MODELING TOXIC ENDPOINTS FOR IMPROVING HUMAN HEALTH RISK ASSESSMENT

A Dissertation

by

ERICA DAWN BRUCE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Civil Engineering

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Approved by:

Chair of Committee, Robin L. Autenrieth
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ABSTRACT

Modeling Toxic Endpoints for Improving Human Health Risk Assessment. (May 2007)

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Risk assessment procedures for mixtures of polycyclic aromatic hydrocarbons (PAHs) present a problem due to the lack of available potency and toxicity data on mixtures and individual compounds. This study examines the toxicity of parent compound PAHs and binary mixtures of PAHs in order to bridge the gap between component assessment and mixture assessment. Seven pure parent compound PAHs and four binary mixtures of PAHs were examined in the *Salmonella*/Microsome Mutagenicity Assay, a Gap Junction Intercellular Communication (GJIC) assay and the 7-ethoxyresorufin-*O*-deethylase assay (EROD). These assays were chosen for their ability to measure specific toxic endpoints related to the carcinogenic process (i.e. initiation, promotion, progression). Data from these assays was used in further studies to build Quantitative Structure-Activity Relationships (QSARs) to estimate toxic endpoints and to test the additive assumption in PAH mixtures. These QSAR models will allow for the development of bioassay based potential potencies (PP_B) or toxic equivalency factors (TEFs) that are derived not only from bioassay data, but also from structure, activity, and physical/chemical properties. These models can be extended to any environmental media to evaluate risk to human health from exposures to PAHs.

DEDICATION

To my husband, Brian

Thank you for your love, support and for our beautiful baby girl, Camden Riley.

You complete me.

ACKNOWLEDGMENTS

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NOMENCLATURE

ADME	Absorption, Distribution, Metabolism, and Excretion
Ah	Aryl hydrocarbon
ATSDR	Agency for Toxic Substances and Diseases Registry
AT_{NC}	Averaging Time for Non-Carcinogens
BAP	Benzo(a)pyrene
BW	Body Weight
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CFDA	Carboxyfluorescein Diacetate
CIC	Complementary Information Index
CPSA	Charged Partial Surface Area
CYP	Cytochrome P450
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ED	Exposure Duration
EF	Exposure Frequency
EROD	Ethoxyresorufin-O-deethylase
ET	Exposure Time
FC	Fraction From Contaminated Source
FRAP	Fluorescence Recovery After Photobleaching
GC/MS	Gas Chromatography/Mass Spectrometry
GFA	Genetic Function Algorithm
GJIC	Gap Junction Intercellular Communication
HABs	Hazardous Algal Blooms
HHRA	Human Health Risk Assessment
IUPAC	International Union of Pure and Applied Chemistry
IR	Ingestion Rate
IRIS	Integrated Risk Information System
LC/MS	Liquid Chromatography/Mass Spectrometry
LOF	Lack-of-Fit
LSE	Least Squares Error
MALDI-TOF	Matrix-assisted Laser Desorption/Ionization Time-of-Flight
MF	Modifying Factor
MGPRs	Manufactured Gas Plant Residues
MTT	3-[4,5-dimethylthiozol-2-yl]-2,5 –triphenyltetrazolium
NOAEL	No Observable Adverse Effects Level
NPL	National Priority List
NSP	Nuerotoxic Shellfish Poisoning
PAH	Polycyclic Aromatic Hydrocarbon

PBTX	Brevetoxin
PLR	Partial Linear Regression
PP	Potential Potency Factor
PP_B	Bioassay Based Potential Potency Factor
ppt	Part Per Thousand
PRESS	Predicted Sum of Squares
QSAR	Quantitative Structure-Activity Relationship
QSTR	Quantitative Structure-Toxicity Relationship
RAGS	Risk Assessment Guidance For Superfund
RCRA	Resource Conservation and Recovery Act
SD	Sum of Squared Deviation
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin
TEF	Toxic Equivalency Factor
TEF_B	Bioassay Based Toxic Equivalency Factor
UF	Uncertainty Factor
USEPA	United States Environmental Protection Agency

CHAPTER I

INTRODUCTION

The Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) provides federal authority for the remediation of hazardous waste sites deemed severe by the U.S. Environmental Protection Agency's (USEPA) National Priority List (NPL) (USEPA, 2006a, 2006b). Currently 1245 hazardous waste sites are on the NPL and pose a risk to human health and the environment. The contaminants on these sites have a potential to cause harm to those exposed and therefore an assessment of the potential hazards must be made. The USEPA guidelines are the industry standard when performing environmental and human health risk assessment (Sims, P. et al., 1974). There are four major steps in this framework, (1) data collection and evaluation, (2) exposure assessment, (3) toxicity assessment, and (4) risk characterization.

There are two basic approaches that the EPA gives guidance for when doing risk assessments of complex mixtures, a whole mixture approach and a component approach (USEPA, 1986, 1993, 2000). These two methods are based on the nature and quality of the available data and the assumption that chemical toxicity among the PAHs in a mixture is additive. An additive response occurs when the combined response or two chemicals is equal to the sum of the two chemicals individually. PAHs in mixtures can also have a synergistic or inhibitory response.

The style and format of this dissertation follows that of the *Journal of Toxicology and Environmental Health – Part B.*

Synergistic responses occur when the combined effect of two chemicals are much greater than the sum of the two chemical individually. While an inhibitory response occurs when two chemicals interfere with one another's actions or one chemical interferes with the other chemical.

Among environmental health scientists, the consensus is that current data will only support a quantitative risk assessment for the carcinogenic effects of PAHs (Schoeny, R. et al., 1998). There are three approaches that are commonly used for evaluating the human health risks associated with the exposure to PAH containing mixtures: the surrogate mixture approach, the comparative potency approach, and the relative potency factor approach.

The surrogate mixture approach is a mixture-based approach that should only be used to estimate the potency of the PAH component of the mixture of concern because benzo (a) pyrene (BAP) is not a consistent indicator of the concentration of "non-PAH" components of a mixture (Versar et al., 2002). An inherent assumption with this method is that any PAH mixture is a dilution of a "surrogate" PAH mixture, where the surrogate mixture is characterized chemically and toxicologically. The assumption that there is "sufficient similarity" both chemically and toxicologically between the mixture of concern and the surrogate mixture can be problematic if the mixture is not well characterized or sufficiency is questionable. A second assumption is that the factor used to measure the extent of dilution of the mixture of concern (i.e. the BAP concentration) is a valid indicator of the concentration of all PAHs in the mixture and will influence the

carcinogenic potential. The assumptions that are required to use this method lead to some specific advantages and disadvantages.

An advantage of using this method is the relative ease for regulatory agencies to apply and its results are expected to be conservative as long as the mixture of concern is less potent than the surrogate mixture. However, the criteria for determining "sufficient similarity" are vague and the selection of the surrogate mixture is difficult. Further, this method does not take into consideration substituted versions of PAHs in the mixture that can represent a significant percent of the total mass.

Comparative potency is also a mixture-based approach that evaluates the potency of the whole mixture rather than just the PAH component(s) of the mixture. A key underlying assumption is that similar mixtures in a data set act in a similar manner toxicologically and the relative potency in an *in vivo* or *in vitro* bioassay is directly proportional to the relative potency in humans (Versar et al., 2002). This proportionality is represented by a scaling factor, k, that is assumed to be the same for different PAH containing mixtures. Further, the mixtures used to derive the scaling factor and the mixtures of concern must be "sufficiently" similar.

The main advantage of this particular mixture-based approach is that it estimates the potency of the whole mixture and it uses existing epidemiological data on human carcinogenicity of mixtures (Schoeny, R., Muller, P., and Mumford, J., 1998). However, mixtures from the same types of sources (i.e. diesel emissions) do not always have similar ratios of PAHs or equivalent toxicological potency in which case they are not considered "sufficiently" similar. Currently, this method is only useful for inhalation

routes of exposure because epidemiological data for human oral and dermal exposures are not yet available. Consequently, it is only feasible to use in cases where inhalation risk will be the dominant contributor to the overall risk estimates. This method is not considered a viable option for mixtures that have originated from unknown sources or that are poorly characterized.

A component- based approach that is commonly used to evaluate the risk of a complex mixture of PAHs is the relative potency factor approach. This method for mixtures risk assessment relies on existing EPA risk assessment information on single chemical toxicity. Generally, it is used to evaluate mixtures of polychlorinated biphenyls (PCBs), dioxins and PAHs (USEPA, 2000). Relative potency factors (RPFs) or toxic equivalency factors (TEFs) are used to adjust a compounds toxicity based on a reference compound in the same class. The term "relative potency factor" refers to toxicity comparisons from an individual experiment, while the term "toxic equivalency factor" is most commonly used for toxicity estimates determined by consensus (USEPA, 2000) Compounds are included for those which TEF values can be determined. Complete representation of all compounds in the mixture is not guaranteed.

This method assumes that the toxicity of individual PAHs is additive and that all PAHs have the same mode of action. Additionally, it assumes that the potency of the carcinogenic PAHs sufficiently reflects the potency of the entire mixture. Commonly in practice, the mixture of concern is from an unknown source or the composition of the mixture is not fully characterized. This method can be applied to such situations with relative simplicity because there are existing TEFs for PAHs. A component-based

approach is generally considered less desirable than a whole mixture approach. The most accurate representation of risk using this method can only be calculated for the oral exposure route because there are currently no cancer slope factors for dermal and inhalation routes. Furthermore, it is not clear whether the toxicity of individual PAHs is additive. The RPF method is the least desirable method for assessing the risk for a complex mixture of PAHs. In most cases, however, this is the method that must be used due to the nature and available data for the PAH mixture of concern (Versar et al., 2002).

The focus of the proposed research is on PAHs and chemical mixtures of PAHs. This group of chemicals represents a class of environmental organic pollutants to which we are regularly exposed (Afghan, B. and Chu, A., 1989). Characterized by two or more fused aromatic rings, PAHs are usually crystalline solid materials that have high melting points, low vapor pressures, low water solubility, and strong adsorption affinity for surfaces (Table 1) (Afghan, B. and Chu, A., 1989; Dabestani, R. and Ivanov, I., 1999). These physico-chemical characteristics make this group of chemicals especially important when considering routes of exposure in a risk assessment. Their characteristics are typical of contaminants that can be in high concentrations in environmental media (i.e., soils).

Pyrolysis of any material containing carbon and hydrogen can lead to the formation of PAHs. The product composition depends primarily on the temperature for a given source material. At combustion temperature below 700°C the majority of the PAHs formed are alkyl substituted PAHs while temperature greater than 1000°C leads to the formation of unsubstituted compounds (Guerin, M. R., 1978). Accordingly, PAHs

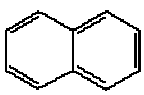
that stem from coal-derived products and crude oils have an abundance of alkyl substituted PAHs.

Classification of PAHs is determined by the arrangement of the rings during formation. There are numerous possibilities for PAH compounds therefore the International Union for Pure and Applied Chemistry (IUPAC) has delineated a set of rules for naming PAH compounds. There are only a select number of compounds that are given trivial names such as fluorene, chrysene, and pyrene (Figure 1). The numbering system is determined by orienting the compound so that the maximum numbers of rings are in a horizontal row and as many rings as possible are above and to the right of that row (Harvey, R. G., 1997). Numbering starts with a carbon atom that is only part of one ring, is in the most counterclockwise position on the ring farthest to the right above the horizontal plane, and continues in a clockwise direction. As not all compounds are given a trivial name, they are assigned a name by adding the name of the substituent to the base trivial name. The base name takes into account the maximum number of rings (Harvey, R. G., 1997).

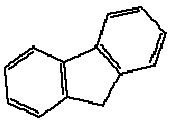
Table 1. Table of physical-chemical properties of selected PAHs.
(ATSDR, 1990a, 2005; IARC, 1983)

PAH	Molecular Weight (g)	Solubility at 25 °C (µg/L)	Vapor Pressure at 25 °C (mm Hg)	Log Kow (Log Koc)	Carcinogenicity
Naphthalene	128.2	12500 to 34000	1.8×10^{-2}	3.37	NC
Acenaphthylene	152.2	3420	10^{-3} - 10^{-4}	4.07 (3.40)	NC
Acenaphthene	154.2			3.98 (3.66)	NC
Fluorene	166.2	800		4.18 (3.86)	NC
Anthracene	178.2	59	2.4×10^{-4}	4.5 (4.15)	NC
Phenanthrene	178.2	435	6.8×10^{-4}	4.46 (4.15)	NC
2-Methylanthracene	192.3	21.3		4.77	NC
9-Methylphenanthrene	192.3	261		4.77	NC
1-Methylphenanthrene	192.3	269		4.77	NC
Fluoranthene	202.3	260		4.90 (4.58)	NC
9,10-Dimethylanthracene	206.3	56		5.13	NC
Benzo[a]fluorene	216.3	45		5.34	NC
Benzo[b]fluorene	216.3	29.6		5.34	NC
Pyrene	202.1	133	6.9×10^{-7}	4.88 (4.58)	NC
Benz[a]anthracene	228.3	11	1.1×10^{-7}	5.63 (5.30)	C
Chrysene	228.3	1.9		5.63 (5.30)	WC
Benzo[b]fluoranthene	252.3	2.4		6.04 (5.74)	C
Benzo[j]fluoranthene	252.3	2.4		6.21	C
Dibenzo[a,h]fluorene	266.3	0.8		6.57	WC
3-Methylcholanthrene	267.3	0.7		6.64	SC
Benzo[ghi]fluoranthene	214.2	0.5		6.78	NC
Benzo[a]pyrene	252.3	3.8	5.5×10^{-9}	6.06 (5.74)	SC

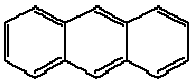
* NC= non-carcinogenic; WC=weakly carcinogenic; C=carcinogenic; SC=strongly carcinogenic; Kow=Octanol /water partition coefficient; Koc= partitioning coefficient for organic carbon



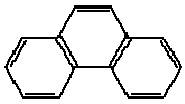
Naphthalene



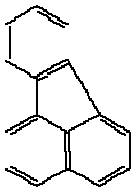
Fluorene



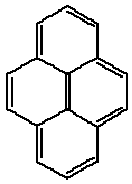
Anthracene



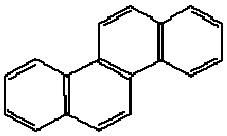
Phenanthrene



Fluoranthene



Pyrene



Chrysene

Figure 1. Polycyclic aromatic hydrocarbons that are used as the basis for the International Union of Pure and Applied Chemists (IUPAC) nomenclature determinations.

The lower molecular weight PAHs (e.g., 2 to 3 ring group of PAHs such as naphthalenes, fluorenes, phenanthrenes, and anthracenes) are acutely toxic to aquatic organisms, whereas the high molecular weight PAHs, 4 to 7 ring (from chrysenes to coronenes) are not due to their solubility. However, several members of the high molecular weight PAHs have been shown to be carcinogenic (Table 1). The greatest concern about PAHs is their carcinogenic potential. Carcinogenicity appears to increase with the molecular size until 4- and 5-ring molecules are reached (Afghan, B. and Chu, A., 1989). The general relationship of structure to carcinogenic potential favors the 4-, 5-, and 6-membered ring PAHs rather than smaller, 3-ring structures, or larger 7-ring structures (Afghan, B. and Chu, A., 1989). For example, one of the most toxic PAHs, Benzo(a) pyrene, is a 5-ring PAH and has an oral cancer slope factor of 7.30, an indicator of a strong carcinogen (USEPA, 2006d).

Although PAHs have been linked to a wide range of toxic effects (i.e., cancer, renal disease, circulatory disorders), the parent compounds are not believed to be the cause of these effects (ATSDR, 1990a). It is the oxidized metabolites and their reactive intermediates that are more biologically active (Pitot, H. C. and Dragan, Y. P., 1996; Sims, P. et al., 1974). Metabolism of the PAH family begins with oxidation by the cytochrome P450-family, specifically, CYP1A1 (Figure 2). This induction process is initiated by the absorption of a PAH by a cell from systematic circulation. Once the PAH is in the cytosol, it binds with the Aryl Hydrocarbon (Ah) receptor. Upon binding with its ligand, the Ah receptor releases two heat shock proteins (hsp 90) which are normally associated with the receptor in its inactive state. This disassociation of the heat

shock proteins allow the Ah receptor-ligand complex to be phosphorylated by tyrosine kinase. Next, the Ah receptor and its

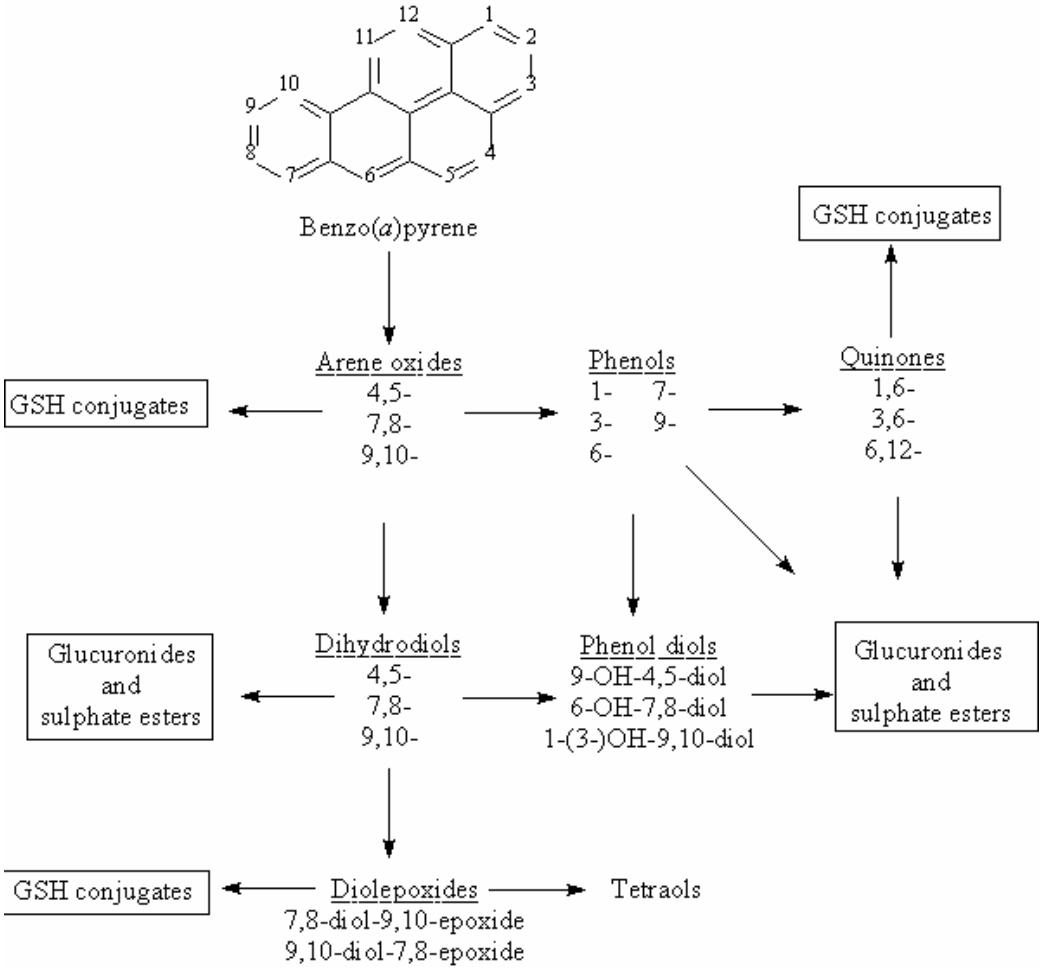


Figure 2. BAP as a model of PAH metabolism (IARC, 1983)

ligand enter the nucleus and form a heterodimer with the Ah receptor –nuclear translocator protein (Arnt). The heterodimer then binds with a Xenobiotic Response

Element (XRE) or the Dioxin Response Element (DRE) in the upstream regulatory region of a gene to enhance transcription (Parkinson, A., 1996).

PAHs are ubiquitous in the environment and are routinely found in food, water, air, and sediment. PAHs have been identified at 600 of the 1,420 USEPA NPL sites(ATSDR, 1996). A product of incomplete combustion, PAHs are prevalent and inconsistent, with respect to composition, in the environment. Wild fires, volcanoes, transportation and smoking are among the many sources of PAHs (Baum, E. J., 1978). The U.S. EPA estimated that 40% of all BAP comes from home wood fires (ATSDR, 1990b). Accordingly, 98% of BAP emissions are to the air and only 1% is to the soil and water. However, this number can be deceiving because it represents emissions only. BAP and other PAHs that are emitted into the air are eventually deposited on the soil and on surface waters. Most PAHs do not dissolve easily in water, therefore binding to particles and depositing in soils are significant transport phases. Some PAHs will eventually migrate through the soil and contaminant groundwater.

Due to the extensive and persistent contamination of media by PAHs, the USEPA has many mechanisms in place to regulate and monitor these compounds. Congress enacted the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), commonly known as Superfund, on December 11, 1980 to authorize two kinds of response actions: short-term removals and long-term remedial responses. Long-term remedial responses can only be conducted on sites that are listed on the USEPA's National Priority List (NPL). The NPL is a list of sites among the known releases or threatened releases of hazardous substances, pollutants, or

contaminants throughout the United States and its territories. Sites on the NPL have a hazard ranking score (HRS) greater than 28 (USEPA, 2006c). Several factors are considered in the HRS including the potential for migration off site, magnitude of contamination, proximity to sensitive receptors, and the toxicity of compounds present.

USEPA and the Agency for Toxic Substance and Disease Registry (ATSDR) must also maintain a list of chemicals and mixtures commonly occurring at NPL sites. If a chemical or mixture is listed, then priority is given to gather and characterize the toxicological properties of these substances. There are currently 275 compounds and mixtures on the ATSDR list of which 26 are PAHs or their source materials (ATSDR, 2005).

The release of PAH containing materials is regulated under the Resource Conservation and Recovery Act (RCRA) (USEPA, 2005). RCRA also designates a code for hazardous wastes based on the hazard they represent. This designation consists of a letter (F, K, D, U, or P) and three numbers. For example, single PAHs are given a U code, meaning they are regulated for toxicity. Most notably, this act provided the framework for the disposal of both hazardous and non-hazardous wastes. The Hazardous and Solid Waste Amendments (HSWA) of 1984 further strengthened the EPA's ability to regulate waste disposal. The HSWA was responsible for the eventual removal of land disposal of hazardous wastes.

Cancers, renal disease, circulatory disorders, reproductive disorders, and immune system dysfunction are among the more common endpoints observed from PAH exposure in mammals (ATSDR, 1990a; Ramos, K. S. et al., 1996). The fields of

toxicology have focused on the high molecular weight PAHs because of their well-characterized genetic toxicity (ATSDR, 1990a, 1996).

Chronic PAH exposure resulted in liver tumor formation yet, cytotoxicity was not observed at low doses in experimental animals (Moslen, M. T., 1996). The most commonly encountered non-cancer effects are enzyme induction and interference with cell-to-cell communication. The induction of CYP1A1 is often used as a measure of the biological activity of Ah receptor ligands. This is an essential set in the induction of Cytochrome p450 enzymes. Till and colleagues (1999) studied PAH induction of CYP1A1 in male Wistar rat hepatocyctes. Induction of this enzyme by PAHs was measured as a function of CYP1A1 catalyzed 7-ethoxyresorufin-O-deethylase (EROD) activity. Some of the most active inducers of this enzyme were chrysene, BAP, indeno (1, 2, 3-cd) pyrene, and benzanthracene. This same study also tested smaller PAH compounds such as anthracene and fluoranthene. Interestingly, they found that the induction potential of the mixture of the tested PAHs was greater (i.e. synergistic) than what was expected based on the individual PAHs (Till, M. et al., 1999). These results imply that the additive assumption used is mixture risk assessment is an inaccurate representation of the true toxicity.

Gap junction intercellular communication (GJIC) is an important cellular mechanism for maintaining homeostasis in multicellular organisms. Downregulation of GJIC is considered an important epigenetic effect (Barhoumi, R. et al., 1993). Several PAHs are known to inhibit GJIC. For example, 1- and 9-methylanthracene each significantly inhibited GJIC at 50% of the control value (IC_{50}) at 22 and 36μM (Upham,

B. L. et al., 1998). In this same study, anthracene and 2-methylanthracene did not inhibit GJIC at concentrations up to 350μM (Upham, B. L., Weis, L. M., and Trosko, J. E., 1998). In similar experiments, BAP significantly suppressed GJIC in Clone 9 hepatocytes following dosing with 0.4μM BAP for 16hrs (Barhoumi, R. et al., 2000). It has been suggested that PAHs that contain a bay region, or which have a methyl group that forms a "bay-like" region, are more potent inhibitors of GJIC than their linear counterparts (Upham, B. L., Weis, L. M., and Trosko, J. E., 1998).

The most sensitive endpoint for PAH toxicity effects is carcinogenicity (Bostrom, C. E. et al., 2002; Schoeny, R., Muller, P., and Mumford, J., 1998; USEPA, 1993) A challenge regularly faced with evaluation of PAH carcinogenicity is their occurrence in the environment as mixtures and rarely as individual compounds. Some of the earliest evidence of carcinogenicity of PAH mixtures occurred over 200 years ago in London, England. A London surgeon, Sir Percivall Pott, described a high frequency of what at the time was know as "soot-wart" in chimney sweeps. "Soot-wart" was later discovered to be cancer of the scrotum due to their regular exposure to soot in the chimneys (Pitot, H. C. and Dragan, Y. P., 1996). The USEPA's Integrated Risk Information System (IRIS) database categorizes seven PAHs as probable human carcinogens (Table 2).

Table 2. Class B2 PAH carcinogens as classified by the USEPA.

Chemical Name	CASN	Number of Rings
Chrysene	218-01-9	4
Benzanthracene	56-55-3	4
Benzo(b)fluoranthene	205-99-2	5
Benzo(k)fluoranthene	207-08-9	5
Benzo(a)pyrene	50-32-8	5
Dibenzo(a,h)anthracene	53-70-3	5
Indeno(1,2,3-cd)pyrene	193-39-5	6

One study examined the carcinogenicity of BAP and two different manufactured gas plant residues (MGPRs) (Rodriguez, L. V. et al., 1997). MGPR, a complex mixture of PAHs, is generated as a by-product of coal gasification. Fifteen-day-old male mice (B6C3F1) were dosed with a single IP injection of BAP or one of two MGPRs. The mice were then sacrificed at 26, 39, and 52 weeks and examined for tumor presence. The most predominant tumors were seen in the liver. At 39 weeks after exposure, mice dosed with the MGPR samples exhibited a liver tumor incidence of between 45% and 82% depending on the amount of BAP present in the samples. Mice that were treated with 250μg BAP showed a tumor incidence of 38% with 1.9 tumors/mouse, while mice that were treated with 375μg BAP exhibited 65% tumor incidence and 1.9 tumors/mouse. These results suggest that BAP is not responsible for the observed tumorgenicity of the MGPRs. Instead, the MGPR tumorgenicity could be due to other

unidentified components of the mixture or synergistic interactions between mixture components (Rodriguez, L. V. et al., 1997). In this case, the observed tumorgenicity of the MGPR was greater than what would have been expected based on the known carcinogenic PAH components of the mixture and on the additive assumption for PAHs.

Additional research is needed to fully understand the carcinogenicity of individual PAHs, PAH mixtures, and substituted PAHs. Improving the methods for risk assessment of PAHs and PAH mixtures is dependent upon increased knowledge of the toxicity and carcinogenicity of these compounds. Methods to estimate toxicity of PAHs and PAH mixtures based on structure and known activity, is an inventive way to begin to further explain the observed toxicity.

Quantitative structure-activity relationships (QSARs) are multi-variant statistical correlations between an expressed chemical property and the key geometric or chemical characteristics of a molecular system. QSARs are constructed by analyzing known and/or calculated property information and a set of descriptors that represents the system attributes (Gombar, V. A., 1998). A QSTR, which is a QSAR developed for a toxicity metric, is a mathematical relationship between a given toxicity metric and numerical descriptors of molecular structure (Gombar, V. A., 1998). These models allow for the prediction of toxicity solely from a chemical structure. For example, Koenemann (1981) developed the following relation:

$$\text{Log } (1/LC50) = 0.871 \log P - 4.87 \qquad \text{Eqn. 1.1}$$

This equation relates the partition coefficient, P, of a chemical and its acute toxicity (LC50) in a guppy. If the only available data for a chemical was the partition

coefficient, the value of the median lethal concentration for a guppy could be calculated using this equation (Koenemann, H., 1981). QSAR models can predict toxicity using empirical relationships if (1) the predictive QSTR model is statistically robust and (2) the QSTR model is not only applicable to the query chemical but also chemicals "similar" to the query chemical (Gombar, V. A., 1998).

Building reliable QSTRs from bioassay data requires constraints in development. The bioassay data must be as uniform as possible because a QSTR can only predict what the bioassay results represent. If a researcher develops a model for mutagenicity using a variety of mutagenicity assays, a variety of species, and different exposure durations and routes, the developed QSTR would not be valid for prediction. The chemical might be mutagenic to some species but not all; likewise, the exposure time for a mutagenic response may be different for some species. It is important that the criteria of uniformity in the bioassay protocols be strictly adhered to so that the developed database from which to derive the QSTR is valid and consistent for that scenario and for similar chemicals in the same bioassay (Debnath, A., 2001).

After selecting bioassay parameters and constraints and choosing information-rich descriptors, the QSTR can now be developed. Building a robust, relevant model begins with identifying a training set of chemicals to be analyzed. The individual models are representative of the activity of similar compounds, for example, the family of PAH compounds or PCBs. Thus a model developed for amino acids would not be valid for estimating toxicity of PAHs. Next, for each of the molecules to be analyzed, their observed biological data is entered and the appropriate descriptors calculated.

There are thousands of descriptors that can be used to describe the biological activity of a compound; spatial, electronic, topological, information-content, thermodynamic, conformational, quantum mechanical, and shape descriptors are just a few (AccelrysSoftwareInc., 2005). The descriptors are analyzed for correlation and uniformity from which appropriate dependent and independent variables are identified. Several statistical methods are available for generating a QSTR equation. These include multiple linear regressions, partial least squares (PLS), simple linear regression, stepwise multiple linear regression, principal components regression (PCR), or genetic function approximations (GFA). Next, validation and analysis of the QSTR equation by applying techniques to identify outliers and leverage points are performed. Characterizing the robustness of the QSTR is accomplished using graphical analysis and cross-validation. The calculated QSTR equation can now be used to predict biological activity of compounds similar in structure to the training set of compounds.

The hypotheses that have driven this research are:

(1) The toxicity of PAHs and substituted PAHs may be estimated using bioassay based QSAR models, and;

(2) The additive assumption does not adequately estimate the true toxicity of PAHs in complex mixtures.

To test these hypotheses there were three objectives investigated. The first research objective was to test seven PAH compounds and four PAH mixtures in a set of three bioassays. Each bioassay measures a different toxic endpoint that is significant in human health risk assessment. Secondly, the data that was generated from each bioassay

was used to generate QSAR models for each bioassay. Each QSAR model can be used to estimate the specific toxic endpoints of each bioassay for PAHs, substituted versions of PAHs and mixtures of PAHs. Finally, the additive assumption for mixtures that is commonly used in risk assessment practices was examined using these models. The results from the specific models was used to determined if the additive effects from individual compounds was the same as the mixture results from the bioassays. The combination of the responses from the three bioassays was used to generate an empirical weighted equation. This equation can be used to estimate toxicity factors, such as relative potency factors or toxic equivalency factors.

CHAPTER II

MODELING TOXIC ENDPOINT FOR IMPROVING HUMAN HEALTH RISK ASSESSMENT OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) – PARENT COMPOUNDS AND SIMPLE MIXTURES

Overview

Risk assessments for mixtures of polycyclic aromatic hydrocarbons (PAHs) are problematic due to the lack of available potency and toxicity data on individual compounds and mixtures. This paper examines the toxicity of parent compounds and designed mixtures of PAHs in order to bridge the gap between component assessment and mixture assessment of this class of ubiquitous compounds. The objective for this research was to test seven parent PAH compounds and four PAH mixtures in a set of three bioassays to evaluate the toxicity of parent compound PAHs and binary mixtures of PAHs. PAHs and mixtures were examined in the *Salmonella*/Microsome Mutagenicity Assay, a Gap Junction Intercellular Communication (GJIC) assay and the 7-ethoxyresorufin-*O*-deethylase (EROD) assay. These assays were chosen for their ability to measure specific toxic endpoints related to the carcinogenic process (i.e. initiation, promotion, progression). Two compounds similar in structure, BAP and benzanthracene, consistently produced positive results in all three bioassays. While a linear PAH, anthracene, produced negative results in all three bioassays. An antagonistic response was observed for the mixtures in all three bioassays. Chemical structure was important in explaining the observed responses. Chemical structure relationships with activity and the steps of the carcinogenic process can be used to

improve estimates of toxicity for compounds and mixtures for human health risk assessments.

Introduction

Chemical exposures for people commonly occur in the presence of multiple chemicals. The effect on toxicity for one chemical in the presence of another is not well understood. Currently, more than 7 million chemicals exist and approximately 70,000 are in common use while 1000 are added each year worldwide (Government.Accountability.Office.(GAO), 1994). Research efforts have focused primarily on the toxicology of individual chemicals. As a result, risk assessment practices using available toxicity data have evaluated individual chemical exposures, ignoring the complexities of multiple chemical exposures. Although the dilemma of predicting the human health risks of exposure to chemical mixtures is not new, the challenges presented by chemical mixtures risk assessment remain unmet. This is due in part to the lack of a clear framework and methodology for mixtures assessment and because there are many individual components of mixtures that are not toxicologically characterized. Rarely do single PAHs occur in the environment; rather exposures are to multiple compounds in mixtures. There are more than 100 PAHs that can be quantified by GC/MS analysis but many more substituted PAHs, degradation products, and parent compounds that cannot yet be identified. Of those that can be identified 16 are routinely quantified by the USEPA (USEPA, 1993). How chemicals interact when an individual is exposed to a mixture is largely unknown.

To estimate the toxicity of mixtures, the USEPA developed standardized approaches to estimate risk (USEPA, 1993). The three approaches commonly used are: (i) toxicity data for the specific mixture of concern; (ii) extrapolating data from a similar mixture; and, (iii) toxicity of the individual components of a mixture (Versar, I. and Associates, B. S., 2002). Although mixture approaches for assessing risk are preferred, mixture specific toxicity data are not always available. In such cases, component-based approaches are the most common technique for assessing risk. In the case of PAHs, individual PAH toxicity data is lacking and component –based methods are necessary to perform risk assessment estimates.

One such component method used for PAHs is the relative potency factor approach, also known as the toxic equivalency factor (TEF) approach. TEFs were first developed for polychlorinated biphenyls (PCBs) as a way to rank their potency relative to 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (Safe, S. H., 1990). Similar approaches have been used to develop TEFs for PAHs. In the case of PAHs, TEFs are developed for cancer effects and therefore encompass a broad range of toxic endpoints. Some of the first researchers to develop TEFs for PAHs were Chen and Chu (1984) and Clement Associates(1988) (Nisbet, I. and LaGoy, P., 1992). These two works are limited in that they only address a small number of the PAHs that are commonly found at hazardous waste sites. Further, they are unreasonably precise. These two works were used as a basis for the commonly used set of TEFs developed by Nisbet and LaGoy (1992) which were order of magnitude estimates.

In 1993, the Office of Environmental Health Assessment (OHEA) issued a provisional guidance document for implementing TEFs for PAHs (USEPA, 1993). The state of knowledge for the implementation of TEFs for PAHs revealed the lack of information about PAH toxicity, interactions, and their promotional effects. Consequently, the TEF method for assessing risk is limited. Under EPA's guidance, the only logical alternative for PAHs is to use order of magnitude estimates called potential potencies (USEPA, 1993). Prior to potential potencies, EPA separated PAHs into carcinogens and non-carcinogens and used the cancer slope factor assigned to BAP for all carcinogenic PAHs (Nisbet, I. C. and LaGoy, P. K., 1992; USEPA, 1993).

Complex mixtures consist of tens, hundreds, or even thousands of constituents. Most often, their composition is not fully known and can change with time. Adequate testing of such mixtures is virtually impossible because the mixture is unavailable for testing, the composition of the mixture is changing, and a sufficient number of doses cannot be applied. Effort to determine if the toxicity of a mixture is different from the sum of the toxicities of the single constituents has been reported in several studies (ATSDR, 1990a; Barata, C. et al., 2005; Bostrom, C. E. et al., 2002; Reeves, W. R. et al., 2001). The toxicity of a mixture depends on the exposure level, the mechanism of action, and the receptor for each of the mixture constituents (Feron, V. J., Woutersen, R. A. et al., 1995; Groten, J. P. et al., 2001; Henschler, D., 1996). Another hurdle of mixture testing is the different types of effects that can occur at high-dose levels and low-dose levels, making low-dose extrapolation dubious (Feron, V. J., Groten, J. P. et al., 1995; Henschler, D., 1996). The two hypotheses that drive this study are: (1) The

toxicity of PAHs, mixtures of PAHs, and substituted PAHs may be estimated using bioassay based TEFs that take into account the process of carcinogenesis; and, (2) the additive assumption does not adequately estimate the true toxicity of PAHs in complex mixtures. To test these hypotheses seven parent PAH compounds and four PAH mixtures were evaluated in a set of three bioassays for their toxicity.

In evaluating PAHs with regard to human health, carcinogenicity drives the risk assessment. In recent years the TEF methodology has been used to assess the risk associated with exposure to a mixture of PAHs (Nisbet, I. C. and LaGoy, P. K., 1992; USEPA, 1993). To develop bioassay based TEFs for PAHs the multistage process of cancer that involves genotoxic and epigenetic events should be considered. In this study, bioassays were chosen for their ability to measure both initiation and promotion related effects of carcinogens. Each bioassay measures a different toxic endpoint that is significant to the process of carcinogenicity (Pitot, H. C. and Dragan, Y. P., 1996; Reeves, W. R. et al., 2001). The limitation of TEFs to accurately predict the toxicity of mixtures has been addressed by targeting initiation and promotion related effects to understand the relationship between mixture composition and toxicity.

Bioassays were chosen for their ability to measure both initiation and promotion related effects of carcinogens. Following initiation, a series of events leads to the initiated cells' promotion and the eventual progression to a rapidly growing malignant cell. A genotoxic event can be responsible for the initiation of cancer, however promotion and progression may include a variety of epigenetic events (Trosko, J. E. et al., 1998). Each compound was tested in the *Salmonella*/microsome assay, the Gap

Junction Intercellular Communication assay (GJIC) and in the ethoxyresorufin-O-deethylase assay (EROD).

The *Salmonella*/microsome assay measures mutations of DNA which are linked to initiating events (Ames, B. N. et al., 1975a; McCann, J. and Ames, B. N., 1976; McCann, J. et al., 1975; Zeiger, E., 1998). Cytochrome P450 mono-oxygenases are active in the metabolism of many xenobiotics. Alkoxyresorufin-o-deethylase substrates have been used to distinguish isoforms of P450 induced by various types of xenobiotics. Induction of the cytochrome P450 family of enzymes has been shown to be an essential step in the activation of carcinogens prior to initiation (Kennedy, S. W. and Jones, S. P., 1994; Szklarz, G. D. and Paulsen, M. D., 2002). The impairment or elimination of a cell's capability to communicate with other cells is believed to promote tumor growth by eliminating signals that instruct an initiated cell to stop dividing (Couch, D. B., 1996; Trosko, J. E. et al., 1998; Trosko, J. E. and Ruch, R. J., 1998). Measuring GJIC between cells serves as an indicator of a PAH's ability to impair this function. Using bioassay based data from specifically targeted endpoints to estimate a TEF or potential potency will allow for a toxicity estimate specific to a chemical's toxic potential in each step of carcinogenesis. TEFs or potential potencies are only estimates of toxicity, however, failing to take into account both initiation and promotion effects limit their ability to predict toxicity. Separation of initiation and promotion effects revealing the relationship between composition and toxicity of these compounds and mixtures of these compounds is a goal of this study.

The multistage carcinogenic process includes multiple mechanisms such as gene and/or chromosomal mutations (genotoxic events), altered gene expression at several (transcriptional, translational, post-translational) levels (epigenetic events), and changed cell survival (necrosis/apoptosis). A toxicant could potentially bring about one or all of these mechanisms. Using three different bioassays, each measuring a specific stage of carcinogenesis, may reveal the dominant mechanisms and lead to more appropriate toxicity estimation.

Materials and Methods

Cell Culture

The liver cell line, Clone 9 (ATCC, CRL 1439, passage 17) was used for the GJIC and EROD experiments. The Clone 9 cell line exhibits gap junctions as detected by electron microscopy and is inducible for EROD (Barhoumi et al., 2000). Cultures were used within 10 passages after being received and maintained in Ham's Nutrient Mixture F-12 containing 10% fetal bovine serum. For EROD measurements, cells were seeded at 90,000 cells/well in 2-well Lab-Tek chamber slides and incubated until they were approximately 80% confluent. For the GJIC assay, cells were seeded in 2-well Lab-Tek Chamber slides at 50,000 cells/cm^2 and incubated for 24 hours before use.

Binary Mixture Preparation

Binary mixtures of BAP with 5-methylchrysene and BAP with chrysene were prepared in DMSO. The ratio of constituents in the mixture was 1 to 1. Each mixture was tested in two separate arrangements such that each chemical was held at a constant concentration and mixed with varying concentrations of the second chemical. For

example, BAP was held at 10mM while varying concentrations (10mM to 0.5mM) of chrysene were added at a 1:1 ratio. The second arrangement of these two chemical held chrysene constant at 10mM while varying BAP concentrations (10mM to 0.5mM) were added at a 1:1 ratio. For the GJIC and EROD assays, these mixtures were diluted (DMSO final concentration less than 0.5%) in medium.

EROD

EROD assays were performed in quadruplicate with Clone 9 cells using a modified version of the 48-well microplate fluorometric assay derived from Donato et al., 1993 and Kennedy et al., 1995. Cells were seeded in 2-well Lab-Tek slides and allowed to incubate 24 hours before treatment was applied. PAHs were dissolved in DMSO (final concentration less than 0.5%) to give final concentrations of 1µM and 10µM. After an exposure period of 24 hours, 50µl of EROD (10µM) was added to the treatment well to start the reaction. The addition of 100µl of fluorescamine (150 µg/ml) stopped the reaction after a 10-minute period. Following a 5-minute period for fluorescence stabilization, the resorufin concentrations were measured using a Zeiss Stallion system (Carl Zeiss, Thornwood, NY). Excitation and emission wavelengths were set to 530/590 nm for measuring EROD. Fluorescence intensity is proportional to resorufin formed and responses were in the linear response range. Results from each treatment were compared using a one-way ANOVA and Tukey's test. Significance was set at $p < 0.05$ (Appendix A).

GJIC

GJIC was measured in the Clone 9 cells by dye coupling. The rate constant of dye transfer between cells obtained using a fluorescence recovery after photobleaching (FRAP) technique used by Barhoumi et al, 1993. Stock solutions of 2mg/ml 5-carboxyfluorescein diacetate (CFDA) in DMSO were diluted to 10µg/ml in medium. PAH solutions (final concentrations ranging from 0.5µM to 10µM) were prepared in DMSO and diluted in medium such that the concentration of DMSO never exceeded 0.1%. Cells were seeded in 2-well Lab-Tek Chamber slides at 50,000 cells/cm^2 and incubated for 24 hours. Cells were dosed with 1µl/ml of chemical treatment at five doses. Following 24 hours of incubation at 37.5^oC the medium was removed; cultures were washed 3 times with PBS. CFDA solution (10µg/ml) in serum free medium without phenol red was added (10µl) and the cells were again incubated at 37.5^oC for 15 min. Loading times below saturation were used to avoid the presence of unconjugated dye that could lead to overestimation of GJIC. After dye was loaded, cultures were washed three times in PBS and maintained in serum-free medium without phenol red. A microscopic field of groups of cells was selected for analysis. Within these groups of cells, single cells served as photobleached negative controls. Similarly, single cells or small isolated groups of nonphotobleached cells were used as positive controls. Positive controls were used to monitor background photobleaching from image scans and increases in fluorescence due to unconjugated dye. Selected cells were photobleached to a level that allowed for observance of fluorescence recovery without damaging the cells. After Cells were scanned for fluorescence recovery and with a Meridian Ultima confocal workstation (Meridian Instruments, Okemos, MI). Data was collected for at least 30

cells in two culture wells per treatment. A rate constant (k) for the fluorescence recovery was estimated by fitting the percent fluorescence intensity at a given time, F(t), to the following equation:

$$F(t) = F_{eq} (1-e^{-kt}) + F(0) \qquad \text{Eqn. 2.1}$$

F_{eq} represents the percent fluorescence recovery of the bleached cell at equilibrium and F(0) represents the percent fluorescence intensity immediately following photobleaching. The value of F_{eq} is dependant on the number of cells contacting each other and their initial level of bleaching. Therefore, data from at least 30 cells from each treatment were pooled to obtain the mean F_{eq} and k values. Extrapolation of fluorescence recovery over time was accomplished using curve fitting regression analysis (GraphPad Software). Results from each treatment were compared using a one-way ANOVA and Tukey's test. Significance was set at $p< 0.05$ (Appendix A).

*Salmonella/*microsome Assay

The *Salmonella*/microsome assay was used to test the mutagenicity of PAH compounds. The *Salmonella typhimurium* strain TA98 was provided by Dr. B. N. Ames (University of California, Berkeley). The samples were dissolved in DMSO (final concentrations ranging from 0.5mM to 30mM) and tested with and without metabolic activation using the S9 fraction of Aroclor 1254-induced Sprague-Dawley rat liver (Molecular Toxicology, Inc., Boone, NC). Each pre-poured plate containing 25 ml of VBX bottom agar, 2.5ml of top agar (0.5mM histidine, 0.5mM biotin) received 1-2 x 10^9 cells, 0.5 ml of 20% S9 (sodium phosphate buffer was used for plates without metabolic activation), and 50µl of chemical, mixture, or control. Each sample was tested on

duplicate plates in two independent experiments. The plates were then incubated for 72 hours at 37°C. The number of revertant colonies for each treatment was determined using an Artek Model 880 automatic colony counter (Dynatek Laboratories, Chantilly, VA). A response was considered positive if the average number of revertants at two or more consecutive concentrations exceeded twice the average number of revertants in the corresponding negative solvent control, and at least two of these consecutive concentrations showed an increasing number of revertants with increasing dose (Chu, K. C. et al., 1981). Media preparation and other methods followed those of Maron and Ames (1983). All bioassays included positive, negative, and solvent controls. Results from each treatment were compared using a one-way ANOVA and Tukey's test. Significance was set at $p < 0.05$ (Appendix A).

Materials

Ham's Nutrient Mixture F-12, Dulbecco's phosphate-buffered saline, serum, Dimethyl Sulfoxide (DMSO), methanol, acetonitrile, all general chemical reagents, and PAH compounds were purchased from Sigma Chemical Co. (St. Louis, MO). Fluorescamine, Resorufin, and 7-Ethoxyresorufin used in the EROD assay were also purchased from Sigma Chemical Co (St. Louis, MO). Tissue culture flasks and dishes were obtained from BD Falcon (Bedford, MA). Coverglass chambers were purchased from Nunc (Naperville, IL). 5-carboxyfluorescein diacetate (CFDA) was purchased from Molecular Probes (Eugene, OR).

Results

Parent Compounds

Salmonella/*Microsome Assay*

A positive response in the *Salmonella*/microsome assay resulted if the average number of revertants at two or more consecutive concentrations exceeded twice the average number of revertants in the corresponding negative solvent control, and at least two of these consecutive concentrations showed an increasing number of revertants with increasing dose (Chu, K. C. et al., 1981). Using this rule, naphthalene and anthracene failed to produce positive results at any of the doses tested with metabolic activation (Table 3). The DMSO control produced 36 ± 7 revertants/plate while, naphthalene, and anthracene produced a maximum of 39 ± 2, and 47± 7 revertants, respectively. Chrysene produced a weakly positive result over three doses tested (Table 3). 5-methylchrysene produced a positive result at the four doses tested greater than 0.5mM. BAP produced positive results at all five doses tested with the minimum response at 30mM producing 243 ± 10 revertants/plate. Benzanthracene also produced positive results at 30, 10, and 3mM. The maximum response was observed at 10mM, which produced 199 ± 2 revertants/plate. Chrysene and 5-methylchrysene were the only two chemicals tested at 20mM because these two compounds would not stay in solution at 30mM. All other compounds (Anthracene, Naphthalene, BAP, and Benzanthracene) tested were at a maximum concentration of 30mM.

Table 3. Total revertants in the *Salmonella*/microsome assay produced by each chemical and DMSO control with metabolic activation.

Dose	Anthracene	Naphthalene	BAP	Benzanthracene	Chrysene	5-methylchrysene
30mM	47 ± 7	39 ± 2	**243 ± 10**	**176 ± 5**	-	-
20mM	-	-	-	-	**87±2**	**101±9**
10mM	51 ± 5	48 ± 7	**303 ± 8**	**199 ± 2**	**80±3**	**112±8**
3mM	48 ± 5	41 ± 3	**322 ± 11**	**193 ± 10**	**73±7**	**84±7**
1mM	53 ± 13	37 ± 0	**303 ± 9**	55 ± 1	58±5	**74±4**
0.5mM	47 ± 4	60 ± 11	**323 ± 5**	48 ± 9	48±2	51±2
0mM	36 ± 7	36 ± 7	36 ± 7	36 ± 7	36 ± 7	36 ± 7

(-) Indicates not tested.

Bold lettering indicates positive responses.

GJIC

Intercellular communication in Clone 9 hepatic cells are reported as a rate of recovery (min^{-1}) from the photobleaching process. A significant reduction in the rate of recovery is relative to a standard and is compared to each treatment. BAP showed strong inhibition at doses of 10, 5, and 2μM, where the rate of recovery (min^{-1}) was 0.3679 ± 0.002, 0.456 ± 0.003, and 0.532 ± 0.005, respectively (Figure 3). Similarly, 5-methylchrysene showed strong inhibition at all of the doses tested. Benzanthracene also showed significant inhibition at doses of 10μM and 5μM where the rate of recovery (min^{-1}) was 0.4441 ± 0.004 and 0.563 ± 0.007, respectively. Chrysene did produce slight inhibition at the two low doses tested (1 and 0.5 μM) and at the highest dose tested (10μM). Phenanthrene and anthracene did not produce any response that was significantly different from DMSO with the exception of phenanthrene at 0.5μM where the rate of recovery (min^{-1}) was 0.543 ± 0.002. However, the 0.5 μM phenanthrene dose is the lowest of these tested.

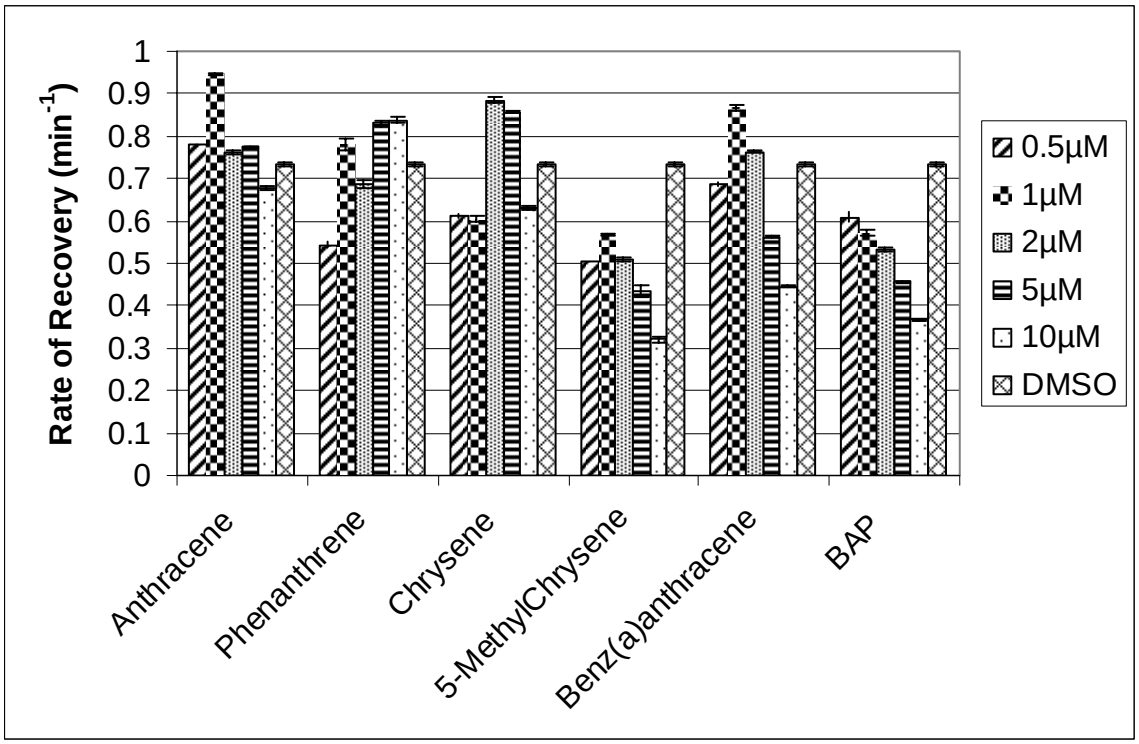


Figure 3. Inhibition of gap junction intercellular communication by parent compound PAHs in Clone 9 cells. Values shown are mean k values for at least 30 cells tested from two culture dishes per treatment.

EROD

The EROD assay measures the rate of the CYP1A-mediated deethylation of the substrate 7-ethoxyresorufin to form the product resorufin as indicated by an increase in fluorescence intensity relative to the control. All of the chemicals tested induced responses that were significant when compared to control at both doses tested (Figure 4). Anthracene and 5-methylchrysene induced significantly lower responses (353.9±3.15 and 369.2±7.2, respectively) than did the other chemicals tested at 10µM (Figure 4). Chrysene, benzanthracene, BAP, and phenanthrene all induced similar responses at

10μM (Figure 4). Phenanthrene and BAP induced slightly lower responses (417.9±6.35 and 436.5±8.93, respectively) than did chrysene and benzanthracene (468.2±4.72 and 467.4±12.48, respectively). At the lower dose tested (1μM), benzanthracene induced the greatest response (517.5±10.08) followed by phenanthrene and chrysene (477.3±2.98 and 399.8±8.47, respectively) (Figure 4). Anthracene, BAP, and 5-methylchrysene induced the three lowest responses (378.4±11.40, 350.5±6.24, and 333.1±5.99, respectively) among the chemicals tested at 1μM. Chrysene, BAP, 5-methylchrysene, and benzanthracene were consistently among the highest inducers at both concentrations tested while anthracene was the lowest inducer tested at both concentrations. BAP induced a higher response at the higher concentration tested than at the lower concentration. One of the useful aspects of CYP1A induction for biomonitoring purposes is the enzyme's tendency to increase in concentration upon chemical exposure. This is observed here with increased expression at the higher concentration of BAP, chrysene, and 5-methylchrysene.

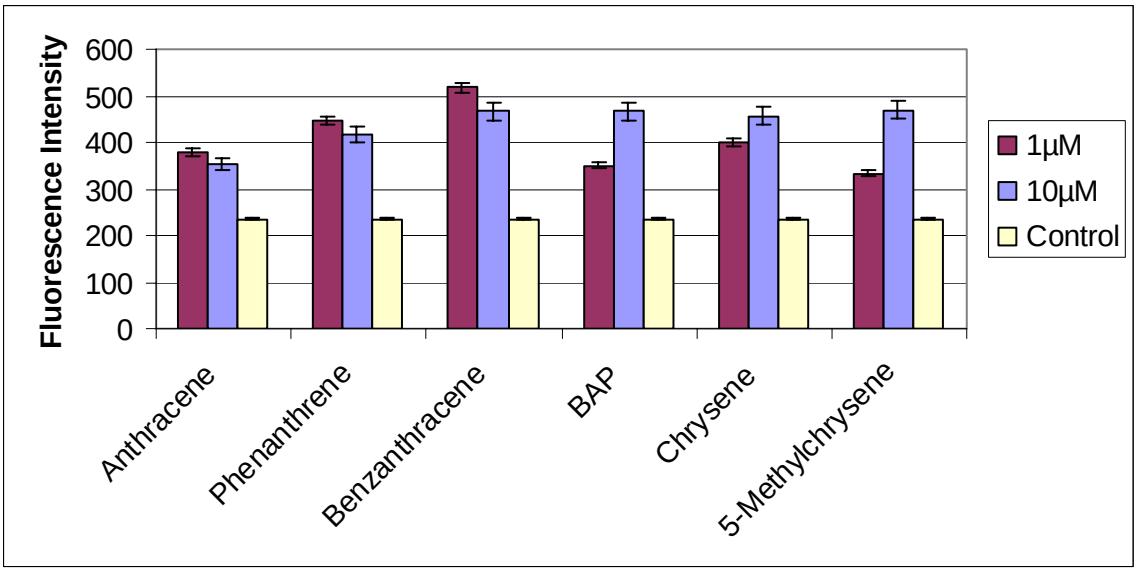


Figure 4. EROD activity for parent compound PAHs in Clone 9 cells. Fluorescence intensity was measured in two culture dishes per treatment with cells at 90% confluency. Values represent the mean response for each treatment.

PAH Mixtures

Salmonella/*Microsome Assay*

Four mixture combinations of BAP, chrysene and 5-methylchrysene were tested in this bioassay (Table 4, Figure 5). Using the 2-fold rule (Chu, K. C. et al., 1981), both arrangements of the BAP and 5-methylchrysene mixtures produced positive results at the three doses tested when compared to the control (Table 4, Figure 5). BAP alone at 30mM produced 292±17 revertants, yet when 5-methylchrysene was added at varying concentrations the response was less than that for BAP alone resulted. The addition of 5-methylchrysene reduced the effective toxicity of BAP in this assay. Less than additive

response in the mixture resulted as compared to the individually tested compounds which was statistically significant in Tukey's Test at the $p<0.05$ level.

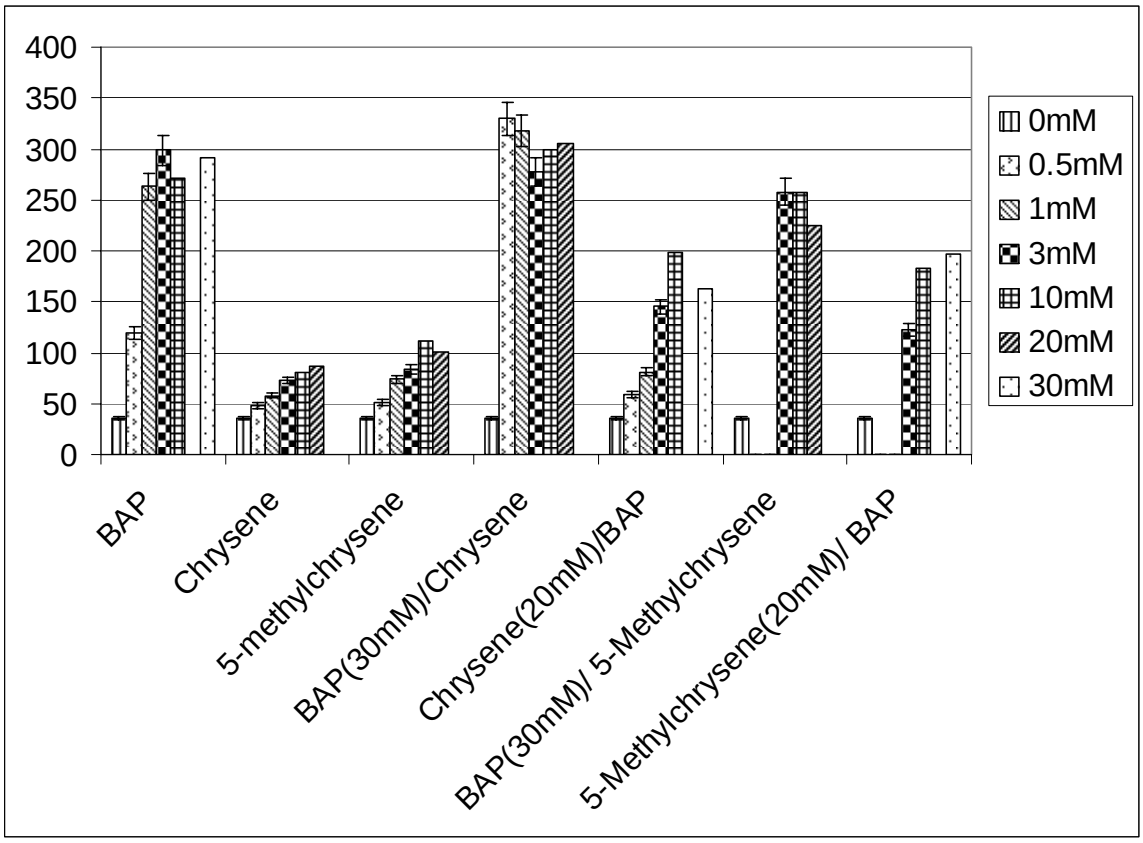


Figure 5. *Salmonella*/microsome assay results for parent compound PAHs and PAH mixtures.

Similarly, when a constant concentration of 5-methylchrysene was amended with varying BAP concentrations, additivity was not observed (Table 4, Figure 5). As a single compound, 5-methylchrysene was mutagenic (101±9 revertants) at 20mM.

Increasing the concentration of BAP in the presence of 20mM 5-methylchrysene did increase the mutagenicity but not in an additive manner. There was approximately a 50% decrease in the expected additive response (393 revertants) and the observed response (197 revertants).

Two mixture arrangements for BAP and Chrysene were also compared and both resulted in positive responses over three consecutive doses (305±13, 299±17, and 278±11, respectively) but the responses were not additive. When varying amounts of Chrysene were added to BAP (30mM), there was a positive response (Table 4, Figure 5) that was not statistically significant from that of BAP alone. The mixture response was significantly lower than that of the expected additive response (Table 4, Figure 5) showing that the additive assumption over-estimated the response in this case.

Varying amounts of BAP added to Chrysene (20mM) show a 50-70% decrease in the expected additive response. While chrysene (20mM) alone resulted in 87±2 revertants, the addition of BAP resulted in an approximate doubling of revertants at the three highest doses test (30mM, 10mM and 3mM, respectively). The results of the mixture response were less than additive. There was an antagonistic effect observed in the mixture when compared to the response of BAP alone, indicating an interaction between Chrysene and BAP in this mixture that was statistically different at $p<0.05$. The additive assumption in this case over-estimated the response by more than double the number of revertants in some cases. At lower doses of BAP (1mM and 0.5mM), the response was similar to that of Chrysene alone. This could indicate that the toxicity expected due to BAP was inhibited by an interaction with Chrysene at these lower doses.

Table 4. Total revertants in the *Salmonella*/microsome assay produced by each mixture and DMSO control with metabolic activation.

Dose[1]	BAP	Chrysene[2]	5-methylchrysene[2]	BAP(30mM) /Chrysene	Chrysene(20mM) /BAP	BAP(30mM)/ 5-Methylchrysene	5-Methylchrysene(20mM)/ BAP
30mM	292±17	-	-	-	**163±17**	-	**197±15**
20mM	-	**87±2**	**101±9**	**305±13**	-	**225±31**	-
10mM	272±10	**80±3**	**112±8**	**299±17**	**198±19**	**257±31**	**183±11**
3mM	299±10	**73±7**	**84±7**	**278±11**	**145±10**	**258±17**	**123±3**
1mM	263±6	58±5	**74±4**	**318±6**	**81±10**	-	-
0.5mM	119±11	48±2	51±2	**330±3**	59±6	-	-
0mM	36 ± 7	36 ± 7	36 ± 7	36 ± 7	36 ± 7	36 ± 7	36 ± 7

[1]Each mixture was tested in two separate arrangements with one chemical being held at a constant concentration and the other chemical at varying concentrations. The dose in this column refers to the amount of the second chemical added to the mixture.

All mixtures were prepared in a 1to1 ratio. (-) Indicates not tested.

[2] Data from individual compound testing as seen in Table1.

Bold lettering indicates positive responses.

Chrysene and 5-methylchrysene were the only two chemicals tested at 20mM because these two compounds would not stay in solution at 30mM. BAP was tested at a highest concentration of 30mM.

GJIC

The response of each mixture arrangement was compared to the control response of 0.822±0.008 min^{-1}. BAP (10μM) mixed with varying concentrations of chrysene showed the most significant inhibition of cellular communication (Figure 6). Increasing concentrations of Chrysene were added to BAP (10μM), resulted in a statistically significant decrease in cellular communication. Generally, the higher the chrysene concentration the slower the rate of recovery was. Only at 0.5μM chrysene was the response similar to the control (Figure 6). The largest decrease in communication was observed for BAP (10μM) mixed with chrysene at 5μM. The rate of recovery for this combination was 0.037±0.005 min^{-1} compared to 0.822±0.008 min^{-1} for the control. When BAP was analyzed alone at 10μM (Figure 6) the rate of recovery was 0.469±0.011 min^{-1}, therefore the reduced communication cannot be attributed to BAP alone.

In comparison, chrysene at 10μM with varying concentrations of BAP responses did not show significant decreases in communication between 10, 5, and 2μM of BAP added (Figure 6). The lowest rate of recovery was 0.607±0.011 min^{-1} and was observed when BAP at 10μM and 5μM was added to chrysene (10μM). This rate was significantly different from the control rate (0.822±0.0083 min^{-1}) at the $p<0.05$ level. Similarly, the rate was significantly different from chrysene tested alone at 10μM that

produced a 0.568±0.006 min^{-1} rate of recovery. These responses indicate that some inhibition between the chemicals in this mixture could have prevented them from fully affecting the cellular communication. When this combination is compared to the previous arrangement, chrysene appears to be the inhibiting compound and not BAP. When chrysene was added in varying concentrations, communication was significantly inhibited except at a concentration of 5µM chrysene. However, when chrysene was held at a constant concentration and varying concentrations of BAP where added, the same inhibition was not observed.

Two arrangements of BAP and 5-methylchrysene mixtures were also tested. When 5-methylchrysene was added in varying concentrations to BAP (10µM), there were significant differences in the rates of recovery at two of the concentrations tested (Figure 6). The lowest rates of recoveries (0.527±0.002 min^{-1} and 0.542±0.005 min^{-1}, respectively) for this arrangement were observed for the addition of 0.5µM and 10µM 5-methylchrysene were added to 10µM BAP. These rates of recovery were lower than that observed for BAP (10µM) (0.603±0.011 min^{-1}, respectively) tested alone. These results suggest that when 5-methylchrysene was mixed with BAP (10µM), a chemical interaction alone was not likely to be causing the observed result, but a physiochemical (i.e. transport, solubility) phenomena could explain the observed results.

When varying concentrations of BAP were added to 10µM of 5-methylchrysene, the rates of recovery were significantly repressed (Figure 6). The lowest rate of communication occurred when BAP at 10µM was mixed with 5-methylchrysene (10µM) (0.421±0.003 min^{-1}).

EROD

EROD activity describes the rate of the CYP1A mediated deethylation of the substrate 7-ethoxyresorufin to form the product resorufin which can be measured fluorometrically. The induction of these enzymes is a vital step in the activation of carcinogens prior to initiation and increase in the fluorescence intensity is directly related to increased toxicity. As in previous experiments with these mixtures, there were two arrangements of each mixture tested. Typically, the additive assumption for mixture responses was not observed in any of the mixtures tested using the EROD assay.

For the mixture of BAP and chrysene at 10μM each, the response (420.3±9.16) was similar to the response observed for each of the chemicals alone (Figure 7). Neither there was an additive effect nor did the chemical mixture show any increase in induction when compared to the experiments with the single chemicals. Similarly, this was observed in the mixture containing BAP and 5-methylchrysene at 10μM. There was not an additive effect observed with these two chemicals. The result (477.0±7.08) was not significantly different from the result produced by BAP alone.

In the case of BAP held at a constant concentration, (10μM) and 5-methylchrysene added at 1μM, the result was similar to that observed for 5-methychrysene tested alone at 1μM (Figure 7). There was not an additive effect observed with this mixture; the result being similar to that of 5-methylchrysene alone might indicate an inhibition of the activity of BAP in the presence of 5-methylchrysene. The mixture arrangement composed of 5-methylchrysene held at a constant

concentration (10μM) and BAP added at 1μM showed the opposite result. The result for the mixture was similar to the result for BAP tested alone at 1μM.

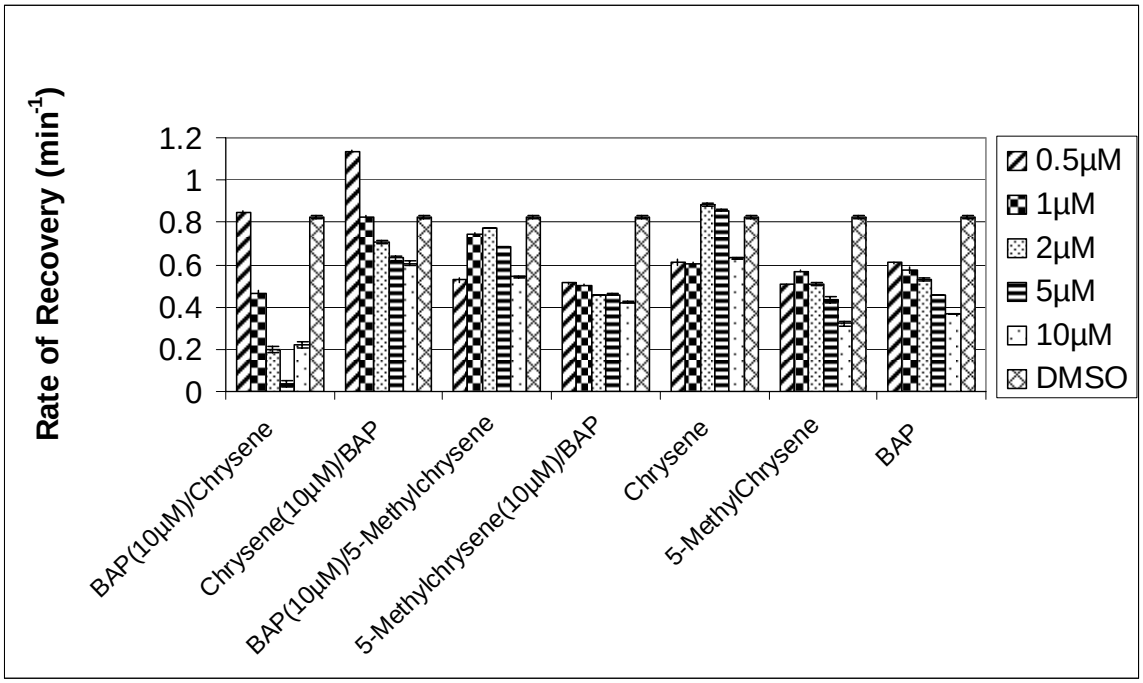


Figure 6. In Inhibition of gap junction intercellular communication by PAH mixtures in Clone 9 cells. Values shown are mean k values for at least 30 cells tested from two culture dishes per treatment.

The mixture of chrysene held at a constant concentration (10μM) and BAP added at 1μM produced similar results as observed in the previous mixture of chrysene and BAP at 10μM each. The result (419.8±14.2) was similar to that observed for chrysene alone at 10μM. BAP did not produce an observable effect on chrysene in this mixture. The mixture arrangement of BAP held at a constant concentration (10μM) combined with chrysene at 1μM showed significant inhibition when compared to the chemicals tested

alone. Enzyme induction in this case (309.2±4.8) was significantly lower than either chemical tested alone and lower than the assumed additive effect (Figure 7).

Discussion

To predict toxicity of carcinogens for assessing human health risk it is important to take into account each stage of carcinogenesis and the effect that two or more chemicals in mixture have on the process. The *Salmonella*/microsome assay is a simple, quick, and inexpensive genotoxicity assay which is one of several required for product safety testing of a variety of materials including drugs, medical devices, food additives, industrial chemicals, and pesticides. The *Salmonella*/microsome assay measures mutations of DNA, important initiating events of the carcinogenic process (Ames, B. N., McCann, J., and Yamasaki, E., 1975a; McCann, J. and Ames, B. N., 1976; McCann, J. et al., 1975; Zeiger, E., 1998). There is considerable evidence, using this test and with few exceptions, that carcinogens are mutagens (Ames, B. N., McCann, J., and Yamasaki, E., 1975a; Ames, B. N. et al., 1975b; McCann, J. and Ames, B. N., 1976; Miller, E. and Miller, J., 1971). However, some disadvantages of this test were encountered.

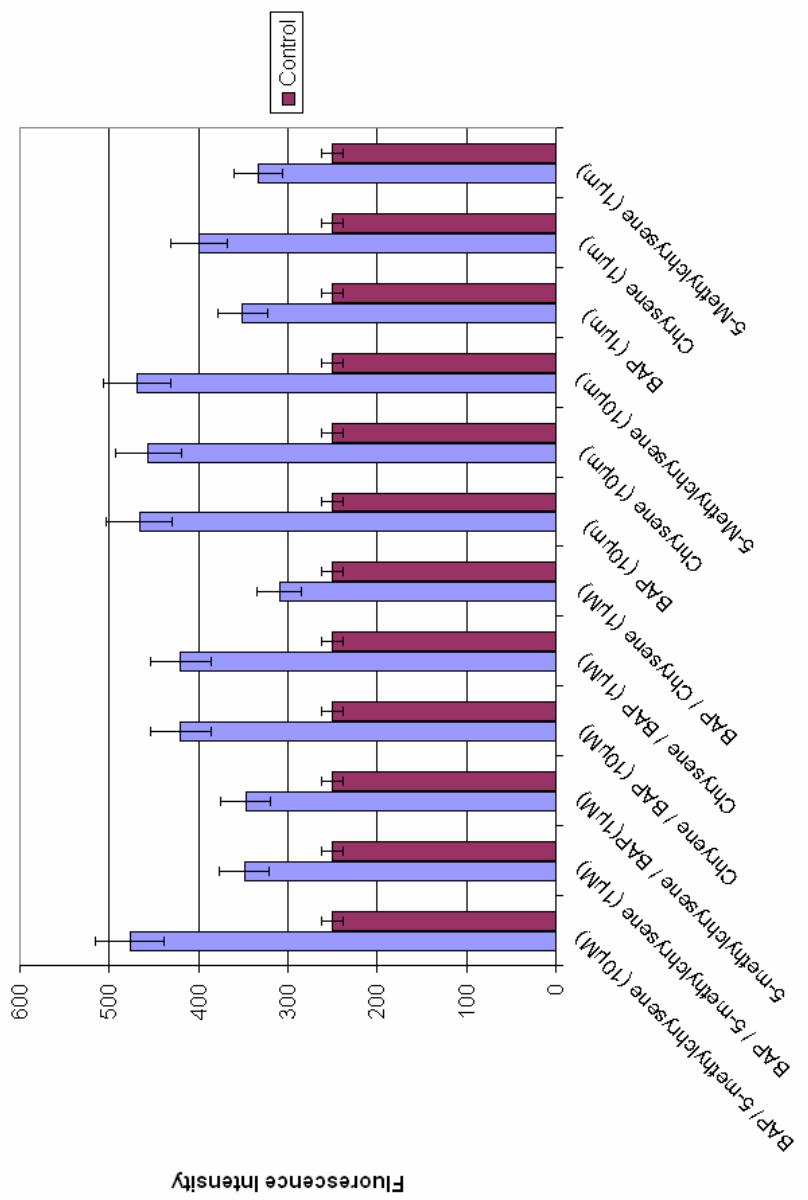


Figure 7. EROD activity for PAH mixtures in Clone 9 cells. Fluorescence intensity was measured in two culture dishes per treatment with cells at 90% confluency. Values represent the mean response for each treatment.

The chemical concentrations needed to elicit a response in this strain of bacteria are not environmentally relevant. In using such high concentrations, solubility limits are exceeded for some of the PAHs tested however the presence of DMSO kept the PAH in solution. For example, chrysene, is known to be a weak carcinogen, was dosed at its solubility limit of 20mM in DMSO and elicited a weak positive response. Another concern with this assay is that the toxicity of some chemicals can mask genotoxic potential. If a compound is toxic to the bacteria, then the bacteria is killed resulting in a false negative result. The results observed in this assay followed the hypothesized response of the chemicals containing a bay region being mutagenic and those linear counterparts being non-mutagenic (Ames, B. N., McCann, J., and Yamasaki, E., 1975a; McCann, J. and Ames, B. N., 1976; McCann, J. et al., 1975). Although chrysene has two bay regions, it is considered a weak carcinogen (McCann, J. et al., 1975; Donnelly, K. C. et al., 2004). Due to the high concentration of chrysene used in this experiment, it is possible that the toxicity masked some of the genotoxic potential in this set of experiments.

Evaluation of the mixture responses in the *Salmonella*/microsome assay yielded slightly antagonistic responses. The mixture of chrysene held constant and BAP added in varying concentrations produced the most antagonistic effects among the mixtures of BAP and chrysene. The expected additive response was as much as 30% higher than the observed response. However, at the two highest doses of BAP and chrysene together, in two different assays, the response was 61% lower than the expected additive response. A similar response was seen when 5-methylchrysene was held constant and BAP was

added in varying concentrations. Antagonistic effects were greater in the mixture of 5-methylchrysene held constant and BAP added. However, the response at the highest doses of the two chemicals did not show significant difference between the two experiments (approximately 13% difference).

A greater antagonistic response in this assay would indicate that the interaction of these two chemicals in mixture reduces their initiation potency. It has been documented that chrysene and BAP both bind with DNA at the N2 position. Competition for binding sites is an explanation for the observed results and was also observed by Warshawsky and Landolph (2006). While the observed effects could indicate mixture interaction effects, the mechanism of this antagonistic behavior is most likely competition for binding sites on the DNA. The *Salmonella*/microsome assay has been historically used to screen carcinogens as mutagens. However, using the results from this assay alone to predict behavior of PAHs in mixture would not be accurate due to the limits of sensitivity in this assay.

The GJIC experiments for the parent compounds with bay-regions were shown to cause inhibition. Many investigators have shown that PAHs containing a bay or "bay-like" region inhibit gap junction communication to a greater extent than do their linear counterparts (Rummel, A. et al., 1999; Weis, L. M. et al., 1998; Blaha, L. et al., 2002; Upham, B. et al., 1996). Bay-like regions, as referred to in this study, represent the angular pocket formed at the top of the benzene ring by a methyl group. The regions are structurally similar to the sterically hindered bay regions that are formed by angular benzene rings. In this study, BAP and 5-methylchrysene were both strong inhibitors of

GJIC and both possess bay or "bay-like" regions. Similar responses, at a lower level of inhibition, were seen for benzanthracene and chrysene. In contrast, chrysene, with two bay regions, produced rates of recoveries higher than expected. This could be due in part to the steric hindrances of the two bay regions of this molecule (Singer, B. and Grunberger, D., 1983). The two bay regions being in such close proximity to each other cause the molecular shape to change due to the strain of the atoms close together which results in the inhibition observed.

The mixture with BAP held constant and at varying concentrations of chrysene resulted in the most inhibited cellular communication. When chrysene at 10, 5, and 2μM was added to BAP (10μM) the communication results indicated a synergistic effect where the presence of both chemicals reduced the toxicity of each. Conversely, the opposite combination of the same chemicals did not produce similar results. Other investigators have shown that structural dependences such as higher molecular mass and higher lipophility (Kow) affect GJIC inhibition (Blaha, L. et al., 2002). Both BAP and chrysene are similar in structure and are known to promote tumors. An apparent effect is related to which compound is held at a constant concentration. Inhibition potency could be contributed to one or many physiochemical characteristics of the individual PAHs in the mixture. Another contributing factor could be the influence of total mixture concentration. The higher the total concentration of the mixture the greater effect on communication was observed (Figure 6).

The mixtures of BAP and 5-methylchrysene were inhibitory to GJIC between cells when 5-methylchrysene was constant at 10μM and BAP was added. There were

not significant differences between concentrations of BAP added, nor was there a dose related response. An additive response was not observed in either arrangement of the mixture. Alone, 5-methylchrysene inhibited communication (0.3212 ± 0.002 min^{-1}) to a greater extent than when mixed with varying concentrations of BAP. An additive assumption to estimate the toxicity of this mixture would have resulted in a large inhibition of communication considering both compounds have bay regions. The observed results suggest that BAP and 5-methylchrysene have an effect on one another that disables or reduces an additive effect on cellular communication. Weiss et al, (1998) found that PAHs with bay regions inhibited cellular communication to a greater extent than did their linear counterparts (Weis, L. M. et al., 1998). In the case of 5-methylchrysene, a methylation of the parent compound, chrysene, creates the "bay-like region." It has been reported in other studies (Upham, B. et al., 1996; Weis, L. M. et al., 1998; Upham, B. L. et al., 1994) that methylated PAHs inhibit gap junction communication more than the unmethylated versions. Considering these previous studies and the results of this study, 5-methylchrysene induces only a slightly additive inhibitory effect on communication when mixed with BAP.

Both 5-methylchrysene and BAP have been shown to be potent inhibitors of GJIC (Blaha, L. et al., 2002; Krutovskikh, V. et al., 1994; Trosko, J. E. et al., 1998). The inhibition observed in this study cannot be attributed solely to BAP or 5-methylchrysene activity individually. Rather, the observed response is most likely contributed to interactions between the two chemicals or a combination of interactions and physiochemical properties of the two chemicals. If 5-methychrysene, with its "bay-

like" region, acted differently than other PAHs containing a bay or "bay-like" regions then a purely additive response would not be expected.

The bay region of 5-methylchrysene is crowded by the methyl substitution and therefore can act as a non-planar compound. The electrostatic and steric interactions between the protons across the crowded bay region can destabilize the planar structure resulting in a helical shaped molecule (Rabinowitz, J. et al., 2002). These interactions between the proton across the bay region and the epoxide oxygen may decrease the reactivity of the diol-epoxide (Rabinowitz, J., Little, S., and Brown, K., 2002). The energy needed to make 5-methylchrysene become planar is relatively small compared to other compounds but small perturbations such as the less ordered aqueous environments can cause 5-methylchrysene to act as if it were non-planar (Rabinowitz, J., Little, S., and Brown, K., 2002). For these reasons, 5-methylchrysene cannot be expected to always act as a planar PAH in *in vitro* biological studies and less toxicity will be observed as was the case in this study.

Several investigators have proposed the mechanism by which GJIC is disrupted. Due to the rapid occurrence of GJIC inhibition many believe that the mechanism of action is at the posttranslational level, specifically a modification of the gap junction proteins (Yamasaki, H., 1995; Rummel, A. et al., 1999; Upham, B. et al., 1996; Blaha, L. et al., 2002). In this study, rapid occurrence of GJIC suppression was also observed suggesting that this mechanism is primarily at the posttranslational level as well.

EROD activity for these compounds and mixtures was not conclusive. The induction of EROD was concentration dependent for BAP, chrysene, and 5-

methylchrysene. However, the same result was not seen for anthracene, phenanthrene, and benzanthracene. Anthracene and phenanthrene induced similar amounts of EROD at both concentrations tested. Both of these compounds are similar in structure and induced relatively similar amounts of EROD. Anthracene has been shown to be a weak inducer of EROD in other studies (Falahatpisheh, M. et al., 2004). It is known that induction of CYP1A is mediated through the binding of xenobiotics to a cytosolic aryl hydrocarbon receptor (AhR) (Safe, S., 2001). Anthracene is not a strong AhR ligand and did not produce responses at either concentration that were significant when compared to control. Although, phenanthrene is also not considered a strong AhR ligand, at the lower concentration of 1μM, one of the highest responses among the chemicals tested was observed.

There is an apparent similarity in protein inducability among structurally related PAHs. The behavior of PAHs in the EROD assay seems to be influenced by the reactivity of the oxidative intermediates that are generated during the course of metabolism (Basu, N. et al., 2001; Safe, S., 2001; Falahatpisheh, M. et al., 2004). Therefore, each compound's metabolic pathway plays an important role in the induction of EROD.

The mixture combinations of BAP and 5-methylchrysene, while showing there was not an additive effect on induction, did reveal increased induction over either of the chemicals tested alone. This could be due in part to a saturation effect of the chemicals in the mixture. There were similar responses with both arrangements of this mixture; increasing the concentration of either chemical did not affect the overall response.

Chrysene and BAP in a mixture at the same concentration showed the same effect as chrysene mixed with a smaller concentration of BAP. Again, although the response was not additive, the slight inhibitory effects on the induction of enzymes cannot be attributed solely to one specific chemical. Induction of the detoxifying enzyme, EROD, encoded by both CYP1A1 and CYP1B1, was inducible by all the chemicals and mixtures tested and should be proportional to the exposure concentration. Other investigators have shown chrysene to inhibit the inducibility by BAP in mixtures and to yield irregular responses in this assay (Falahatpisheh, M. et al., 2004). This could be due in part to chrysene or 5-methychrysene inhibiting the transformation of BAP to metabolites that are more toxic or the competition of the chemicals for metabolizing enzymes (Petrulis, J. R. et al., 2001; Brunstrom, B. et al., 1991; Falahatpisheh, M. et al., 2004). These interactions would cause antagonistic interactions that shield against additional chemical toxicity.

The helical structure of 5-methylchrysene supports a theory of antagonistic interaction. The steric effect at the site of methyl substitution may alter the radioselectivity of oxidation by the metabolizing enzymes. The radioselectivity would direct the metabolism at other regions of the molecule resulting in the production of metabolites that would inhibit the transformation of BAP to its ultimate carcinogen and thus producing more carcinogenic 5-methylchrysene metabolites (Rabinowitz, J., Little, S., and Brown, K., 2002; Schoeny, R., Muller, P., and Mumford, J., 1998; Shappell, N. W. et al., 2003). Shappell, et al (2003) in a study of the metabolites of both chrysene and 5-methylchrysene in trout and rat liver microsomes confirmed this result. They

concluded that metabolizing enzymes of trout and liver microsomes were more efficient in attacking the bay region double bond in chrysene and the non-bay region double bond of 5-methylchrysene resulting in different metabolites. The radioselective differences of these two compounds may result in the ease of enzymatic activation to the ultimate carcinogenic bay region diol-epoxides thus inhibiting the transformation of BAP by the detoxifying enzyme, EROD, to its ultimate carcinogenic metabolite.

CHAPTER III

USING QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS (QSARs) TO PREDICT TOXIC ENDPOINTS FOR POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)

Overview

Quantitative Structure-Activity Relationships (QSARs) offer a reliable, cost-effective alternative to the time, money, and animal lives necessary to determine chemical toxicity by traditional methods. Additionally, humans are exposed to tens of thousands of chemicals in their lifetime necessitating a need to predict chemical toxicity and screen for those posing the greatest risk to human health. This study developed models to predict toxic endpoints for three bioassays specific to the stages of carcinogenesis. The ethoxyresorufin- *O*-deethylase assay (EROD), *Salmonella*/ microsome assay and a gap junction intercellular communication (GJIC) assay were chosen for there ability to measure toxic endpoints specific to activation, induction, and promotion related effects of PAHs. Shape-Electronic, Spatial, Information Content, and Topological descriptors proved to be important descriptors in predicting the toxicity of PAHs in these bioassays. Predicting toxicity for a specific PAH compound, such as a bioassay-based potential potency (PP_B) or a bioassay-based toxic equivalency factor (TEF_B), is possible by combining the predicted behavior from the Quantitative Structure Toxicity Relationship (QSTR) models. These toxicity estimates can then be incorporated into a risk assessment for compounds that lack toxicity data. It is believed

that accurate toxicity predictions can be made by examining each type of endpoint important to the process of carcinogenicity and a clearer understanding between composition and toxicity can be obtained.

Introduction

It is estimated that there is little to no toxicity data available for more than 75% of chemicals in use today (Gombar, V. A., 1998). Experimental testing of new and existing chemicals require approximatly 730 days and $2,000,000 US to conduct carcinogenicity testing for one chemical (Gombar, V. A., 1998). It is imperative in these times of shrinking resources for experimental research, that an efficient, timely, and less costly method be used to test chemicals for environmental and human health risks.

Using quantitative structure-activity relationships (QSARs) to estimate toxicity of chemicals is a practical an efficient approach, however limitations of use must be recognized. QSARs developed for a specific toxicity metric are also referred to as quantitative structure-toxicity relationships (QSTRs), and serve to predict toxicity of a chemical based on that chemical's structure. QSARs can also reveal mechanisms of interaction. One such example from Koenemann (1981) of a QSTR is the relationship between the partitioning coefficient, P, of a chemical and its acute toxicity to a guppy (Equation 3.1). If only given a chemicals' partitioning coefficient, P, can be approximated the value of the mean lethal concentration (LC_{50}) to a guppy.

$$\log (1/LC50) = 0.871(\log P) - 4.87 \qquad \text{Eqn.3.1}$$

There have been QSTRs developed for some toxic endpoints of PAHs (Braga, R. S. et al., 2000; El-Alawi, Y. S. et al., 2002; Govers, H. et al., 1984; Lewis, D. F. V. and Parke, D. V., 1995) and models of physiochemical properties for PAHs (Abraham, M. H. et al., 2005a). The predictive equations are applicable for a family of compounds and may not be appropriate for a different family.

The driving hypothesis of this study was that PAH toxicity can be predicted using QSTRs and can further improve toxicity assessments in human health risk assessment. The objective of this study was to develop QSTRs describing the toxic endpoint of PAHs in three individual bioassays. Each bioassay was chosen based on the importance of the measured endpoint in the process of carcinogenicity. Carcinogenicity is one of the driving force behind human health risk assessment of chemicals such as PAHs. Due to the lack of toxicity information for several parent compound PAHs, methylated versions of the parent compounds, and mixtures of PAHs, QSTRs provide a means to estimate toxicity of these compounds and further reduce the uncertainty otherwise introduced into the risk assessment process. These methods should be used with caution within a specific family of compounds. Each QSTR is specific to the characteristics of the family of compounds to which it was trained. If PAHs are chlorinated and the models were not trained with chlorinated PAHs, then the models may not reliably predict the behavior of chlorinated hydrocarbons.

The three bioassays chosen to generate data to train a QSTR model were the *Salmonella*/microsome assay, a gap junction intercellular communication (GJIC) assay, and the ethoxyresorufin-*O*-deethylase assay (EROD). Results from the three bioassays

were interpreted with the QSTR models for applications in human health risk assessments. The *Salmonella*/microsome assay measures mutations of DNA which are known to be important initiating events (Ames, B. N., McCann, J., and Yamasaki, E., 1975a; McCann, J. and Ames, B. N., 1976; McCann, J. et al., 1975; Zeiger, E., 1998). Cytochrome P450 mono-oxygenases play a major role in the metabolism of many xenobiotics. Alkoxyresorufin-o-deethylase substrates have been used to distinguish isoforms of P450 induced by various types of xenobiotics. Induction of these enzymes of the cytochrome P450 family has been shown to be an essential step in the activation of carcinogens prior to initiation (Kennedy, S. W. and Jones, S. P., 1994; Szklarz, G. D. and Paulsen, M. D., 2002). The impairment or elimination of a cell's capability to communicate with other cells is believed to promote tumor growth by eliminating signals that instruct an initiated cell to stop dividing (Couch, D. B., 1996; Trosko, J. E. et al., 1998; Trosko, J. E. and Ruch, R. J., 1998). Measuring gap junction communication between cells serves as an indicator of a PAH's ability to impair this function. Using the data generated by these three bioassays, three major steps in the process of carcinogenicity are predicted and the combination of these responses for a given chemical can be used to estimate a bioassay based toxic equivalency factor (TEF_B) or potential potency factor (PP_B).

To assess the appropriateness of QSTRs for expressing toxicity as a linear relationship consider the initiation of cancer (Gombar, V. A., 1998). For example, a necessary condition for the initiation of cancer is the formation of covalent conjugates with the macromolecule at the site of action. This process leading to carcinogenesis can

be represented by equation 3.2 where, C is the concentration of a chemical reacting with Z, the concentration of the macromolecule that generates C:Z, the concentration of the covalent conjugate.

$$C + Z \longrightarrow (C:Z) = P \qquad \text{Eqn. 3.2}$$

The rate of the process of initiation could be rewritten as,

$$d(p) / dt = k_3(C:Z) \qquad \text{Eqn. 3.3}$$

where k_3 is the first order rate coefficient. The resulting steady state equation will be,

$$d(p) / dt = k_3 (C) * (Z) \exp(-\Delta G/RT) \qquad \text{Eqn. 3.4}$$

where ΔG is the free energy associated with the formation of the C:Z conjugate. Integration of this equation over a given time period will result in,

$$P = k_3 (C) * (Z) \exp (\Delta G/RT) * t \qquad \text{Eqn. 3.5}$$

It is not feasible to measure C and Z at the reaction site, however if a set of *n* chemicals are assumed to react with the same receptor, the R term could be considered constant. For a fixed value of P/t (i.e. a predefined response such as tumor formation over 5 years of exposure), equation 3.5 can be transformed into equation 6.

$$K = (C_i) \exp (-\Delta G_i / RT) \quad \text{for } i = 1, 2, \ldots, n \qquad \text{Eqn. 3.6}$$

Using a simple log transformation and considering that the concentration C is proportional to some exposure concentration, C', equation 3.6 can be written as,

$$\log (1/ (C'_i)) = \log A'_i - \Delta G_i / RT + K'' \quad \text{for } i = 1,2,\ldots,n \qquad \text{Eqn. 3.7}$$

where the left-hand side of the equation is the toxicity metric, A' is a factor which relates C and C', ΔG is the free energy change associated with the formation of the conjugate and K'' is the transformations on the constants. The terms A' and ΔG are both functions of a chemical's molecular structure. The linear free energy relationship (Equation 3.7) enables the expression of toxicity as a function of descriptors of the molecular structure (Purcell, W. P. et al., 1973). By examining essential steps in the carcinogenic process (i.e. activation, initiation, and promotion), the relationship between chemical composition and toxicity can be obtained for a set of chemicals and applied to predict the toxicity of related chemicals.

Methods and Materials

The datasets presented in Chapter II for PAHs in the *Salmonella/*microsome assay, the gap junction intercellular communication (GJIC) assay, and the ethoxyresorufin-*O*-deethylase (EROD) assay were used as input data to develop QSARs. Biological data is normally skewed, as was the case with these datasets, and requires some form of transformation to normalize the data. In this case, a logarithmic transformation was used to normalize the data.

Cerius^2 4.10 molecular simulation package (Accelrys Inc., San Diego, CA) was used for QSAR development. Each of the PAH molecules was imported into Cerius^2 as a structure SD file for use in the training set. The energy of each molecule was minimized using a CFF91 open forcefield (Maple, J. R. et al., 1994). The training set of

molecules and its corresponding bioassay data was used to create a study table. Descriptors were added to the study table using Descriptor+, a module of the Cerius^2 package. Typically, the descriptors were calculated using MOPAC7 due to the accuracy of this method as compared to other methods in Cerius^2. The final training set contained descriptors from several categories (i.e. conformational, electronic. spatial, among others.). To develop QSARs for each of the bioassays, the corresponding parameter (i.e. revertants, rate of recovery, or fluorescence intensity) was set as the dependent variable and the descriptors as the independent variables.

QSARs were created using a genetic function approximation (GFA) algorithm built in the QSAR+ module of Cerius^2 (Rogers, D. and Hopfinger, A. J., 1994). The GFA algorithm is preferred over other traditional regression methods for two reasons. The GFA algorithm generates better quality equations and it provides, through study of the evolving models, additional information not available from standard regression analysis. The algorithm generated 100 parent equations that include randomly selected descriptors. Regressions were performed on the generated equations and equations were ordered according to their lack-of-fit (LOF) score (Friedman, J. H., 1991). LOF scores are a measure of the statistical fit and an indicator of overfitting. Crossover operations (repeated 20,000 times) used random pairs of parent equations and randomly mixed descriptors to produce offspring equations. The offspring equation substituted the equation with the highest LOF if its LOF score was in the top 100, otherwise it was discarded. The crossover procedures and recombination of equations resulted in an

improved final population of equations that were ordered according to the individual LOF scores.

Statistical analysis was performed on the final 100 equations generated by the GFA for each bioassay. The relevant statistical parameters calculated were the correlation coefficient, R, the coefficient of determination, R^2, and the adjusted value, R^2-adj, the lack-of-fit-score, LOF, and the least squares error, LSE. The significance of the regression equations was tested using the F-test. If the F-value is greater than a standard tabulated value, the equation is considered significant. The significance level for the F-test was set at 0.05.

Validation testing was used to evaluate the generated QSARs for their uncertainties and predictive power using a bootstrap validation test and a randomization test. The bootstrap test calculates the coefficient of determination ($R^2_{bootstrap}$) and the uncertainty associated with it. Repetitive analysis of random samples of the dataset with resampling was used. Fisher's randomization method was used to test the assumption that adequate random regressions exist for a parameter thus testing the validity of the model and the data that was used to generate it. Each bioassay-specific value predicted by the QSAR was randomly reassigned 19 times (0.05 significance level) and statistical parameters were recalculated each time. These parameters were then compared to the non-random QSARs for each bioassay. There are four additional statistics along with the previously mentioned bootstrap coefficient of determination ($R^2_{bootstrap}$) reported from the validation testing. These include the sum of squared deviations of the

dependent variable values from their mean (SD), the predicted sum of squares (PRESS), the cross-validated R^2, and the outliers.

Results

The final QSTRs for each bioassay, potential potency factors, and TEFs are represented by equations 8 through 12. These equations were among the most significant of the GFA equation output for each case. The equations chosen were the lowest three LOF scores among the equations generated for each case. This means that the GFA successfully converged to valid and useful equations. The significant statistics are presented in Table 5 for each of the QSTRs. Equations were selected based on high coefficients of determination and significance of the F-statistic. To insure that equations were not overfitted, R^2 was compared to R^2_{adj}. When observations were not sufficiently independent of one another, even in cases with large numbers of observations, the models generated can have poor explanatory and predictive power (AccelrysSoftwareInc., 2005). A comparison of the R^2 parameter and the $R^2_{bootstrap}$ parameter shows that the calculated R^2 is accurate and the probability of it being low is small insuring the quality of fit for each equation.

Log (Fluorescence Intensity) = 4.31258 + 0.484167 * Kappa-1 + 0.500625 * Jurs-WNSA-3 Eqn. 3.8

Log (Net Revertants) = 8.4492 + 0.0009 * E-DIST-mag Eqn. 3.9

-0.0164 * Jurs-TASA

Log (Rate of Recovery) (min^{-1}) = -4.35035 + 0.426617 * CIC + 5.44241 * Shadow-XYfrac - 0.00709 * Jurs-PNSA-1 Eqn. 3.10

Log (Potential Potency) = -1.8891 – 0.0180 *Vm + 0.0086 * PMI-mag Eqn. 3.11

Log (TEF) = -6.9378 +0.8266 * Jurs-WNSA-3 + 0.0219 * PMI-mag Eqn. 3.12

Comparison of measured and calculated toxic endpoints using equations 3.8-3.12 are shown with 95% confidence intervals for each bioassay in Figures 8-12. The diagonal line on each graph represents the 1:1 relationship. Proximity of the data to the line indicates that the predicted QSAR response was the same as the actual bioassay response.

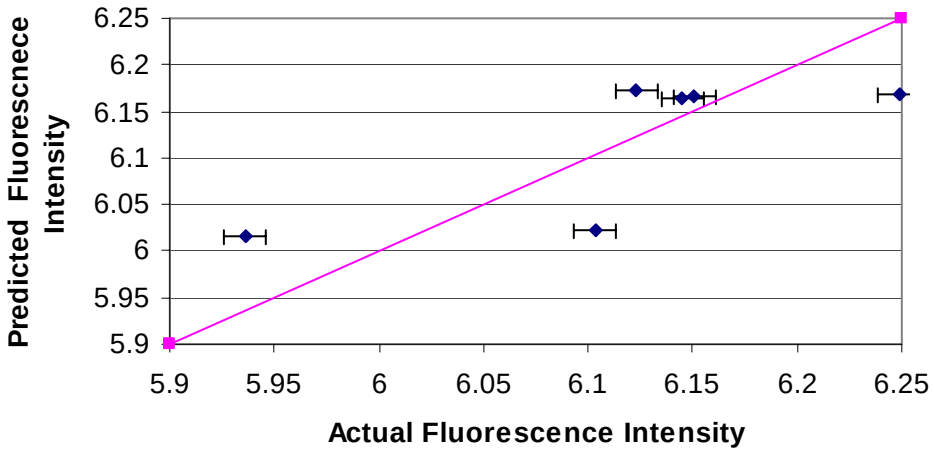


Figure 8. QSTR for EROD activity by PAHs in Clone 9 cells. Actual bioassay response versus QSTR predicited response.

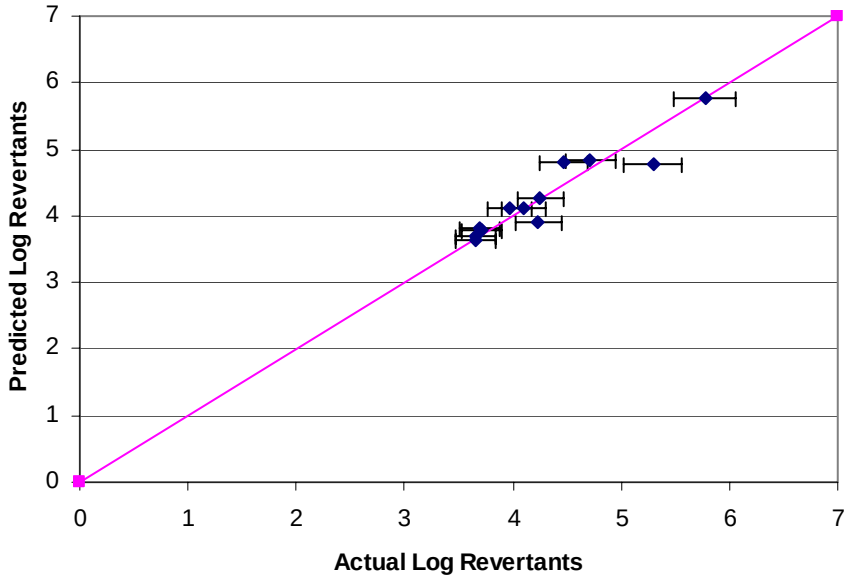


Figure 9. QSTR for revertants in *Salmonella* /microsome assay. Actual bioassay response versus QSTR predicted response.

Table 5. Statistics for the developed QSTRs.

						Validation Test Results			
QSTR	R^2	R^2_{adj}	F	LOF	LSE	$R^2_{bootstrap}$	SD	PRESS	$R^2_{cross\text{-}validated}$
Log (Fluorescence Intensity)	0.78	0.63	5.3	0.017	0.002	0.76	0.052	0.048	0.79
Log (Revertents)	0.89	0.86	37.3	0.102	0.045	0.89	0.505	0.787	0.84
Log (Rate of Recovery)	0.94	0.90	24.3	0.029	0.003	0.94	0.454	0.141	0.96
Log (Potential Potency)	0.86	0.84	38.5	1.35	0.195	0.83	21.6	4.587	0.79
Log (TEF)	0.89	0.87	56.2	1.42	0.833	0.89	127.9	20.469	0.84

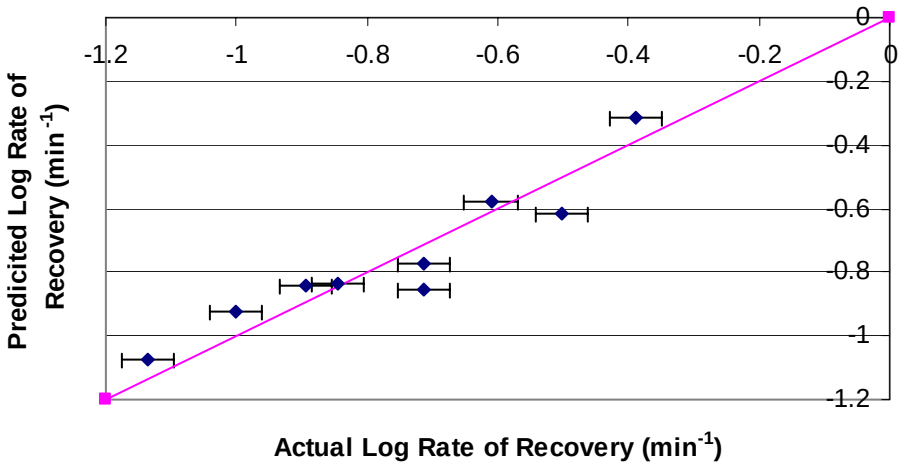


Figure 10. QSTR for rate of recovery of Clone 9 cells in the GJIC assay. Actual bioassay response versus QSTR predicted response.

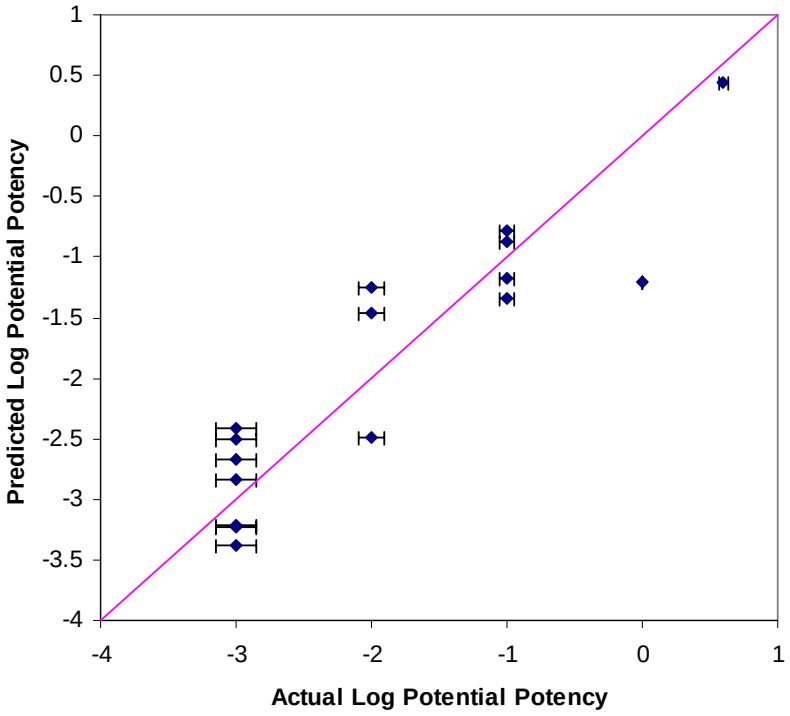


Figure 11. QSAR model of potential potencies for PAHs. Actual USEPA potential potencies versus QSAR predicted potential potencies.

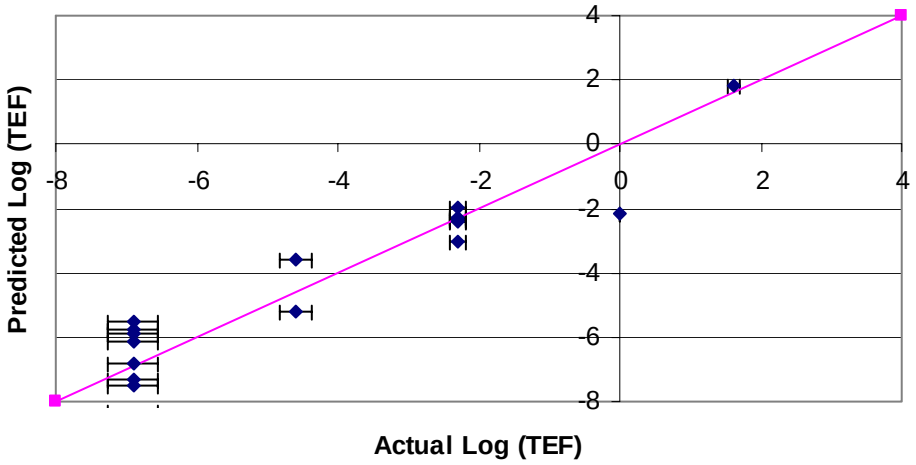


Figure 12. QSAR of TEFs for PAHs. Actual versus QSAR predicted TEFs.

The significant descriptors used in equations 3.8-3.12 are from four categories: Shape-Electronic, Spatial, Information Content, and Topological. The calculated values for the descriptors used in equations 8-12 are presented in Table 6. The shape-electronic descriptors combine shape and electronic information to characterize molecules. The descriptors are calculated by mapping atomic partial charges on solvent-accessible surface areas of individual atoms (AccelrysSoftwareInc., 2005; Stanton, D. T. and Jurs, P. C., 1990). Jurs-TASA, Jurs-PNSA-1, and JURS-WNSA-3 are all shape-electronic descriptors. Jurs-TASA is the total hydrophobic surface area that is the sum of solvent-accessible surface areas of atoms with absolute value of partial charges less than 0.2. Jurs-PNSA-1 is the partial negative surface area that is calculated as the sum of the solvent-accessible surface areas of all negatively charged atoms. Jurs-WNSA-3 is the surface-weighted charged partial surface area calculated as the solvent accessible surface

area multiplied by the partial charge for all negatively charged atoms multiplied by the total molecular solvent accessible surface area divided by 1000 (Stanton, D. T. and Jurs, P. C., 1990; Rohrbaugh, R. H. and Jurs, P. C., 1987).

There are three spatial descriptors that emerge as dominant: Shadow-XYfrac, Vm, and PMI-mag. The Shadow-XYfrac, one of several shadow indices, is a set of geometric descriptors that characterizes the shape of the molecules. The descriptors are calculated by projecting the molecular surface on three mutually perpendicular planes, XY, YZ, and XZ (Rohrbaugh, R. H. and Jurs, P. C., 1987). These descriptors depend not only on conformation but also on the orientation of the molecule. Specifically, Shadow-XYfrac is the fraction of the area of the molecular shadow in the XY plane over the area of the rectangle enclosing the projection of the molecule. Vm is a 3D spatial descriptor that defines the molecular volume inside the contact surface (Hill, T. L., 1960). The molecular volume is calculated as a function of conformation and is related to binding and transport. PMI-mag is the magnitude of the principle moments of inertia about the principle axes of the molecule (Hill, T. L., 1960). Taken together these descriptors capture the influence of a molecule's geometry on its behavior.

Table 6. Descriptors used in QSTR equations to describe toxic endpoints for PAHs.

	Shape-Electronic (Spatial)			Information Content (Topological)		Spatial			Topological
Compound	Jurs-WNSA-3	Jurs-PNSA-1	Jurs-TASA	E-DIST-mag	CIC	Shadow-XYfrac	Vm	PMI-mag	Kappa - 1
15DMN[1]	-6.044	96.127	341.413	1207 891	1.000	0.668	160.972	182.109	8.592
23DMN	-5.475	85.983	347.959	1204 471	1.000	0.753	160.633	207.524	8.592
26DIN[2]	-9.112	80.670	418.001	2274 815	1.250	0.632	228.568	544.393	12.457
26DMN	-6.439	100.716	355.283	1202 975	1.000	0.655	160.907	227.420	8.592
27DMN	5.935	86.225	349.728	1203 781	1.000	0.687	161.228	222.157	8.592
Anthracene	-5.622	127.284	370.765	1978 145	1.593	0.759	170.902	260.409	12.719
BAP	-8.510	152.645	444.348	5145 001	1.857	0.662	230.188	540.647	9.242
Benzanthracene	-7.645	146.606	428.299	3756 841	2.038	0.673	214.827	516.295	12.535
Chrysene	-7.837	148.780	426.315	3758 182	1.973	0.694	213.842	502.487	11.796
Naphthalene	-3.826	104.822	306.931	803.054	2.333	0.724	127.343	119.010	11.796
Phenanthrene	-5.393	122.695	363.662	1982 373	1.800	0.701	170.713	258.141	5.694
Pyrene	-6.324	127.043	382.759	2985 934	1.965	0.660	186.580	287.386	9.242
Fluoranthene	-6.321	132.598	392.259	2985 127	2.250	0.701	187.429	303.558	9.972
Acenaphthene	-3.860	82.843	327.678	1444 975	0.833	0.707	142.386	150.830	7.408
Acenaphthylene	-4.071	110.136	320.222	1444 973	0.833	0.704	139.364	147.773	7.408
Benzo[b]fluoranthene	-8.100	159.407	438.409	5146 590	1.356	0.646	223.516	537.114	12.535
Benzo[g,h,i]perylene	-8.034	150.712	437.498	6785 384	2.419	0.761	237.962	523.153	13.309
Benzo[k]fluoranthene	-8.060	155.543	443.801	5137 065	1.438	0.704	223.852	597.235	12.535
Dibenz[a,h]anthracene	-10.122	175.107	478.422	6168 633	2.273	0.685	249.059	847.368	14.352
Fluorene	-4.818	106.030	347.422	1698 042	1.538	0.716	153.924	221.708	8.320
Indeno[1,2,3-cd]pyrene	-8.088	161.823	453.496	6768 180	1.449	0.668	239.146	613.488	13.309
2-methylnaphthalene	-4.912	98.060	328.013	992.103	0.796	0.657	144.182	167.165	7.639
5-methylchrysene	-8.473	141.534	443.986	4194 965	1.593	0.658	231.147	519.911	12.719

[1] - DMN-dimethyl naphthalene, [2] - DIN-Diisopropyl naphthalene

Two information content descriptors that merged as significant are: E-DIST-mag, and CIC. This set of descriptors is based on their calculations of the representation of molecular structures as graphs, where atoms are represented by vertices and covalent chemical bonds by edges (Bersohn, M., 1983; Muller, W. R., 1987). E-DIST-mag is based on the edge adjacency and the edge distance matrices. Specifically, it is the total edge distance of a molecule divided by the magnitude (Bonchev, D., 1983; Bonchev, D. et al., 1981; Katritzky, A. R. and Gordeeva, E. V., 1993). CIC is referred to as the complementary information index. This descriptor represents the deviation of the information content from its maximum possible value where the information content is an index of the partitioning of the valence and bonds of a chemical's structure. The Kappa indices are a set of topological descriptors that view molecule graphs as connectivity structures to which numerical invariants can be assigned. Kappa indices specifically help to differentiate the molecules according to size, degree of branching, flexibility and overall shape. Specifically, Kappa-1 describes the shape of the molecule in terms of the count of atoms (Kier, L. B., 1990; Kier, L. B. and Hall, L. H., 1991). Each of the descriptors appear in one QSTR except the PMI-mag and Jurs-WNSA-3, which appear in two (Equations 3.10, 3.11 and 3.12).

Discussion

Enzymatic activation is a precursor to the initiation of chemicals to be DNA mutagens. Induction of the enzymes of the cytochrome P450 family has been shown to be an essential step in the activation of carcinogens prior to initiation (Kennedy, S. W. and Jones, S. P., 1994; Szklarz, G. D. and Paulsen, M. D., 2002). PAHs have been

linked to a broad range of toxic effects such as cancers, renal disease, circulatory disorders and immune system dysfunction (ATSDR, 1990a). The parent compound PAHs are not believed to be the cause of these many effects. Rather the oxidized metabolites and their reactive intermediates show a greater level of biological activity than do the unmetabolized parent compounds. Metabolism of PAHs begins with oxidation by enzymes of the cytochrome P450 family, specifically CYP1A1. The induction process begins with PAH absorption by a cell from systematic circulation. Once in the cytosol, PAHs bind with the aryl hydrocarbons (Ah) receptor and release two heat shock proteins (hsp 90). The Ah receptor-ligand complex can then be phosphorylated by tyrosine kinase. This allows the Ah receptor and ligand to enter the nucleus, bind with the Ah receptor-nuclear translocator protein (Arnt) and bind with the xenobiotics response element in the upstream region of a gene to enhance transcription (Parkinson, A., 1996). The absorption and transport of a PAH by the cell, into the cytosol, can be a limiting factor in the metabolic activation process (Parkinson, A., 1996). Similarly, the structure of a molecule will dictate the path of metabolic transformation into more active intermediates.

The two descriptors that are used to represent the induction of P450 enzymes are both consistent with chemical transport and structure. Jurs-WNSA-3 is a spatial descriptor that combines shape and electronic information about a molecule (Stanton, D. T. and Jurs, P. C., 1990). Specifically, it is the surface weighted charged partial surface area. This descriptor us highly correlated with the molecular area which is related to the binding, transport and solubility of a molecule (AccelrysSoftwareInc., 2005). Recently,

the charged partial surface area (CPSA) descriptors have been found to be useful in the study of acute aquatic toxicity where they appear to provide an alternative to energy level measures for describing global and local electrophilicity in cases of non-covalent molecular interactions (Stanton, D. T. et al., 2002). Local electrophilicity is also important in the metabolism of PAHs by the cytochrome P450 family of enzymes (Parkinson, A., 1996). CYP1A1 contains a heme group capable of supplying singlet oxygen for insertion into the double bonds of PAHs. This insertion produces epoxides. Cells attempt to excrete PAHs by increasing their polarity through electrophilic attacks using Phase II enzymes. Unfortunately, this often results in a more biologically active compound. The Jurs-WNSA-3 descriptor illustrates the importance of structure in the chosen position of attack to detoxify a compound by these metabolizing enzymes.

The Kappa-1 index or the Kier's Shape Index-1 describes the induction of the metabolizing enzymes in the QSTR equation is. This index differentiates the shape of a molecule based on the count of atoms. This descriptor is also highly correlated with molecular volume, molecular weight, and molecular area (AccelrysSoftwareInc., 2005). The importance of a molecule's shape and degree of branching and flexibility impacts detoxification/metabolism processes within cells (Vyas, K. P., Levin, W. et al., 1982; Vyas, K. P., Thakker, D. R. et al., 1982; Warshawsky, D. and Landolph, J. R., 2006; Szklarz, G. D. and Paulsen, M. D., 2002). Recent studies demonstrate that absorption, distribution, metabolism, and excretion (ADME) properties are accurately predicted using topological descriptors such as the Kappa-1 index (Votano, J. R. et al., 2004).

Of the three bioassays, the least amount of experimental data was available for the EROD assay. A minimal dataset reduces the predictive power of equation 3.8 somewhat because the more data that is used to train a model the more predictive power results. Equation 3.8 did show a lower coefficient of determination and smaller F-statistic (Table 5). However, the lack-of-fit score was among the top three (Table 5). The result of this minimal dataset reduces the confidence in this equation.

The *Salmonella*/microsome assay measures mutations of DNA, which is an initiation, a critical step in the carcinogenic process (McCann, J. and Ames, B. N., 1976; Zeiger, E., 1998). Mutagenic activity is strongly influenced by several structural factors such as isomeric positioning, conformation, steric hindrances, physical dimension, and the ability to resonance stabilize the electrophilic metabolites (Ames, B. N., McCann, J., and Yamasaki, E., 1975b; Analogues, C. o. P. a. S. and Hazards, B. o. T. a. E. H., 1983; Ashurst, S. W. et al., 1983; Warshawsky, D. and Landolph, J. R., 2006). These factors are consistent with the descriptors used in equation 9 for the number of revertants in the *Salmonella*/microsome assay (Table 6). This result confirms that structure activity relationships can be good predictors of toxic endpoints relating to carcinogenesis.

The E-DIST-mag and Jurs-TASA descriptors in equation 3.9 correlate well with DNA revertants and have significant implication to the process of initiation. E-DIST-mag is an information content descriptor that represents a molecules' physical dimension based on atoms and chemical bonds. Jurs-TASA is a shape-electronic descriptor that characterizes the total hydrophobic surface area of a molecule. Two important aspects in DNA mutation are intercalation and hydrophobicity (lypophilicity) (Pitot, H. C. and

Dragan, Y. P., 1996; Roy, D. R. et al., 2006; Singer, B. and Grunberger, D., 1983). Intercalation of PAHs with DNA is a fundamental process in which aromatic molecules associate directly with DNA by fitting into the minor and major groves of the helix (Geacintov, N. E., 1986). The degree of intercalation is related to the lypophilicity (hydrophobicity) that determines the ease of transport within the cell. Therefore, the negative coefficient of Jurs-TASA means that decreasing the total hydrophobic surface area of a molecule would increase the ease of transport within a cell. Once a mutagen (e.g. a PAH) crosses the cell membrane, the controlling factor for binding with hydrophobic DNA is the hydrophobic nature of the mutagen and lowering of the energy of the system possibly through pi-driven (π-π interaction) intercalation. The process of pi-driven intercalation is most efficient if aromatics are electron deficient. This deficiency facilitates electrostatic attraction between electron rich centers on the nucleotides and the electron deficient centers on the aromatic. PAHs are known to be efficient intercalators with the optimum size being three and four ringed structures.

The positive coefficient for E-DIST-mag means that a greater edge distance will increase the binding affinity resulting in a greater number of revertants. A greater edge distance means there are more covalent chemical bonds and less steric hindrance. Steric effects occur when atoms within a molecule, having and occupying a certain amount of space, are brought too close together (Harvey, R. G., 1991; Cancer), I. I. A. f. R. o., 1983). There is an associated cost in energy due to the electron clouds overlapping affecting the molecular shape and reactivity (Harvey, R. G., 1997).

The spiral shape of DNA is such that one groove is bigger than the other. This has some advantages for compounds or drugs that bind to it because they too are different sizes. Bigger molecules bind in the major grooves of DNA, while small molecules bind in minor grooves. PAHs and their metabolites intercalate between adjacent base pairs or can form adducts. Therefore, less steric hindrance allows a molecule to readily intercalate with the DNA. Similarly, the physical dimension of a molecule with less steric hindrance more readily bonds to the DNA.

The GJIC assay measures the impairment or elimination of a cell's capability to communicate with other cells. This communicative ability between cells controls several important functions such as growth control, homeostasis, and cellular apoptosis. The elimination of this communication between cells is believed to promote tumor growth by eliminating signals that instruct an initiated cell to stop dividing (Couch, D. B., 1996; Trosko, J. E. et al., 1998; Trosko, J. E. and Ruch, R. J., 1998). The structure-toxicity relationship for the rate of recovery in the GJIC assay is expressed in descriptors from three categories: information content, spatial and shape-electronic. CIC is an information content descriptor that corresponds to the steric qualities of a molecule. Steric hindrance or strain will cause a molecule to change its shape and reactivity (Harvey, R. G., 1997). These changes in shape and reactivity can modify the gap junction proteins which can extend the inhibition of GJIC (Rummel, A. et al., 1999).

The rate of recovery for GJIC is embodied in the Shadow-XYfrac. This geometric descriptor characterizes the shape of the molecule and depends not only on the molecular conformation but on orientation as well. Shadow-XYfrac is calculated by

projecting the molecular shape on the three Cartesian planes (i.e. XY, YZ, XZ) and aligning the principle moments of inertia with the X, Y, and Z axes (Rohrbaugh, R. H. and Jurs, P. C., 1987). The significance of this descriptor with regards to GJIC is that positions of methyl substitutions, which change the molecular shadow influence on GJIC (Upham, B. et al., 1996; Upham, B. L. et al., 1998). This descriptor is also influential in describing the binding and transport of molecules as recently shown in a study conducted on numerous chemicals to explain estrogen receptor binding (Hong, H. et al., 2002).

The third descriptor used in the QSTR for GJIC is Jurs-PNSA-1, the partial negative surface area. This descriptor is a charged partial surface area descriptor that encodes spatial and electronic information that relates to the tendency of molecules to engage in polar interactions (Stanton, D. T. and Jurs, P. C., 1990). With a negative coefficient, decreasing values of this descriptor results from decreasing molecular polarity for a molecule more or less likely to be moved through the gap junction of a cell (Equation 3.10). This descriptor correlates with the spatial descriptor, density, which expresses transport behavior of a molecule (AccelrysSoftwareInc., 2005).

Two QSTRs were generated for previously developed TEFs and USEPA potential potencies (PPs) (Nisbet, I. C. and LaGoy, P. K., 1992; USEPA, 1993). These two models were generated to determine the effectiveness of QSTRs to predict TEFs or PPs. Equations 3.11 and 3.12 describe PPs and TEFs and contained only spatial descriptors. Both of these equations contain the descriptor PMI-mag, the magnitude of

the principal moments of inertia about the principal axes of a molecule. This descriptor evaluates the symmetry of the top of the molecule.

The QSTR describing potential potencies (equation 3.11) also contains a molecular volume descriptor (Vm). This descriptor is a 3D spatial descriptor that defines the molecular volume inside the contact surface. The molecular volume is calculated as a function of the molecules' conformation and is related to binding and transport (AccelrysSoftwareInc., 2005).

TEFs are described by not only PMI-mag but also by a shape-electronic descriptor, Jurs-WNSA-3, a surface weighted charged partial surface area. This descriptor refers to a negatively charged surface area and is highly correlated with the molecular area, another descriptor related to binding and transport. The model for the PPs and for the TEFs showed a source of potential error because there were several observations that were the same (Figures 11and12). The PPs and TEFs were order of magnitude estimates and resulted in generating the same estimate for chemicals of different size, shape, and activity. This can result in a model that has poor predictive and explanatory power thus reinforcing the need for a better means to estimate toxicity factors. Evaluating the relevant statistics for equations 11 and 12 reveals poor explanatory and predictive power (Table 5). The lack-of-fit (LOF) scores as well as the sum of squared deviations of the dependent variable values from their mean (SD), for both equations, were high compared to the other equations (Table 5) indicating that the equations do not have much predictive power.

QSTRs can be effective in rapidly assessing chemical toxicity solely from molecular structure. However, not all QSTRs reliably assess toxicity. In fact, the most meaningful QSTRs describe how variation in chemical structure relates to a specific response not overall toxicity. This is demonstrated in the two developed QSTRs for TEFs and PPs (Figures 11 and 12). TEFs and PPs refer to an overall toxicity and not to a specific toxic endpoint. The order of magnitude estimates do not provide enough interpretation of the chemical attributes to make a toxicity estimate solely from these relationships. The currently available TEFs for PAHs from Nisbet and Lagoy (1993) are order of magnitude estimates. The TEFs predicted in this study reflect more precise estimates as they are made up of estimates of toxicity for specific toxic endpoints.

Improved predictions of toxicity for PAH compounds are possible if each compound is evaluated based on its ability to activate, initiate and/or promote cancer. TEF or PP values were estimated based on the QSTR predicted response from each bioassay. A weighting factor was assigned to the appropriate toxic endpoint prediction for the PAHs ability to activate, initiate, and promote (Table 7) generating a TEF_B or PP_B relative to the toxicity of BAP. An example of this concept is illustrated for chrysene in equation 3.13. Chrysene, a complete carcinogen, activates detoxifying enzymes, is an initiator, and is shown to be a promoter of cancer.

$$TEF_B \text{ (Chrysene)} = 0.3(\text{QSTR (EROD)}) + 0.3 \text{ (QSTR (AMES))} + 0.4(\text{QSTR (GJIC)}) \quad \text{Eqn. 3.13}$$

Therefore, each response is given an equal weight except the promotion response (0.4) where chrysene was shown to be a promoter and distant site tumors were observed. This TEF_B would still be an estimate of toxicity that is relative to BAP however; it would be based on specific knowledge of the chemical's ability to act in each stage of carcinogenesis. This would provide an improved estimate of toxicity over the currently available TEFs and PPs. As QSTR models are improved and expanded the estimated toxic endpoint becomes more robust and reliable allowing for better predicted TEFs.

Shape-electronic descriptors were present in each of the QSTRs developed, indicating that these descriptors were effective in representing the toxicity of PAHs in the bioassays modeled. Shape-electronic descriptors are a subset of spatial descriptors that symbolize the tendency of a molecule to engage in polar interactions. Polar interactions are important in many processes of toxic action. Polar and non-polar surface areas are commonly used to determine cell permeability. Recently, modeling transport through the blood brain barrier was characterized using molecular flexibility, hydrophobicity, and polar surface areas (Winkler, D. A. and Burden, F. R., 2004).

Table 7. QSTR predicted TEFs for selected PAHs.

	Coefficient			QSAR Predicted Activity				
Chemical[4]	(EROD)[1] Activator	(AMES)[2] Initiator	(GJIC)[3] Promoter	(EROD)[1] Activator	(AMES)[2] Initiator	(GJIC)[3] Promoter	Calculated Bioassay Based TEF_B	Relative to BAP TEF_B
Acenaphthene	0.5	0	0	5.967	4.376	-0.713	2.983	0.939
Acenaphthylene	0.5	0	0	5.861	4.498	-0.944	2.931	0.923
Anthracene	0.5	0	0	5.936	3.970	-0.389	2.968	0.935
Benzo(g,h,i)perylene	0.5	0	0	6.734	7.381	-0.245	3.367	1.060
Fluoranthene	0.5	0	0	5.976	4.701	-0.844	2.988	0.941
Fluorene	0.5	0	0	5.929	4.280	-0.549	2.964	0.933
Phenanthrene	0.5	0	0	6.103	4.248	-0.611	3.052	0.961
Pyrene	0.5	0	0	5.621	4.859	-0.713	2.811	0.885
5-methylchrysene	0.3	0.4*	0.3	6.151	4.718	-1.136	3.392	1.068
2-methylnaphthalene	0.3	0.4*	0.3	5.552	3.963	-1.130	2.912	0.917
Chrysene	0.33	0.33	0.33	6.123	4.466	-0.503	3.328	1.048
Benzanthracene	0.33	0.33	0.33	6.249	5.293	-0.895	3.514	1.106
BAP	0.3	0.3	0.4*	6.145	5.775	-1.000	3.176	1.000
Naphthalene	0.33	0.33	0.33	3.267	4.094	-0.158	2.377	0.748
Benzo(b)fluoranthene	0.3	0.3	0.4*	6.327	5.891	-1.386	3.111	0.979
Benzo(k)fluoranthene	0.33	0.33	0.33	6.347	5.794	-1.008	3.674	1.157
Dibenzo(a,h) anthracene	0.33	0.33	0.33	6.194	6.155	-0.894	3.780	1.190
Indeno (1,2,3 -cd) pyrene	0.33	0.33	0.33	6.707	7.103	-1.244	4.147	1.306

[1] - Used to test for activation of cytochrome P450 detoxifying enzymes.

[2] - Used to test for initiation properties of PAHs.

[3] - Used to test for promotion properties of PAHs.

[4] - PAHs selected for toxicity, potential for human exposure, frequency at NPL sites, and extent of available toxicity information.

* - More weight is given to this property due to observance of distant site tumors in animal studies or other statistically significant (Fisher's Exact Test with $p>0.05$) experimental findings.

CHAPTER IV

RISK ASSESSMENT OF HARMFUL ALGAL BLOOMS: CHALLENGES AND PROGRESS IN ASSESSING TOXICITY

Overview

There are six recognized poisoning syndromes that result from algal toxins affecting human health through their consumption of contaminated seafood or direct contact with bloom water or inhalation of aerosolized toxin. Human health risk assessments (HHRAs) for harmful algal blooms (HABs) are hindered due to the lack of toxicity factors that describe the toxicological impact of the toxins based on exposure route. A bioassay based reference dose (RfD_B) for an inhalation exposure was developed for use in HHRAs. The reference dose is the first step in assessing the risk to human health from *in vitro* cytotoxicity data rather than from shellfish bed closures. The reference dose developed for PBTX-2 is based on several strains of *Karenia brevis* cultured under different salinities and temperatures. A reference dose of 0.0571 mg/kg-day dose was used in a HHRA to estimate the risk from inhaled aerosolized brevetoxin in a recreational setting. The use of this reference dose showed that there was no significant non-carcinogenic risk from exposure to the brevetoxin in the exposure scenario examined herein.

Introduction

Varieties of harmful algal blooms (HABs) affect human and ecological health every year. Algal blooms are reported in the waters of every U.S. coast and in many other parts of the world. Recently, there has been an increase in the frequency and geographic occurrence of these HABs (Hallengraeff, G. M., 1993; Van Dolah, F. M., 2000; Hallegraeff, G. M., 1993). The increased incidence has magnified the need to assess impact to human and ecological health. Currently, regulatory measures for the six recognized human poisoning syndromes are based mainly on epidemiologic data derived from poisoning incidents. However, these data are seldom accurate and complete, and mainly restricted to acute toxicity. Incident based data provide acute toxicity explanation, but to predict potential impact necessitates a more complete data set generated under controlled experimental conditions. Improved assessment methods for HABs can then be used to develop appropriate regulatory measures.

An HHRA for brevetoxins is hampered by the lack of toxicity factors and measured exposure concentrations. There are unidentified toxins (e.g *Pfiesteria*), in some cases, the indicators for effects and exposure are poorly defined, and there are no available reference doses or toxicity factors for these algal toxins. The significant uncertainties in HHRAs currently include: unidentified toxins, lack of reference doses or toxicity factors, species and toxin variability, measured aerosol concentrations at points of exposure, and unknown environmental triggers causing the blooms.

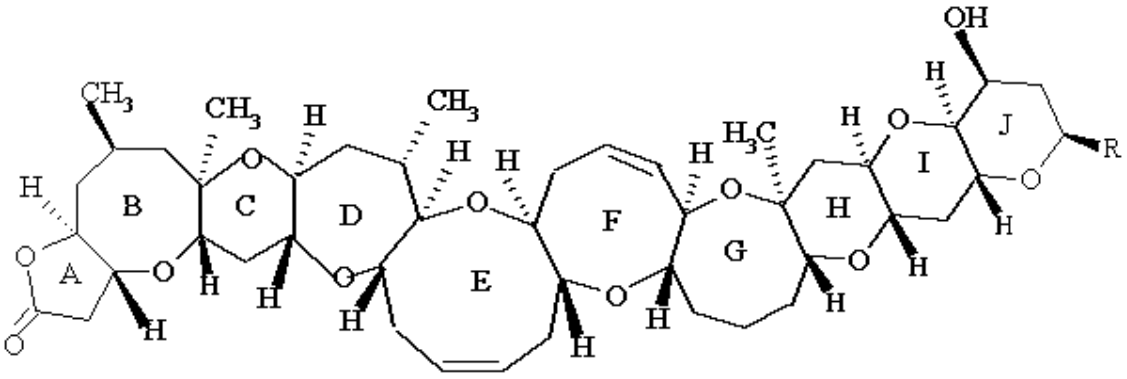
Neurologic or neurotoxic shellfish poisoning (NSP) is caused by polyether brevetoxins produced by the unarmored dinoflagellate *Karenia brevis*. The brevetoxins

are toxic to fish, marine mammals, birds and humans, but not to shellfish. Until the year 1993, neurologic shellfish poisoning was considered endemic only to the Gulf of Mexico and the east coast of Florida, where "red tides" had been reported as early as 1844 (Pierce, R. H., 1986). An unusual feature of *Karenia brevis* is the formation of toxic aerosols generated when wave action causes the release of toxin that then becomes aerosolized. Asthma-like symptoms in humans can result upon exposure. In 1987, a major Florida bloom event was dispersed by the Gulf Stream northward into North Carolina waters where it has since continued to be present. In early 1993, more than 180 human shellfish poisonings were reported from New Zealand caused by an organism similar to *Karenia brevis*. It is likely that this was a member of the hidden plankton flora that was previously present in low concentrations. This plankton flora then developed into bloom proportions triggered by unusual climatic conditions that can range from higher than usual rainfall to lower than usual water temperatures. It has been hypothesized that this bloom was coincident with an El Niño event (Hallegraeff, G. M., 1993).

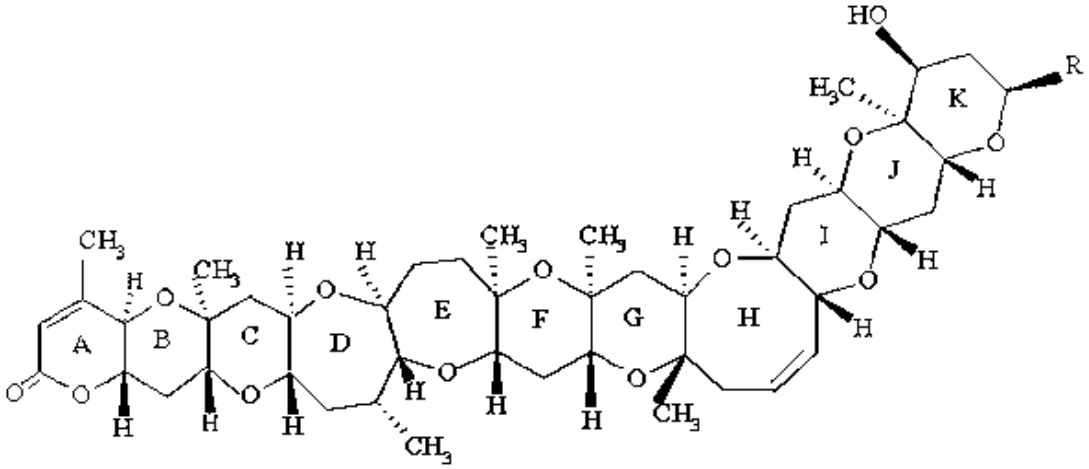
The NSP toxins (PBTX) are tasteless, odorless, heat and acid stable, lipid-soluble, cyclic polyether neurotoxins produced by the marine dinoflagellate *Karenia brevis*. The molecular structure of the brevetoxins consists of 10 to 11 transfused rings with molecular weights are around 900 mass units (Figure 13). Ten brevetoxins have been isolated and identified from field blooms and *Karenia brevis* cultures (Benson, J. M. et al., 1999). These brevetoxins show specific binding to site-5 of voltage-sensitive

Na^+ channels leading to channel activation at normal resting potential causing toxic effects (Cembella, A. D. et al., 1995).

Karenia brevis produces a lipophilic toxin (brevetoxin; PBTX) that alters the normal function of excitable cell membranes. This toxic action is accomplished by inducing persistent activation of sodium channels (Catterall, W. A. and Risk, M., 1981; Baden, D. G., 1989). Brevetoxins are depolarizing substances that open voltage gated sodium (Na^+) ion channels in cell walls. This opening of the channel alters the membrane properties of excitable cell types to enhance the inward flow of Na^+ ions into the cell (Fleming, L. E. et al., 1999). The toxin appears to produce its symptoms by transforming fast sodium channels into slower ones, resulting in persistent activation and repetitive firing (Watters, M. R., 1995).



Type 1 (A) brevetoxins: PBTX-1 R = $CH_2C(=CH_2)CHO$
PBTX-7 R=$CH_2C(=CH_2)CH_2OH$
PBTX-10 R=$CH_2CH(CH_3)CH_2OH$



Type 2 (B) brevetoxins: PBTX-2 R = $CH_2C(=CH_2)CHO$
oxidized PBTX-2 R=$CH_2C(=CH_2)COOH$
PBTX-3 R=$CH_2C(=CH_2)CH2OH$
PBTX-8 R=CH_2COCH_2Cl
PBTX-9 R=$CH_2CH(CH_3)CH2OH$
PBTX-5 the K-ring acetate of PBTX-2
PBTX-6 the H-ring epoxide of PBTX-2

Figure 13. Brevetoxin Structures (Adapted from Hua, et al, 1995)

Conformational analysis revealed that the unsaturated H-ring of brevetoxin B (Figure 13) favors the boat-chair conformation, as does the saturated G-ring of brevetoxin A (Figure 13) (Plakas, S. M. et al., 2002; Poli, M. A. et al., 2000; Radwan, F. F. and Ramsdell, J. S., 2006; Rein, K. S. et al., 1994). After reduction has occurred, the H-ring of brevetoxin B shifts to a crown conformation. This subtle change in conformational preference induces a significant change in the overall shape of the molecule, which is believed to be responsible for the loss of binding affinity and toxicity (Rein, K. S. et al., 1994).

Respiratory problems associated with the inhalation of aerosolized brevetoxins are believed to be due in part to opening of sodium channels. In sheep, bronchospasm could be blocked by atropine while there appears to be a role for mast cells. In sheep the bronchospasm could be effectively blocked by cromolyn, a mast cell stabilizer preventing histamine release and chlorpheniramine, a first generation antihistamine (Abraham, W. M. et al., 2005b). It has been reported that brevetoxin could combine with a separate site on the gates of the sodium channel, causing the release of neurotransmitters from autonomic nerve endings (Fleming, L. E. et al., 1999). In particular, this can release acetylcholine, leading to smooth tracheal contraction, as well as massive mast cell degranulation (Abraham, W. M. et al., 2005; Fleming, L. E. et al., 1999; Fleming, L. E. et al., 2005).Brevetoxins are periodically responsible for marine animal mortalities, non-fatal human effects and occasional economic hardships. Some of the known human health effects include respiratory distress upon exposures to seawater aerosols containing brevetoxins and seafood poisoning (neurotoxic seafood

poisoning; NSP) after consumption of brevetoxin-contaminated shellfish (Aune, T., 1997; Pierce, R. H., 1986). Although these health effects have been observed, the lack of brevetoxin toxicity factors prevents generating reliable HHRAs. The current regulations for states along the Gulf of Mexico is based on shellfish bed closures at concentrations of 5000 *Karenia brevis* cell per liter, reopening is based on shellfish PBTX of <80 μg/100 g.

A bioassay based reference dose for brevetoxins was used to generate data for a HHRA on these HABs. The hypothesis is that brevetoxin toxicity can be estimated using a bioassay based reference dose (RfD_B) for the purpose of an initial attempt to evaluate human health risk. The objectives of this study were to culture algal species that are common to the Gulf of Mexico in different salinities and temperatures to determine how stress affects toxin production. Secondly, these cultures were extracted and there toxicity was evaluated using an *in vitro* cytotoxicity bioassay. Finally, the dose response curves from the bioassay were used to develop bioassay based reference doses to be used in an initial human health risk assessment. The results of the initial human health risk assessment are also presented herein.

Materials and Methods

Experimental Approach

Several species of HABs were grown and maintained for harvesting to be used in the toxicity assays. Each species was maintained at different salinities and temperatures to examine the effects of temperature and salinity on toxin production. Each species was

harvested and counted to estimate toxicity production. These species were used in a traditional cytotoxicity assay, at different concentrations to determine there toxicity. Three controls were used in this assay to determine the cytotoxicity of the brevetoxins produced by these species. The controls used are as follows: cells only, solvent only (ethanol at less than 0.5%), and activator control (oubain and veratridine, O/V). From this data, a reference dose was determined for PBTX-2 to be used in HHRAs. A HHRA was developed to estimate the effects of inhaled brevetoxin on human in a recreational setting.

Cell Culture

Mouse neuroblastoma cells (N2A) (ATCC-CCL131) were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Sigma Co., St. Louis, MO). Streptomycin (Sigma Co., St. Louis, MO.) and Penicillin (Sigma Co., St. Louis, MO.) were also added at 50µg and 50 units per milliliter. Cultures were maintained in an incubator at 37°C with humidified 5% CO_2 and 95% air atmosphere. Cells were harvested for the cytotoxicity assay with a solution of Trypsin (5%) and PBS. The cells were then seeded in a 96-well plate (Costar, Cambridge, MA) at a density of 30,000 cells per well in 100µl of growth medium. Cells were incubated for 24 hours in an incubator exposed to 5% CO_2 before dosing.

Algal Culture

Seven clonal isolates of *Karenia brevis*, five from the Texas coast and two from Florida (Table 8), were maintained in L1 medium (Guillard and Hargraves, 1993) at 22º

C, 70 ± 2 mol photons m^{-2} s^{-1} on 12:12h light:dark cycle, and salinity of 30 ppt. These cultures were grown in the laboratory of Dr. Campbell of the Texas A&M University's Department of Oceanography. Prior to cytotoxicity assays, all clones were acclimated to a range of growth conditions for at least ten generations gradually (at increments of 2 ^{o}C ; 2 salinity units) to experimental conditions (20 and 25 ^{o}C ; salinities of 27 and 35). For each growth condition, glass culture flasks (100 mL) were inoculated with approximately 10 mL of dense, acclimated culture and allowed to grow until mid-stationary phase. All cultures were harvested within 17-20 days to prevent variable toxicity due to external variables (Pierce, R. et al., 2001). Cell pellets from each culture were harvested by gentle centrifugation 3200 g / 5 min / 4º C, to create a soft pellet of cells. Supernatant was then aspirated and the pellet was extracted immediately. The supernatant volume was centrifuged at 3200rpm for approximately 5 minutes. The supernatant was decanted to 5ml and the pellet re-suspended and deposited in its respective culture bottle. The concentrated cell solution was then extracted using 3 washes of equal parts ethyl acetate. The extracted volume of ethyl acetate was dried down in the roto-evaporator unit, transferred to 4ml or 8ml amber glass vials via a pipette, and dried down manually with nitrogen. Cultures were resuspended in ethanol for cytotoxicity testing.

Table 8. *Karenia brevis* clones at Texas A&M University.

Clone	**Collection location and date**	**Origin of isolate**
SP1	Brownsville, TX (Fall, 1999)	T. Villareal
SP2	Brownsville, TX (Fall, 1999)	T. Villareal
TSP3	Brownsville, TX (Fall, 1999)	T. Villareal
NTSP3	Brownsville, TX (variant)	C. Hyatt
TXB3	Brownsville, TX (Fall, 1999)	K. Steidinger
TXB4	Brownsville, TX (Fall, 1999)	K. Steidinger
CCMP2228	Sarasota, Florida (August, 2001)	C. Higham
CCMP 718	St. John's Pass (1953)	W.B. Wilson

MTT Cytotoxicity Assay

Cultured wells received 10µl of each sample at concentrations of 8, 4, and 2 µg/ml. Additionally, 10µl of 10mM Ouabain (Sigma Co., St. Louis, MO.) and 10µl of 1mM Veratridine, pH 2 (Sigma Co., St. Louis, MO.) were added to each well. A cell control, solvent control, and ouabain/veratridine control (O/V) were run for each experiment. Each concentration and control was run with 8 replicates. Cultures were then incubated for an additional 24 hours following treatment in an incubator exposed to 5% CO_2 before running the assay. MTT (3-[4,5-dimethylthiozol-2-yl]-2,5 – triphenyltetrazolium) was prepared as a 2.5mg/ml stock solution in PBS, pH 7.4 and stored at 4°C until use. Following the incubation with samples, the media was removed and 50µl of a 1:4 dilution of MTT and growth media was added to each well. Cultures were incubated for 30 minutes at 37°C in 5% CO_2. Medium was then removed and 100µl of dimethyl-sulfoxide (DMSO) was added to each well. The plates were immediately read on a Biotek Synergy HT Plate Reader (Biotek Instruments Inc.,

Winooski, VT) at a wavelength of 570nm. Results from each treatment were compared using a one-way ANOVA.

Chemical Analysis

Each sample and a set of 9 brevetoxin standards (Brevenal, PBTX-1, 2, 3, 6, 7, 9, 11, PBTX-ca) samples were analyzed using a Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) instrument. MALDI-TOF spectra were recorded in reflected ion mode with an Applied Biosystems Voyager DE-STR mass spectrometer (ABI, Framingham, MA). Positive ions were generated by using a nitrogen laser pulse (λ = 337 nm, 20 Hz) and accelerated under 20 kV using delayed extraction (175 ns) before entering the time-of-flight mass spectrometer. Laser strength was adjusted to provide minimal fragmentation and optimal signal-to-noise ratio. An average of 200 laser shots was used for each spectrum, and data were processed with the accompanying Voyager software package. A dried droplet procedure was used with 2,4,6-trihydroxyacetophenone (THAP). A 1:1:1 mixture of analyte, a 10 mg/mL THAP solution in methanol, and a 1 mg/ml solution of LiCl (to assist with ionization) was spotted in 1μL aliquots on top of a stainless steal plate. The most abundant ions observed for Brevetoxin B (PBTX-2) and Brevetoxin A (PBTX-3) correspond to $(M+Li)^+$ at *m/z* 901 and *m/z* 903, respectively.

Samples were also analyzed via LC/MS. The instrument was optimized using a standard solution of the eight brevetoxins and brevenal used in this study. The APCI ionization technique was used to analyze the samples by both single ion monitoring, for increased sensitivity and by collision induced dissociation, for structure determination.

Results

Cytotoxicity Assay

Toxicity assays performed on algal cultures at combinations of two temperatures and two salinities yielded data from which a reference dose value were determined. This reference dose was used to generate an initial HHRA for the inhalation exposure to brevetoxin. Cultures were determined to be toxic if their response fell below the activator response as indicated by the bold line on each figure. Cultures of *Karenia brevis* raised at 25°C and either 35 ppt or 27 ppt salinity demonstrated significant changes in toxicity as measured by the mouse neuroblastoma cytotoxicity assay. The cytotoxicity assay revealed that only SP1, NTSPE, and TXB3 (grown at 25°C and 27ppt) resulted in a toxic response (Figure 14). A dose response curve for SP1, NTSP3 and TXB3 (25°C, 27 ppt) resulted with the most toxic response being at 8 μg/ml that decreased proportionally (Figure 14). At 25°C and 35ppt, SP2 and NTSP3 elicited a toxic response at all concentrations tested. However, TXB3 and 718 were toxic at 8 and 4 μg/ml and a dose response was observed for both species (Figure 15). The dose response curves for the species at 25°C and 35ppt were not as pronounced as was seen at other temperature and salinity. For example, the higher salinity (35 ppt) resulted in greater toxicity at 20°C.

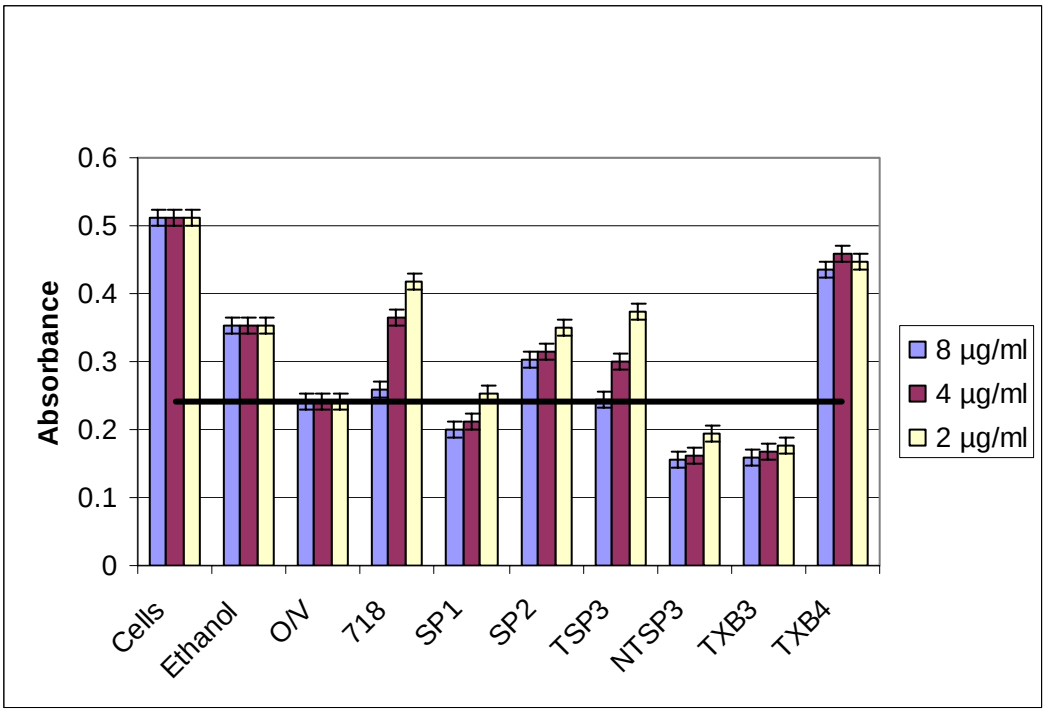


Figure 14. Cytotoxicity of *Karenia brevis* species at 25°C and 27ppt.

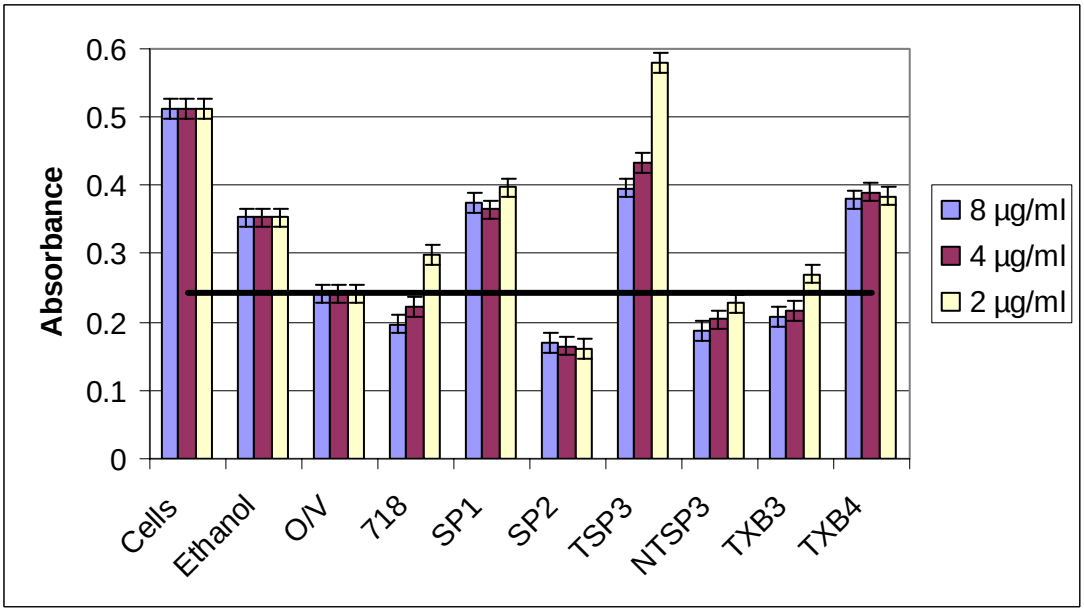


Figure 15. Cytotoxicity of *Karenia brevis* species at 25°C and 35ppt.

For cultures grown at 20°C and 27ppt, three species showed significant toxicity to the N2A cells. Cultures 718, SP2, NTSP3 and TXB3 all showed 30-50% more toxicity than the positive control response of O/V (Figure 16). A dose response curve was observed for TXB4 at this temperature and salinity. At 20°C and 35ppt, there was significant toxic response when compared to controls from 718, SP1, NTSP3 and TXB4. Also, SP2 showed toxic responses for 8 and 4 µg/ml and the toxicity at 2µg/ml was slightly less but within the standard error (Figure 17). Dose response curves were determined for NTSP3 and TXB4 at this temperature and salinity.

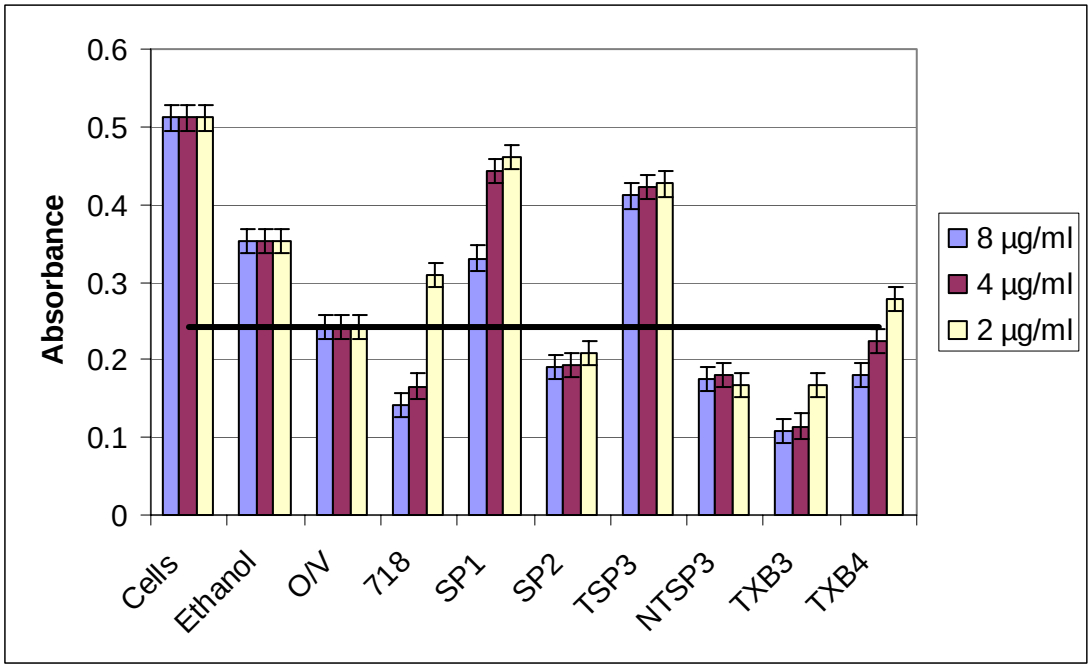


Figure 16. Cytotoxicity of *Karenia brevis* species at 20°C and 27ppt.

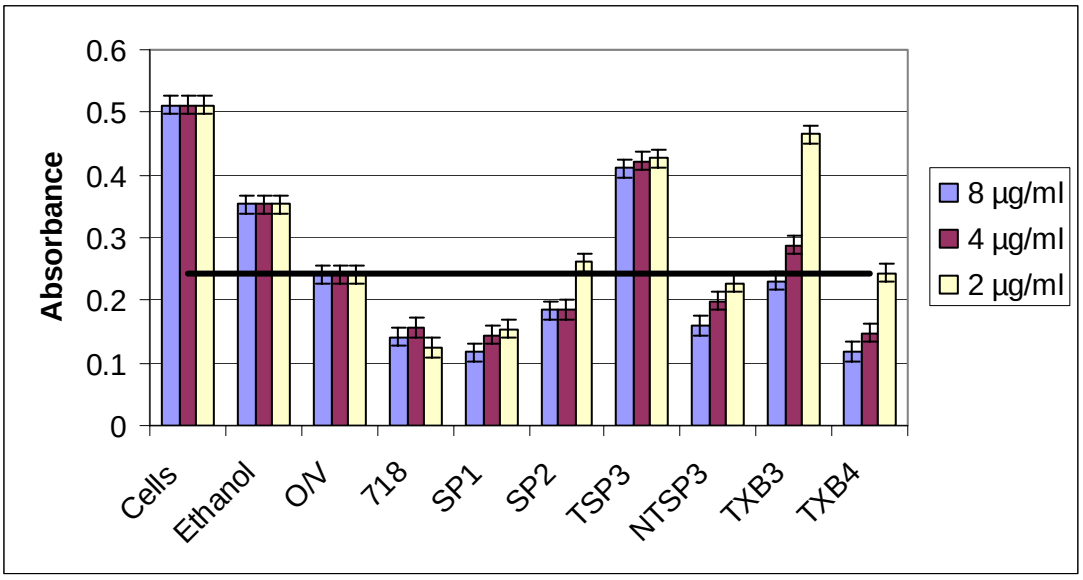


Figure 17. Cytotoxicity of *Karenia brevis* species at 20°C and 35ppt.

At 25°C the toxicity of species of *Karenia brevis* was more obvious at 35 ppt. Species 718 and SP2 showed toxic responses that were not seen in cultures grown at 27 ppt. Similarly, for 20°C the toxic response of species was more pronounced at 35 ppt for 718 and SP1. SP1 showed significant increase in toxicity when compared to cultures grown at 27 ppt. Interestingly, at this salinity (35ppt) NTSP3, TXB3, and TXB4 showed reduced toxicity when compared to cultures grown at 27ppt. The 718 species showed toxic responses at two or more doses at all temperatures and salinities tested in this assay except at 25°C and 27ppt. NTSP3 produced a toxic response at all temperatures and salinities tested in this assay. SP1 elicited the most toxic response at 20°C and 35ppt, however there was not an obvious pattern for toxicity based on temperature or salinity for this species. Similarly, SP2 elicited the most toxic response at 25°C and 35ppt, but

there was not an obvious correlation of toxicity with any one temperature or salinity. TXB3 showed the most toxic responses at 20 and 25°C when the salinity was held at 27ppt. This same species at higher salinity (35ppt) did not produce as significant a response. Similarly, for TXB4 there was a toxic response observed when the temperature was held at 20°C and varying salinities, while at 25°C there was not a toxic response observed at either salinity tested. TSP3 was the only species that did not elicit a toxic response at any of the salinities or temperatures tested.

Chemical Analysis

The chemical analyses preformed in this study determined which species of brevetoxin were present in each of the cultures tested. The MALDI-TOF technique detected brevetoxin species at femtamole quantities of molecules. Although nine standard brevetoxins were scanned for in each of the cultured species only one brevetoxin was identified in the cultures, PBTX-2 (data not shown), using the MALDI-TOF method. There were not any other forms of brevetoxin detected. Nor was brevenal, the brevetoxin inhibitor, detected in the cultured species. PBTX-2 was detected only for the TXB4 culture at all temperatures and salinities. NTSP3 (20°C and both 27 and 35ppt) had detectable amounts of PBTX-2 present. SP2 at 20°C and 27ppt had detectable amounts of PBTX-2, however other variations of salinity and temperature did not yield any other species of brevetoxin or brevenal detectable in this method of analysis. The chemical analyses did not correspond well with the observed cytotoxicity suggesting that PBTX was not the only contributor to overall toxicity.

Data from the LC/MS method detected several other brevetoxin species present in the same cultures as were tested by the MALDI-TOF method as seen in Table 10. PBTX- 1, 2, 3, 7, 11, and brevenal were all detected in the cultures grown at varied temperature and salinity. PBTX- 6, 9, and –ca were not detected in any of the cultures regardless of temperature or salinity. Brevenal, the brevetoxin inhibitor was detected in all the cultures except SP1 grown at 25°C and 27ppt, SP2 and TSP3 grown at 20°C and 27ppt, and SP2 grown at 20°C and 35ppt.

Risk Assessment

A reference dose was calculated using the bioassay based no-observable-adverse-effects-level (NOAEL) from cytotoxicity assay (2µg/ml) to calculate the risk due to inhalation of toxin aerosols in the beach environment. The reference dose is adjusted using body weight and inhalation rate. A reference dose was calculated based on the following equation:

$$RfD_{B\text{-}Inh} = NOAEL / (UF_1 * UF_2 * UF_3 * MF) \qquad \text{Eqn 4.1}$$

where NOAEL was 2000 mg/m^3, the uncertainty factor UF_1 is 10 for variations in population sensitivities, UF_2 is 10 for extrapolation from animals to humans, UF_3 is 10 for using a subchronic NOAEL instead of a chronic NOAEL, and MF was a modifying factor (10) based on a qualitative professional assessment of the uncertainties in this study. The scenario used in the risk assessment was a recreational exposure for adults and children. The exposure factors used to calculate the risk are listed in Table 9. The

concentration of PBTX-2 in air was assumed to be 2.7E-5 mg /m^3 based on work reported by Cheng et. al (2005) which evaluated particle size distribution, concentration and toxic profile for PBTX in a beach environment. This value was the highest daily mean observed during the previously mentioned study (Cheng, Y. S. et al., 2005). The risk was calculated using the following equation:

$$Risk = [EPC * IR * ET * EF * ED * FC / (BW * AT_{NC})] / RfD_{B\text{-}Inh} \quad \text{Eqn 4.2}$$

where EPC is the expected concentration, IR is the ingestion rate, ET is the exposure time, ED is the exposure duration, FC is the fraction from the contaminated source, BW is the body weight, AT_{NC} is the averaging time from non-carcinogens, and $RfD_{B\text{-}Inh}$ is the bioassay based reference dose for inhalation developed in this study (Table 9).

Table 9. Exposure factors used in HHRA.

Adult			
Abbreviation	**Factor**	**Value**	**Units**
IR	Inhalation Rate	0.833	m^3/hour
EF	Exposure Frequency	56	days/year
ED	Exposure Duration	30	yr
BW	Body Weight	70	kg
AT_{NC}	Averaging Time - Non-carcinogen	10950	days
FC	Fraction from Contaminated Source	1	unitless
ET	Exposure Time	6	hours/day
Child			
Abbreviation	**Factor**	**Value**	**Units**
IR	Inhalation Rate	0.5	m^3/hour
EF	Exposure Frequency	56	days/year
ED	Exposure Duration	6	yr
BW	Body Weight	15	kg
AT_{NC}	Averaging Time - Non-carcinogen	2190	days
FC	Fraction from Contaminated Source	1	unitless
ET	Exposure Time	6	hours/day

Discussion

Cytotoxicity Assays

Each of the tested clones has been reported to be capable of producing different forms of brevetoxin and are found in typical algal blooms in the Gulf of Mexico (Baden, D. G., 1989). Each clone was evaluated in the bioassay and toxin profiles were examined. It is known that brevetoxins exert their effects by interaction with voltage sensitive sodium channels in excitable membranes (Dickey, R. et al., 1999; Manger, R. L. et al., 1995; Manger, R. L. et al., 1993). The brevetoxins responsible for NSP bind to a certain class of biological receptors namely to voltage-sensitive Na^+ channels. This

highly specific interaction with naturally occurring receptors forms the basis of this bioassay. Detection is based on the functional activity of the toxin rather than on recognition of a structural component. Moreover, the affinity of a toxin for its receptor is directly proportional to its toxic potency. Thus, for a mixture of toxins (i.e. PBTX-2 and PBTX-3), a receptor-based assay will yield a response representative of the integrated potencies of those toxins present (Cembella, A. D. et al., 1995). This neuroblastoma cell assay cannot distinguish between individual brevetoxins (Hua, Y. et al., 1995). Any modification to a toxin molecule that would alter its ability to bind to the receptor and thus its detection in a receptor-based assay, compromises its ability to elicit a toxic response. Another consideration is the toxicity of other components in the cultures that could cause cytotoxicity. The results from the bioassay analysis showed some toxicity even when brevetoxin was not detected in the culture (i.e. TXB3, Figures 14, 15, 16). Therefore, some other component caused cytotoxicity in the cells.

At a temperature of 25°C toxicity was observed in cultures SP1, NTSP3, and TXB4 when the salinity was 27ppt (Figures 14 and 15). When the salinity was increase to 35ppt at this same temperature those cultures (SP1, NTSP3, and TXB4) showed a decrease in toxicity. However, for the SP2 culture, toxicity increased at the higher salinity of 35ppt. These results indicate that for the the species SP1, NTSP3, and TXB4 the brevetoxin produced by these cultures was less at 35ppt. For the SP2 culture, the increase in toxicity at 35 ppt would suggest that an increase in salinity could affect strains of *Karenia brevis* differently. Therefore, in a bloom of mixed cultures there

could be different interactions due to temperature and salinity that affect the overall toxicity.

For cultures grown at 20°C, the species 718, SP1, and TXB4 showed an increase in toxicity at 35ppt when compared to 27ppt (Figure16 and 17). At this temperature (20°C) there was more toxicity observed for cultures SP2, NTSP3, and TXB3 at 27ppt. These results indicate that the effects of temperature and salinity can be different depending on the different species of *Karenia brevis.* The toxic effects of the same cultures were different at the temperatures and salinities in this study. For the same cultures (SP1, NTSP3, and TXB4) at 25°C the toxicity decreased as salinity increased. However, at 20°C, as salinity increased the toxic response increased in some cases (718, SP1, and TXB4). This would indicate a definite relationship between temperature and salinity that affects the amount of toxin produced. Other researchers have shown differential physiologic effects with many of the natural toxins and their derivatives. Researchers have hypothesized that metabolism or modification of toxin structure modulates both the specific toxicity and potentially the molecular mechanism of toxic action (Baden, D. G. et al., 2005). Similarly, the salinity and temperature could affect the specific toxicity or the mechanism of toxic action as seen in this study.

This assay was also run using two additional cell lines; Clone 9 rat liver cells (ATCC, CRL-1439, passage 17) and a human neuroblastoma cell line (ATCC, CRL-2266, passage 23). These two cell lines showed no toxic response to any of the cultures tested (data not shown). The Clone 9 cell line and the human neuroblastoma cell lines

may not have sodium channels that are affected in the same manner as the mouse neuroblastoma cell line. These toxins may not affect these two cell lines or the response was too low to detect given the sensitivity of the assay.

The cytotoxicity results were used to determine the NOAEL for those cultures with demonstrated toxic effects. Nine brevetoxin standards (PBTX-1, 2, 3, 6, 7, 9, 11, -ca, and brevenal) where analyzed for in each extracted culture. A bioassay based reference dose for PBTX-2, the brevetoxin species detected in all of the cultures, to be used in human health risk assessment was developed using a NOAEL of 2µg toxin/ml. The NOAEL was derived from the results of the cytotoxicity assay dose response curves in this study. HHRAs for non-carcinogens compare environmental exposure concentrations to a reference dose that is based on the NOAEL.

Chemical Analysis

Analytical techniques for the identification of the brevetoxins and determining quantify the toxins are limited by availability of standards and the need for advanced analytical methods. The MALDI-TOF method only identified PBTX-2 in some of the cultures examined. Initial results using the LC/MS method revealed all except three (PBTX-6, -9, -ca) of the nine brevetoxin standards looked for to be present in some or all of the cultures (Table 10). The brevetoxins identified for each of the algal species and their respective culture conditions are summarized in Table 10. No distinct pattern of brevetoxin production based on temperature and salinity of these cultures was revealed. Temperature and salinity had no effect of the production of PBTX-6, -9, and –ca which

were not detected in any of the cultures (Table 10). Similarly, temperature and salinity had no effect on the production of PBTX-2, one of the toxins found in all the cultures tested. Brevenal was detected in all except four of the cultures tested (Table 10), yet there was observed toxicity in the cytotoxicity assay in these four cultures (Figures 14-17). Brevenal is supposed to be a natural inhibitor of brevetoxins. That toxicity was observed in the presence of this blocking agent indicates that there is another compound or mechanism that is also eliciting toxicity in these cultures. Initial results using this LC/MS method also revealed two unidentified peaks that are being further examined (data not shown).

Risk Assessment

Karenia brevis produces brevetoxins that are readily aerosolized by wave action releasing the toxins into the air. The most likely immediate route of exposure for humans is inhalation. Similarities in the mechanism of toxic action of NSP and the inhalation of aerosolized brevetoxin justify the bioassay based reference dose for inhalation. A reference dose was developed for an inhalation scenario to evaluate the risk to humans from the inhalation of aerosolized brevetoxins. The reference dose for PBTX-2 aerosols was 0.0571 mg / kg-day, derived from the NOAEL divided by the appropriate uncertainty and modifying factors. An estimated aerosol concentration of concentration of 2.7E-5 mg / m^3 was used based on work done by Cheng et.al (2005). For an adult in a recreational setting the inhalation exposure scenario risk was estimated to be 5.17E-6 while that for a child was 1.45E-5. Both estimates are well below the

Table 10. Brevetoxin species identified by LC/MS in *Karenia brevis* cultures at various temperatures and salinities.

Species	PBTX-1	PBTX-2	PBTX-3	PBTX-6	PBTX-7	PBTX-9	PBTX-11	PBTX-ca	Brevenal
MW	867	895	897	911	869	899	867	911	657
Retention Time	45.5	43.99	42.12	41.74	43.7	42.62	46.39	42.6	41.23
ID	CID 1	CID 2	CID 3	CID 6	CID 7	CID 9	CID 11	CID Ca	CID Brev
25°C, 27ppt									
718	Y	Y	Y	N	Y	N	Y	N	Y
SP1	N	Y	Y	N	N	N	N	N	N
SP2	Y	Y	Y	N	Y	N	Y	N	Y
TSP3	Y	Y	Y	N	Y	N	N	N	Y
NTSP3	Y	Y	Y	N	Y	N	N	N	Y
TXB3	Y	Y	Y	N	Y	N	N	N	Y
TXB4	?	Y	Y	N	?	N	N	N	Y
25°C, 35ppt									
718	?	Y	Y	N	N	N	N	N	Y
SP1	N	Y	N	N	N	N	N	N	Y
SP2	Y	Y	Y	N	Y	N	Y	N	Y
TSP3	Y	Y	Y	N	Y	N	N	N	Y
NTSP3	Y	Y	Y	N	Y	N	N	N	Y
TXB3	Y	Y	Y	N	Y	N	Y	N	Y
TXB4	Y	Y	Y	N	Y	N	Y	N	Y
20°C, 27ppt									
718	Y	Y	Y	N	Y	N	N	N	Y
SP1	Y	Y	N	N	N	N	N	N	Y
SP2	Y	Y	Y	N	Y	N	Y	N	N
TSP3	Y	Y	Y	N	Y	N	Y	N	N
NTSP3	Y	Y	Y	N	Y	N	Y	N	Y
TXB3	Y	Y	Y	N	Y	N	N	N	Y
TXB4	Y	Y	Y	N	Y	N	Y	N	Y
20°C, 35ppt									
718	Y	Y	Y	N	Y	N	N	N	Y
SP1	Y	Y	Y	N	N	N	Y	N	Y
SP2	Y	Y	Y	N	Y	N	N	N	N
TSP3	Y	Y	Y	N	Y	N	Y	N	Y
NTSP3	Y	Y	Y	N	Y	N	Y	N	Y
TXB3	Y	Y	Y	N	Y	N	N	N	Y
TXB4	Y	Y	Y	N	Y	N	Y	N	Y
Y- Present in culture									
N- Not present in culture									
?- Not clearly identified									

hazard index of 1.0 indicating that no adverse effects to humans at the concentration encountered in the evaluated scenario. While this attempt at calculating the human health risk from exposures to aerosolized brevetoxin shows no risk at the concentration tested here, this is a first attempt at calculating a reference dose for these toxins. The reference dose is traditionally calculated from a chronic animal study but the US Environmental Protection Agency (USEPA) has made provisions in the Risk Assessment Guidelines for Superfund (RAGS) to account for reference doses calculated from acute testing (USEPA, 1989). The bioassay based reference dose for inhalation developed in this study does provide a first step at evaluating human health effects from something other than the current bed closure or tissue concentration guidelines set up for the ingestion route of exposure.

CHAPTER V

CONCLUSIONS

By comparing the response of single PAH compounds and binary mixtures of PAHs, some insight was gained into how the structure of the compound can affect the carcinogenic process. Structural differences can influence the carcinogenicity by simple effects on cellular uptake to metabolism and binding with critical proteins in the cell. We observed results that were not expected in some instances based on the bay region structure of some of the compounds. Similarly, it was determined that compounds in mixtures affect each other producing a less than additive response (i.e. BAP mixed with 5-methylchrysene); although additivity is a common assumption in the practice of risk assessment. These responses reveal highly complex interactions that complicate predicting toxicity of PAHs or PAH mixtures.

Attempts to define toxicity of PAHs and PAH mixtures relative to BAP have been complicated by the fact that some carcinogenic PAHs are capable of both initiating and promoting tumors (ATSDR, 1990a). Benzanthracene, chrysene, and BAP are reported to both initiate and promote (ATSDR, 1996). The capability of these compounds to act via a distinct pathway to produce cancer results adds a great deal of uncertainty when attempting to predict toxicity based solely on responses in biological systems. For this reason, the focus of this research was on three different assays each specific to a part of the carcinogenic process. By evaluating a chemical's response in each assay, an estimate of toxicity can be based on the observed response in each bioassay. Taking into consideration the response of a chemical in each step of the

carcinogenic process (i.e. activation, initiation, and promotion), the mechanism, and toxic action of each chemical can be approximated. Together these responses could be used to develop a bioassay based toxicity factor.

The lack of previous success in using a ranking system like TEFs or PP_B for carcinogenic risk assessment of PAHs may be due to the complexity of behaviors exhibited among PAHs. Success with the use of TEFs for dioxin-like compounds is due in part to toxicity being caused by a single, well-defined endpoint of AhR affinity. The toxicity of PAHs and PAH mixtures may best be predicted by deriving a TEF or PP_B from bioassays that measure specific toxic endpoints of the carcinogenic process while taking into consideration relevant physiochemical properties as well as structural properties of these compounds. This study makes such estimates for PAHs using QSTR models that incorporate toxic responses for activation, initiation, and promotion stages of carcinogenesis, as well as structural properties of the compounds. The predicted TEFs in this study provided a more precise estimate for PAH toxicity because they are based on specific toxic endpoints and are not order of magnitude estimates. Using bioassay data and physiochemical properties to build QSTRs for classes of compounds which lack sufficient toxicity data will allow for more comprehensive risk assessments to be completed. This method also reduces the time and expense of laboratory experiments for all the members of a class of chemicals.

This study developed QSTRs for three bioassays relating to specific endpoints important in the carcinogenic process for PAHs. Application of QSTRs for assessing toxicity in a variety of bioassays can provide a reliable estimate of dose ranges for

animal testing and for toxicity factors for human health risk assessment. QSTRs can also be used to prioritize costly and time intensive experimentation by predicting those compounds most likely to cause adverse effects. There are limits to this approach for predicting toxicity from structure activity relationships. It is important to train a QSAR model with chemicals within the same family and realize that the predictive power can only be applied within the family. Also important is the quantity and diversity of the data used to create the model. As much data as is available for each toxic endpoint should be used from as many compounds as possible from within the family. The addition of more data to the training set for each bioassay will increase the explanatory and predictive power of each model. Realizing the limitations of this procedure and carefully designing and training, the models can result in a robust QSTR that can lead to a more effective way to estimate toxicity and predict toxic response. Using QSTRs developed from experimental data in this study allowed for a bioassay based TEF prediction.

Until this study, there has been insufficient data on which to base a human health risk assessment for brevetoxin exposure through an inhalation pathway. Current risk management along the coasts of the Gulf of Mexico, is based on shellfish bed closures at 5000 *K. brevis* cells / liter with reopening based on determination of PBTX in shellfish at <80 µg/100 g. Methods to develop a toxicity factor for these toxins were necessary to provide the basis of a risk assessment for brevetoxins. Cell based detection methods for brevetoxins similar to the one used in this study provide a convenient method to evaluate biological activity of algal toxins. Although *in vitro* methods cannot replace entirely the

data derived from animal studies, these methods do offer the potential to reduce cost, time, and the reliance upon animal testing. This study employed a widely used cytotoxicity assay to predict an initial toxicity factor for one of the most prevalent forms of brevetoxin (PBTx-2). The reference dose developed is a first attempt that provides a starting point for predicting human health effects from aerosolized brevetoxins.

Effects of the various exposure routes on humans are difficult to assess because toxicity data for brevetoxins are limited. There are some acute studies in mice and data from poisoning cases in humans and (marine) mammals that are available but data on acute dermal and inhalation studies are lacking. In addition, analytical reference materials are important to further develop and improve the analytical methodology and to allow analytical quality assurance. Currently, varieties of obstacles stand in the way of reliable assessment of brevetoxin occurrence and exposure and thus the establishment of meaningful regulations. In this study a bioassay based RfD was developed for PBTX-2 and an initial HHRA was done to estimate the risk associated with exposure to inhaled aerosolized brevetoxin. The developed RfD was used to calculate a non-carcinogenic risk for adults and children in a recreational scenario. Using the developed RfD, the result observed was that there was no risk due to the inhaled aerosolized brevetoxin for the concentration used in this scenario. This RfD was a bioassay based reference dose however, it provides an initial estimate to complete a HHRA.

Risk assessment in general is a science that can be improved upon for many classes of chemicals. Chemical analysis, bioassays, and QSARs can be used to improve the reliability and reduce the uncertainty in a risk assessment. The combination of these

techniques to develop improved methods of toxicity assessment is a promising avenue for those classes of chemicals that are currently lacking sufficient toxicity data.

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APPENDIX A

GJIC Statistical Analysis

Error						
	10uM	5uM	2uM	1uM	0.5uM	DMSO
5-MethylChrysene	0.002926	0.003118	0.003048	0.003722	0.004146	0.004659
Anthracene	0.007044	0.01282	0.009483	0.008985	0.005957	0.004659
Benz(a)anthracene	0.00389	0.007859	0.006742	0.00235	0.003743	0.004659
BAP	0.001696	0.003048	0.005957	0.01282	0.007235	0.004659
Chrysene	0.003838	0.00643	0.004511	0.000972	0.002487	0.004659
Phenanthrene	0.01143	0.007235	0.003582	0.00238	0.002406	0.004659

Error						
	10uM	5uM	2uM	1uM	0.5uM	DMSO
BAP(10uM)/Chrysene	0.0137	0.0119	0.0161	0.01421	0.0045	0.0081
Chrysene(10uM)/BAP	0.011	0.01266	0.008	0.00374	0.00309	0.0081
BAP(10uM)/5-Methylchrysene	0.0056	0.00159	0.0013	0.00453	0.00794	0.0081
5-Methylchrysene(10uM)/BAP	0.0026	0.00187	0.0005	0.00105	0.00284	0.0081
Chrysene	0.0016	0.00132	0.0045	0.00794	0.01421	0.0081
5-MethylChrysene	0.011	0.01266	0.008	0.00374	0.00453	0.0081
BAP	0.0011	0.00284	0.0081	0.01421	0.0045	0.0081

EROD Statistics

BAP	
Mean	436.4652
Standard Error	8.930544
Median	447.56
Mode	#N/A
Standard Deviation	65.62583
Sample Variance	4306.749
Kurtosis	-0.49572
Skewness	-0.37711
Range	255.51
Minimum	306.14
Maximum	561.65
Sum	23569.12
Count	54
Largest(1)	561.65

Smallest(1)	306.14
Confidence Level(95.0%)	17.9124

Phen	
Mean	417.9332
Standard Error	6.35214
Median	404.01
Mode	#N/A
Standard Deviation	44.91641
Sample Variance	2017.484
Kurtosis	-0.00619
Skewness	1.01274
Range	164.69
Minimum	364.58
Maximum	529.27
Sum	20896.66
Count	50
Largest(1)	529.27
Smallest(1)	364.58
Confidence Level(95.0%)	12.7651

5-MC	
Mean	369.1677
Standard Error	7.208234
Median	372.04
Mode	#N/A
Standard Deviation	42.64448
Sample Variance	1818.552
Kurtosis	-0.89621
Skewness	0.017192
Range	156.46
Minimum	311.08
Maximum	467.54
Sum	12920.87
Count	35
Largest(1)	467.54
Smallest(1)	311.08
Confidence Level(95.0%)	14.64889

DMSO	
Mean	314.585
Standard Error	4.947319
Median	301.69
Mode	#N/A
Standard Deviation	32.06229
Sample Variance	1027.99
Kurtosis	-0.39328
Skewness	0.926212
Range	116.31
Minimum	275.16
Maximum	391.47
Sum	13212.57
Count	42
Largest(1)	391.47
Smallest(1)	275.16
Confidence Level(95.0%)	9.991313

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	2322	387.0641	3876.4
Column 2	6	1502	250.3554	9.7E-28

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	56067.77	1	56067.77	28.9277	0.000311	4.964603
Within Groups	19382.01	10	1938.201			
Total	75449.78	11				

Tukeys Test

M1	476.9574
M2	250.3554
Msw	19382.01
n	6

Statistic=	3.986941
critical value	4.91

BAP (10uM)/5-MC (10uM)	
Mean	476.9574
Standard Error	7.076193
Median	482.885
Mode	481.86
Standard Deviation	64.85438
Sample Variance	4206.09
Kurtosis	0.198314
Skewness	-0.3067
Range	294.46
Minimum	344.61
Maximum	639.07
Sum	40064.42
Count	84
Largest(1)	639.07
Smallest(1)	344.61
Confidence Level(95.0%)	14.07426

Chryene 10uM)/BAP (10uM)	
Mean	420.2751
Standard Error	9.1573
Median	398.6
Mode	#N/A
Standard Deviation	77.16079
Sample Variance	5953.787
Kurtosis	5.942661
Skewness	2.170431
Range	407.55
Minimum	335.28
Maximum	742.83
Sum	29839.53
Count	71
Largest(1)	742.83
Smallest(1)	335.28
Confidence Level(95.0%)	18.26366

BAP (10uM)/5-MC (1uM)	
Mean	348.7192
Standard Error	4.159283
Median	344.725
Mode	#N/A
Standard Deviation	28.81636
Sample Variance	830.3824
Kurtosis	3.313031
Skewness	1.505429
Range	130.15
Minimum	313.83
Maximum	443.98
Sum	16738.52
Count	48
Largest(1)	443.98
Smallest(1)	313.83
Confidence Level(95.0%)	8.367397

5-MC (10uM)/BAP (1uM)	
Mean	347.237
Standard Error	4.13452
Median	343.425
Mode	#N/A
Standard Deviation	28.04168
Sample Variance	786.3359
Kurtosis	-0.65609
Skewness	0.175493
Range	106.36
Minimum	295.5
Maximum	401.86
Sum	15972.9
Count	46
Largest(1)	401.86
Smallest(1)	295.5
Confidence Level(95.0%)	8.327351

Chrysene (10uM)/BAP (1uM)	
Mean	419.9706
Standard Error	14.17117
Median	460.33
Mode	#N/A
Standard Deviation	97.15265
Sample Variance	9438.637
Kurtosis	-1.87709
Skewness	0.067739
Range	252.61
Minimum	315.68
Maximum	568.29
Sum	19738.62
Count	47
Largest(1)	568.29
Smallest(1)	315.68
Confidence Level(95.0%)	28.52508

BAP (10uM) /Chrysene (1uM)	
Mean	309.2254
Standard Error	4.821829
Median	300.215
Mode	#N/A
Standard Deviation	34.09548
Sample Variance	1162.502
Kurtosis	2.351046
Skewness	1.477162
Range	152.08
Minimum	270.09
Maximum	422.17
Sum	15461.27
Count	50
Largest(1)	422.17
Smallest(1)	270.09
Confidence Level(95.0%)	9.689828

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Row 1	2	867.9878	433.9939	2343.699
Row 2	2	732.2671	366.1336	300.6438
Row 3	2	984.9206	492.4603	1252.348
Row 4	2	786.9811	393.4905	3693.639
Row 5	2	865.278	432.639	432.5206
Row 6	2	702.314	351.157	648.7717
Row 7	2	469.3907	234.6953	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	80738.76	6	13456.46	10.86247	0.003007	3.865969
Within Groups	8671.621	7	1238.803			
Total	89410.38	13				

Tukeys Test

M1	393.4905
M2	432.639
Msw	1238.803
n	2
Statistic=	1.572999
critical value	3.34

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