ABSTRACT

Investigating the Mixture Effects of Polycyclic Aromatic Hydrocarbons through *In Vitro* Toxicity Assessments using Clone-9 Liver Cells

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The Environmental Protection Agency is reevaluating current methods for assessing the toxicity of polycyclic aromatic hydrocarbons (PAHs), including the assumption of toxic additivity in mixtures. This study was aimed at testing mixture interactions using select PAH congeners through *in vitro* cell culture experimentation, and modeling the toxicity using quantitative structure-activity relationships (QSAR). Clone-9 rat liver cells were used to analyze cellular proliferation, viability, and genotoxicity of 15 PAHs in single doses and in binary mixtures. Tests revealed that many mixtures have non-additive toxicity, but display varying mixture effects depending on the mixture composition. QSARs were developed using viability data to predict toxic activity both in single PAH congeners and in binary mixtures. Effective concentrations inhibiting 50% of the cell populations were successfully modelled, with r^2= 0.90, 0.99 and 0.84, respectively. Our findings suggest that PAH mixtures have complex interactions, and continued mixture research will strengthen toxicological assessments of PAHs.

Investigating the Mixture Effects of Polycyclic Aromatic Hydrocarbons through *In Vitro* Toxicity Assessments using Clone-9 Liver Cells

by

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A Thesis

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Accepted by the Graduate School

December 2014

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LIST OF ABBREVIATIONS

AHH	Aryl Hydrocarbon Hydroxylase
AhR	Aryl Hydrocarbon Receptor
AO	Acridine Orange
ATSDR	Agency for Toxic Substances & Disease Registry
BaP	Benzo[a]pyrene
BCF	Bioconcentration Factors
CERCLA	Comprehensive Environmental Response, Compensation, and Liability Act
CFDA	5-Carboxyfluorescein Diacetate Acetoxymethyl Ester
COC	Chemical of Concern
CYP	Cytochrome P450 Monooxygenase
DAPI	4’,6-Diamidino-2-Phenylindole Dihydrochloride
DDR	DNA Damage Response
DLC	Dioxin-like Compound
DMSO	Dimethyl Sulfoxide
EC_{50}	Effective Concentration for 50% of test Organisms
ECFP	Extended Connectivity Fingerprint
EPA	U.S. Environmental Protection Agency
GFA	Genetic Function Approximation
GJIC	Gap Junction Intercellular Communication
HHRA	Human Health Risk Assessment

IARC	International Agency for Research on Cancer
JG	Janus Green B Dye
K_{ow}	Octanol/Water Partitioning Coefficient
K-9	Clone 9 Rat Liver Cells
LC_{50}	Lethal Concentration for 50% of test Organisms
LDH	Lactate Dehydrogenase
LOAEL	Lowest Observed Adverse Effect Level
MCL	Maximum Contaminant Level
mg	Milligram
MGP	Manufactured Gas Plant
MN	Micronucleus
NOAEL	No Observed Adverse Effect Level
NIOSH	National Institute for Occupational Safety and Health
NPL	National Priorities List
OSHA	Occupational Safety and Health Administration
PAH	Polycyclic Aromatic Hydrocarbon
PEL	Permissible Exposure Level
PM	Particulate Matter
PPB	Parts Per Billion
PPM	Parts Per Million
QSAR	Quantitative Structure Activity Relationship
RCRA	Resource Conservation and Recovery Act
REACH	Registration, Evaluation, Authorization, and Restriction of Chemicals

REL	Recommended Exposure Limit
RfD	Reference Dose
ROS	Reactive Oxygen Species
RPF	Relative Potency Factor
SRM	Standard Reference Material
STEL	Short-Term Exposure Limit
TEF	Toxic Equivalency Factor

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DEDICATION

To my parents, Barry and Yvette Gray, and to my new husband Christopher Gaskill. This journey has been strengthened by your endless encouragement and love.

CHAPTER ONE

Introduction

There are thousands of chemical compounds that have been identified in environmental matrices, and most of them have deficient toxicity information.[1] PAHs are a class of over a hundred parent compounds and various substituted congeners. Polycyclic aromatic hydrocarbons (PAHs) are among the dominant species of environmental pollutants and exhibit mutagenic and carcinogenic toxicity in animal and epidemiological studies.[2] PAHs are cyclic hydrocarbons containing two or more fused rings. The count and configuration of the rings cause variations in the chemical properties and environmental partitioning of each congener.[3] Many PAHs have structural bay regions and fjord regions that can affect metabolic activity. These regions can cause electrostatic attractive or repulsive forces near the active sites of enzymes.[4]

Sources

PAHs are released into the atmosphere via natural and anthropogenic mechanisms that involve high pressure or temperature reactions.[5] They are naturally emitted by volcanic activity and wildfires.[6] PAHs are present in many natural fossil fuel mixtures, such as coal and oil, and are released into the environment by the incomplete combustion of fossil fuels or other organic materials.[7] Oil spills also contribute significantly to the release of PAHs into environmental matrices.[8] The burning of coal, gas, wood, or tobacco is likely to produce PAHs. The most common anthropogenic sources of PAHs include automobile exhaust, industrial processes, grilling foods, and the incineration of

waste. Industrial processes that release PAHs include coal tar, coke and asphalt production.[9] Crude oil contains about 1 to 3% of PAH mixtures.[10] Of that PAH portion, as much as 90% has been recorded to be methylated congeners.[10] PAHs can also be present in small amounts in dyes, plastics, personal care products, medicines and pesticides.[11] PAHs are made up of fused benzene rings, without any substituted molecules or moieties (table 1.1). Substituted congeners may contain structural oxygen, nitrogen or functional groups that deviate from the pure hydrocarbon backbone. Methylated PAH congeners – also known as alkyl homologs- are common PAHs that can have multiple alkyl group substitutions branching from the ring structures.[1] Methylated congeners are very common in petroleum products, and may outnumber the parent congeners.[12] Contamination in soils and sediments are often caused by industrial spills or dumping, tire degradation, oil leakage, coal tar sealants and creosote contact.[13] Contamination of water and sediment may occur from marine creosote sites, oil spills, boat leakage, and motor oils.[14]

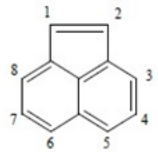
The composition of environmental PAH mixtures may vary based on two major sources: petrogenic or pyrogenic emissions. Recent studies have observed correlations in the composition of PAH mixtures with their origin, including the presence or absence of congeners and chemical ratios (see tables A.1-A.4 in Appendix). Other factors that may contribute to the variation in environmental mixtures include location, presence of human development, proximity to emission sources, degradation, deposition, and climate.[3]

Chemistry

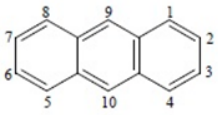
Figure 1.1 shows the structures of various parent and methylated PAH congeners. PAHs have the ability to persist, bioaccumulate, and cause toxicity. Methylated PAHs

Table 1.1 Various physical and chemical properties of common PAHs.[15]

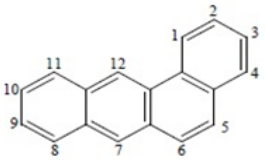
CAS #	Chemical	Abbr.	Rings	Molar Mass (g/mol)	Water Solubility (g/m^3)	IARC Cancer Group	Log K_{ow}
83-32-9	Acenaphthene	AN	3	154.2	3.8	3	3.92
208-96-8	Acenaphthylene	ACY	3	152.2	16.1		4.07
120-12-7	Anthracene	AC	3	178.2	0.045	3	4.50
56-55-3	Benz[a]anthracene	BaA	4	228.3	0.011	2B	5.91
50-32-8	Benzo[a]pyrene	BaP	5	252.3	0.0038	1	6.06
205-99-2	Benzo[b]fluoranthene	BbF	5	252.3	0.0015	2B	5.78
192-97-2	Benzo[e]pyrene	BeP	5	252.3	0.004	3	6.44
191-24-2	Benzo[ghi]perylene	BgP	6	276.3	0.0003	3	6.78
207-08-9	Benzo[k]fluoranthene	BkF	5	252.3	0.008	2B	6.11
218-01-9	Chrysene	CH	4	228.3		2B	5.86
191-07-1	Coronene	Co	6	300.4	0.0001	3	6.50
53-70-3	Dibenz[a,h]anthracene	DB	5	278.4	0.0006	2A	6.75
191-30-0	Dibenz[a,l]pyrene	DBP	6	302.4		2A	7.71
206-44-0	Fluoranthene	Fla	4	202.3	0.26	3	4.90
86-73-7	Fluorene	FL	3	166.2	1.9	3	4.18
193-39-5	Indeno[1,2,3c,d]pyrene	IP	6	276.3	0.062	2B	6.58
91-20-3	Naphthalene	Nap	2	128.2	31.0	2B	3.37
85-01-8	Phenanthrene	Phe	3	178.2	4.57	3	4.52
129-00-0	Pyrene	Pyr	4	202.3	0.132	3	4.88
779-02-2	9-Methylanthracene	9-AC	3	192.3			5.07
1576-67-6	3,6-Dimethyl-phenanthrene	3,6-Phe	3	206.3			5.44
57-97-6	7,12-Dimethyl-benz[a]anthracene	7,12-BaA	4	256.3	0.05		5.80
781-43-1	9,10-Dimethylanthracene	9,10-AC	3	206.3			5.13



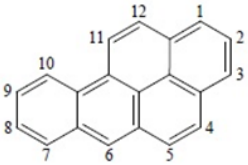
Acenaphthylene



Anthracene



Benz[a]anthracene



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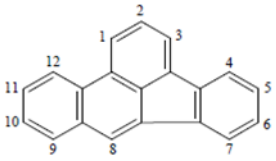

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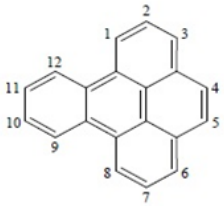
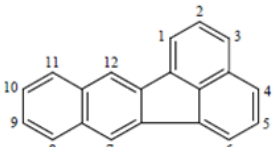
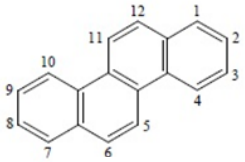


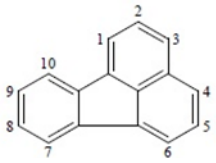
Benzo[e]pyrene



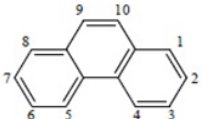
Benzo[k]fluoranthene



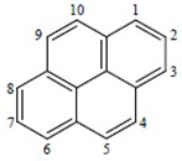
Chrysene



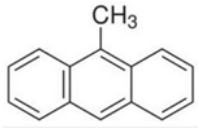
Fluoranthene



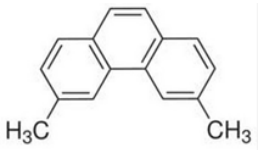
Phenanthrene



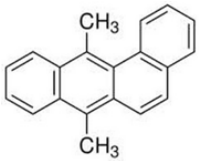
Pyrene



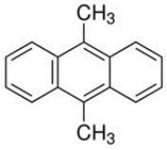
9-Methylanthracene



3,6-Dimethylphenanthrene



7,12-Dimethylbenz[a]anthracene



9,10-Dimethylanthracene

Figure 1.1 Common parent and methylated PAH congeners.[4]

are more persistent, bioaccumulative, and possibly more toxic than parent congeners.[16] As a class, they have high lipophilicity, high melting and boiling points, and low water solubility (table 1.1).[4] They are chemically stable, photodegradable, and interact with other environmental components such as nitrogen oxides or ozone.[17] PAHs have bioconcentration factors (BCF) values ranging from 10 to 10,000.[11] PAHs with four or fewer fused rings have high volatility and tend to remain gaseous in the atmosphere, whereas PAHs with more than four rings adsorb onto suspended particulate matter.[8] Consequently, PAHs are commonly sampled as PM.

Reason for Concern

PAHs are ranked #9 on the Agency for Toxic Substances and Disease Registry's (ATSDR) Substance Priority List of 2013.[11] Benzo[a]pyrene (BaP) is the most heavily studied PAH and is ranked #8.[11] BaP is classified as a known human carcinogen, the only congener in the group with that title and sufficient supporting data.[18] Several other PAHs were classified as probable or possible human carcinogens in 2010 by the International Agency for Research on Cancer (IARC), as seen in table 1.1.[13] PAHs have the potential to exhibit both cancer and non-cancer toxicological effects. PAHs are implicated for reproductive, cardiovascular, liver, and bone marrow toxicity as well as immunosuppression, birth defects, endocrine disruption and nervous system disorders.[19]

Approximately 600 of over 1400 national priority sites (NPL) are contaminated with high levels of PAHs.[11] Exposures are likely to occur in areas near municipal incinerators, coal gasification plants, wood treatment facilities, and smokehouses.[3] Furthermore, PAHs have been detected in environmental media, including stormwater runoff,

sediments, surface waters, air, indoor air, foodstuffs, paved terrains, and biological samples (table 1.2).[20] PAH concentrations have been increasing in freshwater sediments due to stormwater discharge from urbanization.[21] PAH concentrations are highly impacted by the characteristics of the sediments into which they are deposited.[20]

Human Exposures

Because PAHs are ubiquitous in the environment, humans are expected to come into contact with PAHs on a daily basis in food, air, dust, soil and water.[3] In addition, sources of PAHs raise concern for wildlife exposure, environmental contamination, and bioaccumulation.[8] Common exposure sources for humans are motor vehicle exhaust, food, water, and smoking.[3] Exposure can vary widely based on diet, lifestyle choices, and occupation.[3] Inhalation is often considered the most significant exposure route for occupationally exposed populations and smoking individuals. Ingestion is the dominant pathway for the general non-smoking, and non-occupationally exposed population. The public regularly encounters PAHs in the air they breathe, but they appear to be more concentrated in certain consumed foods and settled dust.[22] PAHs in food is caused by various food processes and storage practices that involve heat, such as toasting grains and wine preservation.

The average daily intake for the non-smoking population is approximately 0.16-1.6 µg from food, 0.027 µg from water, and 0.207 µg from urban air.[23] An adult male is expected to have a daily exposure rate of 3 µg of carcinogenic PAHs per day.[24] A 1991 assessment of BaP concentrations found that the average ingestion of BaP for the general U.S. population is 2.2 µg/day.[25] Before consumption, food may become contaminated through traditional food preparation and processing like baking, frying, roasting,

smoking, and drying.[9] Barbequed or smoked foods have an average of 100 µg/kg, and drinking water contains approximately 23 ng/L, with a range of 0.2-1000 ng/L.[26] An estimated 70% of PAH exposure occurs through food intake for the non-smoking individual.[27] In polluted air, the average concentration of BaP is 100 ng/cm^3.[4] Studies have found that non-smokers have BaP concentrations of approximately 0.025 ng/g in lung tissue originating from ambient air exposure.[28] Smokers have approximately 1.0 ng/g of BaP in lung tissue.[4]

Table 1.2 Total PAH concentrations in commonly encountered media, in ppm unless stated otherwise. The PAHs sampled are the most common identified PAHs (see tables A.1-A.4).

Exposure Source	Mixture Concentration (ppm)
Urban air [29]	19.30
Rural air [29]	1.20
Drinking water [30]	24.00 ppb
Average U.S. Diet [27]	2.00
Foodstuffs [31]	18.18
Charbroiled/Smoked Meat [3]	0.02
Grilled beef [32]	0.12
Smoked fish [3]	2.00 ppb
Non-coal tar Paved Lot [22]	14.10
Coal Tar Paved Lot [22]	4,816.60
House Dust [22]	27.40
House Dust (adjacent to coal tar lot)[22]	124.30
Urban Sediments[33]	20.00
Superfund Sediment (Eagle Harbor, WA) [34]	3.20

For the non-smoking individual, exposure from the air may come from wood stoves, home heating devices, vehicle and engine exhaust, incineration (garbage), or natural fires.[3] Indoor dust is a potential exposure source for the general population in households and work environments. Dust can be incidentally ingested or inhaled. PAHs in dust come from many origins like power generators, food preparation, tobacco smoke, nearby coal tar sources, and even home improvement products.[9] Some researchers claim that PAHs in

indoor environments may be more dangerous to human health because they do not degrade as quickly as outdoor environmental PAHs.[9] Furthermore, they gradually accumulate and become more concentrated in dust.[9]

Children are a susceptible subpopulation because their exposures are higher, and they are prone to adverse developmental and immunological effects. They have higher risk than adults because they touch many accessible items and put them in their mouths.[13] Children also have more contact with dusty floors. A recent study documented very high levels of PAHs in house dust near paved public areas.[13] Coal-tar based pavement sealants (CTS) are applied to driveways, parking lots, and playgrounds, are the source of high PAH concentrations in those areas.[22] Seven PAHs, classified as probable human carcinogens, were analyzed in house dusts from homes adjacent to lots with and without CTS. Results showed that homes adjacent to CTS lots contained significantly higher concentrations of PAHs in dust, including harmful carcinogens.[22]

Occupational exposures. Certain occupations can lead to PAH exposures that are higher than those encountered in the general population. Laborers in the following occupational categories are associated with high exposure risks: aluminum, asphalt, and black carbon production, coal gasification, oil refineries, roofing and road construction, rubber and tire manufacturing, among others.[2] The Occupational Safety and Health Administration (OSHA) has developed permissible exposure levels (PELs) of PAHs in the workplace (table 1.3).[35] The established exposure limits are not specific to each congener, since many lack sufficient data to establish reliable limits.[3] The established PEL for total PAHs is 0.2 mg/m^3 concentration in air.[35]

Table 1.3 Recommended PELs for PAHs, according to body weight. [3]

Chemical	Suggested Maximum Intake (mg/kg/day)
Acenaphthene	0.06
Anthracene	0.30
Fluoranthene	0.04
Fluorene	0.04
Pyrene	0.03

The health hazards that PAHs may pose to the general population and to susceptible subpopulations (laborers and children) is addressed in human health risk assessments established under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), which also manages NPL sites. CERCLA and RCRA are important legislations that give federal agencies the authority to manage, mitigate, and regulate the handling and release of hazardous substances. Human health risk assessments, remedial investigations, feasibility studies, hazard assessments and risk management investigate and mediate the sources of chemical contamination, the pathways of human exposures, outline the toxicological effects of chemicals, investigate source/site remediation, manage and monitor the health hazards. By characterizing the nature of human exposures to chemicals, HHRAs can extrapolate the probabilities of developing adverse human health effects.

CHAPTER TWO

Literature Review

Historical research exists mainly for mixtures containing PAHs, leading to the early discovery of BaP as a common constituent in toxic PAH-containing mixtures. The first human health study that involved PAHs proved to be a major breakthrough for occupational medicine and safety.[36] Sir Percival Pott, an English surgeon, first reported a connection between occupational chemical exposures and the incidence of scrotal cancer.[37] The select subpopulation of chimney sweeps in London were experiencing high incidence of scrotal cancer, which he suspected was related to their common occupation.[37] The incidence rate of scrotal cancer in chimney sweeps was approximately 200 times larger than the incidence rate of other workers not exposed to tar or soot.[37] In later years, trends were established for people with occupations involving combustion processes and their by-products. Workers were experiencing high rates of lung cancers, gastrointestinal cancers, scrotal cancers, among others.[36] This research marked the beginnings of investigations surrounding chemical mixtures and their potentially harmful components. Such research led to the eventual development of occupational safety protocols and protective equipment for high risk laborers.

Current Health Regulations

Governmental agencies have developed policies, standards and regulations to reduce risk of PAH exposures in the environment and workplace. The primary avenue to monitor and regulate risk associated with PAHs is to enact regulations and policies that

limit PAH emissions from their major sources.[14] Currently, federal PAH standards exist for occupational exposure conditions and for drinking water.[30] The current Maximum Contaminant Level (MCL), established by the Environmental Protection Agency, is currently set at 0.2 µg/L for BaP in drinking water (table 2.1).[30] In their sampling studies, drinking water concentrations remain approximately 100-fold lower than this MCL. Federal government developed maximum intake recommendations for several PAHs (table 1.3).[38] The federal government has also mandated that the EPA be notified and regulatory be taken if certain PAHs are released into the atmosphere in a 24 hour period (table 2.2).[38]

Table 2.1 Available MCLs for various PAHs in drinking water.[3]

Chemical	MCL (µg/L)
Benz[a]anthracene	0.1
Benzo[a]pyrene	0.2
Benzo[b]fluoranthene	0.2
Benzo[k]fluoranthene	0.2
Chrysene	0.2
Indeno[1,2,3-cd]pyrene	0.4
Total PAHs	0.2

For occupational-related hazards, OSHA has the jurisdiction to enforce and regulate a PAH PEL of 0.2 mg/m³ (for air) during an eight hour period in the workplace.[39] The National Institute for Occupational Safety and Health (NIOSH) set occupational exposure limits for the high-risk occupations (listed previously), since coal exposures increase the risk of lung and skin cancers.[39] For example, the NIOSH REL mandates that workers should not be exposed to air that contains PAH concentrations of 0.1 mg/m³ (air) in a 10 hour workday within a workweek.[39]

Table 2.2 Mandated point source emission levels for EPA notification.[3]

Chemical	Quantity Released (lbs)
Acenaphthene	100
Acenaphthylene	5,000
Anthracene	5,000
Benz[a]anthracene	10
Benzo[a]pyrene	1
Benzo[b]fluoranthene	1
Benzo[k]fluoranthene	5,000
Benzo[g,h,i]perylene	5,000
Chrysene	100
Dibenz[a,h]anthracene	1
Fluoranthene	100
Fluorene	5,000
Indeno[1,2,3-c,d]pyrene	100
Phenanthrene	5,000
Pyrene	5,000

Dealing with PAH contamination and proposing intervention strategies begins with the National Priorities List, as mentioned earlier, and are funded for cleanup by CERCLA.[15] They pose significant health risks depending, on the toxic effects, genetics or sensitivities, the duration and concentration of exposures, and the route of the exposure.[11] A common cleanup protocol involves dredging and collecting contaminated media (e.g. soils, sediments), burying the materials and capping the land for containment. In some instances, the area above the buried materials can be recovered for further uses.

Environmental PAH Mixtures

While fully characterizing individual PAH species is ideal, environmental PAH exposures involve mixtures, without exception.[32] Environmental PAH exposures occur in complex mixtures, therefore understanding mixture interactions at the molecular level is important to better predict human health hazards. Cumulative toxicity of PAH mixtures on organisms also varies greatly based on several factors, including alkylation,

species' mechanisms of biotransformation, route of exposure, transfer through the food chain, photoactivation, and environmental stressors.[1]

If the preferred method of assessing PAH toxicity is the whole mixtures approach, as outlined above, then knowledge of prevalent environmental mixtures (and their sources) is crucial. In addition, obtaining knowledge about PAHs' routes of entry into the environment and their eventual fate are important considerations for managing human health hazards. Several studies have investigated the components of PAH mixtures in various environmental media, as is summarized in tables A.1-A.4.

Environmental partitioning of PAH mixtures is affected by many parameters and poses concerns for researchers. Researchers are struggling to find correlations between emission sources and environmental concentrations because PAH emission mixtures vary widely depending on sources and environmental influential factors. Research supports that the prevalent phases of PAHs, their component ratios, and media concentrations may range significantly depending on the season, rainfall, and wind patterns of a specific region. For example, some studies have observed larger concentrations of particulate-phase PAHs during the winter months.[40]

One study compiled the total expected PAH emissions in the United States, with approximately 16% originating from residential heating, 13% from open burning or combustion, 25% from mobile engine exhaust, 41% from industrial sources, 5% from power generation and <1 % from incineration.[11] Studies have found that mixtures containing large proportions of high molecular weight PAHs are likely originating from combustion sources.[41] Conversely, mixtures containing higher proportions of low

molecular weight PAHs originate from petrogenic sources, such as extracted coal deposits.[41]

Automobile emissions are expected to release high proportions of benzo[g,h,i]perylene and pyrene when compared to other PAHs.[11] Diesel exhaust contains primarily 3 to 4 ring PAHs, with high concentrations of phenanthrene, anthracene, acenaphthene, fluorene and phenanthrene particulates or vapors.[29] Wood combustion displays more production of acenaphthylene than other PAHs.[2] Coal tar pitch has high concentrations of phenanthrene and pyrene when compared to BaP and benzo[g,h,i]perylene, which can be 20 to 80 times less concentrated.[42] Fluoranthene and phenanthrene represent a very large portion of many environmental PAH mixtures.[43]

Environmental PAH mixtures seem to vary largely based on emission source, partitioning, biological activity (e.g. microbial organisms) and influential ecological factors. These influential factors likely cause fluctuations in PAH concentrations within mixtures. The greater scope of PAH research may find that individual toxicity characterization is helpful in predicting mixture interactions and environmental mixtures that pose hazards to human health. A whole mixtures approach may not be pragmatic when the composition of ambient PAHs seems to vary widely and inconsistently.

Current Approaches for Assessing PAH Mixtures

The assessment of PAH toxicity is crucial for identifying the risks posed to humans, as well as the management of health hazards. Experts have developed and ranked methods to analyze the toxicity of PAHs, including the congeners that lack applicable data. Current approaches for assessing PAH toxicity fall into two categories: component-based and mixture-based methodologies. The toxicity of chemical mixtures is often

difficult to evaluate because of composition inconsistencies and interactions, thus whole mixtures is often preferred.[44] Mixture based methodologies include the use of mixture analogs or similar mixtures, characterizing the components, and using a reliable toxic equivalence method.[44] The EPA has ranked different approaches based on the consistency of available data, while also concomitantly acknowledging the limitations of each approach in publications.[45]

Currently, the most favorable method is obtaining toxicity data for whole PAH mixtures.[45] A whole mixtures approach accounts for any mixture effects that may occur between components because it examines the toxicity of the entire mixture altogether. This approach does not require identification of the components and their individual toxicities, which is convenient for this class of compounds. The greatest limitation with the whole mixtures approach is that the composition of PAH mixtures vary widely depending on the emission source and the containing environmental media (tables A.1-A.4).[45] There are very few studies that include methylated PAHs in their analysis. Another limitation is the lack of experimental data for specific PAH mixtures that may serve as reference materials.[44] Furthermore, a whole mixtures approach does not contribute to our knowledge of the interactions that may be occurring between components, but research on simple mixtures (two or three PAHs in controlled mixtures) may elucidate such relationships. Until robust data is available for the major PAH components in mixtures, whole mixtures may produce the most accurate toxicity estimates.

The mixture comparative potency method uses data on similar mixtures to extrapolate the cancer potency in humans.[1] The approach factors the sources of PAHs

into account, assuming that mixtures originating from the same processes have comparable toxic activity.[46] Available *in vivo* or *in vitro* bioassay data on mixtures is used to find the proportional relative potency in humans.[46] The data is used to determine a potency scaling factor in humans for that mixture, or set of mixtures.[45] This approach does not require characterizing the components in mixtures, but only bioassay data on the similar mixtures. The assumption of potency proportionality from the bioassay data to humans introduces uncertainty when using this approach. Also, the approach is not valid if there are other contributing sources of PAH exposures. Another limitation is the overall lack of data on potencies dependent upon different routes of exposure.

The relative potency factor approach (RPF) focuses on the potencies of the major components in the PAH mixtures. An index chemical is used to estimate toxicity of PAHs based on the reference compound's characterization and toxicity data.[47] The RPF approach is used almost exclusively for cancer assessments.[48] RPFs are derived from a small amount of data (perhaps one study). RPFs are similar to toxic equivalency factors (TEFs), but TEFs are more robust consensus estimates that are generated from a wide array of research studies, and have been developed for dioxin like compounds.[47] BaP is used as the reference compound to calculate the relative potency of other PAHs, and then summed to get a total potency value for the PAH mixture.[1] The RPF approach is different from Toxic Equivalency Factor methodology because it focuses on the ratio of a PAH's potency for inducing cancer, although it can be used for non-cancer endpoints.[49] Toxic Equivalency Factors (TEFs) are used to predict the toxicity of a mixture of a class of compounds when using a reference chemical that is thoroughly researched.[49] RPFs are less reliable than established TEF values because they are developed from a less robust

set of toxicological information. Researchers acknowledge that, without understanding the mechanisms of action, neither RPFs nor TEFs are complex enough to consistently predict the toxicities resulting from PAH exposures.[44] A simplified ranking system inadequately addresses the many variables involved in PAH toxicities.[44] Additionally, the current RPFs and TEFs do not include various methylated PAHs, which are in some instances more toxic than their parent compound and also suspected of inducing cancer.[44]

The World Health Organization and the UK's Expert Panel on Air Quality Standards established standards in order to utilize BaP as the RPF index.[32] The first assumption for the RPF method states that any given PAH is proportionally potent to BaP's quantifiable toxicity so it can be used as a reference measure.[31] Secondly, the compounds in mixtures are assumed to be stable for consistent assessment.[31] Finally, the PAHs in mixtures are assumed to have the same mode of actions (carcinogenicity) relative to BaP, allowing for additive toxicity.[50] This assumption of toxic additivity has been the center of much debate, since specific data on PAHs are limited.[1]

Human Health Risk Assessment

Human Health Risk Assessments (HHRA) characterize the hazards of chemical compounds, identify exposure pathways, and produce a quantified level of risk for proper mitigation of such hazards to human health.[1] The HHRA provides a measure of the probability of an adverse effect resulting from exposures to the specified hazards. In short, HHRAs seek to answer questions about potential hazards to human health, and characterize the levels of risk involved with specific circumstances, hazards or chemicals.[51] The human health risk assessment process involves several steps:

(1) site research and early planning

(2) hazard identification

(3) exposure assessment

(4) dose-response assessment

(5) risk characterization[51]

Risk management, mitigation and public communications will follow these steps for remedial action. An HHRA is usually designed for a specific location or circumstance. Assessing the toxicity of PAHs is a fundamental part of HHRAs that seek to mitigate PAH hazards, as part of the dose-response assessment.[51] The human health risk assessment process employs a specific component-based method for calculating PAH hazards that involves the use of developed RPFs for PAHs.[47] Several assumptions are made for this approach, including the assumption that biotransformation pathways are consistent, and that the mixtures are additive with similar dose-response characteristics. Experts acknowledge that this method leaves many uncertainties, since RPFs for PAHs have been developed without sufficient experimental data, similar to the limitations with other approaches.[44]

Quantitative Structure Activity Relationships

QSAR models are helpful tools for predicting the chemical activity of classes of environmental contaminants, usually with deficient information.[52] QSAR models are mathematical functions that are incorporated into specialized computer software programs.[53] The models use the physical characteristics of a class of contaminants as molecular descriptors to predict a chosen endpoint, such as toxic responses.[52] The following equation is an example of a QSAR linear function.[52]

$$\text{Activity} = (a\ x_1) + (b\ x_2) + (c\ x_3)\ldots + (n\ x_i)$$

"x_1" and "x_2" are assigned molecular descriptors that may be molecular weight, number of double bonds, and the sum of electronegativity, to name a few.[53] QSAR models may have several descriptors, but strong models have as few as possible while maintaining a strong correlation. "a", "b" and "c" are specific parameters that the QSAR software generates. Molecular descriptors represent the independent variables in the function, while the activity is the variable dependent upon the chosen descriptors.[50] The activity can be a wide range of biological responses such as site toxicity, proliferation, bioaccumulation factors, among others. Several studies have sought to analyze the predictive potential of QSAR for characterizing PAHs. Studies have modelled carcinogenesis, mutagenicity, photo-induced toxicity, dermal absorption, catalysis, environmental partitioning and biodegradation of PAHs.[54] Many molecular descriptors, including quantum chemical properties (energies of molecular orbitals), ionization potential, dipole moments, boiling points, partitioning coefficients, and retention times in reverse-phase liquid chromatography were found to be significant descriptors.[54] However, the weight of importance for descriptors depends heavily on the type of endpoint being modelled.

QSAR modeling is useful for prioritizing the aims of research bioassays. For example, QSARs can be used to estimating chemical concentrations that cause a toxic effect in 50% of a test species, or EC_{50}.[52] QSAR models are also invaluable for pharmaceutical companies during drug discovery and development of new medicines. Specific software like Accelrys' Discovery Studio and Toxicity Estimation Software Tool provide quick and information-rich interfaces that can compute simple or detailed QSAR models.[52] QSAR models may contribute to understanding the mechanisms of action for

PAHs, interactions between enzymes and chemicals, and identifying the most important hazards associated with PAHs. QSARs contribute to decision-making about chemical screening.[55] Such information adds to the diminutive known toxicity information about PAHs and their behavior in a biological system.

Adverse Health Effects

Biotransformation

The ultimate toxic endpoint of PAHs varies across studies, depending largely upon the route of exposure. Many times, the site of absorption is also the site of toxicity, such as is the case with smokers, who have high incidence rates of lung and larynx cancers.[4] Phase I metabolism involves the oxidation of parent PAHs, which produces reactive intermediates.[56] The liver is the most significant site for toxicity because this is often where biotransformation occurs. However, several other tissues are capable of transforming BaP, including intestinal mucosa, lung, thyroid, striated muscle, renal cortex, testes, placenta, leukocytes, lymphocytes, and monocytes.[56]

After exposure and subsequent absorption, PAHs will bind to aryl hydrocarbon receptors (AhR), which will activate gene expression to produce more enzymes that can metabolize these PAHs. PAHs in their parent form do not interact with cellular macromolecules (such as DNA).[57] Rather, they need to be activated by Phase I enzymes, including Cytochrome P450 isozymes (CYP 1A1, 1A2, and 1B1), epoxide hydrolases, peroxidases, and aldo-keto reductases.[58] Phase I enzymes add functional groups that are more reactive than the parent compound.[4] Phase II further metabolizes the compound by adding hydrophilic groups via conjugation reactions in order to facilitate elimination.[4]

The most likely metabolic pathway includes the oxidation of a PAH by cytochrome P450 monooxygenases (CYPs) during Phase I metabolism.[4] After it has been oxidized, there are a number of different avenues that PAHs can be further biotransformed. A common carcinogenic avenue involves the hydrolysis of the oxidized PAH by epoxide hydrolase, followed by further oxidation by CYPs.[4] This pathway creates diol epoxides, which can form bulky adducts. Other harmful metabolites are phenols and quinones, which generate reactive oxygen species like hydroxyl radicals, superoxide anions, and hydrogen peroxide.[4] These products will produce oxidative stress in the host organism. Although CYP enzymes may generate reactive metabolites, there are a variety of other enzymes that are involved in PAH metabolic pathways (table 2.3).[59]

Table 2.3 Major enzymes that biotransform PAHs.[4]

Enzyme	Reaction Type	Metabolite
Quinone Reductase	Reduction	Semiquinones
Peroxidases	Oxidation	Radical Cations, Phenols
CYP 450s (1,2,3 families)	Oxidation	Phenols, Arene Oxides, DEs
Phenol Monooxygenase	Oxidation	O-quinones
Dihydridiol Dehydrogenase	Oxidation	Dihydrodiols, O-quinones
CYP 448 with AHH	Oxidation	Arene oxides
Epoxide Hydrolase	Hydrolysis	Diol epoxides
Uridin 5'-diphosphate Glucuronosyltransferases	Conjugation/ Glucuronidation	Conjugates
Glutathione S-transferases	Glutathione Conjugation	Conjugates
Sulfotransferases	Conjugation/ Sulfation	Conjugates

Genotoxicity and Carcinogenesis

The most significant result of exposure to PAH mixtures is the development of cancer, which complicates identifying the health hazards because of the latency period.

PAH exposures are known to cause cancer in the kidney, bladder, bone marrow, lymph nodes, lung, bronchi, liver, skin, and mammary glands.[2] A human study observed an increase in the development of cancers when PAHs were inhaled or exposed dermally.[25] Animal studies have indicated that PAH inhalation, dermal absorption and ingestion showed high incidence of tumor formation.[60]

The carcinogenic potency of a PAH depends on the activation of reactive intermediates by Phase I enzymes, the rate of detoxification by Phase II enzymes, and the rate of elimination of the parent compound. The PAH metabolites can form adducts with biological macromolecules in the body, including DNA, hemoglobin, globin and serum proteins.[11] The primary mechanism of inducing cancer is through DNA adduction and subsequent mutations.[57] The generation of reactive oxygen species is also a possible pathway.[61] Three major genotoxic reactive metabolites are diol epoxides formed by CYP enzymes, radical cations formed by peroxidases, and O-quinones formed by dihydrodiol dehydrogenases.[62]

According to the EPA's IRIS database, some PAHs are capable of both inducing and promoting cancer. These compounds are called complete carcinogens. They include benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene, chrysene, and dibenz[a,h]-anthracene.[11] The genotoxicity of BaP has been verified in many studies using the Comet assay, measuring protein biomarkers, measuring DNA adducts, animal studies, as well as epidemiological studies.[18] Studies have found that fluoranthene and pyrene are not genotoxic in various *in vitro* assays on human cell lines.[16] Benzo[g,h,i]perylene has shown evidence for genotoxicity and carcinogenicity.[46]

BaP and dibenz[a,l]pyrene (DBP) are currently thought to be highly mutagenic and carcinogenic, potentially the most potent compounds within the class.[63] A rodent study revealed that DBP can induce the formation of DNA adducts that easily evade cellular repair with a frequency significantly higher than that of BaP.[63] The evidence reveals that DBP may be several orders of magnitude more potent than BaP, and possibly the most potent carcinogen in the class.[63]

There is limited research on methyl PAHs, but published studies have found that 9,10-dimethylanthracene (9,10-Ac), among other methylated congeners, have carcinogenic potency.[64] Methylated chrysenes are suspected of having carcinogenic properties but research is not yet definitive.[44] 7,12-dimethylbenz[a]anthracene (7,12-BaA) has been found to metabolize easily into diol epoxide intermediates, causing the rate of DNA adduction formation to be much higher than that of BaP.[65] 7,12-BaA and 9,10-Ac have been used as models for carcinogenesis in animal and cell systems.[66] These findings have brought up further questions about the genotoxicity and tumorigenicity of other methyl congeners. Namely, 9-methylanthracene (9-Ac), as well as its parent anthracene, have shown no evidence of genotoxicity. There appears to be a significant biochemical relationship between genotoxicity and PAH substitution. One study concludes that the congeners' stereoselectivities, which determines the ratio of stereoisomer metabolites, may be a source of the varying genotoxicities.[67]

Published research has validated the *in vitro* Micronucleus (MN) test as a genotoxicity assay when compared to other common genotoxicity screening procedures, such as the widely used Comet assay and the chromosome aberration test. The MN test examines DNA aberrations in the form of micronuclei, which are pieces of DNA that

become fragmented and isolated outside of the main nucleus. Micronuclei are small fragments of chromosomal DNA that fail to migrate properly, forming small aggregates of inappropriate DNA after mitotic division.[1] MN can be caused by chromosome breakage or chromosome loss. Using fluorescent DNA probes, micronuclei are easily identified within cells. BaP and 7,12-BaA were tested and found to be positive with the MN test, indicating they are genotoxic.[68] Positive results mean that the test compound caused an increase in the frequency of micronuclei in dosed cells when compared to untreated cells.

When genotoxic metabolites are generated, many cells initiate damage response signal pathways to repair DNA.[69] The response is a complicated signal transduction cascade that attempts to maintain homeostasis and prevent mutations, but the pathways vary based upon circumstance.[69] The signal cascade may be a potential mechanism for nullifying the reactive PAH metabolites from generating mutations and inducing carcinogenesis. If the cell is unable to repair the DNA, it may initiate the apoptosis signaling cascade to prevent abnormal cell division.

Non-Carcinogenic Toxicity

There are various factors that influence the toxicity of PAHs, including the presence of important enzymes in specific organs, the rate of bioactivation, the rate of detoxification, and the affinity of a PAH by an appropriate receptor, such as AhR. Acute exposure can result in headaches, vomiting, nausea, and site irritation.[3] Some animal studies have observed toxicity in neurological, reproductive, immune, lymph, and developmental processes.[70] According to the ATSDR's characterization of PAH exposures, other adverse effects include bronchitis, irritation at site(s) of contact,

dermatitis, burns, erythema, and others.[3] During pregnancies in mice, individuals that were fed high levels of benzo[a]pyrene showed various infertility effects.[71]. Their offspring exhibited these issues as well. Birth defects and decreased body weight were seen in offspring.[71]

In vitro cytotoxicity can be a very useful biomarker to examine the effects that contaminants may have on cell function. Testing cell viability is a widely accepted strategy to examine cellular responses to chemicals. There various methods for testing cytotoxicity, including testing cell membrane integrity, testing the presence of certain proteins, testing ATP content, leakage of lactate dehydrogenase (LDH), testing for genetic alterations, examining cellular communications, among others.[72] The most appropriate cytotoxicity test is dependent upon the mechanisms of cell damage and death for the chemical.

Literature evaluating toxicity of PAHs highlights their weak non-cancer potency but several studies have shown oxidative stress and cell death.[61] Researchers observe that when a cell line is capable of metabolizing the PAH, the cells display acute cytotoxic responses. Responses can range from suppression of cellular growth and proliferation, induction of apoptosis signaling cascades, cell cycle arrest, inhibition of immune response, and inhibition of normal DNA repair processes.[73]

A study by Oh et al.(2004) investigated the toxicity of BaP on human lymphocyte cells and found that cytoxicity was observed in the cells at concentrations of 0.631 mg/L or higher.[58] The study performed a 48 hour BaP exposure and observed dose-dependent increase in the levels of LDH, a biomarker used to analyze cellular stress and

cytotoxicity.[58] The study also found significant DNA damage at the lowest levels that also displayed elevated LDH levels.[58]

While BaP studies have been conducted, there is little to no data available for other PAH congeners regarding cytotoxicity. There is a need for investigating the non-cancer toxic effects in animal or cell culture models. One study examined the effects of fluoranthene on the viability of cultured rat sertoli cells and found that lactate levels were significantly increased at concentrations of 10^{-8} M or higher.[74] The study also exposed cells to Fla and other PAHs (BaP, BbF, BaA) to examine the induction of apoptosis.[74] Cells exposed to BaP and BbF showed evidence of early stages of apoptosis.[74]

Some studies have compared the toxicity of parent compounds- such as BaA- to their metabolites, and have found that the parent compounds display much lower toxicity effects. The primary cytotoxic effect of BaP is the induction of apoptosis, as seen in the *in vitro* cell placental model.[75] Sources say that BaP can also impair the function of cellular lysosomes and cellular membranes.[61]

Phenanthrene displays dose-dependent cytotoxicity in the form of cellular growth inhibition.[76] Phenanthrene interferes with the cell cycle, resulting in eventual death [76]. A different study did not observe toxicity in placental cells by neither phenanthrene nor anthracene.[75]

Methylated congeners appear to affect test organisms as severely or sometimes more severely than parent compounds, with demonstrated higher mortalities and cytotoxicity at similar concentrations. One study observed that methylated PAHs accumulate more quickly than their parent congeners in earthworms.[77] The higher the degree of

substitution, the higher the observed toxicity, which could explain the difference in toxicity with methylated anthracenes.[77]

Cytotoxicity of PAHs appears highly variable, depending on *in vivo* or *in vitro* methodologies. *In vivo* experiments may be more helpful in determining the cellular responses from whole organisms and multiples mechanisms of action. *In vivo* experiments, however, are costly and dependent upon the appropriateness and viability of the study organisms. *In vitro* responses seem to vary depending on cell type, exposure scenarios, and the selection of analytical assays. Cell type has a large influence on the ability of the cell to respond to the doses. Exposure protocols can range for acute single dosing procedures, to multiple dosing events, and even the inclusion of recovery periods.

Toxicity of Mixtures

There have been recent studies that examine potential mixture effects in PAH cocktails.[63] More interestingly, the only trend that has yet to be established is that PAH interactions appear erratic, with clear impacts on toxicity of test organisms that do not equal the sum of the congeners.[63] One study dosed liver cells with environmental PAH mixtures extracted from dust that was collected from different locations. The samples were taken from various workplaces, including schools, shopping malls, offices, and factories.[9] The study found that cytotoxicity was observed in a dose-dependent manner, and a source-dependent manner.[9] The highest toxicity was caused by the PAH samples from a manufacturing plant.[9] They observed relationships with the LC_{50} as the concentrations of the mixtures increased.[9] However, the study observed different ranges of LC_{50} values depending on the source and site of the dust sampling.[63] This is suggesting that two phenomena are occurring 1) that the mixtures have different toxicities

and 2) that the component interactions in each mixture are responsible for the differing toxicities.

One *in vitro* cell culture study compared the DNA damage of a PAH mixture extracted from soil samples to a solitary BaP solution.[78] The results showed that the BaP-exposed cells were able to recover from and repair DNA damage, however, the cells exposed to the PAH mixture showed significantly more DNA damage and inability to recover and repair themselves.[63] Consequently, this suggests a more than additive relationship may be occurring between PAH compounds in a complex mixture.

A study by Tzekova et al. (2004) dosed rats with binary mixtures of BaP and Pyr in a chronic exposure scenario, varying the ratio of BaP to Pyr.[79] They examined the electrophilic tissue burden, where proteins serve as nucleophilic sinks to reactive PAH metabolites. The reactive metabolites formed adducts with various proteins, rather than DNA or RNA. They observed a linear relationship between BaP and protein adducts, corresponding with dose concentrations.[79] They also noted that Pyr did not have an apparent effect on the genotoxicity of BaP, neither enhancing nor inhibiting the rate of protein adducts.[79] These results suggest that Pyr neither synergizes nor antagonizes the formation of protein adducts by BaP.

Conversely, some studies have revealed results that indicate antagonism or less than additive genotoxicity can occur in mixtures.[80] Marston et al. performed a tumor induction study in rats and concluded that PAH mixture interactions may serve to inhibit carcinogenic activation.[80] They observed fewer total DNA adducts in mice that were co-treated with a standardized coal tar mixture when compared to mice that were treated only with BaP.[80] However, certain exposure periods resulted in insignificant DNA

adducts levels between the two treatments.[80] BaP and dibenzo[a,l]pyrene (DBP) displayed significantly higher levels of adduct formation in breast cancer MCF-7 cells than in trials where they were co-treated with a standard reference coal tar mixture, indicating antagonistic interactions within the complex mixture.[81] A study previously mentioned saw similar results in mice, where the same coal tar mixture was co-treated with DBP and revealed significantly fewer adduct formations when compared to the mice treated only with DBP.[80]

The mechanism is believed to be very complex, with outcomes ranging from antagonism to potentiation depending on which PAH congeners interact at any given time, and their concentrations.[80] A different study exhibited this complexity, when they varied binary PAH mixtures in mice and saw conflicting results.[82] The tumor induction study found that benzo[e]pyrene (BeP) enhanced BaP's potency in mouse skin treatments, but it appeared to reduce the potency of 7,12-BaA in duplicate treatments.[82] According to research, fluoranthene and pyrene are also circumstantial enhancers of BaP potency.[80] Tarantini et al. found specific relationships between PAHs when co-treated with BaP in human liver cancer HEPG2 cells.[57] It was reported that benzo[b]fluoranthene, dibenz[a,h]anthracene, indeno[1,2,3-cd]pyrene enhanced the formation of BAP-DNA adducts in co-treatments.[57] Benzo[k]fluoranthene reduced the amount of adducts in co-treatments.[57]

As discussed, several studies have found that non-carcinogenic PAHs can enhance the potency of various carcinogenic PAHs likes BaP or benz[a]anthracene because of mixture interactions.[80] Different studies have reported that mixture interaction may serve to decrease the cytotoxic potency of certain PAHs, specifically the most potent

congeners. For these confounding results, there is much need for research on single congeners and binary interactions between congeners to start characterizing the possible PAH mixture effects and resulting toxicity.

Implications of Characterizing Mixture Effects

Various regulatory agencies, both national and international, have responded to chemical production and emissions by enforcing the screening and prohibition of new chemicals that lack toxicity data for humans and wildlife.[83] Examples of such regulations include the Environmental Protection Agency's Toxic Substances Control Act, the European Union's Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), and the Organisation for Economic Cooperation and Development's Screening Information Data Set.[83] However, PAHs fall into a unique category of unknown chemicals because they are not intentionally produced for chemical application. They are still labeled as priority pollutants requiring research, but complications may interfere with their regulation because they are inevitable by-products of industrial processes.[45]

Understanding PAHs and potential mixture interactions may help decrease the cause for alarm, or perhaps highlight the urgency for PAH emissions management for the purpose of reducing health hazards.[45] Such knowledge can be applied in HHRAs and Quantitative Structure Activity Relationships Modeling (QSAR) that can utilize basic properties of PAHs to predict various activity like toxic action.[52] QSAR modeling is an important start for understanding new chemicals, and is widely used during the chemical screening process.[55]

CHAPTER THREE

Research Objectives

The studies discussed above have highlighted several data gaps in PAH research, including little knowledge on the toxic potential of single PAHs, the confounding data for PAH interactions, and high variability in the composition of environmental PAH mixtures. Relatively little research has been conducted on complex mixtures, simple PAH mixtures or even many single PAHs. Essentially gathering this sort of data will contribute to thorough PAH characterization, and to understanding complex mixture interactions. Furthermore, evidence suggest that current toxicity factors- like RPFs or TEFs- that use BaP as an index are not entirely reliable for estimating mixture toxicities.[44]

Complex mixtures are difficult to research with financial and time limitations. The stepping stone to understanding PAH mixture toxicities is studying the toxicological properties of single congeners, and simple mixtures (binary and ternary).[57] One avenue that can provide helpful toxicological information is *in vitro* cell culture testing. *In vitro* experiments allow researchers to identify biological mechanisms of action for various health conditions in organisms or for chemical exposures, such as PAH toxicity.

Fifteen PAHs were selected for *in vitro* cell culture toxicity assessments. The research will seek to evaluate the toxicity and mixture effects of data-deficient PAH compounds. BaP will be used to compare the difference in cell viability and relative toxicities of different congeners. Some PAH congeners have a larger research database, such as phenanthrene and benz[a]anthracene, and available literature will be compared with the results when applicable.

The liver is the most important organ involved in xenobiotic metabolization, and specifically for PAHs.[84] As discussed, non-smoking individuals will likely encounter PAHs through ingestion as the primary exposure route. Oftentimes, this results in the liver being the first site of metabolic action and potential toxicity. The liver is equipped with suitable concentrations of specific enzymes capable of activating and detoxifying PAHs.[73] Cultured liver cells, or hepatocytes, maintain the same metabolic activity that is observed in a complete liver organ.[84] Thus, they are a representative model of *in vitro* xenobiotic metabolization, appropriate for the ingestion pathway. Clone 9 cells are immortalized normal epithelial liver cells from the liver of a 4 week old Sprague-Dawley male rat.[84] They have a history of use in various laboratories for hepatotoxicity and carcinogenesis experimentation, and will be used in these experiments to model the hepatic metabolism of PAHs.[84] Published research has found that they retain approximately 10% metabolic activity of the whole rat liver, and retain a normal rat karyotype.[85] Clone 9 cells (K-9) grow in a neat monolayer, and have a doubling time of 18 to 24 hours.[86] These cells are surface-adherent and lack locomotion, but they do form junctional complexes between cell membranes to communicate with each other.[86]

The goal of this research is to characterize possible mixture effects of PAHs. Specific objectives of this thesis research include the following:

(1) Investigate *in vitro* cytotoxicity and genotoxicity after exposure to single PAHs in varying concentrations.

(2) Investigate *in vitro* cytotoxicity and genotoxicity after exposure to binary PAH mixtures in varying concentrations.

(3) Compare the cytotoxic and genotoxic responses by evaluating the dose-response relationships of the single PAHs and binary mixtures.

(4) Perform an analysis of chemical mixture effects by comparing the single PAH data to the binary mixture data.

(5) Develop a QSAR model using the dose-response data of the analyte list and assess the model's power to predict PAH toxicity.

The overall goal of this research is to compare single PAH toxicity that we observe in K-9 cells to the toxicities observed in binary PAH mixtures. The research will examine if mixture effects are detectable in these simple mixtures when compared to the toxicity that is produced by each component alone. This type of data is useful for toxicological profiles of chemical contaminants, like those of the Agency for Toxic Substances and Disease Registry (ATSDR) for reference doses, cancer slope factors, RPFs, TEFs, effective concentrations or doses (EC_x , LD_x), minimum or no-effect levels, (NOAELs and LOAELs), among others.[11] *In vitro* mixture data can be used to approximate relative potencies and EC_{50} by comparing the single dose-response curves to that of simple mixtures (and how they differ). *In vitro* and *in vivo* data can be scaled to estimate the potential responses in humans.

The research aims to determine how reliable the component-based additivity model is for determining toxicity of PAH mixtures. The alternative methods employ the use of individual PAH dose-response data, using standardized reference mixtures, or sufficiently similar mixtures, as discussed previously. If this research confirms that complex mixture effects are occurring, it may highlight the inaccuracies of toxicity additivity assumptions, and research on mixtures dose-response data could be more appropriate.

CHAPTER FOUR

Materials and Methods

Materials

Rat liver Clone 9 cells were obtained from the American Type Culture Collection (Manassas, VA) CRL 1439 passage 17, for *in vitro* cell culture. Nutrient HAMs F-12 mixture, serum-free Dulbecco's Modified Eagle Medium F-12, Dulbecco's phosphate buffer saline (PBS), HEPES, penicillin-streptomycin hybri-max, sodium bicarbonate, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Trypsin-EDTA solution was obtained from Atlanta Biologicals (Lawrenceville, GA). Fetal bovine serum (FBS) was obtained from Equitech-Bio (Kerrville, TX).

Acenaphthylene, anthracene, benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[e]pyrene, benzo[k]fluoranthene, chrysene, fluoranthene, phenanthrene, pyrene, 9-methylanthracene, 3,6-dimethylphenanthrene, 7,12-dimethylbenz[a]anthracene, and 9,10-dimethylanthracene neat standards (purity > 97%) were purchased from AccuStandard (New Haven, CT). Janus green B dye, 5-Carboxyfluorescein diacetate acetoxymethyl ester (CFDA), acridine orange base and DAPI were obtained from Sigma-Aldrich (St. Louis, MO).

Methods

Chemical Preparation

All 15 PAHs were dissolved in DMSO in sterilized amber vials. Serial dilution was performed to create stock solutions with lower concentrations. All solutions were prepared at concentrations appropriate for a final concentration of 0.5% DMSO in the cellular media. All PAHs are considered hazardous and were handled following the NIH Guidelines for the use of chemical carcinogens.[87]

Cell Culture

Cells were maintained in Nutrient HAMs F-12 mixture, with 10% fetal bovine serum, 1% penicillin-streptomycin solution, and 1420 M HEPEs buffering agent. Cells were cultured in an incubator at 37° C in 5% CO_2 and 90% humidity. The cells were grown to confluence in culture-treated sterile 75 cm^2 flasks. Once confluent, the cells were rinsed with PBS, detached with 0.25% trypsin and 0.53 mM EDTA solution, centrifuged, and counted using a Beckman Coulter particle counter. The cells were resuspended in media and then re-seeded in transparent 96 multiwell plates or black 96 multiwell plates at approximately 5,000 cells/well. For the MN assay, cells were re-seeded in 6 or 12 multiwell plates at approximately 100,000 cells/well and 50,000 cells/well. The cells were allotted 24 hours to adhere to the plates and reach 50% confluence.

Chemical Treatments

Trials were completed within 10 cell passages of being received from ATCC. The well plates were divided into different treatment groups, including control, vehicle

control, and chemical treatments. The dosing treatments in the cell media were made up of 0.5% DMSO solvent with the dissolved PAH test agent. Previous trials in our lab and in literature have shown that a 0.5% DMSO concentration in the cell media does not have a significant impact on K-9 cell viability or growth.[57] All trials utilized a 24 hour exposure period.

For the single PAH dosing trials, treatments were randomized across the plates, with a control, vehicle control, and PAH doses ranging from 0.25- 10 mg/L. For the chemicals in the binary mixtures, BaP, together with one of the other 14 PAHs in the analyte list, were dissolved in 0.5% DMSO in the cell culture media. The binary mixtures were divided into two treatments: mixtures where BaP was held constant (1 ppm) and the concentration of the other PAH varied (0.25-10 mg/L); and mixtures where BaP varied (1-10 mg/L) and the other PAH remained constant (1 mg/L). These concentrations were chosen because they are high enough to induce a toxic response in this specific cell line.

Cellular Viability and Proliferation

Janus Green B dye (JG) is an exclusion dye- similar to the Trypan blue stain- that can permeate the membranes of cells that have compromised viability.[88] The JG dye will stain damaged and dead cells, yielding helpful information about the cellular proliferation and toxicity.[88] JG dye will be used to assess cellular proliferation and viability of cells exposed to PAHs. CFDA will also be used to assess the viability of cells. By assessing both cell viability and proliferation, the results will provide information about how a PAH affects the cells' ability to grow, divide, and maintain homeostasis.

After 24 hours of chemical exposure, cells were assessed with the aforementioned assays. The cell media was aspirated from all cell wells, followed by two gentle rinses of PBS to remove any impurities and residual chemical from the wells. JG proliferation and viability assays were performed simultaneously in clear 96 well plates. For proliferation, the wells were fixed in ethanol for 90 seconds. JG dye was made up in 1 mg/mL PBS final solution and then all wells were dyed for 60 seconds. The wells were rinsed twice more in PBS and then aspirated to completely remove all liquid. 100 µL of ethanol and 100 µL of nanopurified water were added to each well to homogenize the well colors for more accurate absorbance. The plates were quantified using a BioTek microplate spectrophotometer at 590 nm.

The CFDA assay is similar to the JG assay; however, it is performed in glass-bottom black 96 well plates. All cell media was aspirated and every well was rinsed twice with warm PBS++ (supplemented with calcium and magnesium). CFDA was dissolved in DMSO, and then added to warm, serum-free DMEM media, making up a final concentration of 4µM on the cells. The plate was then incubated for 30 minutes to allow the CFDA to permeate the cells. The cells were then rinsed twice and resuspended with warm serum-free DMEM media. The plates were immediately quantified using a Fluoroskan Ascent microplate fluorometer, with emission/excitation at 485/538 nm.

Genotoxicity

The next step for this research was to test the genotoxicity of the selected 15 PAH congeners in single and binary mixtures. The objectives for these tests include (1) observing the frequency of mutations by quantifying micronuclei after one cell division,

(2) observing the rate and mechanism of cell death and (3) comparing the frequency results of single trials to the frequencies in mixtures.

The Micronucleus (MN) Test was used for the evaluation of carcinogenic potency. The MN assay measures genotoxicity by detecting micronuclei in cells during or after mitotic division. MN are quantified through the microscope or a fluorometer when dyed with a fluorescent nucleus stain. This project utilized two probes: the 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) DNA probe, and Acridine orange RNA/DNA probe to illuminate nuclei, micronuclei, cytoplasmic material, and apoptotic bodies for scoring. A cytokinesis blocker was not used because it increases the chance of producing a false positive result and may inhibit scoring. Cytokinesis blockers are also less appropriate for immortalized cell lines because cells have undergone several cell divisions before experimentation.

Protocol and scoring for the MN Test were adapted from Fenech et al 2003.[89] All trials were performed at cell passage 24. Before chemical treatment, the cells were submerged in serum-free warm DMEM cell media for two hours. This process of serum starvation has two purposes: to synchronize the cell cycles of the population, and to allow for maximum absorption of the chemical post-starvation. Several experiments were performed to find the optimal time frame in which the cell growth cycles could be synchronized in serum-free media to harmonize MN scoring criteria. Two hours in serum-free media proved to be adequate for cell synchronization and would not cause decline in viability. At this time, the majority of cells are in the G_0 mononucleated cell quiescent phase. The cells were then dosed in regular HAMs F12 media with chemical and given 24 hours to complete at least one cell division. Single trials include control

groups, solvent control groups, and two treatment groups (1 and 10 mg/L). Chrysene was tested at 0.25 mg/L and 1 mg/L to achieve solution. Binary mixtures were tested at ratios of 1:1, 1:10 and 10:1 (BaP:PAH). Chrysene ratios were 1:0.25, 1:1, and 10:1.

After a 2 hour starvation period and a 24 hour exposure period, the DAPI probe was prepared at 300 nM in warm PBS stock solution. Acridine orange (AO) was dissolved in 0.1 M citric acid and then 0.2 M Na_2HPO_4 was added to make up the AO stain solution. Clear 6 well plates or 12 well plates were utilized in these trials and dosing protocols were the same, but adjusted for different well volumes. The wells were rinsed once with warm PBS and then incubated with AO stain solution for 30 minutes. The wells were rinsed again with warm PBS and then fixed with ethanol for 90 seconds. The wells were rinsed once more with PBS and then incubated with the DAPI dye solution for five minutes. Wells were rinsed once more and resuspended in PBS. The plates were then imaged using fluorescence microscopy at 20x magnification and scored for data analysis. Plates were stored at 4°C for future analysis.

Micronucleus scoring was performed on a high definition Zeiss fluorescent light microscope using a Zeiss 20x Neofluor objective with dimensions 434 µm by 324 µm per image. The following information was obtained from the microscopy scoring: cell sample population, proportion of micronuclei present in the samples, abnormal nuclei, and the proportion of apoptotic or necrotic cells in the samples. Nuclear fragments were scored as micronuclei if they were less than one third the diameter of the main nuclei. Abnormal, apoptotic, and necrotic cells were excluded from the MN statistical analyses, but were observed only to quantify cell death within the chemical treatments. Apoptotic bodies were confirmed as cells undergoing apoptosis using the AO phase. Cells that

were undergoing mitosis or did not have a defined nuclear boundary were also excluded from scoring. Approximately 2,000 cells were scored for each trial in duplicate although specific densities varied. Examples of photomicrographs that were scored are shown in Figures 4.1-4.3 below.

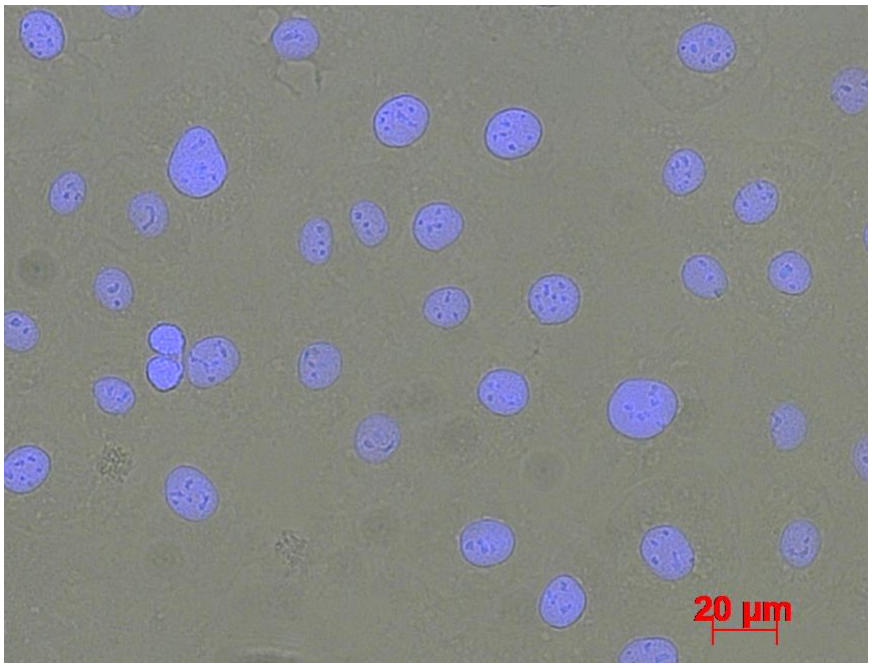


Figure 4.1. Normal K-9 cells were image at 20x magnification using multi-phase of DAPI fluorescence and bright field phase. The blue illuminations are stained nuclei.

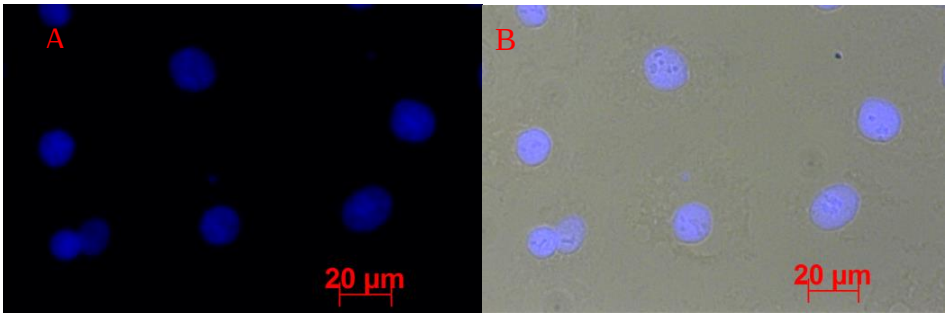


Figure 4.2. A magnified K-9 cell is shown with two different contrasts. A) only DAPI is shown where MN are identified. B) DAPI+ brightfield is used to illuminate cell boundaries.

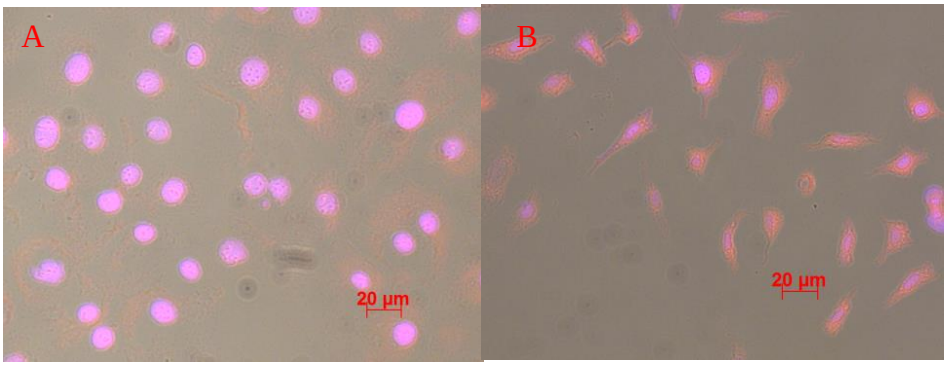


Figure 4.3. Two examples of cells that do not meet scoring criteria. These cells are shown in multi-phase with DAPI + Acridine Orange + bright field phase. A- The conjoined cells have abnormal nuclei and will not be scored. B- Many of these cells are undergoing cell death because their DNA was seen outside their nuclei. AO was used to confirm.

Statistical and Data Analyses

All statistical analyses were performed using Microsoft Excel and Sigmaplot 11.0 software from Systat. A student's t test was used for all proliferation and viability data. The treatment groups were each compared directly to their respective unmodified control and solvent control groups. After verifying the data is normally distributed and that the variances are equal, a two-way student's t-test was performed to compare means of the treatments with the solvent control groups, assigning an alpha value of 0.05 to determine statistical significance.

MN count data was converted into proportions of micronucleated cells per 1,000 cells in each sample, as described by Matsushima et al (1999) and Fenech et al (2003).[89] For the MN assay, a different statistical test was necessary for the smaller data sets that were neither normally distributed nor had equal variances.[68] The Mann-Whitney rank test was chosen because it has been used effectively in other published work with micronuclei tests.[90] First, all control cell subpopulations were compared to the solvent

control subpopulations. In binary trials, each concentration ratio was compared to the solvent control population to produce a p-value for each mixture.

Mixture analysis. In 1986, the EPA published guidelines for the risk assessment of chemical mixtures. The guidelines contain models for analyzing the interactions of chemicals within simple mixtures.[91] The equation below shows an adapted model that was first developed by Finney in 1942 to determine if a mixture's dose-response relationship displays a synergistic, antagonistic or additive chemical interaction when the mixture is compared to the dose-response information of the individual components.[92] The data required log transformation using probit analysis and was performed using StatsDirect statistical software.[91]

$$Y = a_1 + b \log(f_1 + p f_2 + K [p f_1 f_2]^{0.05}) + b \log Z$$

Where:

a_1 = y-intercept of the dose-response equation for the index chemical (1)

b = slope of the dose-response equation for the mixture

f_i = proportion of the i^{th} chemical with respect to the index chemical

p = potency of i^{th} chemical with respect to the index chemical

Z = sum concentration of the chemicals in the mixture

Another method of assessing mixture interactions was employed to be able to incorporate the full dose-response curves, whereas the probit analysis is sensitive to specific mixture ratios. The toxicities of each mixture component were added together to form the Expected Additive response of the mixture.[93]. The expected response was then compared to the actual response. The results were in agreement for both methods of mixture analysis.

QSAR Model

QSAR models are designed to use experimental data sets that are preferably uniform, large, and specific to one biological response.[55] The chosen biological response was the effective chemical concentration that reduced the percentage of viable cells by 50% (EC_{50}), which was approximated using linear regression of the CFDA data. The CFDA data was chosen for developing these models because it is normally distributed and contains no anomalies among the treatments. Linear regressions were performed for every dataset, and the slopes of the curves were used to approximate the EC_{50} of each. The $\log K_{OW}$ values were also used as descriptors because previous research has indicated they may be an important tool to predict PAH activity.[94]

The software chooses 80% of the 15 PAHs to include in the training set. The remaining PAHs are used to test the accuracy of each model (the test set). Discovery Accelrys has dozens of molecular descriptors that can be used as independent variables. Molecular, topological, and partitioning descriptors were included as the most accurate to develop a model. The software generates an equation by using the descriptors (independent variables) and the EC_{50} data (dependent variable) from the training set, and then that equation can be used to predict the EC_{50} of many other congeners in the class. [50]

The genetic function approximation algorithm was used to determine the best predictive model for PAHs' EC_{50}. The appropriateness of each model is determined using various fitness parameters such as correlation coefficients (r^2 and r^2 adjusted) and Friedman lack of fit measure. The fitness depends on the appropriateness of molecular descriptors. Some descriptors do not contribute to the accuracy of the model, and the software will recognize this and eliminate them.

Three models were developed: one employing the EC_{50} of single PAHs, another employing the EC_{50} of the binary mixtures, and another employing the EC_{50} of the reversed binary mixtures.[93] The mixture models account for the observed mixture interactions occurring in the viability trials. After the best regression is developed using the molecular structures, the algorithms for the mixtures are modified to incorporate the component ratios within the mixture, as outlined by Altenburger et al (2003).[93]

CHAPTER FIVE

Results

Cellular Toxicity

Single Compounds

Proliferation. The proliferation data for all 15 single compounds are displayed in Figure 5.1. The figure displays the mean optical density (absorbance) of the JG dye. The absorbance reading corresponds with the cell population within the samples and gives information about the sample populations' ability to grow and proliferate. The separate control groups correspond with different plates (one per chemical), and may vary between trials. Statistical significance was indicated by one asterisk above specific bars.

Anthracene, benzo[a]pyrene, benzo[e]pyrene, benzo[k]fluoranthene, and chrysene did not produce results that were not statistically different from the control: all p-values were larger than 0.05 for all treatments. Acenaphthylene, benz[a]anthracene, fluoranthene and 9-methyl-anthracene displayed partial decline, with p-values less than 0.05 in 1 or 2 treatments. Benzo[b]fluoranthene, 3,6-dimethylphenanthrene, 7,12-dimethylbenz[a]-anthracene, and 9,10-dimethylanthracene displayed a severe statistical decline, where p-values were less than 0.05 in 3 or 4 treatments. Phenanthrene showed a statistical increase in proliferation in two subsequent treatments. Pyrene also displayed a statistical increase in one isolated treatment.

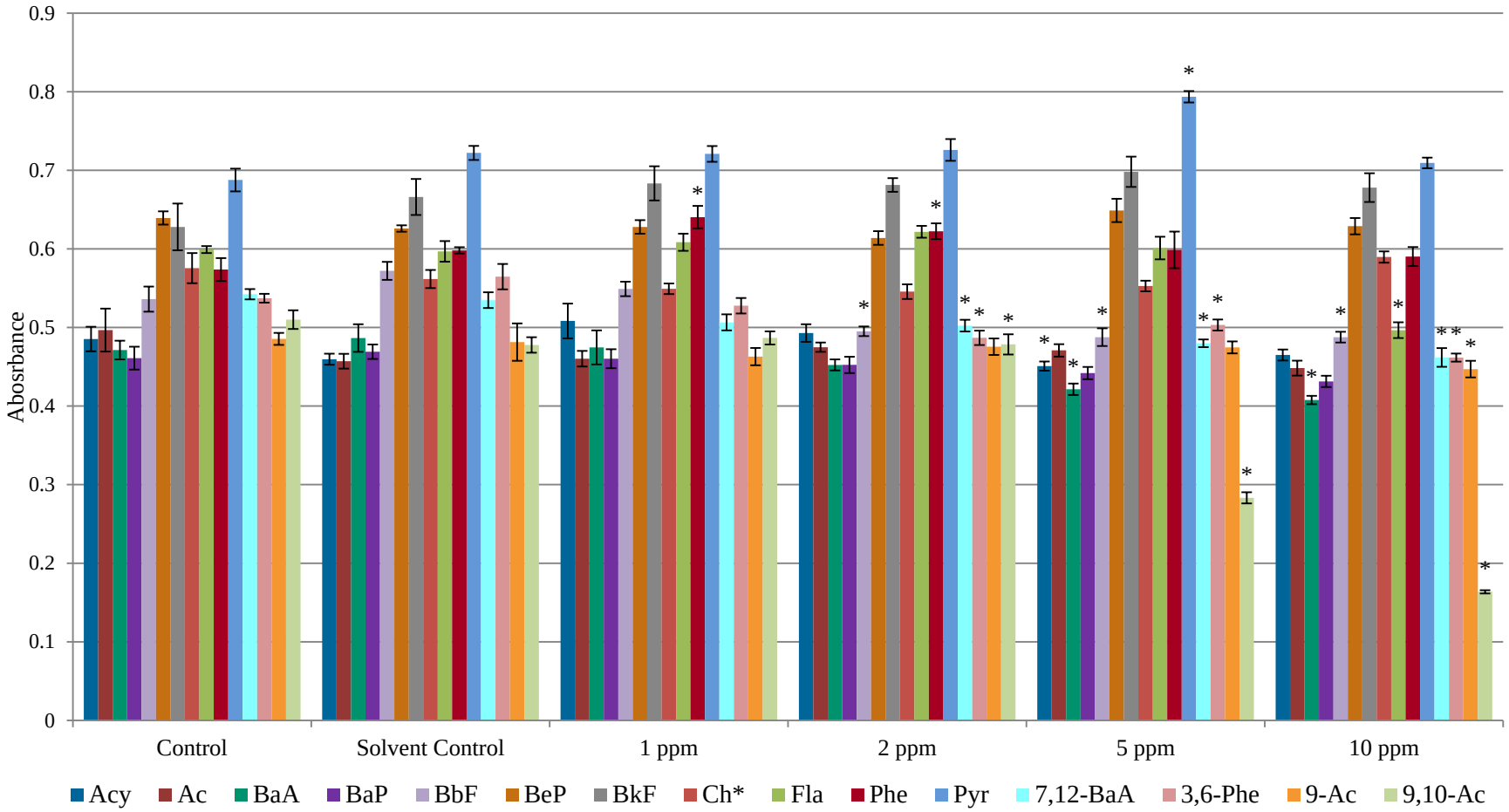


Figure 5.1 The mean absorbance of dyed cells indicates cellular proliferation after 24 hours of chemical exposure to 15 PAHs. Large values correspond with larger populations of cells. Asterisks above treatments indicate P-value < 0.05.

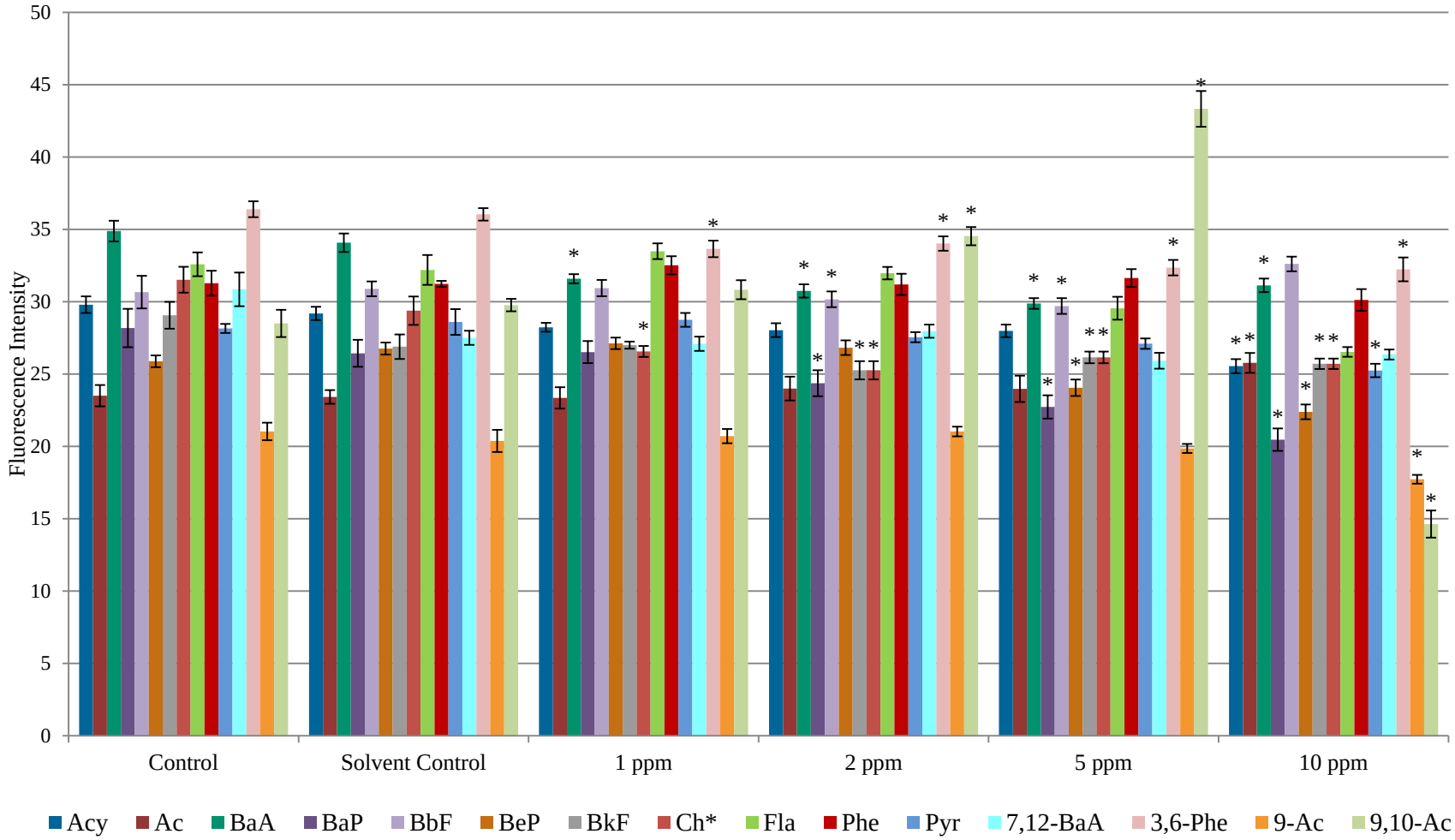


Figure 5.2 The mean fluorescence intensity of cells indicate cellular viability after 24 hours of chemical exposure to 15 PAHs. A strong signal corresponds with a larger population of viable cells. Asterisks above treatments indicate P-value <0.05.

Viability. Refer to Figure 5.2 on the previous page to see the viability results of all 15 single parent compounds in four different dosing treatments. The results displayed in Figure 5.2 are derived from the CFDA fluorescent probe. The fluorescence intensity is correlated with the cell viability; the higher the signal intensity, the more viable the cells. Phenanthrene did not show any significant response in any treatments. Acenaphthylene, benzo[b]fluoranthene, benzo[e]pyrene, fluoranthene, pyrene and 9-methylanthracene showed statistical declines (p-values <0.05) in 1 or 2 treatments.

Benz[a]anthracene, benzo[a]pyrene, benzo[k]fluoranthene, chrysene, 7,12-dimethyl-benz[a]anthracene, 3,6-dimethylphenanthrene showed severe statistical declines in 3 or 4 treatments (p-values < 0.05). Anthracene displayed an increase in viability in the highest treatment. 9,10-dimethylanthracene displayed a statistical increase in viability at 2 ppm and 5 ppm, followed by a sharp statistical decline at 10 ppm.

Binary Mixtures

The data for binary mixtures is arranged for each assay and chemical. The first data set in each figure -entitled "Single"- displays the data for the single compound data (solid color) to display the dose-response trend of each PAH compound. The second data set – entitled "Binary"- includes binary mixtures where BaP is held constant at 1 ppm, and the other PAH in the mixture varies in concentration. The ratios (BaP:PAH) for these mixtures are 1:1, 1:2, 1:5, and 1:10. The third data sets- entitled "Reverse Binary"- include the reverse of the binary mixtures, and the ratios are 1:1, 2:1, 5:1, and 10:1. Each treatment group (single, binary, and reverse binary) contains their own control group per plate, causing a variation in cell densities. The horizontal axis is labeled according to the chemical that is varied. The "BaP 1 ppm" label refers to a treatment where the cells

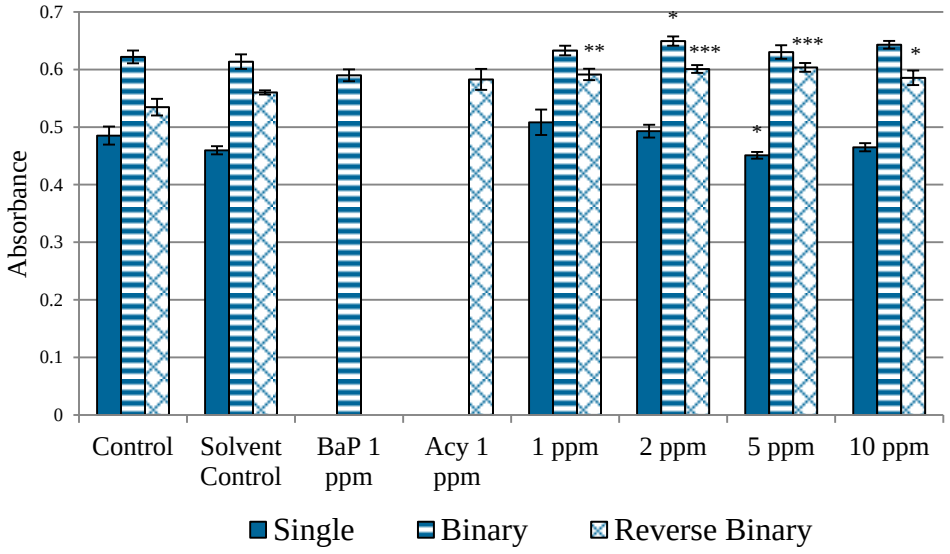
received only BaP at 1 ppm, and is used a reference. The "PAH 1 ppm" label refers to a treatment where the cells received only that specific PAH at 1 ppm, and statistically agrees with the single data. Figure A.2 in the Appendix displays a detailed description for interpreting the mixture charts.

Proliferation. Anthracene, benzo[a]pyrene, benzo[e]pyrene, benzo[k]fluoranthene and chrysene showed no statistical difference from control groups in single trials (figure 5.3). Binary trials showed different trends, however. The statistical differences observed in Anthracene trials were increases seen in three treatments of the reverse binary trial. The only significant response seen in benzo[e]pyrene mixtures occurred in the reverse binary trial, where the two highest treatments displayed significant declines. All benzo[k]fluoranthene mixture data had no trending effect on the proliferation of the cells. The chrysene binary trial showed a decline in the highest treatment. The reverse binary trial showed an increase in proliferation in the two highest treatments.

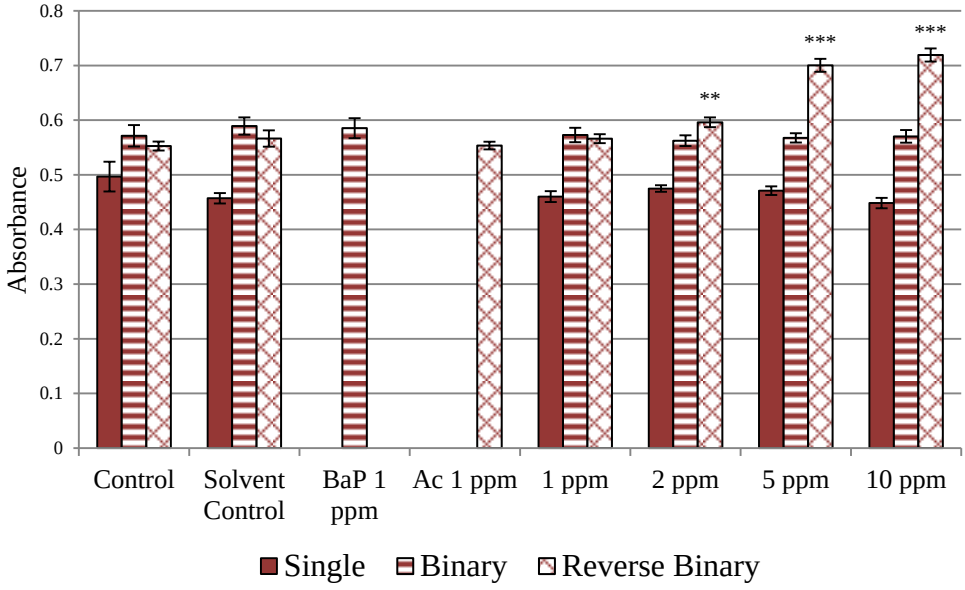
Acenaphthylene, benz[a]anthracene, fluoranthene, and 9-methylanthracene all displayed a statistical decline in single trials in one or two treatments. Acenaphthylene mixtures did not see any statistical decline but several treatments showed statistical increases, especially in the reversed binary trials (figure 5.3a). The mixture data of benz[a]anthracene showed no evidence of a decline in proliferative capability of cells (figure 5.3c). Statistical increases in proliferation were observed in 2 treatments of the benz[a]anthracene mixture data. In fluoranthene mixtures, no decline in proliferative capability was observed. The binary trial showed an increase in the highest treatment, and the reverse binary trial showed an increase in the two highest treatments (figure 5.3h).

In mixture trials of 9-methylanthracene, the only significant statistical difference was a decline in the highest treatment group (figure 5.3m).

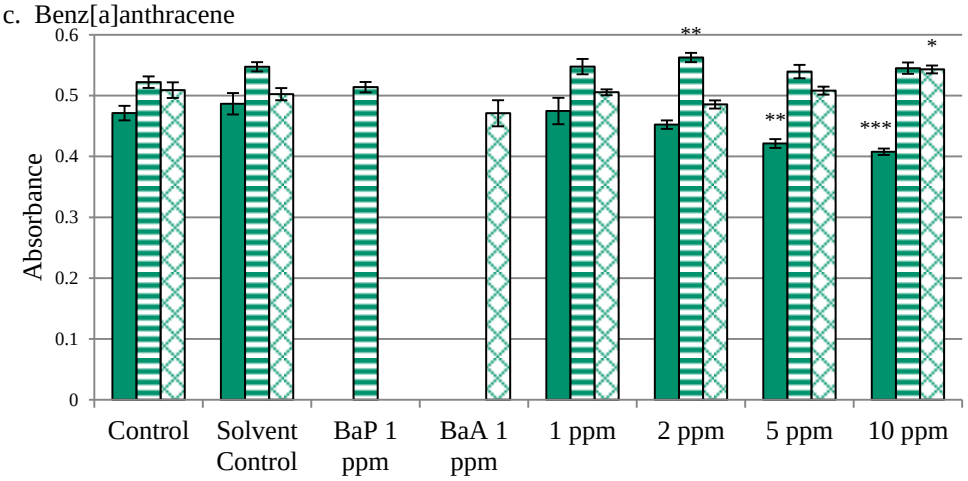
a. Acenaphthylene



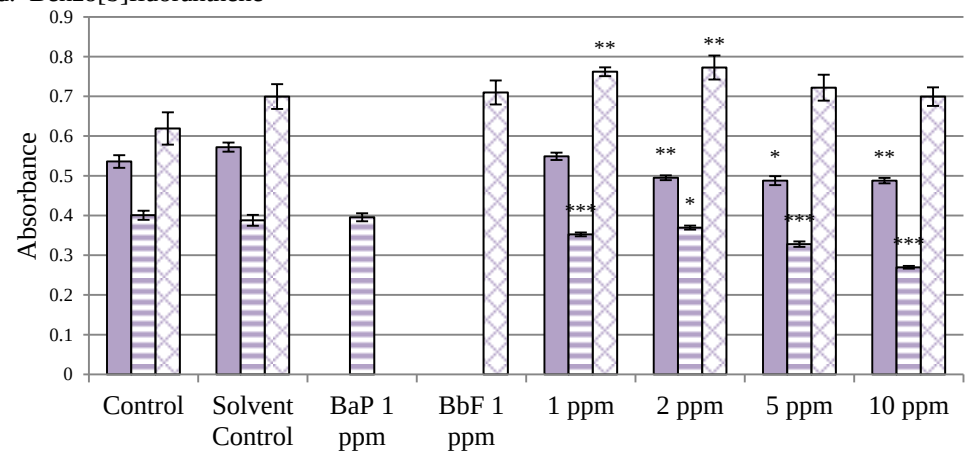
b. Anthracene



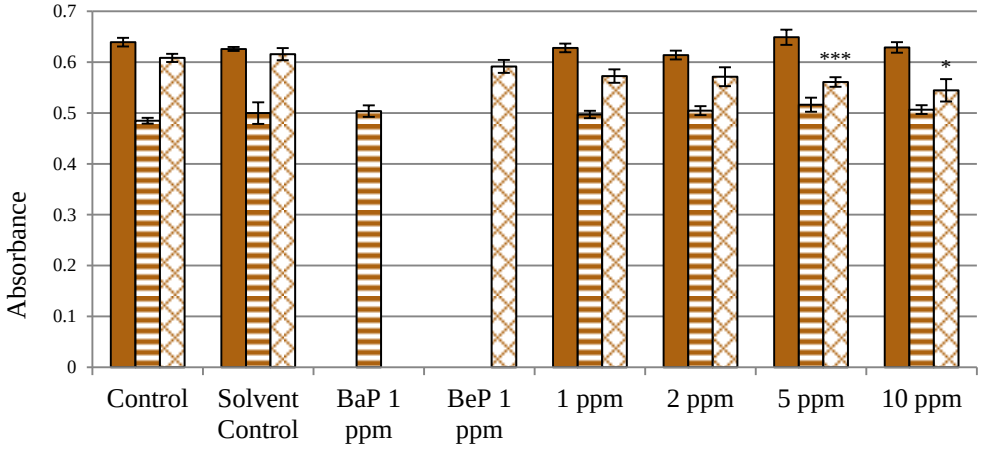
c. Benz[a]anthracene



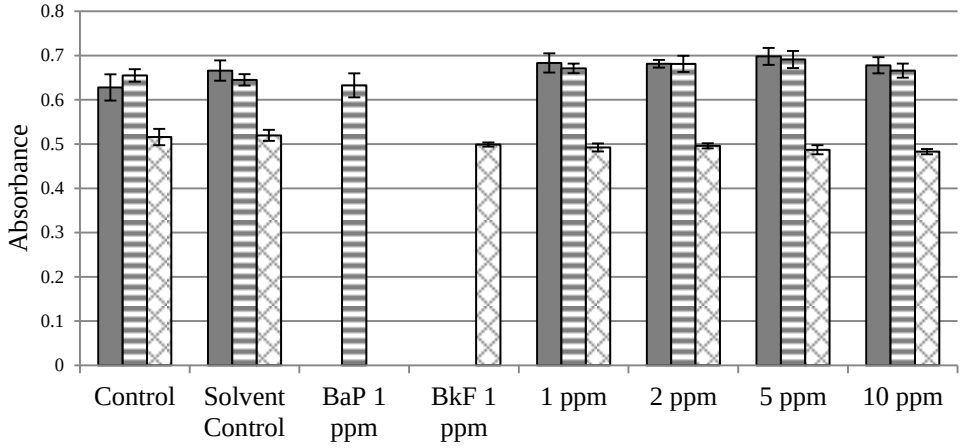
d. Benzo[b]fluoranthene



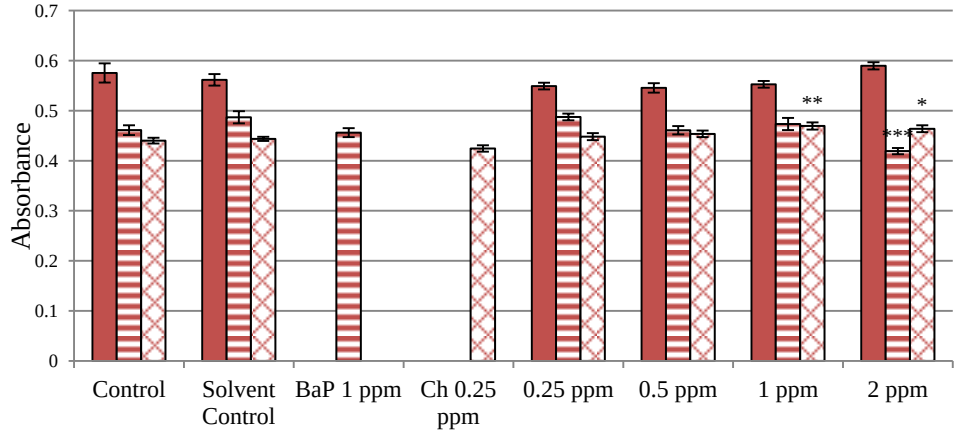
e. Benzo[e]pyrene



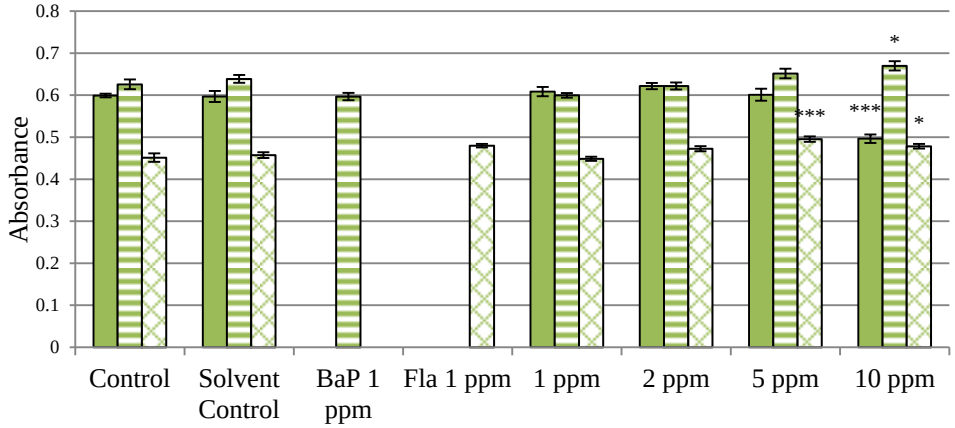
f. Benzo[k]fluoranthene



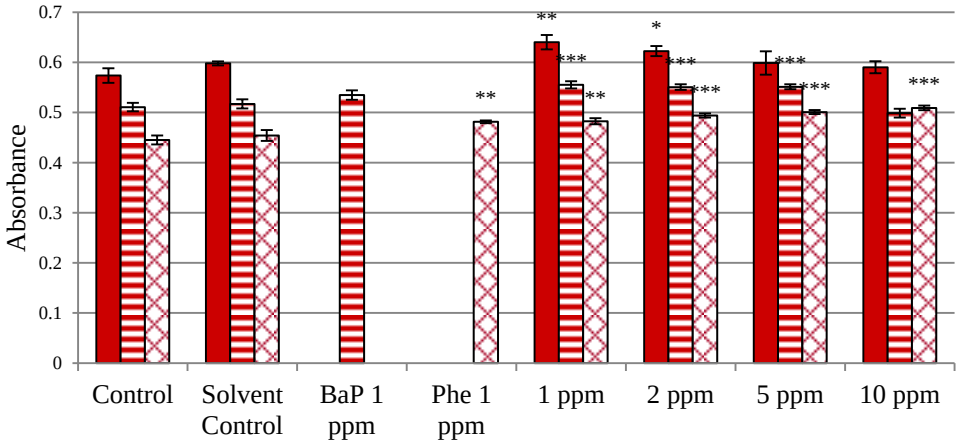
g. Chrysene



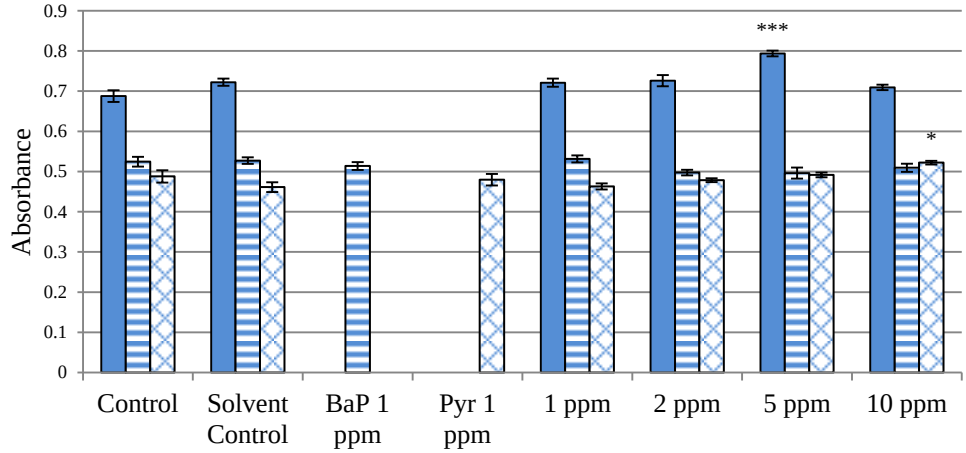
h. Fluoranthene



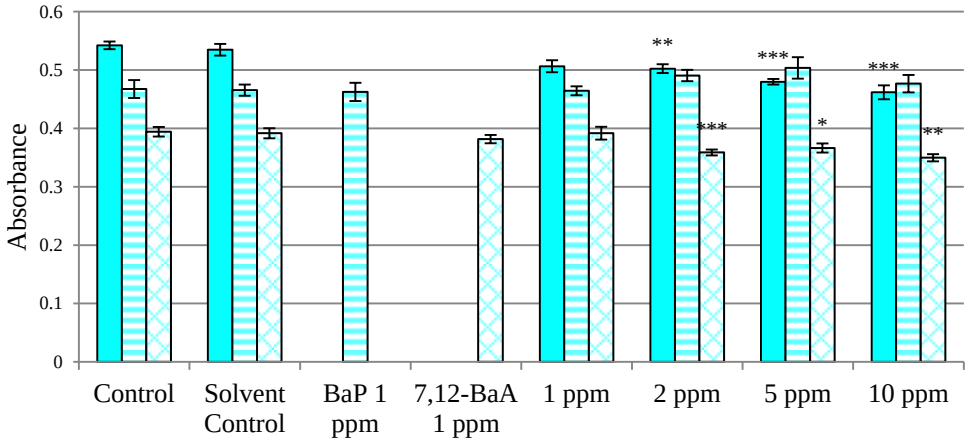
i. Phenanthrene



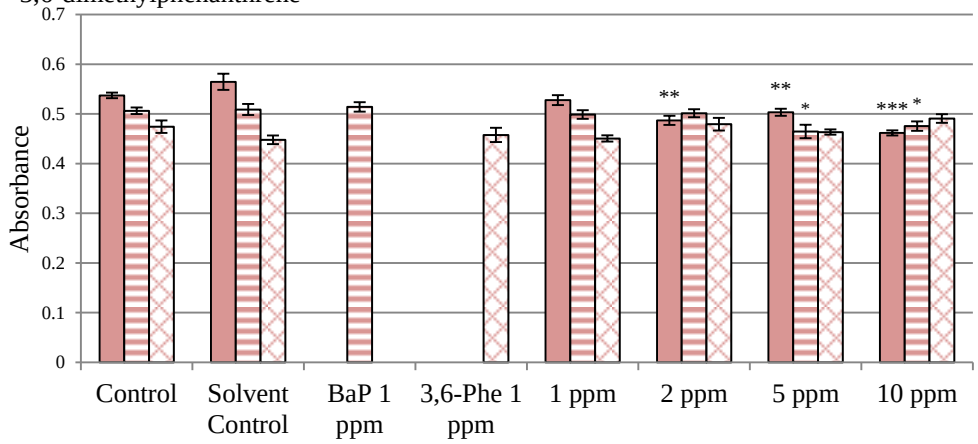
j. Pyrene



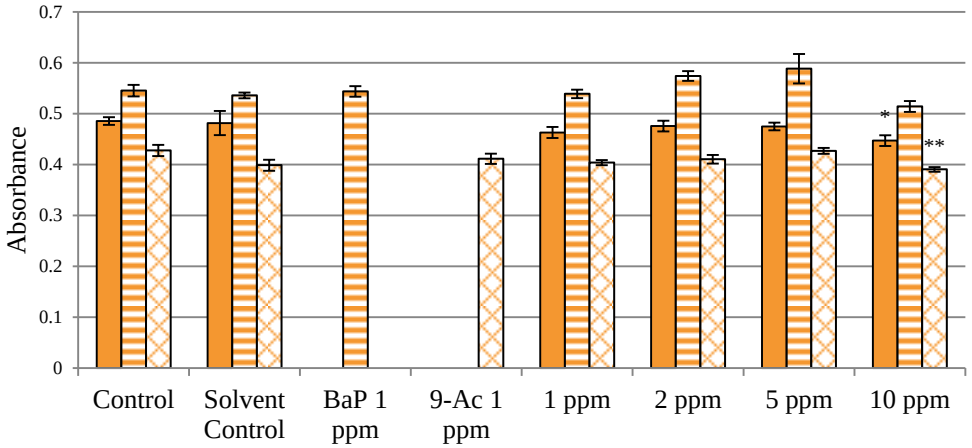
k. 7,12-dimethylbenz[a]anthracene



l. 3,6-dimethylphenanthrene



m. 9-methylanthracene



n. 9,10-dimethylanthracene

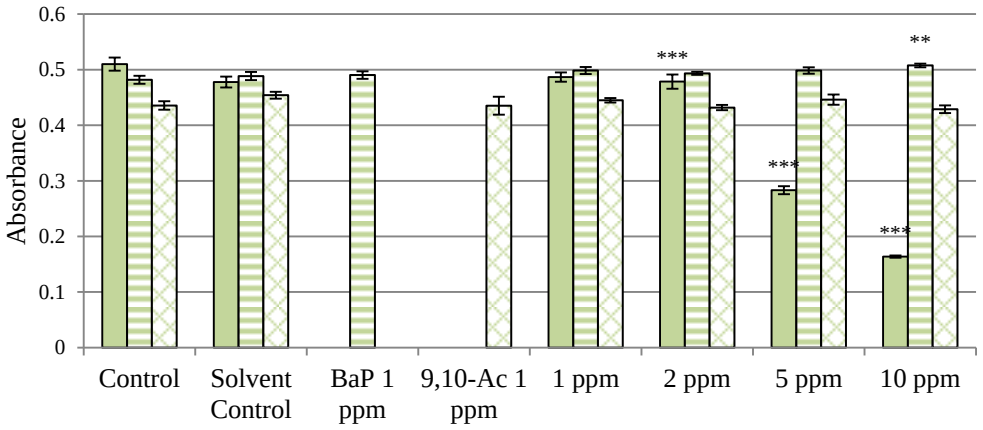


Figure 5.3 The mean absorbance of cells indicate cellular proliferation after 24 hours of chemical exposure to 14 PAHs in binary mixtures with BaP. One asterisk corresponds with a P-value <0.05, two with a P-value < 0.01, and three with a P-value < 0.001. The legends in a and b apply throughout.

Some single PAHs, as mentioned previously, caused a severe decline in proliferation and include benzo[b]fluoranthene, 7,12-dimethylbenz[a]anthracene, 3,6-dimethyl-phenanthrene, and 9,10-dimethylanthracene. The benzo[b]fluoranthene binary trial showed a severe response, with all four treatments significantly impairing the growth of cells. The BbF reverse binary trial did not cause a decline, but the cell populations in two subsequent treatments were significantly higher than the control groups. 7,12-dimethylbenz[a]anthracene binary trial (figure 5.3k) did not have any treatment groups that were significantly different from the control. In the 7,12-BaA reverse binary trial the three highest treatments showed severe decline. 3,6-dimethylphenanthrene trials showed a less severe response, with declines in the two highest treatment groups in binary (figure 5.3l). The reverse binary trial showed no decline in proliferation. Both binary trials of 9,10-dimethylanthracene displayed a diminished effect on proliferation, with significance only in the highest treatment group in the first trial (figure 5.3n). Phenanthrene and pyrene caused significant increases in proliferation of cells exposed to single concentrations. Similarly, all phenanthrene mixtures caused an increase in proliferation in the majority of treatments, as seen in figure 5.3i. Pyrene mixtures trials showed no decline in proliferation but one treatment showed significant increase.

A summary of the results has been provided to show the overall trends of the mixture data compared to the single compound dose-response relationships (table 5.1). The data has been categorized according to statistical significance: statistical increase in at least one treatment (increase), no statistical difference from control (no change), and statistically significant declines (1-2 treatments, and 3-4 treatments).

Table 5.1 A summary of the proliferation data arranged by statistical significance of treatments groups for single PAHs, binary and reverse binary mixtures.

Chemical		Increase	No Change	Decline 1-2 Treatments	Decline 3-4 Treatments
BaP	Single			✓	
	Single			✓	
Acy	Binary	✓	✓		
	R.B	✓	✓		
	Single		✓		
Ac	Binary		✓		
	R.B	✓	✓		
	Single			✓	
BaA	Binary	✓	✓		
	R.B	✓	✓		
	Single				✓
BbF	Binary				✓
	R.B	✓	✓		
	Single		✓		
BeP	Binary		✓		
	R.B			✓	
	Single		✓		
BkF	Binary		✓		
	R.B		✓		
	Single		✓		
Ch	Binary			✓	
	R.B	✓	✓		
	Single			✓	
Fla	Binary	✓	✓		
	R.B	✓	✓		
	Single		✓		
Phe	Binary	✓	✓		
	R.B	✓	✓		
	Single			✓	
Pyr	Binary		✓		
	R.B	✓	✓		

Table 5.1 continued

Chemical		Increase	No Change	Decline 1-2 Treatments	Decline 3-4 Treatments
	Single				✓
7,12-BaA	Binary		✓		
	R.B				✓
	Single				✓
3,6-Phe	Binary			✓	
	R.B		✓		
	Single			✓	
9-Ac	Binary		✓		
	R.B			✓	
	Single				✓
9,10-Ac	Binary			✓	
	R.B		✓		

Viability. Figure 5.4a-n begins on page 62 and displays all the viability results for the single, binary and reversed binary trials, similar to the proliferation charts. Acenaphthylene, anthracene, benzo[b]fluoranthene, benzo[e]pyrene, fluoranthene, pyrene, and 9-methylanthracene displayed statistical declines in one or two treatments. Acy binary data showed a strong decline in three treatment groups, and reverse binary in two treatment groups (figure 5.4a). The anthracene binary data reveals that the cells appeared as viable as the control cells in all treatments except for the 10 ppm treatment (figure 5.4b). Reverse binary data showed a dose dependent decline in viability in the three highest treatments. Benzo[b]fluoranthene in binary trial did not inhibit the viability of cells in any treatment group (figure 5.4d). When the concentration of BaP varied, however, there was a severe response, with viability severely inhibited in the three highest treatments of BbF. In both binary mixtures of benzo[e]pyrene, the three highest treatment groups were significantly less viable than the control groups (figure 5.4e). The

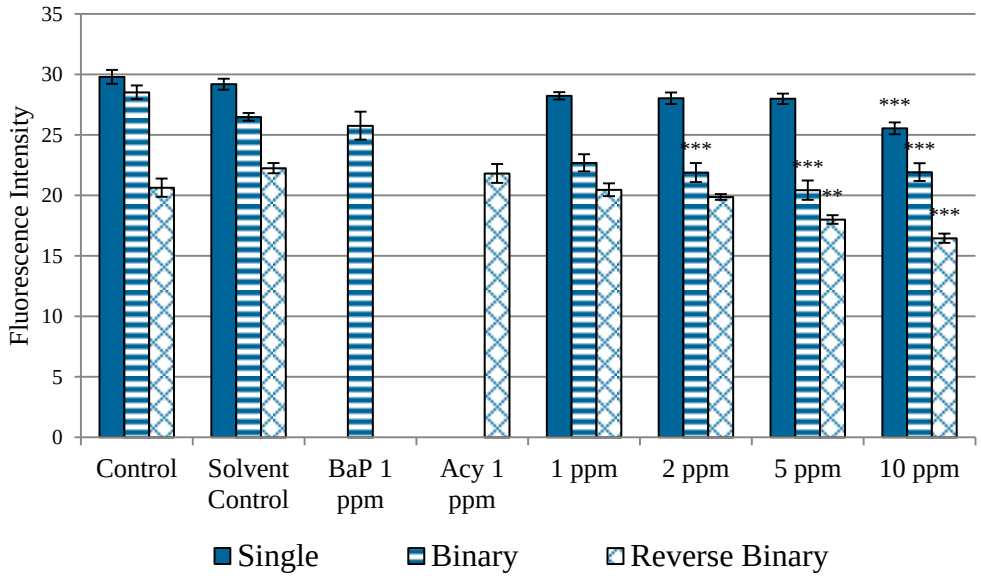
fluoranthene binary trial caused a decline in the three highest treatment groups, and the reverse binary trial in the two highest treatment groups (figure 5.4h). Pyrene binary trials did not display severe response, with declines in the two highest treatment groups of the reverse trial. In both binary trials of 9-methylanthracene, the three highest treatment groups were statistically lower than the control groups.

Benz[a]anthracene, benzo[k]fluoranthene, chrysene, 7,12-dimethylbenz[a]anthracene, and 3,6-dimethylphenanthrene showed statistical declines in three or four treatment groups. In figure 5.4c, both the binary and reverse binary of benz[a]anthracene trials showed decline in the three highest treatments. Benzo[k]fluoranthene binary mixtures caused a severe statistical decline in viability in three treatments, and the reverse binary trial showed the two highest treatments significantly lower than control groups. In figure 5.4g, both chrysene trials showed a decline in the three highest treatment groups. In 7,12-dimethylbenz[a]anthracene mixtures (figure 5.4k), all treatment groups had significantly fewer viable cells than their control groups. 3,6-dimethylphenanthrene showed a similar trend, where the mixtures showed a significant statistical decline in the three highest treatments (figure 5.4l).

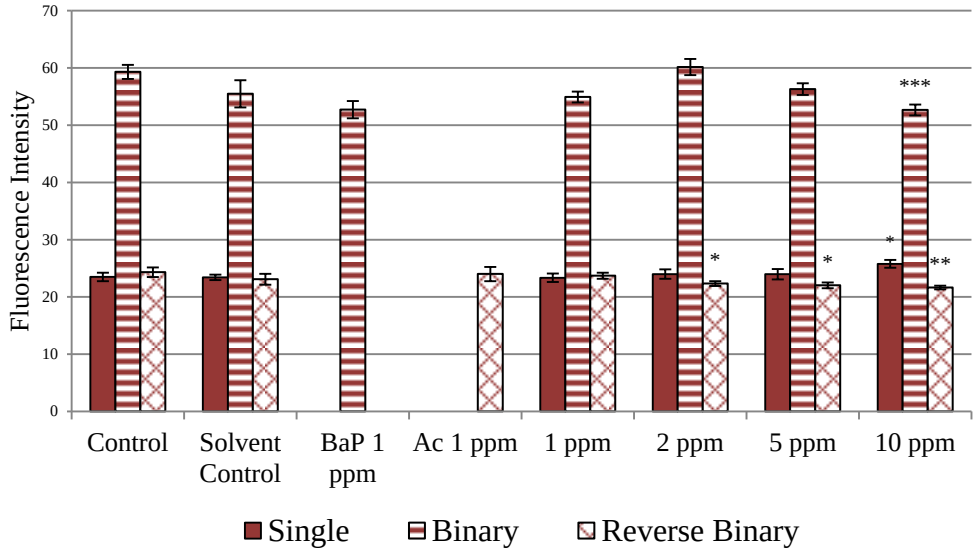
The single trial of 9,10-dimethylanthracene did not display a dose-dependent relationship (figure 5.4n). The treatment groups showed a statistical increase in proliferation, followed by a sharp statistical decline and rapid death in the highest treatment group. The binary trial of showed a statistical increase in the two highest treatments. The reverse binary trials, however, displayed a dose-dependent relationship with a significant statistical decline in the three highest treatments.

A summary of the viability data has been provided that displays the overall trends of the treatment groups from single PAHs and mixtures (table 5.1). As before, the data has been categorized according to statistical significance: statistical increase in at least one treatment (increase), no statistical difference from control (no change), and statistically significant declines (1-2 treatments, and 3-4 treatments).

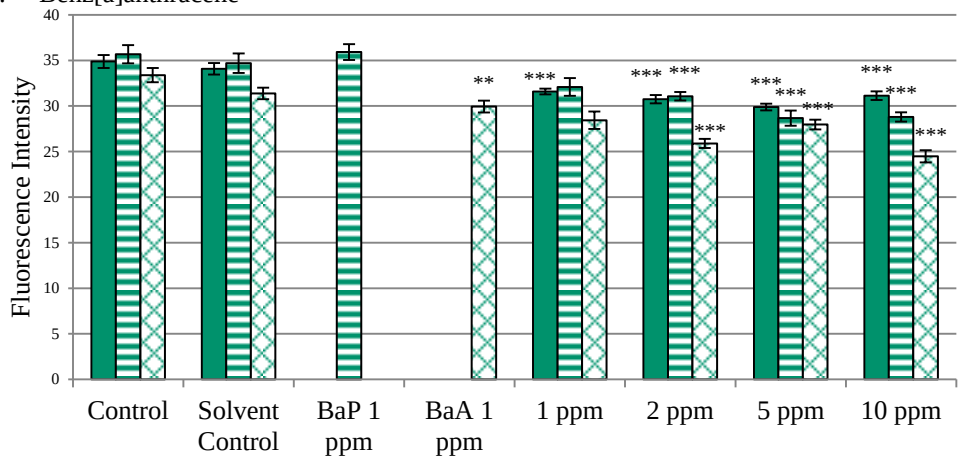
a. Acenaphthylene



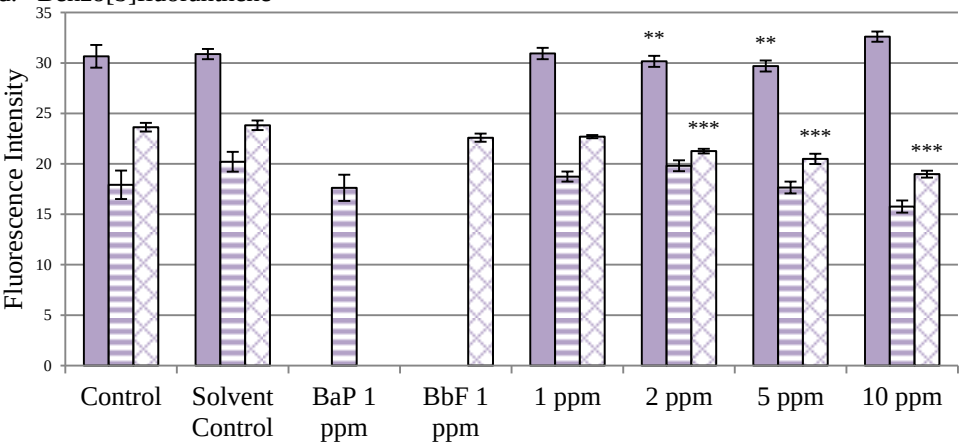
b. Anthracene



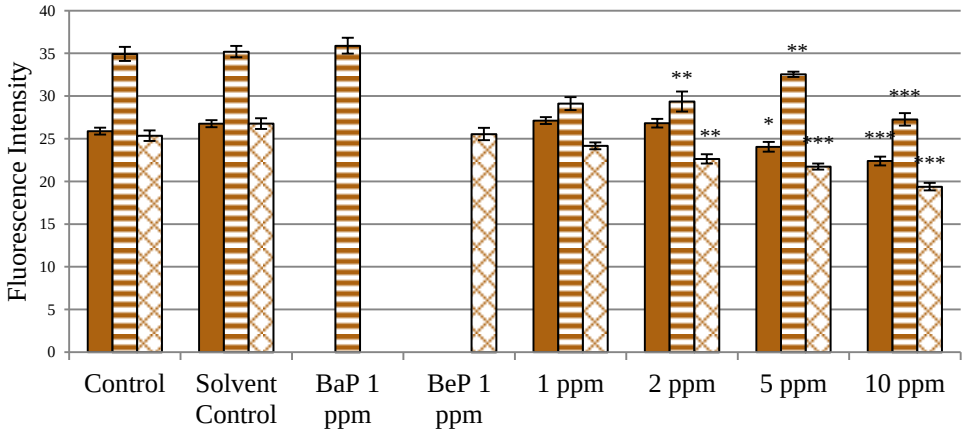
c. Benz[a]anthracene



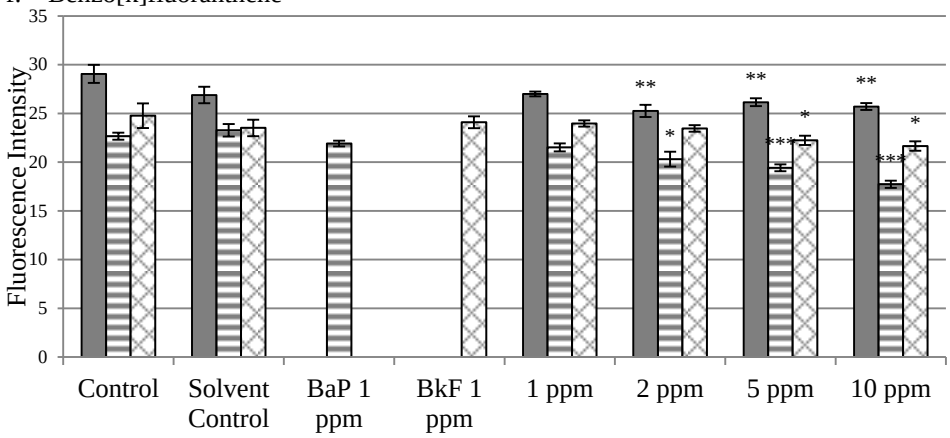
d. Benzo[b]fluoranthene



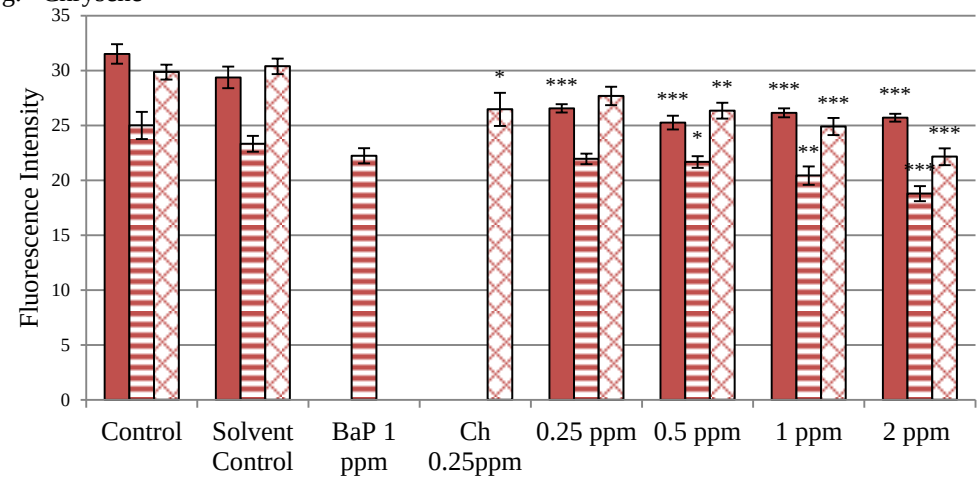
e. Benzo[e]pyrene



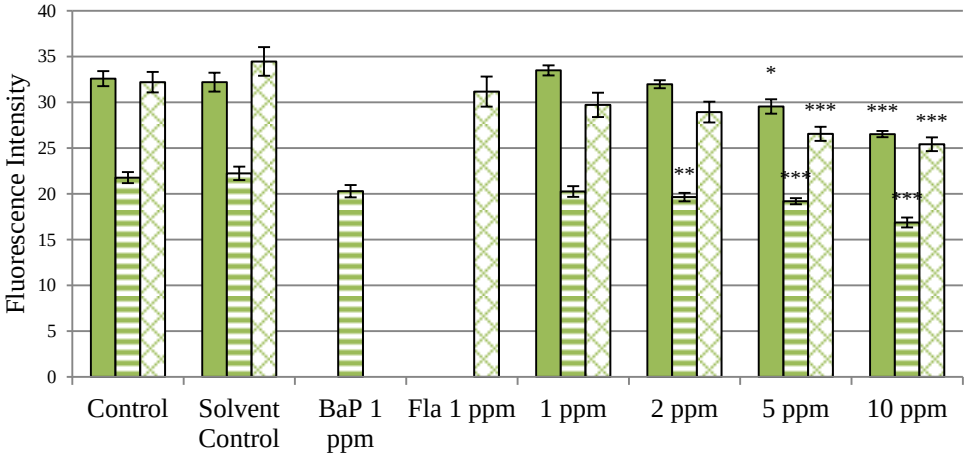
f. Benzo[k]fluoranthene



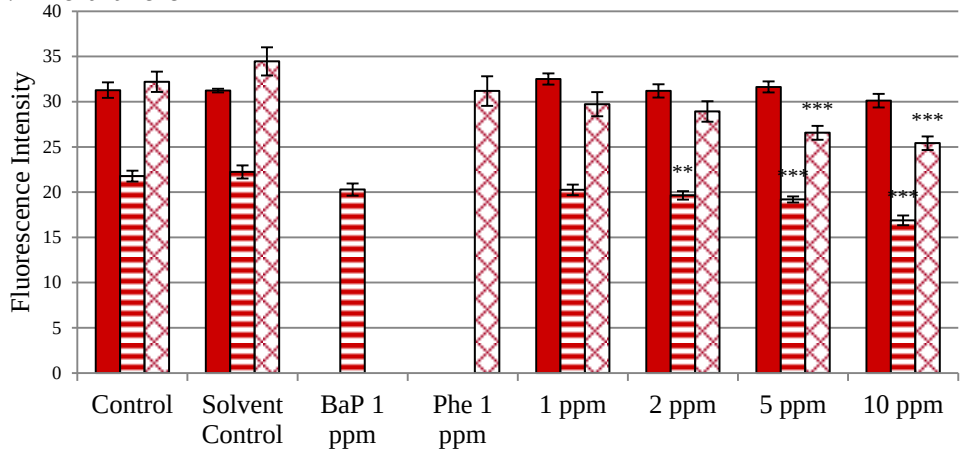
g. Chrysene



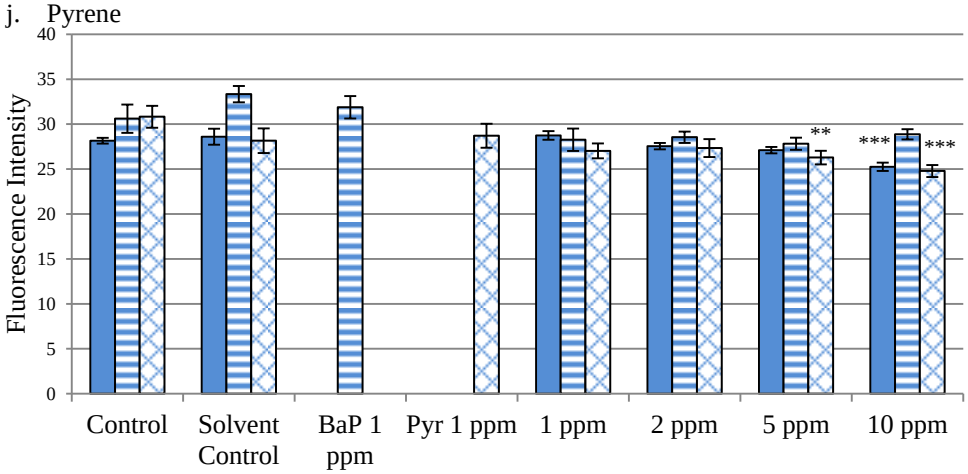
h. Fluoranthene



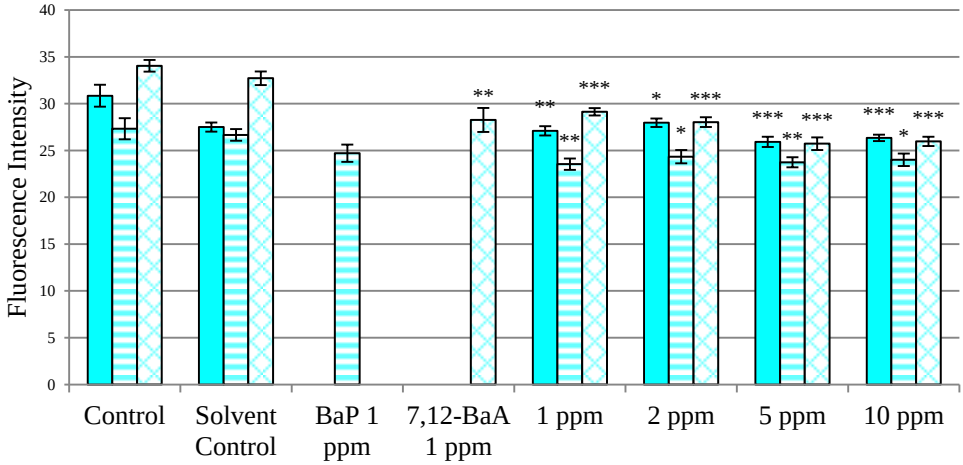
i. Phenanthrene



j. Pyrene



k. 7,12-dimethylbenz[a]anthracene



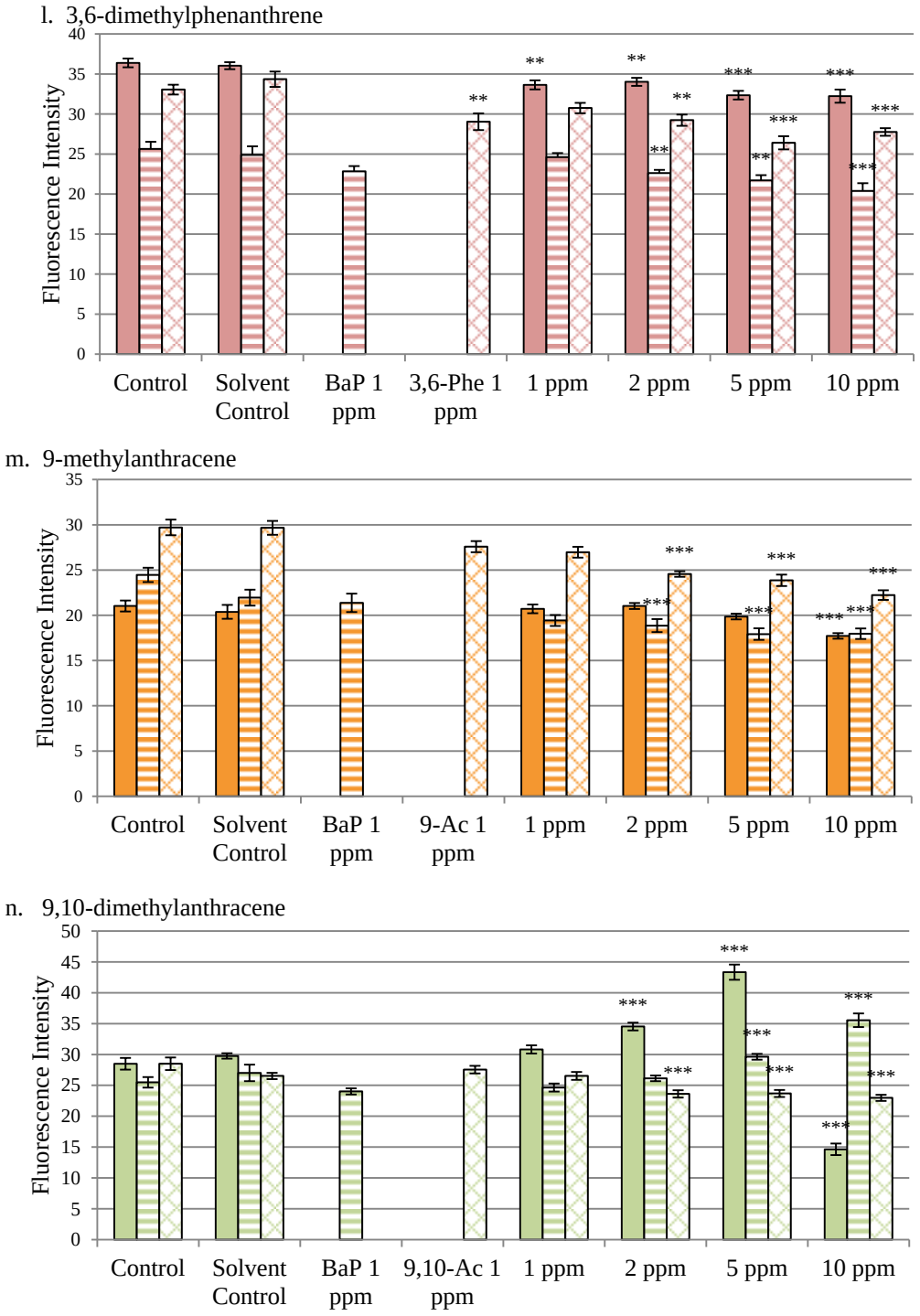


Figure 5.4 The mean fluorescence intensity of cells indicate cellular viability after 24 hours of chemical exposures. Greater intensity corresponds with a larger population of viable cells. As before, asterisks denote statistical significance.

Table 5.2 A summary of the viability data arranged by statistical significance of treatments groups for single PAHs, binary and reverse binary mixtures.

Chemical		Increase	No Change	Decline 1-2 Treatments	Decline 3-4 Treatments
BaP	Single				
	Single			✓	
Acy	Binary				✓
	R.B			✓	
	Single	✓	✓		
Ac	Binary			✓	
	R.B				✓
	Single				✓
BaA	Binary				✓
	R.B				✓
	Single			✓	
BbF	Binary		✓		
	R.B				✓
	Single			✓	
BeP	Binary				✓
	R.B				✓
	Single				✓
BkF	Binary				✓
	R.B			✓	
	Single				✓
Ch	Binary				✓
	R.B				✓
	Single			✓	
Fla	Binary				✓
	R.B			✓	
	Single		✓		
Phe	Binary				✓
	R.B			✓	
	Single			✓	
Pyr	Binary		✓		
	R.B			✓	

Table 5.2 continued

Chemical		Increase	No Change	Decline 1-2 Treatments	Decline 3-4 Treatments
7,12-BaA	Single				✓
	Binary				✓
	R.B				✓
3,6-Phe	Single				✓
	Binary				✓
	R.B				✓
9-Ac	Single			✓	
	Binary				✓
	R.B				✓
9,10-Ac	Single	✓		✓	
	Binary	✓			
	R.B				✓

Genotoxicity

The results for the Micronucleus assay are displayed below in Table 5.3. The table shows the average micronuclei frequency per 1,000 cells in the sample population in all treatment groups. The data shown is for the lowest and highest tested concentrations in the binary mixtures, with ratios 1:1, 1:10 and 10:1 (BaP:PAH). If a p-value <0.05, the treatment was assigned a positive result and the chemical or mixture is interpreted as causing significant genotoxicity when compared to the control group. Negative results were assigned when the frequencies of the treatments were insignificant to the control groups. “N” next to p-values indicate there was a significant decrease in MN.

Table 5.3 The results of the MN assay in single PAH concentrations and various combinations of binary mixtures of PAHs.

Chemical	Treatment (BaP:PAH)	MN Freq/ 1,000 Cells	Cells Scored	Dying Cells (%)	Abnormal Nuclei (%)	P-value	Result +/-
Control		0.01303	7739	0.28	0.62		
Solvent Control		0.01313	4809	0.93	0.19	0.979	Negative
BaP	1 ppm	0.02191	1278	0.47	0.23	0.001	Positive
	10 ppm	0.01829	1148	0.17	0.26	0.001	Positive
Acy	1 ppm	0.01873	995	1.18	0.89	0.062	Negative
	10 ppm	0.04020	776	1.77	0.38	0.003	Positive
	1:1	0.00462	1733	0.84	1.80	0.830	Negative
	1:10	0.00695	1870	0.68	1.26	0.640	Negative
	10:1	0.00851	1997	0.15	0.10	0.061	Negative
Ac	1 ppm	0.04103	819	0.24	0.73	0.004	Positive
	10 ppm	0.05054	689	1.54	2.10	0.037	Positive
	1:1	0.01359	1840	0.00	0.49	0.001	Positive
	1:10	0.00627	1754	0.11	0.23	0.246	Negative
	10:1	0.01151	956	0.62	0.62	0.321	Negative
BaA	1 ppm	0.02386	936	0.42	2.08	0.029	Positive
	10 ppm	0.03704	880	0.66	1.88	0.001	Positive
	1:1	0.03166	1611	0.74	0.55	0.001	Positive
	1:10	0.03972	1435	1.23	0.82	0.001	Positive
	10:1	0.00644	1707	0.18	0.23	0.106	Negative
BbF	1 ppm	0.07078	1049	0.75	0.94	0.001	Positive
	10 ppm	0.20588	669	3.01	5.47	0.001	Positive
	1:1	0.03777	1509	5.67	0.25	0.001	Positive
	1:10	0.08935	1052	4.16	4.76	0.001	Positive
	10:1	0.01752	1884	0.37	0.16	0.001	Positive
BeP	1 ppm	0.01691	946	1.94	1.63	0.422	Negative
	10 ppm	0.02389	921	1.60	0.21	0.409	Negative
	1:1	0.01050	1904	0.05	0.31	0.008	Positive
	1:10	0.00813	1107	0.00	0.00	0.150	Negative
	10:1	0.00787	1907	0.11	0.11	0.536	Negative
BkF	1 ppm	0.01869	963	0.00	0.93	0.833	Negative
	10 ppm	0.02036	786	0.75	0.88	0.055	Negative
	1:1	0.00853	1290	0.23	0.69	0.154	Negative
	1:10	0.01289	1241	0.08	0.72	0.160	Negative
	10:1	0.01457	961	0.21	0.23	0.057	Negative
Ch	0.25 ppm	0.01278	2034	0.20	0.39	0.001	Positive
	1 ppm	0.01126	2043	0.05	0.15	0.001	Positive
	1: 0.25	0.01680	1257	0.32	0.47	0.126	Negative
	1:1	0.00820	1707	0.23	0.93	0.768	Negative

Table 5.3 continued

Chemical	Treatment (BaP:PAH)	MN Freq/ 1,000 Cells	Cells Scored	Dying Cells (%)	Abnormal Nuclei (%)	P-value	Result +/-
Ch	10:1	0.00796	1758	0.00	0.45	0.995	Negative
Fla	1 ppm	0.01419	916	0.22	0.11	0.188	Negative
	10 ppm	0.02605	499	0.00	0.40	0.003 N	Negative
	1:1	0.02407	1370	0.93	0.65	0.001	Positive
	1:10	0.03813	813	0.60	1.21	0.430	Negative
	10:1	0.00438	914	0.11	0.61	0.001 N	Negative
	1 ppm	0.02326	903	0.65	1.20	0.737	Negative
	10 ppm	0.02827	672	0.00	0.89	0.103	Negative
Phe	1:1	0.02535	1223	1.20	1.12	0.001	Positive
	1:10	0.01276	1019	0.39	0.87	0.025 N	Negative
	10:1	0.01312	1143	0.09	0.69	0.048 N	Negative
	1 ppm	0.01476	1016	0.58	0.87	0.462	Negative
	10 ppm	0.01084	830	1.74	1.74	0.001 N	Negative
Pyr	1:1	0.01844	1410	0.07	0.07	0.007	Positive
	1:10	0.01168	1027	0.00	0.48	0.001 N	Negative
	10:1	0.01275	941	0.00	2.18	0.783	Negative
	1 ppm	0.03333	870	1.24	0.79	0.020	Positive
	10 ppm	0.03521	852	0.35	0.32	0.031	Positive
7,12-BaA	1:1	0.01799	1056	0.99	24.01	0.536	Negative
	1:10	0.01629	921	0.37	31.96	0.002 N	Negative
	10:1	0.02483	886	1.45	0.00	0.038	Positive
	1 ppm	0.02886	1490	0.20	0.53	0.001	Positive
	10 ppm	0.02722	1580	0.31	0.57	0.001	Positive
3,6-Phe	1:1	0.00383	1304	0.14	39.49	0.016	Positive
	1:10	0.00000	1163	No Result	49.30	None	None
	10:1	0.01736	1037	0.48	0.67	0.014	Positive
	1 ppm	0.01232	1055	1.20	1.39	0.134	Negative
	10 ppm	0.01402	1070	0.37	0.92	0.612	Negative
9-Ac	1:1	0.02182	1283	0.23	0.16	0.003	Positive
	1:10	0.03383	1212	0.49	0.33	0.001	Positive
	10:1	0.01729	1157	0.60	0.17	0.001	Positive
	1 ppm	0.01704	1643	0.24	0.78	0.001	Positive
	10 ppm	0.02270	881	0.99	1.98	0.008	Positive
9,10-Ac	1:1	0.01794	2341	0.04	0.64	0.001	Positive
	1:10	0.03133	1532	0.26	0.65	0.001	Positive
	10:1	0.01862	1504	0.00	0.53	0.001	Positive

Analysis of Mixture Interactions

Mixture analysis is most reliable with binary mixtures that vary in chemical concentrations, and where one of the components has a well characterized response. The viability data (figure 5.4) was used to assess possible chemical interactions occurring between the PAHs in the binary mixtures using probit analysis. The viability data is normally distributed and has no anomalies among the treatment groups, making for a homogenous mixture analysis. Table 5.4 describes the findings from the analysis of mixture interactions using the viability data. The genotoxicity was also used to analyze mixture interactions that may affect the frequency of MN. The frequencies of single PAHs were used to derive the expected additive response, and then compared to the observed response. Table 5.5 is a summary of the observed mixture interactions for both cell viability and genotoxicity to compare the effects seen in each assay.

Table 5.4 An analysis of the chemical interactions occurring in various combinations of binary mixtures of BaP with one other PAH. The binary ratios were 1:1, 1:2, 1:5, 1:10. Reversed Binary (R.B.) ratios are 1:1, 2:1, 5:1, and 10:1.

Chemical		Probit Analysis	EC_{50} (mg/L)	Coefficient of Interaction	Mixture Interaction
BaP		Y= 0.877106x – 1.46518	18.9		
	Single	Y= 0.53839x - 1.712537	41.7		
Acy	Binary	Y= 0.146303x - 0.870472	22.7	Positive	Synergism
	R.B	Y= 1.45409x - 2.239469	23.4	Negative	Antagonism
	Single	Y = -0.5172x - 22.568	192.8		
Ac	Binary	Y = 0.8748x - 58.974	61.1	Negative	Antagonism
	R.B	Y= 0.567599 - 1.739012	49.5	Negative	Antagonism
	Single	Y= 0.103465x - 1.247856	46.3		
BaA	Binary	Y= 0.441253x - 1.250688	29.2	0	Additive
	R.B	Y= 0.288691x - 0.989015	23.5	Negative	Antagonism
	Single	Y = -0.2657x - 30.016	39.9		
BbF	Binary	Y = 0.5403x - 19.597	32.1	Positive	Synergism
	R.B	Y= 0.793158x - 1.638115	26.8	Negative	Antagonism

Table 5.4 continued

Chemical		Probit Analysis Model	EC_{50} (mg/L)	Coefficient of Interaction	Mixture Interaction
	Single	Y = -1.0056x + 28.227	33.9		
BeP	Binary	Y= 0.0662x - 1.049963	37.0	Negative	Antagonism
	R.B	Y= 0.864445 - 1.604017	22.4	Negative	Antagonism
	Single	Y= 0.173731x - 1.333384	61.6		
BkF	Binary	Y= 0.792498x - 1.580075	24.4	Positive	Synergism
	R.B	Y= 0.708984x - 1.821039	40.9	Negative	Antagonism
	Single	Y= 0.802351x - 1.759228	7.8		
Ch	Binary	Y= 0.564492x - 0.880382	4.5	Positive	Synergism
	R.B	Y=0.775085x - 1.452072	20.8	Negative	Antagonism
	Single	Y= 1.821373x - 2.674363	24.4		
Fla	Binary	Y= 0.607218x - 1.358935	25.5	Negative	Antagonism
	R.B	Y= 0.156566x - 0.864463	29.5	Negative	Antagonism
	Single	Y = 1.675877x -3.509529	96.0		
Phe	Binary	Y = 0.694216x - 1.52407	24.9	Positive	Synergism
	R.B	Y= 0.634535x - 1.420482	25.3	Negative	Antagonism
	Single	Y= 1.386925x - 2.664515	45.0		
Pyr	Binary	Y= -0.065631x - 1.42109	169.0	Negative	Antagonism
	R.B	Y= 0.434323x - 1.317695	30.2	Negative	Antagonism
	Single	Y= 0.207015x - 1.226186	44.3		
7,12-BaA	Binary	Y= -0.0069x - 1.165239	68.4	Negative	Antagonism
	R.B	Y= 0.374306x - 1.035549	24.6	Negative	Antagonism
	Single	Y= 0.301699x - 1.491397	53.3		
3,6-Phe	Binary	Y= 0.783634x - 1.574041	25.5	0	Additive
	R.B	Y= 0.580996x - 1.390005	28.8	Negative	Antagonism
	Single	Y= 1.364107x - 2.430972	32.2		
9-Ac	Binary	Y= 0.227773x - 0.894214	26.3	Positive	Synergism
	R.B	Y= 0.57517x - 1.230876	22.2	0	Additive
	Single	Y = 0.6035x - 32.166	13.6		
9,10-Ac	Binary	Y = -2.5126x - 20.748	93.7	Negative	Antagonism
	R.B	Y= 0.475158x - 1.288458	29.8	Negative	Antagonism

Table 5.5 A summary of mixture interactions observed for all 14 PAHs for cytotoxicity (viability) and genotoxicity (MN test). Ad = addition; A = antagonism; S = synergism

Chemical	Cytotoxicity	Genotoxicity
Acenaphthylene	A, S	A
Anthracene	A	A
Benz[a]anthracene	Ad, A	Ad, A
Benzo[b]fluoranthene	A, S	A
Benzo[e]pyrene	A	A
Benzo[k]fluoranthene	A, S	A
Chrysene	A, S	A
Fluoranthene	A	Ad, A
Phenanthrene	A, S	Ad, A
Pyrene	A	Ad, A
7,12-dimethyl-benz[a]anthracene	A	A
3,6-dimethyl-phenanthrene	Ad, A	A
9-methylanthracene	Ad, S	Ad, A
9,10-dimethyl-anthracene	A, S	A

Quantitative Structure-Activity Relationships

QSAR can be a useful tool as a form of alternative toxicology. Because many PAHs lack toxicological data, the following models were developed to assess the potential efficacy of QSAR to predict toxic activity of the lesser well known PAH congeners, such as the methylated PAHs. The genetic function approximation (or GFA) was used to develop QSAR algorithms that best fit the data. Discovery studio performs thousands of regression analyses using a wide variety of descriptors to find the best selection that fits the data. The software then compare the experimental values to the predicted EC_{50} values to assess the accuracy of the models (results shown in figures). A maximum of four descriptors per model was assigned to avoid over-fitting the data using too many descriptors. Figures 5.5-5.7 below display the best models developed by the software's GFA algorithm. Lastly, tables 5.6 and 5.7 show how the equations are modified to incorporate the component fractions from their EC_{50} values. These modified equations

can utilize the descriptor values of each component in a mixture to predict the toxic activity for that specific mixture. These equations are different from the single PAH model because they are incorporating the non-additive mixture interactions that were observed during toxicity experiments (recall table 5.2). The algorithms incorporate the single, binary and reversed binary data, while also accounting for the observed mixture interactions that one would expect with specific chemical ratios.

Figure 5.5 shows the algorithm and the regression of the best QSAR model to predict the EC_{50} of single PAHs. The model found the extended connectivity fingerprints (ECFPs), molecular weight, and the $\log K_{OW}$ to be significant factors for predicting the EC_{50} of these 15 PAHs. ECFPs are a very diverse set of topological descriptors that can describe an infinite array of molecular substructures and connectivity between atoms of a chemical. The models' values are derived from the stereochemical arrangement of atoms within a chemical structure.

Figures 5.6 and 5.7 display the models predicting the EC_{50} of binary mixtures where the components are known. Bonding information content (BIC) is an index describing the number of bonds and types of bonds in a chemical. The complementary information content (CIC) is a similar descriptor that relates molecular bonding to the atom makeup of a chemical. The CHI descriptor is another topological descriptor that interprets the angles of atom connectivity within the molecular graph of a chemical. Finally, the subgraph count (SC) is a descriptor relating to fractionated portion of a molecule, such as a string of hydrocarbon chains in a PAH. Topological descriptors are derived from the molecular graphs of specific chemicals, and weighted according to chemical composition, bond angles, and dimensional characteristics. Topological descriptors, especially with

planar PAHs, are often significant molecular indices for predicting the biological index of a chemical class.

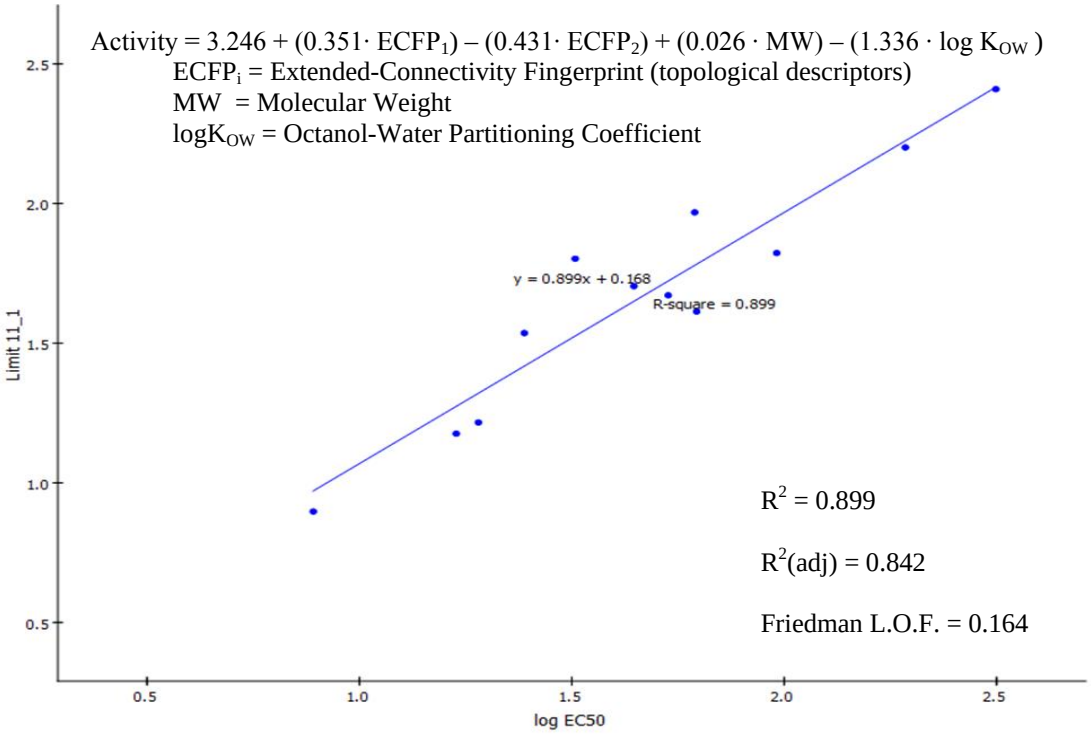


Figure 5.5 A QSAR designed to predict the EC_{50} of single PAH congeners. The x-axis consists of the experimental data, and the y-axis correlates the predicted values after fitting for the best descriptors in the equation.

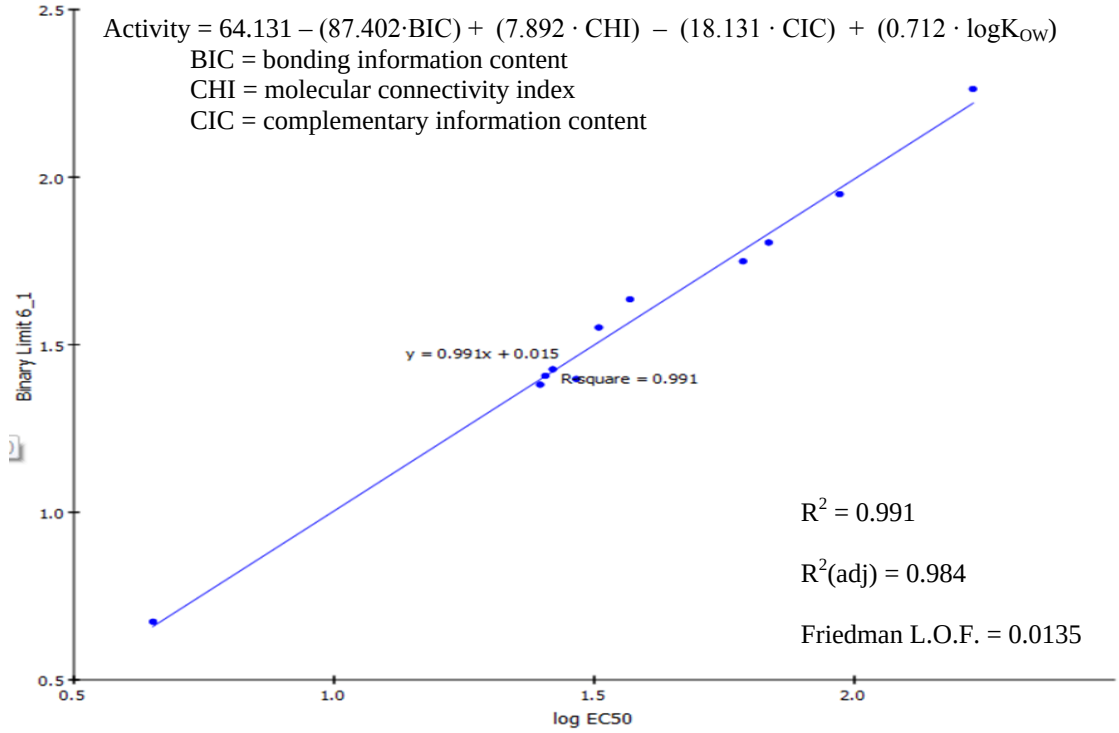


Figure 5.6 The QSAR binary model displays the relationship of the experimental EC_{50} values (x-axis) to the predicted EC_{50} values generated by the model (y-axis).

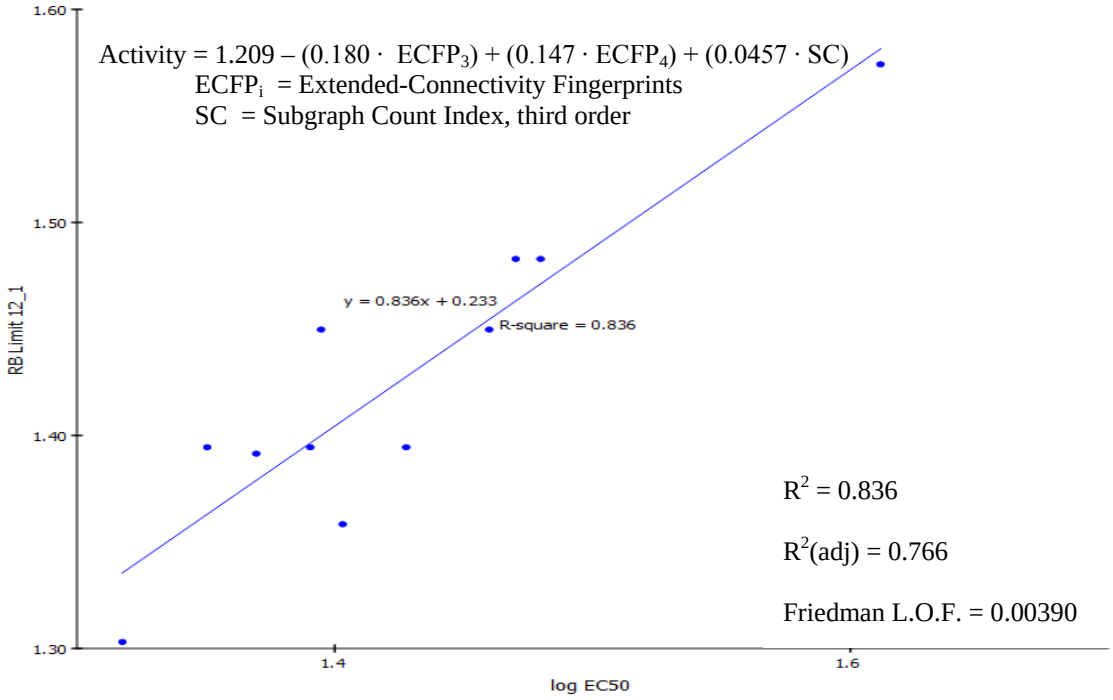


Figure 5.7 The QSAR reversed binary model displays the relationship of the experimental EC_{50} values (x-axis) to the predicted EC_{50} values generated by the model (y-axis).

Table 5.6 Modified QSAR models for Binary Mixtures to account for the component ratios.

$$\text{Activity} = 64.131 - \sum [\ (87.402 \cdot \text{BIC}) + (7.892 \cdot \text{CHI}) - (18.131 \cdot \text{CIC}) + (0.712 \cdot \log K_{OW})\ ]_{\text{Components}}$$

Chemical	BaP Fraction	Modified Algorithms for Binary Mixtures:
Acenaphthylene	0.04	Activity = 64.131 + [-(87.4 * 0.04 * BIC) + (7.89 * 0.04 * CHI) - (18.1 * 0.04 * CIC) + (0.7 * 0.04 * logKOW)] $_{BaP}$ + [-(87.4 * 0.96 * BIC) + (7.89 * 0.96 * CHI) - (18.1 * 0.96 * CIC) + (0.7 * 0.96 * logKOW)] $_{Acy}$
Anthracene	0.02	Activity = 64.131 + [-(87.4 * 0.02 * BIC) + (7.89 * 0.02 * CHI) - (18.1 * 0.02 * CIC) +(0.7 * 0.02 * logKOW)]$_{BaP}$+ [-(87.4 * 0.98 * BIC) + (7.89 * 0.98 * CHI) - (18.1 * 0.98 * CIC) + (0.7 * 0.98 * logKOW)]$_{Ac}$
Benz[a]anthracene	0.03	Activity = 64.131 + [-(87.4 * 0.03 * BIC) + (7.89 * 0.03 * CHI) - (18.1 * 0.03 * CIC) + (0.7 * 0.03 * logKOW)]$_{BaP}$+ [-(87.4 * 0.97 * BIC) + (7.89 * 0.97 * CHI) - (18.1 * 0.97 * CIC) + (0.7 * 0.97 * logKOW)] $_{BaA}$
Benzo[b]fluoranthene	0.03	Activity = 64.131 + [-(87.4 * 0.030 * BIC) + (7.89 * 0.030 * CHI) - (18.1 * 0.030 * CIC) + (0.7 * 0.030*logKOW)]$_{BaP}$+ [-(87.4 * 0.97 * BIC) + (7.89 * 0.97 * CHI) - (18.1 * 0.97 * CIC) + (0.7 * 0.97 * logKOW)] $_{BbF}$
Benzo[e]pyrene	0.03	Activity = 64.131 + [-(87.4 * 0.03 * BIC) + (7.89 * 0.03 * CHI) - (18.1 * 0.03 * CIC) + (0.7 * 0.03 * logKOW)]$_{BaP}$+ [-(87.4 * 0.97 * BIC) + (7.89 * 0.97 * CHI) - (18.1 * 0.97 * CIC) + (0.7 * 0.97 * logKOW)] $_{BeP}$
Benzo[k]fluoranthene	0.04	Activity = 64.131 + [-(87.4 * 0.04 * BIC) + (7.89 * 0.04 * CHI) - (18.1 * 0.04 * CIC) + (0.7 * 0.04 * logKOW)]$_{BaP}$+ [-(87.4 * 0.96 * BIC) + (7.89 * 0.96 * CHI) - (18.1 * 0.96 * CIC) + (0.7 * 0.96 * logKOW)] $_{BkF}$
Chrysene	0.22	Activity = 64.131 + [-(87.4 * 0.22 * BIC) + (7.89 * 0.22 * CHI) - (18.1 * 0.22 * CIC) +(0.7 * 0.22 * logKOW)]$_{BaP}$+ [-(87.4 * 0.88 * BIC) + (7.89 * 0.88 * CHI) - (18.1 * 0.88 * CIC) + (0.7 * 0.88 * logKOW)] $_{Ch}$
Fluoranthene	0.04	Activity = 64.131 + [-(87.4 * 0.04 * BIC) + (7.89 * 0.04 * CHI) - (18.1 * 0.04 * CIC) + (0.7 * 0.04 * logKOW)]$_{BaP}$+ [-(87.4 * 0.96 * BIC) + (7.89 * 0.96 * CHI) - (18.1 * 0.96 * CIC) + (0.7 * 0.96 * logKOW)] $_{Fla}$
Phenanthrene	0.04	Activity = 64.131 + [-(87.4 * 0.04 * BIC) + (7.89 * 0.04 * CHI) - (18.1 * 0.04 * CIC) + (0.7 * 0.04 * logKOW)]$_{BaP}$+ [-(87.4 * 0.96 * BIC) + (7.89 * 0.96 * CHI) - (18.1 * 0.96 * CIC) + (0.7 * 0.96 * logKOW)] $_{Phe}$
Pyrene	0.01	Activity = 64.131 + [-(87.4 * 0.006 * BIC) + (7.89 * 0.006 * CHI) - (18.1 * 0.006 * CIC) + (0.7 * 0.006*logKOW)]$_{BaP}$+ [-(87.4 * 0.994 * BIC) + (7.89 * 0.994 * CHI) - (18.1 * 0.994 * CIC) + (0.7 * 0.994* logKOW)] $_{Pyr}$
7,12-Benz[a]anthracene	0.01	Activity = 64.131 + [-(87.4 * 0.01 * BIC) + (7.89 * 0.01 * CHI) - (18.1 * 0.01 * CIC) + (0.7 * 0.01 * logKOW)]$_{BaP}$+ [-(87.4 * 0.99 * BIC) + (7.89 * 0.99 * CHI) - (18.1 * 0.99 * CIC) + (0.7 * 0.99* logKOW)] $_{7,12\text{-}BaA}$
3,6-phenanthrene	0.04	Activity = 64.131 + [-(87.4 * 0.04 * BIC) + (7.89 * 0.04 * CHI) - (18.1 * 0.04 * CIC) + (0.7 * 0.04 * logKOW)]$_{BaP}$+ [-(87.4 * 0.96 * BIC) + (7.89 * 0.96 * CHI) - (18.1 * 0.96 * CIC) + (0.7 * 0.96* logKOW)] $_{3,6\text{-}Phe}$
9-anthracene	0.04	Activity = 64.131 + [-(87.4 * 0.04 * BIC) + (7.89 * 0.04 * CHI) - (18.1 * 0.04 * CIC) + (0.7 * 0.04 * logKOW)]$_{BaP}$+ [-(87.4 * 0.96 * BIC) + (7.89 * 0.96 * CHI) - (18.1 * 0.96 * CIC) + (0.7 * 0.96* logKOW)] $_{9\text{-}Ac}$
9,10-anthracene	0.01	Activity = 64.131 + [-(87.4 * 0.01 * BIC) + (7.89 * 0.01 * CHI) - (18.1 * 0.01 * CIC) + (0.7 * 0.01 * logKOW)]$_{BaP}$+ [-(87.4 * 0.99 * BIC) + (7.89 * 0.99 * CHI) - (18.1 * 0.99 * CIC) + (0.7 * 0.99* logKOW)] $_{9,10\text{-}Ac}$

Table 5.7 Modified QSAR models for Reversed Binary mixtures to account for the component ratios.

			Activity = $1.209 - \sum [(0.180 \cdot \text{ECFP3}) + (0.147 \cdot \text{ECFP4}) + (0.0457 \cdot \text{SC})]_{\text{Components}}$
Chemical	BaP Fraction	PAH Fraction	Modified Algorithms for Reversed Binary Mixtures:
Acenaphthylene	0.96	0.04	Activity = 1.209 + [-(0.180 * 0.96 * ECFP3) + (0.147 * 0.96 * ECFP4) + (0.0457 * 0.96 * SC)] $_{BaP}$ + [-(0.180 * 0.04 * ECFP3) + (0.147 * 0.04 * ECFP4) + (0.0457 * 0.04 * SC)] $_{Acy}$
Anthracene	0.98	0.02	Activity = 1.209 + [-(0.180 * 0.98 * ECFP3) + (0.147 * 0.98 * ECFP4) + (0.0457 * 0.98 * SC)] $_{BaP}$ + [-(0.180 * 0.02 * ECFP3) + (0.147 * 0.02 * ECFP4) + (0.0457 * 0.02 * SC)] $_{Ac}$
Benz[a]anthracene	0.96	0.04	Activity = 1.209 + [-(0.180 * 0.96 * ECFP3) + (0.147 * 0.96 * ECFP4) + (0.0457 * 0.96 * SC)] $_{BaP}$ + (0.180 * 0.04 * ECFP3) + (0.147 * 0.04 * ECFP4) + (0.0457 * 0.04 * SC) $_{BaA}$
Benzo[b]fluoranthene	0.96	0.04	Activity = 1.209 + [-(0.180 * 0.96 * ECFP3) + (0.147 * 0.96 * ECFP4) + (0.0457 * 0.96 * SC)] $_{BaP}$ + [-(0.180 * 0.04 * ECFP3) + (0.147 * 0.04 * ECFP4) + (0.0457 * 0.04 * SC)] $_{BbF}$
Benzo[e]pyrene	0.96	0.04	Activity = 1.209 + [-(0.180 * 0.96 * ECFP3) + (0.147 * 0.96 * ECFP4) + (0.0457 * 0.96 * SC)] $_{BaP}$ + [-(0.180 * 0.04 * ECFP3) + (0.147 * 0.04 * ECFP4) + (0.0457 * 0.04 * SC)] $_{BeP}$
Benzo[k]fluoranthene	0.98	0.02	Activity = 1.209 + [-(0.180 * 0.98 * ECFP3) + (0.147 * 0.98 * ECFP4) + (0.0457 * 0.98 * SC)] $_{BaP}$ + [-(0.180 * 0.02 * ECFP3) + (0.147 * 0.02 * ECFP4) + (0.0457 * 0.02 * SC)] $_{BkF}$
Chrysene	0.95	0.05	Activity = 1.209 + [-(0.180 * 0.95 * ECFP3) + (0.147 * 0.95 * ECFP4) + (0.0457 * 0.95 * SC)] $_{BaP}$ + [-(0.180 * 0.05 * ECFP3) + (0.147 * 0.05 * ECFP4) + (0.0457 * 0.05 * SC)] $_{Ch}$
Fluoranthene	0.97	0.03	Activity = 1.209 + [-(0.180 * 0.97 * ECFP3) + (0.147 * 0.97 * ECFP4) + (0.0457 * 0.97 * SC)] $_{BaP}$ + [-(0.180 * 0.03 * ECFP3) + (0.147 * 0.03 * ECFP4) + (0.0457 * 0.03 * SC)] $_{Fla}$
Phenanthrene	0.96	0.04	Activity = 1.209 + [-(0.180 * 0.96 * ECFP3) + (0.147 * 0.96 * ECFP4) + (0.0457 * 0.96 * SC)] $_{BaP}$ + [-(0.180 * 0.04 * ECFP3) + (0.147 * 0.04 * ECFP4) + (0.0457 * 0.04 * SC)] $_{Phe}$
Pyrene	0.97	0.03	Activity = 1.209 + [-(0.180 * 0.97 * ECFP3) + (0.147 * 0.97 * ECFP4) + (0.0457 * 0.97 * SC)] $_{BaP}$ + [-(0.180 * 0.03 * ECFP3) + (0.147 * 0.03 * ECFP4) + (0.0457 * 0.03 * SC)] $_{Pyr}$
7,12-Benz[a]anthracene	0.96	0.04	Activity = 1.209 + [-(0.180 * 0.96 * ECFP3) + (0.147 * 0.96 * ECFP4) + (0.0457 * 0.96 * SC)] $_{BaP}$ + [-(0.180 * 0.04 * ECFP3) + (0.147 * 0.04 * ECFP4) + (0.0457 * 0.04 * SC)] $_{7,12\text{-}BaA}$
3,6-phenanthrene	0.97	0.03	Activity = 1.209 + [-(0.180 * 0.97 * ECFP3) + (0.147 * 0.97 * ECFP4) + (0.0457 * 0.97 * SC)] $_{BaP}$ + [-(0.180 * 0.03 * ECFP3) + (0.147 * 0.03 * ECFP4) + (0.0457 * 0.03 * SC)] $_{3,6\text{-}Phe}$
9-anthracene	0.95	0.05	Activity = 1.209 + [-(0.180 * 0.95 * ECFP3) + (0.147 * 0.95 * ECFP4) + (0.0457 * 0.95 * SC)] $_{BaP}$ + [-(0.180 * 0.05 * ECFP3) + (0.147 * 0.05 * ECFP4) + (0.0457 * 0.05 * SC)] $_{9\text{-}Ac}$
9,10-anthracene	0.97	0.03	Activity = 1.209 + [-(0.180 * 0.97 * ECFP3) + (0.147 * 0.97 * ECFP4) + (0.0457 * 0.97 * SC)] $_{BaP}$ + [-(0.180 * 0.03 * ECFP3) + (0.147 * 0.03 * ECFP4) + (0.0457 * 0.03 * SC)] $_{9,10\text{-}Ac}$

CHAPTER SIX

Discussion and Conclusions

Recall that the overarching research objective from these studies is to analyze PAH mixtures and determine mixture interactions. Testing simple mixtures may benefit our understanding of basic interactions that could occur between PAHs congeners within complex mixtures. Since environmental PAH mixtures are complex and variable, characterizing interactions may serve to improve the accuracy of human health risk assessments. The previous chapter outlined the various results from our studies, including proliferation, viability, genotoxicity, mixture analyses, and the QSAR models. Mixture analyses were performed using the viability and genotoxicity data to determine mixture interactions. Tables 5.4 and 5.5 show that the majority of the mixtures exhibited an antagonistic relationship, and few mixtures showed additivity. When additivity was seen, it was coupled with another interaction in a mixture with different ratios.

Mixtures

Factors that can affect the toxicity of mixtures include altered solubility, altered receptor interaction, altered bioavailability, gene expression, and the favoring of certain metabolic pathways at enzymatic saturation. Chemicals in mixtures should act by the same mechanism if the toxicity is to conform to the additivity assumption.[94] Mixture interactions, however, are often caused by competition between toxicants at receptor sites.[91] When doses are low, particularly in acute scenarios, the competitive mechanism that affects toxicity may not be an influential factor because the concentrations are not

high enough to saturate receptor sites.[91] Furthermore, if two toxicants act independently but have low concentrations, they may still simulate toxic additivity, but actually have a more complex relationship.[91] This study, however, did not use low enough concentrations so we can thereby assume that the sites of metabolic action are saturated and competitive mixture interactions should occur. Because metabolic pathways are saturated, and independent action is unlikely because of known biotransformation pathways, the interactions seen in tables 5.4 and 5.5 are the result of competition at receptor sites. As expected, our findings did display non-additive complex interactions occurring in mixture analyses.

Various studies using environmental mixtures that fall below their toxic thresholds were found to be synergistically toxic.[94] High doses are known to saturate the metabolic pathways where the chemicals may compete with each other for biotransformation, usually at the cytochrome P450 enzyme junction.[94] In this case, antagonism is the expected relationship between chemicals in the mixture because the enzymes cannot produce toxic intermediates quickly enough to demonstrate toxic additivity. One publication performed a weight of evidence analysis and concluded that the threshold for competitive inhibition of chemicals and mixtures may be the single most important factor for resulting toxicity.[94] In the case of PAHS, since there are numerous metabolic pathways that can lead to either toxicity or elimination, it is possible that antagonism will occur because the elimination pathways are favored at saturation over the toxic pathways.[95] This concept is what is hypothesized to be occurring in these experiments (recall figures 5.3 and 5.4 for all mixtures). Our findings, and those of other discussed studies, align with this hypothesis. Our mixture analyses observed mostly antagonism,

indicating that such favoritism is evident. Further tests can be performed to confirm the specific mechanisms of actions, such as inducible enzymes and metabolite formation, but our current findings provide much information about possible interactions in PAH mixtures.

As mentioned, our experiments revealed an inhibitory trend, where antagonism was observed in nearly all combinations of mixtures for both cell viability and genotoxicity. As discussed in Chapter Two, there have been very few published studies that examined cytotoxicity because most research is focused on cancer as the endpoint. However cytotoxicity data is useful because they indicate the first signs that a cell has endured an insult or injury (inhibition of cell growth/function, loss of membrane integrity, LDH leakage, etc). The cytotoxicity data was normally distributed and provided excellent congruent data points between and within all treatment groups. The mixture analysis revealed very few treatments that had additive toxicity. One such example is the benz[a]anthracene viability trial (refer to figure 5.4c) when BaP concentrations were held constant in the mixture. Figure 6.1 shows a comparison between the expected additive response to the observed response. The figure shows that they are closely aligned with each other. The reversed binary mixture, however, displayed a weak antagonistic response in figure 5.4c.

Some PAHs displayed opposing mixture effects depending the PAH in the mixture that varied. Acenaphthylene, benzo[b]fluoranthene, benzo[k]fluoranthene, chrysene, phenanthrene, 9-methylanthracene and 9,10-dimethylanthracene all showed both antagonism and synergism in binary mixtures. Acenaphthylene, for example, shows a clear synergistic response in mixtures where the concentration of acenaphthylene

gradually increases and BaP is held at 1 mg/L. Figures 6.2 and 6.3 display the two mixture trials where synergism and antagonism is observed in each.

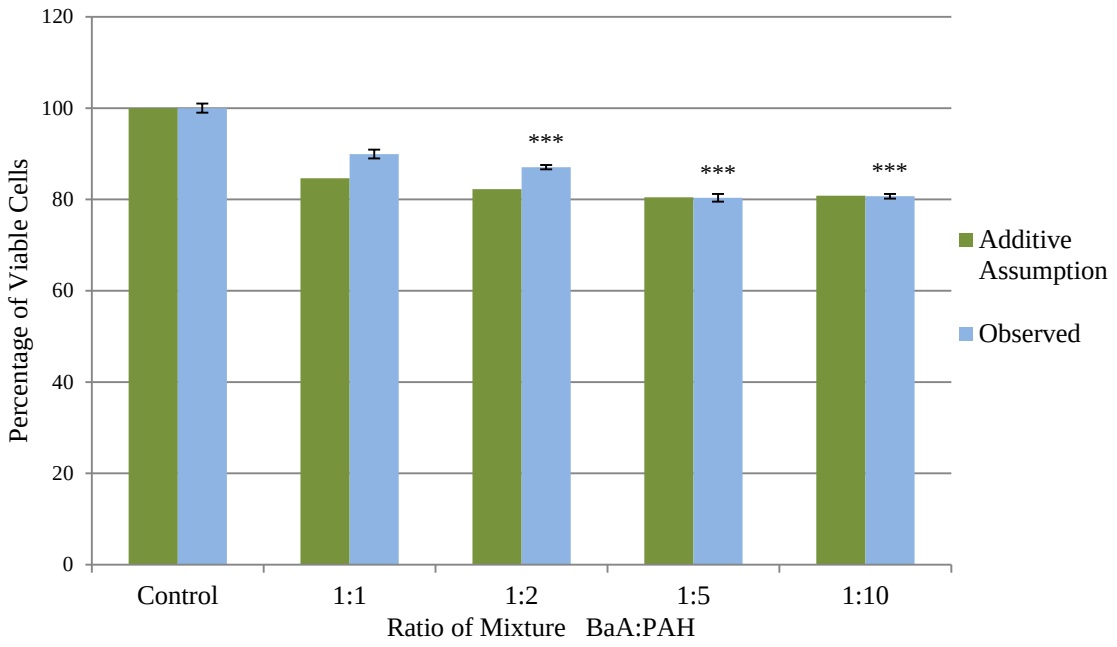


Figure 6.1 A comparison of the expected additive response using the single chemical trial to the actual observed response in the binary mixture trial with benz[a]anthracene and BaP.

The varying mixture effects may be the result of competition at receptor sites due to the ability of the component concentrations to outcompete each other. With the exception of 9,10-dimethylanthracene, all the binary trials showed synergism and the reversed binary showed antagonism. In binary trials, it follows that the PAH outcompetes BaP at receptor sites because the concentration is higher. The reverse is true for the binary trials. This observation may be very important for elucidating the interactions between PAHs in complex mixtures. It could indicate that the varying affinities for the AhR binding site, which varies amongst PAHs, is a possible factor for

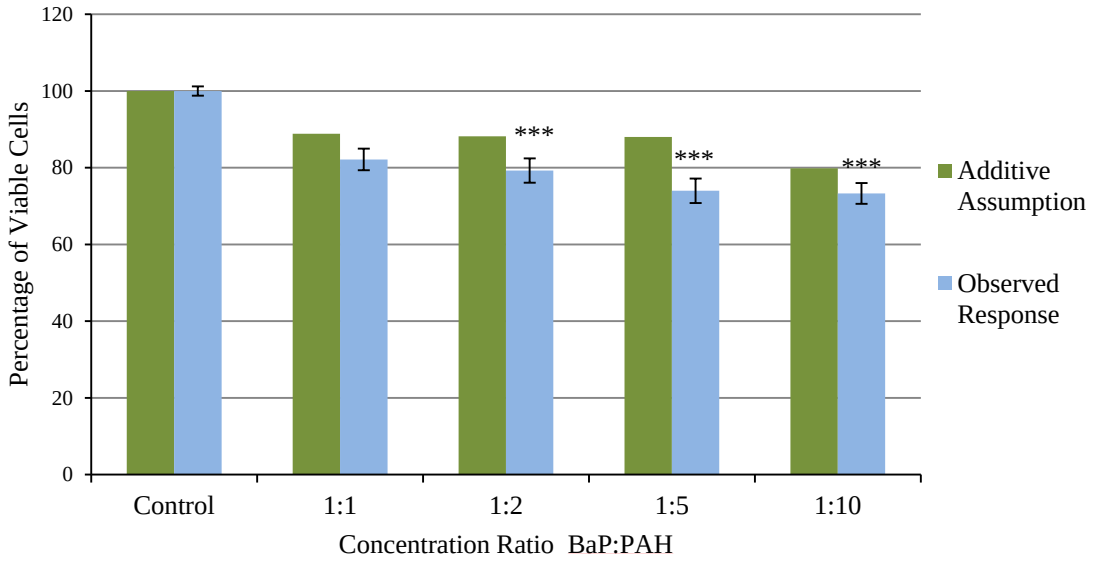


Figure 6.2 Acenaphthylene displays a synergistic toxicity when compared to the expected additive response. In this mixture, BaP concentrations are held constant, and acenaphthylene varies.

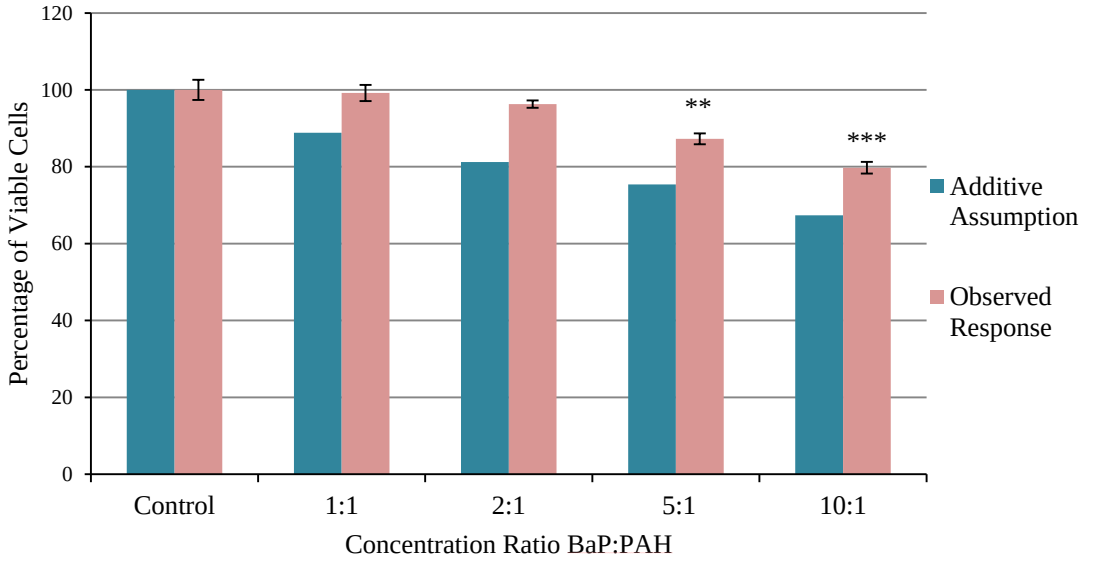


Figure 6.3 The reverse binary mixture of acenaphthylene shows a clear antagonistic response when compared to both the expected additive response and the response of BaP alone. BaP concentrations vary and acenaphthylene is held constant.

predicting possible mixture interactions because it affects the rate of the appearance of toxic intermediates.[95] Other studies have observed non-additive toxicities and have attributed interactions to competition at receptor sites.[96] Mahadevan et al. (2005) for example, observed less than additive mixture toxicity by measuring the development of DNA adducts.[97] Tarantini et al (2010) performed several genotoxicity tests and found that benzo[k]fluoranthene significantly inhibited the adduct formation with BaP.[57] Benzo[b]fluoranthene, however, increased the rate of DNA adduct formation.[57] Staal et al (2008) found that the majority of the genotoxic response in mixtures with BaP, BbF and fluoranthene were antagonistic, and the carcinogenic potency of PAHs in mixtures was significantly less than the predicted additive potency.[98] A different study observed antagonistic responses in human lung cancer cells (A549), in which the genotoxicity seen in complex mixtures were lower than single BaP and BkF trials.[99]

In agreement with these other studies, our genotoxicity results showed mainly antagonistic relationships between BaP and other PAHs. Synergism was not observed in any trials. Additive responses were seen when the frequency of MN in the mixture was approximately equivalent to that of the sum frequencies of the individual components, but addition was always accompanied by another interaction depending on composition. Since BaP should always exert a statistically significant positive effect, any mixtures that yielded negative results (statistically insignificant) were automatically scored as antagonism[57]. The majority of the mixture results showed that inhibition was a common effect, thereby reducing the genotoxicity.

The MN test is time intensive and requires expensive fluorescence microscopy to score individual cells, which may limit sample size and scoring confidence. Despite

limitations, this research scored an adequate sample size and furthermore the results were congruent with other published literature. For example, carcinogenic PAHs BaP, benz[a]anthracene, benzo[b]fluoranthene, and chrysene showed positive results in the MN Test. In addition, anthracene, 7,12-dimethylbenz[a]anthracene and 9,10-dimethylanthracene were positive and in agreement with other published work that tested their genotoxicities.[68] Research on 7,12-dimethylbenz[a]anthracene (DMBA) has seen much higher potencies than BaP.[100] This compound has a high rate of diol epoxide formation, but also has a decreased rate of induction of CYP1A1 when compared to BaP.[101] This means DMBA has a large potential for genotoxicity, but a concomitant possibility of reduced toxic action if the metabolizing enzymes are neither present nor inducible.

PAHs that have not yet been classified as carcinogenic were negative and include acenaphthylene, benzo[e]pyrene, fluoranthene, phenanthrene, and pyrene. Most of these PAHs have animal bioassay data that showed no tumor formation in test organisms (mice, rats, etc), although there are a few exceptions.[11] Discrepancies in the MN results exist with benzo[k]fluoranthene. Benzo[k]fluoranthene has been classified as a probable human carcinogen given surmounting evidence in animal research.[11] However, the results of our MN test did not show a statistical increase in MN in single exposures to benzo[k]fluoranthene. This is likely because of random skewed sampling of the treatment groups, which can be symptomatic of small sample sizes. The sampled cells may not have been representative of the total cell population. It is likely that the weakly carcinogenic benzo[k]fluoranthene did not significantly induce the formation of MN, indicating that the assay may not have captured all genotoxic effects.

The chosen exposure period of 24 hours (with a single occurrence) could be an influential factor affecting genotoxicity. Since human exposures are chronic, many animal studies mimic chronic exposure scenarios but it is often less applicable *in vitro*. *In vitro* research often employs acute scenarios to examine DNA mutation, which is the first step of carcinogenesis. Tarantini et al (2010) study performed an *in vitro* time course study with BAP and binary mixtures with BbF and BkF and noted that the formation of DNA adducts peaked 8 hours after exposure and plateaued.[57] Tarantini et al. (2010) also concluded that binary mixtures did not affect the overall frequency of adducts.[57] This may indicate that the chosen chemical exposure period may influence the rate of adduct formation, but not necessarily the frequency. Another study made an observation with a time-course study using an exposure period of 120 hours, stating that complex standard reference material coal tar mixture (#1597) caused a significant decline in BaP-DNA adducts when compared to cells exposed only to BaP.[97] Inhibition was most prominent during the first 48 hours of the exposure period.[97] Given that our studies utilized a 24 hour exposure period, it follows that many interactions were inhibitory.

Following BaP exposure, some cells significantly down-regulate proteins that are involved in metastasis and tumor suppression.[85] For example, one study found that down-regulated proteins were involved with apoptosis, cell structure, metabolism, and DNA synthesis.[58] Proteins involved with cell proliferation, growth and differentiation were also up-regulated.[58] Cells are inhibited from apoptosis and vulnerable to decreased cell function, mutations, tumorigenesis and metastasis that would otherwise be regulated normally.[102]

One limitation that was encountered in this study's genotoxicity testing was cell death. The concentrations that were used to score MN were meant to mimic hazardous contamination at NPL sites. Scoring of benz[a]anthracene, benzo[b]fluoranthene, and 3,6-dimethylphenanthrene proved difficult because many cells were shrinking and dying, although these cells were not in the majority. Cells exposed to 3,6-dimethylphenanthrene mixtures were often not scored because there was a lot of cellular debris, and cells did not have defined cellular and nuclear membranes.

An important factor to consider is the ability of the K-9 liver cells to express metabolizing enzymes. Kang et al. (2010) found that immortalized liver cells have higher concentrations of the CYP and Phase II enzymes necessary to metabolize and detoxify PAHs, which may contribute to higher proportions of detoxified PAHs when compared to other cell lines.[9] The study also found that the PAH mixtures were significantly cytotoxic to the cell lines tested, although much uncertainty exists because the PAH mixtures used to dose the cells were extracted from dust samples still containing other contaminants like heavy metals.[9] While our studies cannot confirm the specific receptor sites, it does address the overall goal of testing the additivity assumption. As discussed previously, the AhR, and CYP enzymes have been implicated as key predictors for the metabolic fate (and subsequent interactions) that occur in PAH mixtures. The additive assumption is not a accurate method of determining mixture toxicity for human exposures because it was observed infrequently in these trials and in similar studies.[80, 95] In addition, mixture composition is a very important factor that can affect the type of mixture interaction that will occur, which is evident by wide mixture variability and other studies' findings.[2, 96]

A common pitfall for estimating PAH toxicity is the erratic variations of environmental mixtures and the many possible biotransformation pathways for PAHs in organisms. Toxic action could result from receptor competition, impairing cellular uptake, binding to critical proteins, impairing various cellular functions and communications, metabolic interactions, or inducing mutations, among others. Binary mixture data is a necessary first step in elucidating potential relationships between PAHs in complex mixtures. More complex mixtures, such as ternary or quaternary, can further improve current knowledge on complex PAH interactions. For example, Tarantini et al (2010) saw complex genotoxic responses when comparing binary and ternary mixtures.[57] Mammalian studies on multicomponent mixtures have also seen a vast difference in toxic response and mixture interactions.[91] Binary mixtures provide instrumental information about the types of relationships between PAHs and supplements existing research on complex mixtures.

Quantitative Structure-Activity Relationships

Alternative toxicology is a useful tool for describing the toxicity of mixtures. QSAR modeling saves time, money, and experimental materials when a robust set of data is already compiled. Figure 5.5 in the previous chapter displays the model that predicts the EC_{50} of single PAHs based on the molecular structure of the analytes. The analyte list contained 15 congeners and produced a strong model with acceptable predictive power. With a correlation coefficient of about 90% and a lack of fit (LOF) probability of 0.16, this means the model has an acceptable probability of accurately predicting the EC_{50}'s of PAHs, including methylated congeners. This promising model illustrates that QSAR could be a viable tool to bridge the data gaps for PAHs as a class of compounds.

Recall that figures 5.6 and 5.7 show the QSAR models for the mixtures' EC_{50}. While these models have less predictive power (evidenced by the smaller LOF scores), they also achieved strong correlations between the mixture EC_{50} and the molecular structures. These models are unique because they are incorporating the mixtures interactions that have been previously observed in these same trials (table 5.5), something that has not yet been addressed with most congeners. Some researchers have investigated the development of binary mixtures in QSAR modeling and have found that some simple alterations in the model equation may increase the predictive power.[93] For example, in the equations for the binary mixtures, a simple molar fraction or component ratio needs to be added into the polynomials to account for the mixture composition and then the model equations of the components are summed to together (as seen in tables 5.6 and 5.7). Such modifications give the model flexibility by properly weighting the components in the mixture and allowing for customization. The descriptors that apply for each component will then also apply to the summed model for the whole mixture. The algorithms are considerably strengthened when the mixture interactions and component ratios are accounted for, making toxicity predictions much more realistic. While our methods are designed to apply to simple binary PAH mixtures, they can be tailorable to model more complex PAH mixtures. The pursuit of this toxicological avenue would save research time, resources, and funds while continuing to improve the accuracy of human health risk assessments regarding complex PAH exposures.

Another important factor in the QSAR models is the variation of descriptors for each model. The QSAR learner finds the strongest correlation between specific descriptors and the EC_{50}. The output revealed that the only intersection among the descriptors was

$\log K_{OW}$ between the single and binary model. Every other descriptor was different among all three models. As mentioned in the previous chapter, the algorithm descriptors include extended connectivity fingerprints, molecular weight, $\log K_{OW}$, bonding information content, complementary information content, CHI, and subgraph count. The majority of these descriptors are two-dimensional topological descriptors that define important features about PAH structures, such as bonds and stereoelectronic features. The only partitioning value that was found to be significant is the octanol-water partitioning, which other studies have found to be a significant factor because it predicts the fate of PAHs in a biological system (water or lipid partitioning).[54] These descriptors relate to the planar characteristics (2D) of PAHs, and the orientation about molecular bonds, highlighting that the planar nature of PAHs may affect the eventual toxicological fate of these PAHs in organisms.

The wide variability of descriptors suggests that different molecular characteristics in mixture components may have shifting importance on the toxicity of the whole mixture, which suggests that QSAR mixtures models that incorporate interactions are more robust. The toxicity of a single compound may be altered when a second or third competing compound is added to the exposure scenario. The varying mixture effects seen in the cytotoxicity and genotoxicity data corresponds with this inference.

Conclusions

Many studies and agencies are currently revising the methods for assessing human health risks associated with PAH exposures. Due to the fact that humans are chronically exposed to PAH mixtures, it is important that PAHs be toxicologically characterized. The component-based methods include the assumptions that PAHs are biotransformed

through similar metabolic pathways, and that toxicity is additive in mixtures. It has been observed, both by other researchers and in this study, however, that PAHs can exert their effects through a convoluted array of metabolic pathways, and that toxic addition is rare. The mixture-based methods possess their own set of limitations, including a wide variation of environmental mixture composition, as well as various environmental factors affecting fate, partitioning and degradation. It follows that there are many challenges for accurately assessing risks associated with PAH exposures.

The main objectives of this research were to elucidate the types of toxicological interactions occurring within simple PAH mixtures, and compare the observed effects to toxic addition. In summation, our *in vitro* studies saw minimal toxic additivity, with non-additive effects dominating most tested mixtures. The composition of these mixtures and which component varied influenced the type of interaction that occurred. This study confirmed that toxic addition is improbable in simple mixtures and more complex PAH mixtures are likely to see non-additive responses when complex interactions occur between components.

The implications of our findings highlight the weaknesses of current approaches to estimating PAH toxicity. Current methodologies for human health risk assessments could be improved in various ways. First, environmental mixtures could be further studied to understand how the emission source affects mixture components, and how environmental matrices cause fluctuations in mixture compositions. The whole mixtures approach could be strengthened if toxicological data is available for a wider array of complex mixtures. Secondly, toxicological research could be performed on the vast number of PAH congeners that are not well known, as well as simple mixtures to begin to

understand interactions. Methylated PAHs, for example, have very little toxicological data available to incorporate into risk assessments. This study also saw success with the *in vitro* MN test, which could aid in focusing animal carcinogenesis studies on genotoxic chemicals. The consideration of *in vitro* research for prioritizing animal studies will cut down on research costs, material consumption, and animal lives. Such streamlining could improve the accuracy of risk estimation for the development of cancers in humans.

These suggestions can be time-consuming and costly. This is undoubtedly why many data gaps remain for PAHs. Alternative toxicology can be most useful under these restrictions, saving time, funding, and expensive research materials. Developing QSAR models and improving QSAR software with newfound PAH mixture data will provide a cost-effective and timely supply of information regarding behavior of the entire class of PAHs. Such information adds to the little known facts about PAHs and their behavior in a biological system. It is possible PAH models can give valuable information about the mechanisms of biochemical action for PAHs. Furthermore, PAH QSAR models could expand the resources for monitoring techniques and screening, especially with the development of mixture models. QSAR may be a significant addition to traditional toxicological research, further focusing the experiments needed, reducing the time and costs associated with this research, as well as bridging the data gaps for risk assessments.

APPENDIX

Table A.1 Mixture compositions across various studies quantifying PAHs in urban air samples.

	Urban Air							
Sample	Non-smokers' Home [11]	Smokers' Home [11]	Rooftop samples [63]	Outdoor samples [103]	Winter Samples [104]	Sumer samples [104]	Winter samples [40]	Summer samples [40]
Acenaphthylene	12.9	15.4	-	-	-	-	-	-
Anthracene	2.1	3.0	0.7	-	4.7	0.0	-	-
Benz[a]anthracene	0.3	0.3	4.4	10.3	3.6	3.6	4.4	
Benzo[a]pyrene	0.4	0.3	4.7	11.7	4.9	3.9	3.2	7.3
Benzo[e]pyrene	0.8	1.2	6.6	-	3.3	2.5	35.6	6.8
Benzo[b]fluoranthene	0.8	0.7	9.9	7.0	7.0	6.8	5.5	10.4
Benzo[ghi]perylene	0.5	0.5	6.1	15.9	12.8	2.2	7.2	21.4
Benzo[j]fluoranthene	-	-	-	-	-	-	2.9	4.7
Benzo[k]fluoranthene	-	-	4.0	5.6	2.3	3.0	1.9	7.3
Chrysene	0.9	0.8	-	11.2	6.6	11.0	17.0	-
Coronene	-	-	3.7	-	26.7	24.3	5.2	18.2
Dibenz[ah]anthracene	-	-	0.8	4.7	-	-	-	-
Fluoranthene	9.0	6.2	10.5	5.6	5.7	14.7	-	-
Fluorene	-	-	-	4.7	-	-	-	-
Indeno[123cd]pyrene	0.3	0.3	4.8	3.3	12.8	2.2	7.9	24.0
Naphthalene	-	-	-	-	-	-	-	-
Phenanthrene	66.7	67.7	7.7	3.7	-	-	-	-
Pyrene	5.4	3.7	9.2	16.4	9.7	25.8	-	-
% of Mixture Unaccounted	0.0	0.0	26.8	0.0	0.0	0.0	9.1	0.0

- Not Analyzed
ND Not Detected

Table A.2 Mixture compositions across various studies quantifying PAHs in various environmental media.

	Indoor Dust				Cigarette Smoke	Smoky Room
Sample	Vacuum dust[105]	Dust samples [9]	Non-smoking households [106]	Smoking households [106]	µg/100 cigarettes [11]	Air filter samples [11]
Acenaphthene	4.6	0.3	0.7	0.5	-	-
Acenaphthylene	5.1	0.2	0.4	0.4	-	-
Anthracene	4.3	0.5	1.3	1.3	13.2	-
Benz[a]anthracene	2.8	3.6	2.5	3.1	4.3	7.9
Benzo[a]pyrene	1.7	6.1	5.4	4.8	4.4	60.1
Benzo[e]pyrene	-	-	-	-	1.4	1.4
Benzo[b]fluoranthene	4.9	7.9	9.6	9.3	1.2	0.4
Benzo[ghi]perylene	6.3	14.6	13.4	13.3	2.2	-
Benzo[j]fluoranthene	-	-	-	-	1.2	2.8
Benzo[k]fluoranthene	4.2	7.9	9.6	9.3	0.7	2.8
Chrysene	3.7	8.7	6.0	7.6	5.4	1.3
Dibenz[ah]anthracene	1.3	1.0	0.9	0.8	0.2	1.0
Fluoranthene	13.7	13.9	10.0	11.6	15.3	7.8
Fluorene	7.3	0.5	1.3	1.2		-
Indeno[123cd]pyrene	2.8	9.4	4.8	5.9	1.3	0.1
Naphthalene	4.5	1.9	1.4	1.3	-	-
Phenanthrene	24.9	12.5	12.5	13.3	35.2	6.9
Pyrene	8.0	11.2	10.6	12.8	15.2	5.2
% of Mixture Unaccounted	0	0	9.5	3.2	0	0

- Not analyzed
ND Not detected

Table A.3 Mixture compositions across various studies quantifying PAHs in sediments and soils.

	Urban Sediments					Soils		
Sample	Sediments highway runoff [107]	Willowfield [107]	Urban Creek Sediments[20]	Urban Background Sediments [33]	Urban Sediments (point-source) [33]	Rural Soil [11]	Agricultural Soil [11]	Urban Soil [11]
Acenaphthene	0.0	0.2	-	2.0	4.9	1.3	0.8	-
Acenaphthylene	0.0	0.1	-	1.0	0.2	-	0.7	-
Anthracene	0.0	0.4	3.7	3.2	2.8	-	1.5	-
Benz[a]anthracene	0.1	5.1	5.3	5.6	6.7	3.9	7.6	0.7
Benzo[a]pyrene	0.1	8.3	19.8	9.4	2.3	1.6	0.6	0.6
Benzo[e]pyrene	-	-	-	9.8	2.3	-	7.2	0.2
Benzo[b]fluoranthene	0.2	20.0	6.7	9.8	2.9	15.6	7.9	59.5
Benzo[ghi]perylene	0.1	6.1	9.2	8.6	1.0	7.8	9.0	3.6
Benzo[j]fluoranthene	-	-	-	3.8	1.2		-	-
Benzo[k]fluoranthene	0.0	3.8	-	3.8	1.2	7.8	7.9	1.2
Chrysene	0.1	9.2	7.8	-	6.7	29.8	10.6	1.0
Dibenz[ah]anthracene	0.0	2.8	16.5	2.1	0.3	-	-	-
Fluoranthene	0.2	15.0	8.1	13.9	32.0	0.2	16.3	0.8
Fluorene	0.0	0.4	2.5	0.9	4.9	-	1.3	-
Indeno[123cd]pyrene	0.1	11.7	8.6	10.1	1.2	7.8	8.6	31.7
Naphthalene	0.0	1.2	2.3	0.2	0.0	-	-	-
Phenanthrene	0.1	4.8	2.7	4.9	7.6	23.4	6.5	-
Pyrene	0.1	11.7	6.8	10.9	21.8	0.8	13.5	0.6
% of Mixture Unaccounted	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

- Not analyzed
ND Not detected

Table A.4 Mixture compositions across various studies quantifying PAHs in drinking water and various foodstuffs.

	Drinking Water			Foodstuffs						
Sample	Post-Treatment [108]	Faucet water [26]	Faucet water [26]	Japanese Green Tea [43]	Blended Coffee [43]	Burnt Bread [43]	Overall Dietary Intake [31]	Oil & Fat Products [31]	Dairy Products [31]	Meat & Products [31]
Acenaphthene	9.3	6.7	6.6	5.0	9.0	12.5	-	-	-	-
Acenaphthylene	ND	4.5	7.6	-	-	-	5.4	6.6	7.2	5.4
Anthracene	4.0	6.2	2.4	8.7	12.9	ND	7.0	6.6	7.2	8.7
Benz[a]anthracene	7.9	2.6	6.4	4.6	ND	ND	1.9	0.3	2.8	1.3
Benzo[a]pyrene	ND	2.5	10.8	4.3	ND	ND	1.4	0.3	2.8	0.4
Benzo[b]fluoranthene	8.0	5.3	4.3	5.4	ND	ND	1.6	0.3	2.8	0.6
Benzo[ghi]perylene	ND	ND	1.4	ND	ND	ND	1.3	0.3	2.8	0.2
Benzo[k]fluoranthene	11.3	5.5	3.7	3.0	4.8	ND	1.3	0.3	2.8	0.2
Chrysene	11.9	5.7	6.1	3.1	3.3	4.8	2.0	0.3	2.8	1.4
Dibenz[ah]anthracene	ND	1.3	0.6	ND	ND	ND	1.1	0.3	2.8	0.1
Fluoranthene	6.2	9.1	2.2	16.1	ND	20.6	9.7	6.6	7.2	13.4
Fluorene	6.4	7.6	7.8	ND	ND	ND	5.3	6.6	7.2	5.2
Indeno[123cd]pyrene	ND	ND	0.9	ND	ND	ND	1.2	0.3	2.8	0.2
Naphthalene	15.3	29.5	22.7	18.1	34.4	41.7	16.8	33.3	34.8	6.4
Phenanthrene	3.0	7.1	10.3	16.8	20.2	ND	22.9	6.6	7.2	40.9
Pyrene	16.7	6.3	6.3	14.8	15.4	20.9	10.7	6.6	7.2	14.8
% of Mixture Unaccounted	0.0	0.0	0.0	0.0	0.0	0.0	10.5	25.2	76.1	0.0

- Not analyzed
ND Not detected

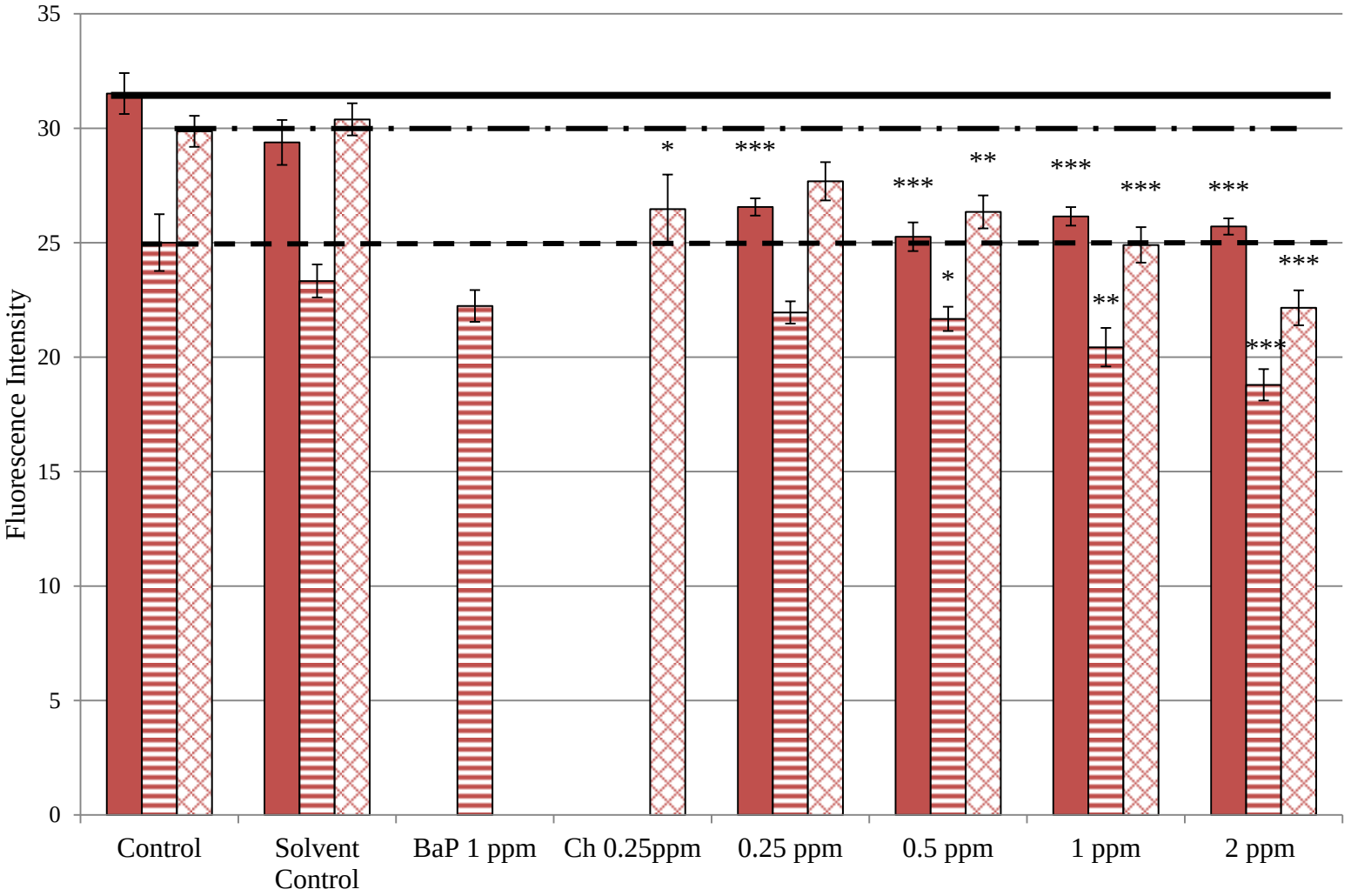


Figure A.1 An enlarged figure of Chrysene mixture data for viability displays reference bars for each control group. Because the mixtures were performed at separate times, population densities vary. Therefore, the treatment groups are statistically analyzed using their own control groups within each 96 well plate.

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