eluted with a water:acetonitrile gradient from 50-100 % acetonitrile over 40 min (Figure 6.2). Using a library of PAHs and oxyPAHs, several of

Figure 6.2 Normal phase HPLC chromatogram of the Hamilton Harbor sediment extracts. First dimension HPLC analysis of a reference site and contaminated site. HPLC conditions as described in materials and methods. The whole extracts were separated into 6 fractions and collected for 2nd dimension analysis. Fraction 1 (2-4 min retention time [RT]), fraction 2 (4-7 min RT), fraction 3 (7-12 min RT), fraction 4 (12-17 min RT), fraction 5 (17- 22 min RT), and fraction 6 (22-30 min RT).

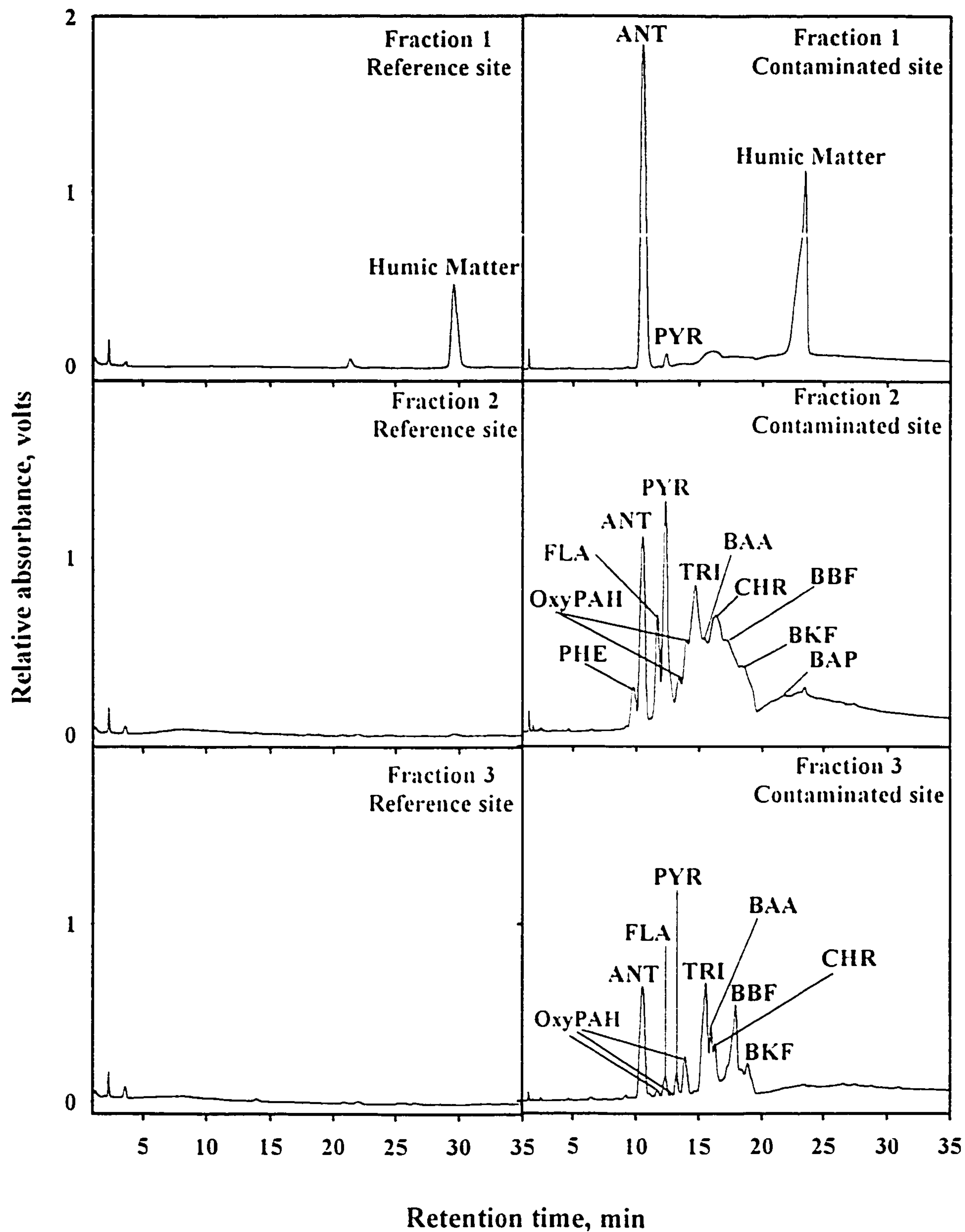

Whole Extract
Reference Site
Whole Extract
Contaminated Site
Relative absorbance, volts
0
1
2
3
4
Fr 1
Fr 2
Fr 3
Fr 4
Fr 5
Fr 6
0
5
10
15
20
25
30
Retention time, min

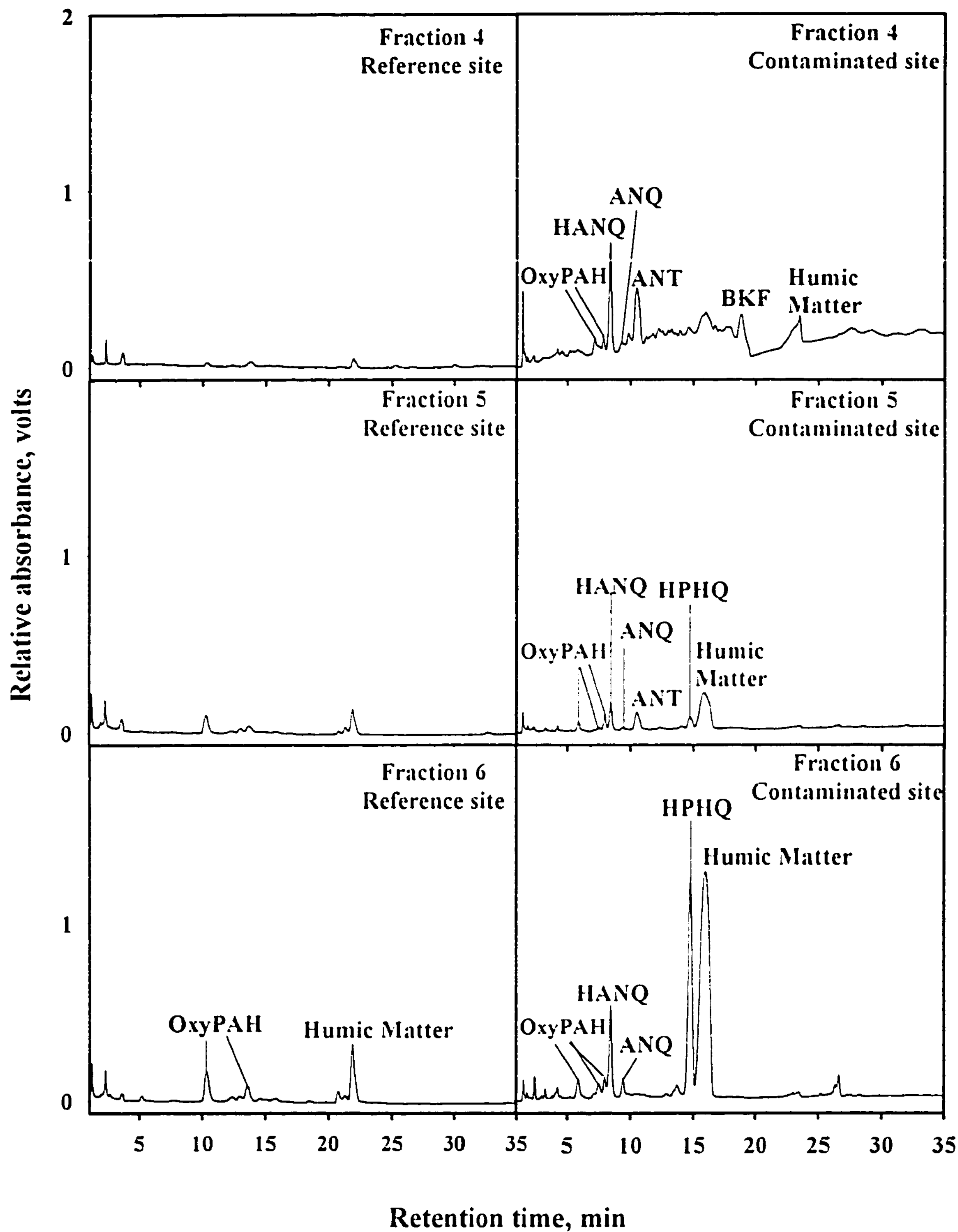
the individual peaks were identified based on absorbance spectra (with a diode array detector) and retention time. Contaminated fractions 1 to 3 predominantly contained intact PAHs, and fractions 4 to 6 were found to contain mostly modified PAHs (Figure 6.2 and Table 6.1). Note the reference site contained no intact PAHs and only small amounts of oxyPAHs. Humic material was detected in fractions 1 and 6 from both the reference and contaminated sediments. Humic material was present in different amounts in contaminated sediments and reference sediments from Hamilton Harbor (Figure 6.3).

The total amounts of modified PAHs (mostly oxyPAHs) were found at lower concentration than intact PAHs. However, the amounts of oxyPAHs were relatively high, and they were readily detected. The concentration range for the oxyPAHs was 0.1 to 0.8 mg/kg of wet sediment, which is comparable to a concentration range of 0.2 to 3 mg/kg for intact PAHs (the high end of this range is due to the large amount of ANT in fraction 1) (Table 6.1 and Figure 6.3). Anthraquinone (ANQ), Hydroxyanthraquinone (HANQ), and Hydroxyphenanthrenequinone (HPHQ) have been identified. About 10 other possible oxyPAHs were found, but have not yet been positively identified. Reverse phase HPLC analysis has revealed modified PAHs in all the polar sediment fractions (Fractions 4-6). Intact PAHs were found in fractions 1 through 5, with the majority in fractions 1 to 3. These data are consistent with other studies regarding the presence of modified PAHs in the environment (Legzdins *et al.*, 1995, Enya *et al.*, 1998, Kosian *et al.*, 1998), indicating their ubiquitous presence. It also shows the complexity of the mixture of PAHs and oxyPAHs in contaminated sediments.

Mohawk Lake sediment contained mixtures of intact PAHs and oxyPAHs as did Hamilton Harbor sediment (Table 6.2). Fractions 1-4 contain intact PAHs and fractions 3-6 contained oxyPAHs. The highest content of PAHs was in the fraction 3. That fraction contained Anthraquinone (ANQ), Benzo(a)anthracene (BAA), Benzo(a)pyrene (BAP), Chrysene (CHR), Hydroxyphenanthrenequinone (HPHQ) and phenanthrene (PHE), (Table 6.2). Anthracene appeared in low concentration only in fraction 1. Fractions 5 and 6 contained compound with absorbance spectra consistent with their identity as oxyPAHs, but they could not be specifically identified. The Mohawk Lake and Hamilton Harbor sediments had some notable differences. Mohawk Lake had less total PAH and oxyPAH

Figure 6.3 Reverse-phase HPLC chromatograms from the analyses of PAH fractions collected from the normal-phase HPLC separation (see Figure 6.2). Reference and contaminated sediments are from Hamilton Harbor are shown. The HPLC chromatograms are for the 6 fractions from the contaminated site and the 6 fractions from reference site. HPLC conditions are described in materials and methods. Peaks were detected with a diode array detector collecting data from 210 nm to 400 nm. Peak identities are based on absorbance spectra and retention time in a library of PAHs and oxyPAHs.


Fraction 1
Reference site
Humic Matter
ANT
Fraction 1
Contaminated site
Humic Matter
PYR
Fraction 2
Reference site
Fraction 2
Contaminated site
PYR
ANT
FLA
BAA
TRI
CHR
BBF
OxyPAH
BKF
PHE
BAP
Fraction 3
Reference site
Fraction 3
Contaminated site
PYR
BAA
FLA
TRI
CHR
ANT
BBF
OxyPAH
BKF
Relative absorbance, volts
Retention time, min
0
1
2
5
10
15
20
25
30
35



Fraction 4
Reference site
Fraction 4
Contaminated site
ANQ
HANQ
OxyPAH
ANT
BKF
Humic
Matter
Fraction 5
Reference site
Fraction 5
Contaminated site
HANQ
HPHQ
ANQ
OxyPAH
Humic
Matter
ANT
Fraction 6
Reference site
Fraction 6
Contaminated site
HPHQ
Humic Matter
HANQ
OxyPAH
Humic Matter
OxyPAH
ANQ
Relative absorbance, volts
Retention time, min
0
1
2
5
10
15
20
25
30
35

Table 6.1 Components of fractions from contaminated Hamilton Harbour sediment. (-) No peak present, (+) Low relative amount, (++) Medium relative amount, (+++) High relative amount.

Toxicant	Abb.	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6
Anthracene	ANT	+++	++	+	+	+	-
Anthraquinone	ANQ	-	-	-	+	+	+
Benzo(a)anthracene	BAA	-	+	+	-	-	-
Benzo(a)pyrene	BAP	-	++	-	-	-	-
Benzo(b)fluoranthene	BBF	-	+	+	+	-	-
Benzo(k)fluoranthene	BKF	-	+	+	+	-	-
Benzo(g,h,i)perylene	BGP	-	+	++	+	-	-
Chrysene	CHR	-	+	+	-	-	-
Fluoranthene	FLA	-	+	+	-	-	-
Hydroxyanthraquinone	HANQ	-	-	-	+	+	+
Hydroxyphenanthrenequinone	HPHQ	-	-	-	-	+	++
Indol(1,2,3-cd)pyrene	I(1)PYR	-	-	++	+	-	-
Phenanthrene	PHE	-	+	-	-	-	-
Pyrene	PYR	+	++	+	-	-	-
Triphenylene	TRI	-	+	+	-	-	-
Unknown oxyPAHs	UOPAH	-	+	+	+	+++	+++

Table 6.2 Components of fractions from contaminated Mohawk Lake (Brantford) sediment. (-) No peak present, (+) Low relative amount, (++) Medium relative amount, (+++) High relative amount.

Toxicant	Abb.	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6
Anthracene	ANT	+	-	+	-	-	-
Anthraquinone	ANQ	-	-	+	-	-	-
Benzo(a)anthracene	BAA	-	-	+	-	-	-
Benzo(a)pyrene	BAP	-	-	+	+	-	-
Benzo(b)fluoranthene	BBF	-	-	-	+	-	-
Benzo(k)fluoranthene	BKF	-	-	-	+	-	-
Benzo(g,h,i)perylene	BGP	-	-	-	+	-	-
Chrysene	CHR	-	+	-	-	-	-
Fluoranthene	FLA	+	+	+	-	-	-
Hydroxyanthraquinone	HANQ	-	-	-	-	+	+
Hydroxyphenanthrenequinone	HPHQ	-	-	+	-	+	++
Indol(1,2,3-cd)pyrene	I(1)PYR	-	-	-	+	-	-
Phenanthrene	PHE	+	+	+	-	-	-
Pyrene	PYR	+	+	+	-	-	-
Triphenylene	TRI	-	-	-	-	-	-
Unknown oxyPAHs	UOPAH	-	-	-	+	++	+++

than Hamilton Harbor. However, the ratio of oxyPAH to intact PAH was higher in Mohawk Lake. This could be related to the fact that contaminant sources are still active in Hamilton Lake, where as the Mohawk Lake contamination occurred several years ago (the factories on the Lake have been closed for many years). Moreover, the hydroxyanthraquinones, which were present in fractions 4, 5, and fraction 6 in Hamilton Harbor, were not present in the Mohawk Lake fractions (Table 6.2). As well, hydroxyphenanthrenequinone (HPHQ) was present in the fraction 3 in Mohawk Lake instead of fractions 5 and 6 in Hamilton Harbor. The reason for this is unclear sediment extracts showed that the intact and oxyPAHs were present in both Hamilton Harbor and Mohawk Lake contaminated sediments (Table 6.1 and 6.2).

6.4.2 Short- and long-term toxicity evaluation

Whole sediment extracts, and fractions 1 to 6 from both contaminated and reference sites from Hamilton Harbor and Mohawk Lake were tested for photoinduced toxicity. All concentrations are given as mg equivalents of extracted sediments per liter (mg EQS/L). This represents the toxicity of a given amount of sediment if fully extracted and presented to the test organism (*V. fischeri*). Both the short-term (15 min) assay and long-term (18 h) assay were used.

6.4.2.1 Short-term toxicity

Whole sediment extracts and the six fractions (contaminated and reference site) from both Hamilton Harbor and Mohawk Lake isolated by normal-phase HPLC were tested for toxicity. Short-term toxicity was measured with *V. fischeri* for the contaminated and reference sites. The reference site whole extracts and fractions had no toxic activity (data not shown). The whole extract and six fractions from the Hamilton Harbor contaminated sediment were more toxic than those from the Mohawk Lake contaminated sediment (Figure 6.4 and Table 6.3). The highest inhibition of luminescence of all the samples tested from Hamilton Harbor was from the whole extract followed by fraction 1

Figure 6.4 Dose response curves for short-term toxicity of extracts from Hamilton Harbor (Left) and Mohawk Lake (Right). Whole extract and fractions 1 through 6 were tested. Assays were carried out in SSR and darkness. Inhibition of luminescence is plotted against concentration (in mgEQS/L) The data points are means and error bars 95% confidence intervals (n = 6).

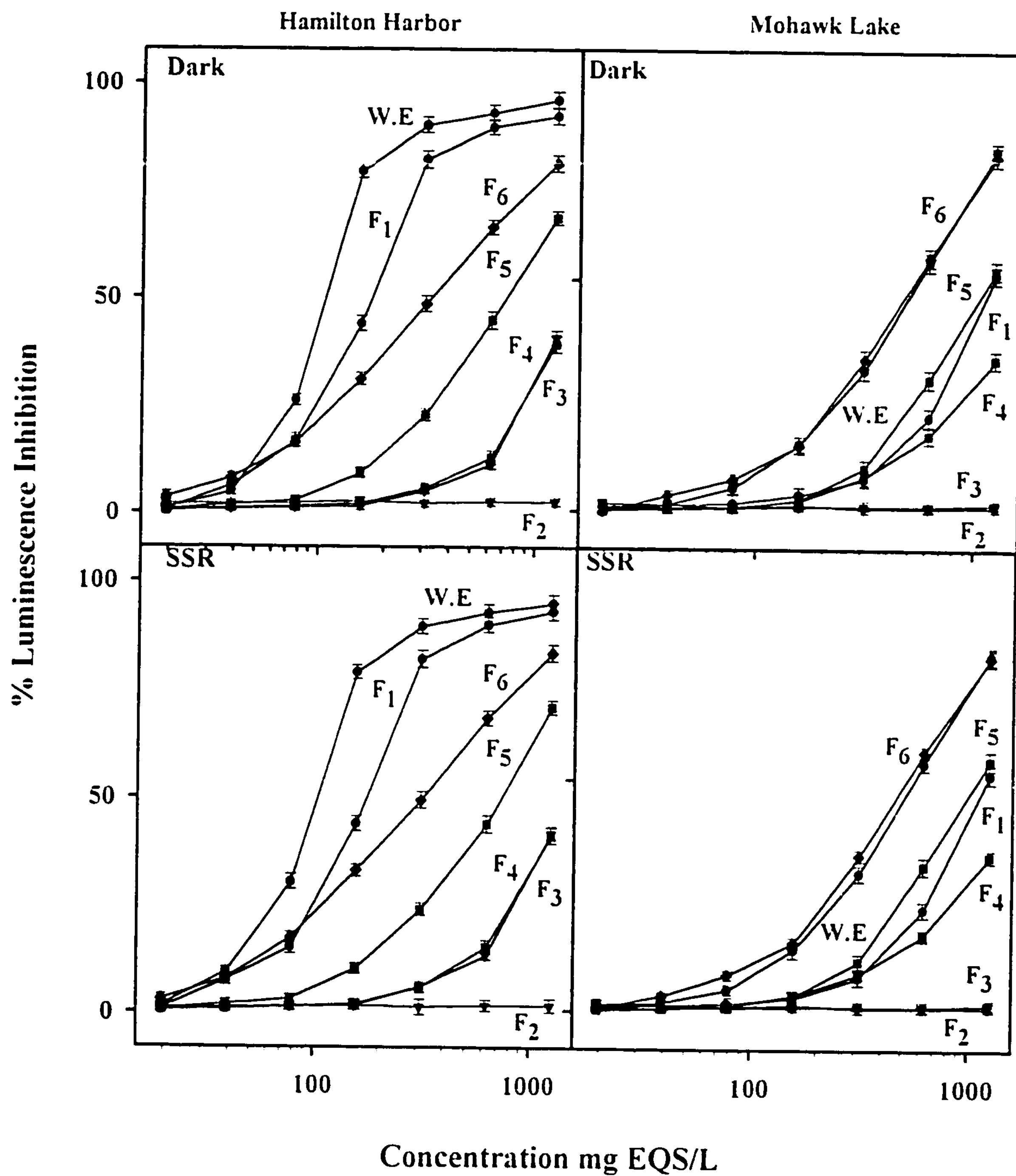

Hamilton Harbor
Mohawk Lake
Dark
Dark
SSR
SSR
W.E
F1
F2
F3
F4
F5
F6
100
50
0
100
1000
% Luminescence Inhibition
Concentration mg EQS/L

Table 6.3 Short-term toxicity to *V. fischeri* of whole extracts and isolated fractions from Hamilton Harbor and Mohawk Lake sediments. The values are EC50s (mgEQS/L) for inhibition of luminescence. All experiment are the average of 6 replicates ± 95 % confidence intervals.

	Hamilton Harbor Sediment		**Mohawk Sediment**	
	Luminescent Inhibition		**Luminescent Inhibition**	
Test Material	**Dark**	**SSR**	**Dark**	**SSR**
Whole Extract	106.6 ± 0.81	102.8 ± 0.65	538.5 ± 0.79	577.9 ± 0.81
Fraction 1	169.5 ± 0.79	173.7 ± 0.73	1226.1 ± 0.99	1201.5 ± 0.98
Fraction 2	N/D	N/D	N/D	N/D
Fraction 3	1486 ± 0.97	1484.1 ± 0.99	N/D	N/D
Fraction 4	1585.7 ± 0.99	1531.0 ± 0.98	2134.1 ± 0.99	2126.0 ± 0.99
Fraction 5	725.7 ± 0.73	721.0 ± 0.85	1150.6 ± 0.97	1041.5 ± 0.98
Fraction 6	331.0 ± 0.58	330.4 ± 0.66	525.4 ± 0.85	479.4 ± 0.78

*N/D: No Toxicity Detected

then fraction 6 in darkness and SSR. Fraction 2 did not show any toxicity to *V. fischeri*. Fractions 3, 4 and 5 had intermediate toxicity (Figure 6.3 and 6.4). None of the fractions showed photoinduced toxicity in the short-term assay.

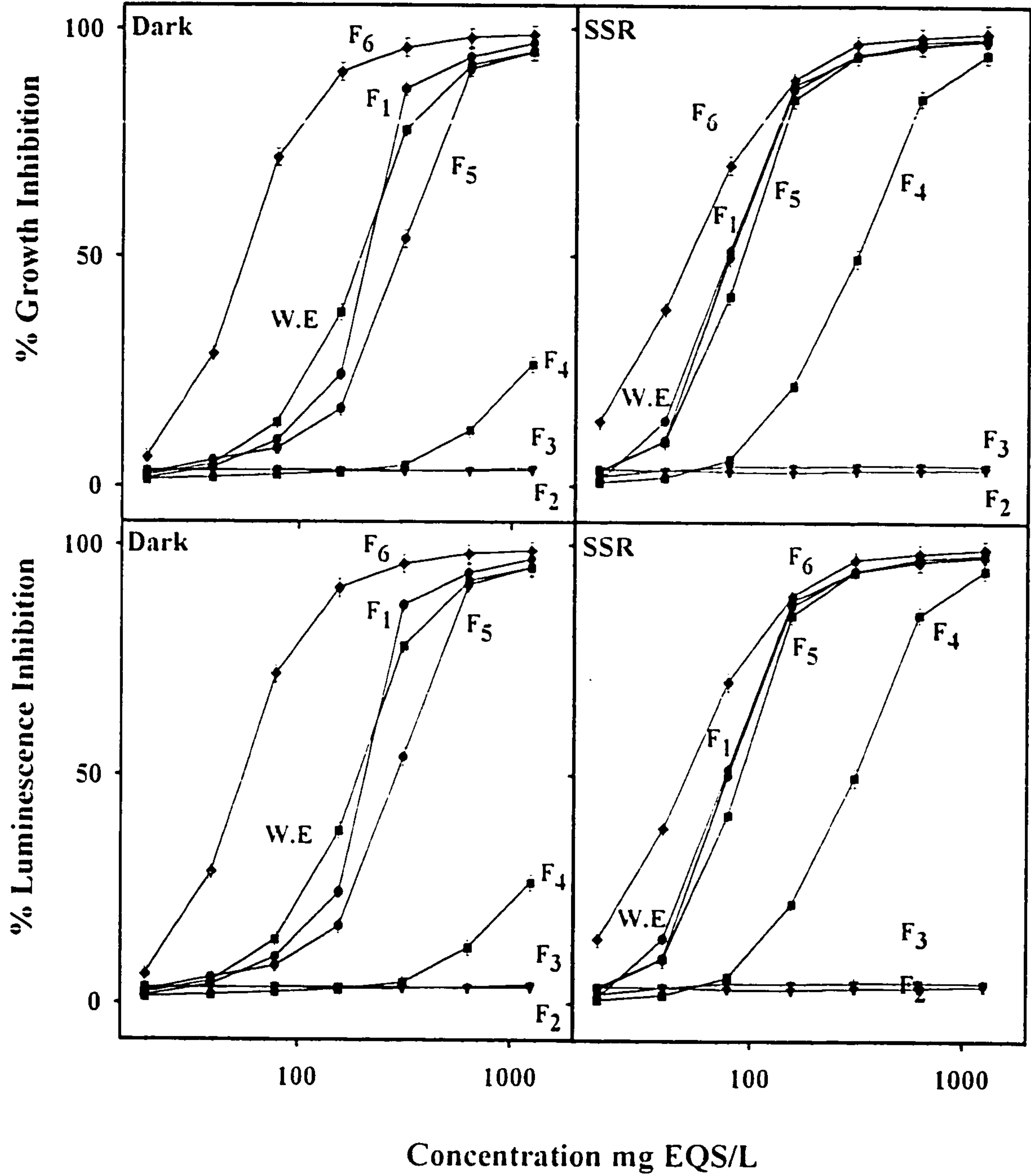
For Mohawk Lake, Fraction 6 and the whole extract had equal toxicity (Figure 6.4). Fractions 1,4 and 5 had intermediate toxicity, and fractions 2 and 3 had no effect. The whole extract and fractions from Mohawk sediments showed lower toxic impact on *V. fischeri* than the Hamilton Harbor samples. In all cases, the EC50s were higher with the Mohawk Lake sample than Hamilton Harbor samples (Table 6.3). It is, however, interesting to note there was less of a difference in toxicity between the two sites for the oxyPAH fractions than for the intact PAH fractions. As well, with Mohawk Lake the toxic impacts from the whole extract were on par with the toxicity of fraction 6 (Figure 6.4 and Table 6.3). As was the case with Hamilton Harbor, there was no influence of light on the toxicity of the Mohawk Lake sample

6.4.2.2 Long-term toxicity

Whole sediment extracts and fractions 1 to 6 from both Hamilton Harbor and Mohawk Lake were tested for long-term toxicity in darkness and SSR. Results for the six fractions from both Hamilton Harbor and Mohawk Lake are quite striking. The highest inhibition of luminescence and growth of all samples tested was produced by fraction 6, and it was more toxic than the whole extract (Figure 6.5, Figure 6.6 and Table 6.4). The fractions containing compounds of higher polarity than intact PAHs (fractions 4 and 5), which are the modified PAHs, were as or more toxic than fractions that contain only intact PAHs (Fractions 1, 2 and 3). Note also that for Mohawk Lake, fractions 5 and fraction 6 had similar levels of toxicity.

Photoinduced toxicity was observed in the long-term assay. SSR increased the toxicity of all fractions from Hamilton Harbor except Fractions 2 and 3, which had no oxicity under either condition. In the case of Mohawk Lake, only fraction 3 did not show toxicity in SSR. This is consistent with other reports on the toxicity of PAHs exposed to UV radiation (Oris and Giesy 1985, Kagan *et al.*, 1985, Newsted and Giesy 1987; Ankley

Figure 6.5 Dose response curves for the long-term toxicity of extracts from Hamilton Harbor. Whole extract and fractions 1 through 6 were tested. Assays were carried out in SSR and darkness. Inhibition of luminescence and growth are plotted against concentration (in mgEQS/L). The data points are means and error bars are 95% confidence intervals (n = 6).



Long -term toxicity Hamilton Harbour
Dark
SSR
Dark
SSR
% Growth Inhibition
% Luminescence Inhibition
100
50
0
100
50
0
F_6
F_1
F_5
W.E
F_4
F_3
F_2
100
1000
100
1000
Concentration mg EQS/L

Figure 6.6 Dose response curves for the long-term toxicity of extracts from Mohawk Lake. Whole extract and isolated fractions 1 through 6 were tested. Assays were carried out in SSR and darkness. Inhibition of luminescence and growth are plotted against concentration (in mgEQS/L). The data points are means and error bars are 95% confidence intervals (n = 6).

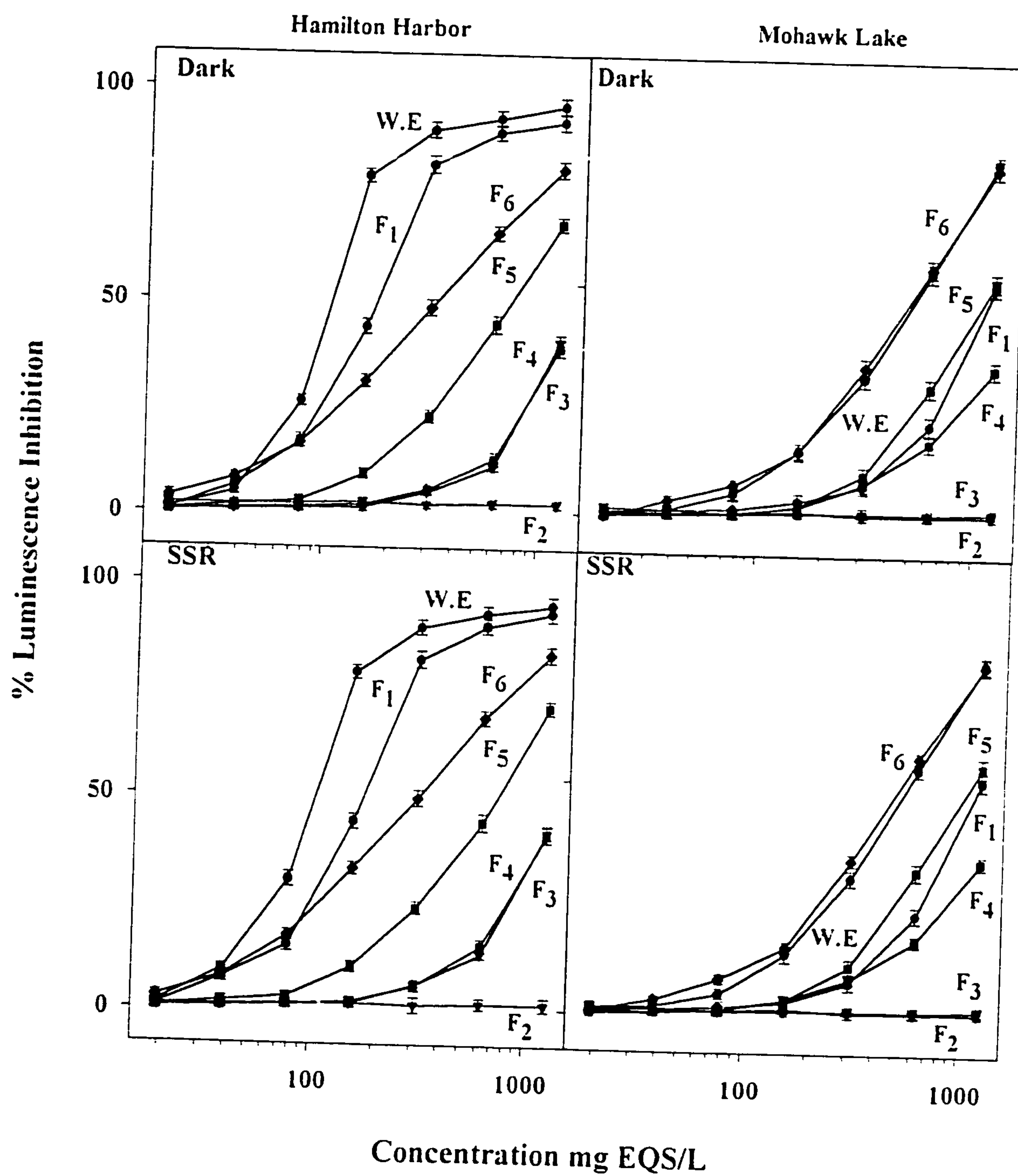

Hamilton Harbor
Mohawk Lake
Dark
Dark
SSR
SSR
W.E
F1
F2
F3
F4
F5
F6
100
50
0
% Luminescence Inhibition
100
1000
Concentration mg EQS/L

Table 6.4 Long-term toxicity of whole extract and fractions from Hamilton Harbor and Mohawk Lake sediments to *V. fischeri*. The values are EC50s (mgEQS/L) for inhibition of luminescence and growth. All experiment are the average of 6 replicates ± 95 % confidence intervals.

Test	Hamilton Harbor Sediment				Mohawk Lake Sediment			
	Luminescent Inhibition		Growth Inhibition		Luminescent Inhibition		Growth Inhibition	
Material	Dark	SSR	Dark	SSR	Dark	SSR	Dark	SSR
W. Extract	203.1 ± 0.56	76.7 ± 0.04	201.1 ± 0.61	75.9 ± 0.05	659.1 ± 0.69	456.3 ± 0.48	656.1 ± 0.78	454.9 ± 0.52
Fraction 1	286.9 ± 0.49	79.4 ± 0.05	284.9 ± 0.72	77.8 ± 0.04	N/D	1253.7 ± 0.95	N/D	1249.9 ± 0.99
Fraction 2	N/D	N/D	N/D	N/D	N/D	1003.9 ± 0.96	N/D	2995.8 ± 0.89
Fraction 3	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Fraction 4	2590.9 ± 0.99	299.9 ± 0.65	2581.9 ± 0.95	296.9 ± 0.75	N/D	305.2 ± 0.63	N/D	301.2 ± 0.56
Fraction 5	187.7 ± 0.39	87.7 ± 0.04	185.7 ± 0.41	86.8 ± 0.06	179.5 ± 0.29	88.5 ± 0.04	173.5 ± 0.32	87.6 ± 0.04
Fraction 6	55.5 ± 0.03	50.7 ± 0.02	54.5 ± 0.04	49.6 ± 0.03	182.0 ± 0.32	82.1 ± 0.04	180.0 ± 0.37	81.4 ± 0.03

*N/D: No Toxicity Detected

Table 6.5 Long-term toxicity of whole extract and isolated fractions from Hamilton Harbor and Mohawk Lake reference sediment to *V. fischeri*. The values are EC50s (mg EQS/L) for inhibition of growth.

	Hamilton Harbor Sediment		**Mohawk Lake Sediment**	
	Growth Inhibition		**Growth Inhibition**	
Test Material	**Dark**	**SSR**	**Dark**	**SSR**
Whole Extract	N/D	6552	N/D	N/D
Friction 1	N/D	N/D	N/D	N/D
Fraction 2	N/D	N/D	N/D	N/D
Fraction 3	N/D	N/D	N/D	N/D
Fraction 4	N/D	N/D	N/D	N/D
Fraction 5	N/D	>6000	N/D	N/D
Fraction 6	7993	3000	N/D	N/D

*N/D: No Toxicity Detected

et al., 1994, Huang *et al.*, 1993). It is also consistent with earlier chapters, where photoinduced toxicity was only observed in the long-term assay.

Long-term toxicity was also assessed for the reference sites from both Hamilton Harbor and Mohawk Lake (Table 6.5). There was no toxicity to *V. fischeri* from whole extract and all the fractions for the reference site from Mohawk Lake in darkness and SSR. Only the whole extract and fraction 6 from of reference site from Hamilton Harbor had toxic activity, but the toxicity was very low. Thus, the toxicity of the above-contaminated samples can be assumed to be due to the PAHs present.

6.5 DISCUSSION

6.5.1 PAH identification

Whole sediment extracts and the fractions from contaminated and reference site were analyzed for PAHs compound with a two-dimensional HPLC method. By using absorbance spectra (with a diode array detector) and retention times from the library data it was possible to identify several PAHs and oxyPAHs in the fractions (Table 6.1 and 6.2). Fractions 1 to 3 predominantly contained intact PAHs and fractions 4 to 6 were enriched with modified PAHs (mostly oxyPAHs). The contaminated sediments from both Hamilton Harbor and Mohawk Lake, were found to contain both intact and oxyPAHs, while the reference sediments contained little of these two classes of compounds. There were significant amounts of oxyPAHs in both contaminated sites. This is consistent with other studies, when modified PAHs found in PAHs contaminate (Legzdins *et al.*, 1995; Enya *et al.*, 1997; Kosian *et al.*, 1998; Huang *et al.* 1999). Thus, it seems likely that oxyPAHs will be found in most PAH-contaminated sites.

The fractionation and analysis technique developed for this study was based on two-dimensional HPLC. In the first dimension a normal-phase HPLC column was used. With this column, hydrophobic compounds bind less strongly than hydrophilic compounds. Thus, the intact PAHs came off the column (Fractions 1- 3) before the oxyPAHs (Fractions 4-6). These six fractions were used for further analysis via reverse-phase HPLC

to identify specific compounds, and for toxicity tests. The intact PAHs, which were predominantly in fractions 1-3, were to a large extent priority PAHs found at most PAH contaminate sites (e.g. ANT, PHE, BAP, BAP, PYR, etc).

The oxyPAHs were quinones and hydroxyquinones (primarily in fractions 4-6). Because these are known photoproducts of PAH exposure to sunlight, it is possible that the oxyPAHs found in the contaminated sediments arose via photooxidation of intact PAHs released into the environment There are two ways this could come about. First, the surface exposed sediment receive enough UVA radiation and blue light to cause some photomodification. Second, as particulate matter migrates in the water column it is exposes to solar radiation. These particles are dynamically moving in and out of sediment, thus allowing photomodified PAHs to accumulate in the sediment.

By comparing the HPLC analyses for contaminated sediments from Hamilton Harbor and Mohawk Lake, further support for photooxidative generation of modified PAHs can be realized. Hamilton Harbor had a higher ratio of intact PAHs to oxyPAHs than Mohawk Lake. Interestingly, the sources of PAHs are still active in Hamilton Harbor, but industrial activity on Mohawk Lake ceased more than a decade ago. Meanwhile, the intact PAHs in Mohawk Lake were available for photooxidation at the surface of the sediments and on particular matter cycling into the water column. This could allow for a net conversion of intact PAH to photomodified PAHs, where as Hamilton Harbor would be in a dynamic equilibrium with new inputs of PAHs. It is thus logical that a higher ratio of modified PAHs was observed in Mohawk Lake than Hamilton Harbor.

6.5.2 Toxicity of the sediment extracts

Contaminated and reference sediments from both Hamilton Harbor and Mohawk Lake were tested for photoinduced short- and long-term toxicity. The whole extract and all of the fractions from the reference site of Mohawk Lake did show any toxicity impact to *V. fischeri*. An examination of the HPLC data for the Mohawk Lake reference site revealed there was no trace of intact PAHs or oxyPAHs. Interestingly, the toxicity data from the Hamilton Harbor reference site revealed low impacts from the whole extract and

fraction 6. Note there is a low level of some unidentifiable oxyPAHs in fraction 6. That might be related to the fact that Hamilton Harbor still has active industries, which are major sources of contamination. Within the constrained space of the Harbor, contamination could migrate from site to site, giving a low level of oxyPAHs at the reference site. Strikingly, only oxyPAHs were formed at the reference site. It is possible that sediment particulate matter migrated from contaminated sites to the reference site. During this period, the particles would be exposed to sunlight. Thus, by the time the particle reached the reference site, predominantly modified PAHs would be present. Note this migration is not possible for Mohawk Lake because the reference site is upstream from the contaminated site.

The short-term toxicity assay data showed that the Hamilton Harbor contaminated sediment whole extract and fractions were more toxic than those from the Mohawk Lake contaminated site. By comparing the HPLC data between Mohawk Lake and Hamilton Harbor it can be seen that Hamilton Harbor has more intact PAHs and oxyPAHs than Mohawk Lake. However, fraction 2 from Hamilton Harbor did not show any toxicity to *V. fischeri*. Interestingly, the HPLC data revealed this fraction contained a high relative amount of intact PAHs. It is unclear why the PAHs in these fractions are not toxic.

The long-term toxicity data for the whole extracts and the fractions generally showed increased toxic impacts on *V. fischeri* in SSR compared to darkness. Of the fractions that showed long-term toxicity, fraction 6 had the smallest difference between darkness and SSR. This may be related to fact that fraction 6 contains only oxyPAHs.

The photoinduced long-term toxicity impacts for fraction 6 from both contaminated sites are quite striking because they had the greatest impact of all the samples tested, including the whole extracts. An explanation for the difference between fraction 6 and the whole extract might be the presence of humic matter in the whole extract, which is known to decrease PAH toxicity (Gensemer *et al.*, 1998). Nonetheless, it is striking that the most toxic fractions from both sites are those that contain modified PAHs.

The whole extract and all the fractions from the Hamilton Harbor contaminated site were more toxic to *V. fischeri* than those from Mohawk Lake. This is consistent with

the higher level of PAH contamination in Hamilton Harbor. However, it is interacting to directly compare the toxicity of selected fractions from Mohawk Lake and Hamilton Harbor. The ratio of the EC50s for fraction 6 of Mohawk Lake to Hamilton Harbor is only 1.6 (82:51), while the ratio for fraction 1 is 16 (1253:79). Thus, the relative toxic impact of fraction 6 for Mohawk Lake is greater than that of fraction 6 for Hamilton Harbor. This is striking in light of the fact that the relative amount of oxyPAH to intact PAH is also greater in Mohawk Lake. Clearly, oxyPAHs are important for evaluating overall risk posed by PAH-contaminated sediment.

The long-term toxicity of the polar fractions (5 and 6) in darkness was greater or equal to that of the whole extract or the non-polar fraction 1. This is consistent with existing evidence that modified PAHs need neither biological activation nor UV radiation to exhibit direct toxicity (Huang *et al.*, 1993, Huang *et al.*, 1995, Ren *et al.*, 1996, McConkey *et al.*, 1997, Mallakin *et al.*, 1999). Thus, it is clear that modified PAHs, whether generated in the laboratory or in the field, pose a direct hazard to the biosphere.

Furthermore, it is clear from this study that modified PAHs are ubiquitous contaminants in the environment. That is, modified PAHs were found at the sites sampled here, and they have been reported at several other sites (Huang *et al.*, 1999). Even more importantly they can account for a significant portion of the toxicity of PAH contaminated sediments. Thus, of the environmental assessment process.

CONCLUSION AND FUTURE DIRECTIONS

Polycyclic aromatic hydrocarbons (PAHs) are a group of chemicals whose toxicology is difficult to study. First, PAHs are structurally and chemically, diverse. Second, PAHs occur in the environment as complex mixtures. PAHs can elicit toxicity in a variety of ways. Two ways in which PAHs can be toxic to aquatic organisms have been studied with marine bacteria *Vibrio fischeri* in this thesis: their direct toxicity was studied using the short-term assay, and their phototoxicity was studied in the presence of simulated solar radiation (SSR) using the long-term assay. The short-term toxicity of PAHs was dependent on water solubility and lipophilicity as two physico-chemical parameters. Water solubility and lipophilicity work in apparent opposite ways. With PAHs the higher their water solubility the lower their lipophilicity, thus, there is great potential taken up into their cellular target sites, the membranes. However, the low lipophilicity allows only a small portion of the available molecules to accumulate and taken up by organism. Therefore, the high lipophilicity favors the cellular uptake of a large portion of the available PAHs molecules. If a PAH is more water-soluble than another PAH which is more lipophilic, the net results in the cell is the same.

Toxicity of most PAHs to aquatic organisms can be greatly enhanced upon exposure of the target organism and/or the chemicals to the ultraviolet (UV) radiation present in sunlight. Photoinduced long-term toxicity of PAHs was found to depend on physico-chemical parameters, such as water solubility and lipophilicity, and additionally, the ability of each PAH to absorb UV radiation and undergo photochemical reactions. There are two major mechanisms involved in the photoinduced toxicity of PAHs:

photosensitization and photomodification. In the former, production of singlet oxygen, which initiated oxidative reactions, such as lipid peroxidation. Photooxidation of PAHs results in new compounds (usually oxygenated PAHs) that are often more toxic than their parent PAHs. Oxygenated PAHs are capable of redox cycling which can interrupt electron transport chains in biological processes (e.g. photosynthesis and respiration). Many new questions arose from the results of this work related to the short- and long-term toxicity of PAHs and the way in which PAHs are toxic.

In an effort to examine the photomodification and photosensitization processes of PAHs, microbial toxicity assays were developed to measure short- and long-term photoinduced toxicity. The test organism was the luminescent bacterium *Vibrio fischeri* (strain NRRL B-11177). The short-term assay developed was based on inhibition of luminescence after a 15-minute incubation with a test chemical. The long-term assay involved returning the cells to the incubator after the short-term endpoint was measured and growing them for 18 hours with the test chemical, followed by measurement of luminescent intensity and growth. The sensitivities of the assays were found to correlate well with other bioassays and they were effective at screening a large number of compounds. Both assays could be preformed in darkness or simulated solar radiation (SSR) to examine the effects of light on PAH toxicity. The short-term and long-term assays were tested with representative intact PAHs and modified PAHs. With the short-term assay the toxicity of all the chemicals was the same in SSR or darkness. This means photoinduced toxicity is not apparent in a short-term exposure. However, with the long-term assay, SSR did enhance PAH toxicity.

Having developed the *V. fischeri* assay, a quantitative structure-activity relationship (QSAR) previously developed for the aquatic plant *Lemna gibba* was applied to the bacteria. Summing two factors, one for photosensitization and one for photomodification resulted in predictive values that showed strong correlation to the *V. fischeri* toxicity data. Thus, a QSAR model derived for plants accurately described the toxicity of PAHs to a bacterial species. This indicates that the bipartite mechanism of PAH photoinduced toxicity is broadly applicable. QSARs are useful for understanding the mechanisms of action of chemicals and predicting toxicity of untested chemicals. The QSAR model not only served in the prediction of toxicity, but also explained the physico-chemical mechanisms of photoinduced toxicity. This could be a very important model of action of photoinduced toxicity to living organism

The *V. fischeri* short- and long-term assays when applied to assessment of PAH-contaminated sediments collected from Hamilton Harbor, ON, and Mohawk Lake, Brantford, ON. They were fractionated and found to contain PAHs and oxyPAHs. Strikingly, the oxyPAH containing fractions were observed to be the most toxic samples. Thus, oxyPAHs in the environment have a hazard potential. The developed assay procedures are simple, inexpensive and rapid. Both individual PAHs as well as a complex mixture were tested reliably, making the assays a useful tool for testing effluents or environmental samples, as well as for toxic identification evaluations. Furthermore, the short- and long-term assays using luminescent bacteria have the potential to lead to new sampling and monitoring techniques.

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