ENVIRONMENTAL TOXICITY OF COMPLEX CHEMICAL MIXTURES

A Dissertation

by

ANNIKA MARGARET GILLESPIE

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

DOCTOR OF PHILOSOPHY

May 2006

Major Subject: Toxicology

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ABSTRACT

Environmental Toxicity of Complex Chemical Mixtures.

(May 2006)

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Complex chemical mixtures may be released into the environment from a variety of sources including hazardous waste sites. Components of chemical mixtures and their metabolites may be genotoxic leading to cancer and heritable gene mutations. Chemical analysis alone does not always provide the most accurate information from which to estimate the risk of adverse effects associated with exposure to mixtures. Current methods to estimate the human health risk for complex mixtures assume additive effects of the components. Although it is assumed that this approach is protective of human and ecological health, it is also recognized that chemical mixtures may induce a variety of interactions including potentiation, synergism, and antagonism. A combined testing protocol, using chemical analysis coupled with a battery of *in vitro*, *in vivo,* and *in situ* bioassays, provides the most accurate information from which to estimate risk. Such a combined testing protocol provides information to describe the major organic and inorganic constituents, as well as the pharmacokinetics and potential interactions of chemical mixtures. This research was conducted to investigate the potential genotoxic effects of complex chemical mixtures of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated aromatics (PCA) using microbial bioassays (Salmonella/microsome assay and the *E. coli* prophage induction assay), the ^{32}P-postlabeling assay in mice, and *in situ* measurements of genotoxicity using flow cytometry. Samples of environmental media and wildlife tissues were collected from four National Priority List Superfund sites within the United States. In general, chemical analysis was not always predictive of mixture toxicity. Although biodegradation reduced the concentration of total and carcinogenic PAHs in soils and groundwater, the genotoxicity of extracts from

environmental media did not display a corresponding reduction. Mixtures of polychlorinated biphenyls (PCBs) extracted from sediments were found to inhibit the genotoxicity of PAH mixtures when administered dermally to rodents. This inhibition exhibited a dose-response relationship, with the adduct frequency reduced at increasing doses of sediment extract. Finally, PAH concentrations in environmental media and tissues were found to correlate with DNA damage in wildlife receptors. An integrated approach, combining *in vitro* and *in vivo* methods to characterize genotoxicity provides more accurate information from which to estimate uptake and risk associated with exposure to complex mixtures and should be considered in both the human and ecological risk assessment process.

To the animals who gave their lives for this research

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This research would not have been accomplished without numerous collaborators and supporters. I would first like to thank my dissertation committee: Dr. K.C. Donnelly, Dr. Robin Autenrieth, Dr. Bruce Duncan, Dr. John Bickham and Dr. Tom McDonald. Dr. Donnelly was the mastermind behind each project, was always available when I had questions (there were many!), and was dedicated to helping in the field. Dr. Autenrieth taught me what I know about human health risk assessment and encouraged me to do my best. Dr. Duncan brought a unique perspective to the research with his background as a senior ecologist for the U.S. EPA Region 10. His refreshing and enthusiastic attitude motivated me to learn more about ecological risk assessment—he more than deserved all the cookies we brought him! Dr. Bickham's research in evolutionary toxicology is both fascinating and inspiring. Not only did Dr. Bickham help me with the flow cytometry method, his research allowed me to bridge the gap between my training as a behavioral ecologist and my new pathway in environmental toxicology. I could not have accomplished the chemical analysis without the help of Dr. Tom McDonald. Dr. McDonald is one of the most warm-hearted people I have met science. He is a humble and gifted analytical chemist, a devoted professor, and an asset to any graduate student committee.

In addition to my committee, there were several people within the Donnelly lab who were with me from the beginning. Annamarie Bokelmann was the first person to show me the ropes in the lab. She taught me how to work the equipment and run the bioassays. Her sense of humor made me feel welcome and comfortable in my new surroundings. Carolyn Kotzot was one of our star student workers. She had a very positive attitude and helped me juggle my technician duties and student responsibilities by keeping the lab clean and organized. She is one of the most introspective and caring people that I know. Dr. Ling-Yu He kept me company in the field lab and spent many of her own hours helping to extract my field samples. She cooked the best Chinese food and was always eager to send me home with leftovers for my husband and me to enjoy.

Dr. Leslie Cizmas was my role model in the lab. Leslie is a highly motivated and hardworking woman with a realistic attitude. There were often times when she helped me work through equations while also giving me the tools to work independently. Rebecca Lingenfelter loves animals as much as I do and treated my cat and iguana with great care whenever I had to work in the field or went home for the holidays. When the science got tough, it was always easy to talk literature or politics with Rebecca. Another person who shared my love for nature is Christine Naspinski. I treasure our talks about bird watching and scientific theory. Tracie Phillips was my other half in the lab. We often joked that we shared a brain because we worked so well together and seemed to always know what the other was thinking! I could not have kept my sanity without her and the long hours we spent postlabeling in Houston and packing for Montana. Nancy White is a dear friend and truly made me feel like I was at home in Texas. She offered emotional support and always helped me keep a positive perspective on life. I never tired of coming into work at 7am every morning because I knew Nancy would be there to share morning tea with me. The Donnelly lab in general and all of the students and staff I had the pleasure of working with made my job and research worthwhile.

There were also other labs that played key roles in this research. Dr. Guo-Dong Zhou's lab at the Institute of Biosciences and Technology in Houston was ^{32}P-postlabeling-central. We progressed from postlabeling 36 samples a week in 2003 to doing 80 samples a week by 2005! This could not have been done without the enthusiastic guidance from Dr. Zhou, or without the assistance of Molly Smith who prepared the lab each time for our visits. During our weeks in Houston, Dr. Safe and Lorna Safe offered their hospitality and gave us a roof over our heads while we were away from home. Dr. Phillips' lab was where I received support and benchtop space to extract field samples and perform DNA extractions. Henry Heubner was always willing to answer my questions, and Melinda Wiles taught me how to do a solid phase extraction method for my water samples. In addition to the Phillips' lab, I also received valuable instruction on how to perform tissue extractions from the staff at B& B Laboratories. Dr. Tom McDonald, Dr. Sue McDonald, Juan Ramirez, Eduardo Nieto, and Rebecca

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At the center of my life through all of this were family, friends, and my husband Joseph Gillespie. Joe and I tackled the ups and downs of graduate school and dissertation writing together and made it through with barely a scratch! Joe, you are a brilliant, motivated scientist and loving, devoted husband. Had we not moved to Texas, I might not have followed this fulfilling path.

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NOMENCLATURE

ATSDR = Agency for Toxic Substances and Disease Registry

BaP = benzo[a]pyrene

CDC = Center for Disease Control

CDI = cumulative daily intake

COC = contaminant of concern

COPC = contaminant of potential concern

CSF = cancer slope factor

ERA = ecological risk assessment

HHRA = human health risk assessment

IRIS = Integrated Risk Information System

NPL = National Priority List

PAH = polycyclic aromatic hydrocarbon

PCA = polychlorinated aromatic

PCB = polychlorinated biphenyl

PCP = pentachlorophenol

QSAR = quantitative structure activity relationship

QSTR = quantitative structure toxicity relationship

RBC = risk-based concentration

RfD = reference dose

RI = remedial investigation

TEF = toxicity equivalence factor

USEPA or U.S. EPA = United States Environmental Protection Agency

CHAPTER I

INTRODUCTION

1.1 Overview

Complex chemical mixtures are present in environmental media, food and drinking water. Two of the most common classes of chemical mixtures in the environment are polycyclic aromatic hydrocarbons (PAHs) and polychlorinated aromatic compounds (PCAs). These compounds are common contaminants of surface waters and sediments. The research described in this dissertation was conducted to investigate novel methods for assessing the ecological risk of chemical mixtures. A battery of biological test methods has been coupled with chemical analysis to provide an improved understanding of the potential for exposure and adverse effects of chemical mixtures on ecological receptors. The introductory chapter of this dissertation will review sources and adverse effects associated with mixtures of PAHs and PCAs; and, discuss the current state of knowledge with regards to ecological risk assessment.

1.1.1 Environmental Mixtures

The toxicity of complex environmental mixtures including smoke and soot was recognized as early as the Middle Ages by Paracelsus (Gallo, 2001). A few centuries later, the industrial revolution became associated with a number of occupational diseases, many of which were attributable to complex mixture exposures. In 1775, Percival Pott observed a relationship between scrotal cancer and exposure by chimney sweeps to PAH mixtures. Since World War II and the marked increase in drug, munitions, pesticide, synthetic fiber, and industrial chemical production, the discipline of toxicology has grown exponentially (Gallo, 2001).

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This dissertation follows the style of Environmental and Molecular Mutagenesis.

1.1.2 Environmental Toxicology

Environmental toxicology is a discipline which assimilates knowledge from several areas of science. Ecology, chemistry, molecular biology, genetics and mathematics are all important in understanding the potential impact that chemicals may have on biological systems (Landis and Yu, 1995). Ecology is the study of species interactions within ecosystems and provides information to understand how toxins affect the structure and function of ecosystems. Chemistry is an important component of ecotoxicology to provide information about the composition and concentration of contaminants in environmental media and tissues. Molecular biology may be used to examine the effects of toxins on organisms at the molecular level. Molecular genetics and microbiology also examine effects at the microscopic level and may help to elucidate the environmental fate and transformation of environmental contaminants. Through genetics and microbiology also comes the tools for helping to cleanup and restore ecosystems (i.e. bioremediation). The adaptation of species to environmental change can be measured using evolutionary biology concepts. Statistics may be employed to interpret data and test hypotheses (this can be achieved through mathematical and computer modeling). Risk assessment is an essential part of environmental toxicology and helps to guide research and to formulate testable hypotheses (Landis and Yu, 1995).

The science of environmental toxicology grew from the use of testing pesticides in the late 1940s to the cleanup of wildlife kills, polluted lakes, and burning rivers of the 1960s (Landis and Yu, 1995). After the establishment of the U.S. Environmental Protection Agency (U.S. EPA) and the National Environmental Policy Act, the field of environmental toxicology began to develop rapidly. A variety of technologies, including molecular biology, chemistry, and genetics have been used in the research described in this dissertation to provide a more comprehensive investigation of the impact of environmental mixtures on ecological receptors. This dissertation research is an example of how the different disciplines within environmental toxicology (as described above) can be integrated to understand complex mixture genotoxicity. In Chapter II, the

research focuses on investigating changes in genotoxicity and chemical concentrations in complex soil mixtures undergoing bioremediation. For this chapter, molecular biology (microbial bioassays), chemical analysis, and statistics were used in the research. A basic background about the remedial techniques (microbiology) used on-site was also essential to the study. These data were collected to facilitate an assessment of the risk associated with remedial contaminants in treated soil and groundwater. Data have been generated to determine if the residual contaminants in soil and groundwater after microbial degradation elicited a genotoxic response *in vitro*. Data have also been obtained to determine if chemical analysis provides an accurate estimate of adverse effects associated with exposure to complex environmental mixtures. Chapter III has investigated contaminant mixture interactions from extracted sediments. This research combined chemical analysis, molecular biology and statistics to test the interactions of contaminated sediment mixtures using both *in vitro* and *in vivo* techniques. The research described in Chapter III was also conducted to determine if genotoxicity could be predicted by chemical analysis. The results of the current research, as well as that of previous studies, indicate that complex environmental mixtures are capable of inducing genetic damage in controlled laboratory experiments. The fourth Chapter expands upon this information and combines ecology, chemistry, molecular biology, and statistics. The research described in Chapter IV involved an *in situ* study of wildlife exposed to complex chemical mixtures. For the field research, it was important to obtain information that could be used to compare environmental media and tissue contaminant concentrations; and, to determine if these data were predictive of genetic damage as an indicator of risk. Studies conducted for Chapter IV required an understanding of the basic ecology, behavior and life histories of the species collected as well as the impact of seasonal variability on contaminant migration and species exposure.

1.1.3 Sources of Environmental Contamination

The U.S. Environmental Protection Agency estimated that in 2002, a total of 4.79 billion pounds of toxic chemicals were released to environmental media from industrial

facilities in the United States (USEPA, 2002). Two of the most common classes of chemicals released from industrial facilities in the United States are complex mixtures of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated aromatic (PCAs) compounds. Included in these mixtures are chemicals which are considered probable human carcinogens (USEPA, 2002); and, many compounds which are persistent in the environment and thus may come in contact with human or wildlife receptors. Environmental contaminants can enter ecosystems by several routes and depending on physical and chemical properties eventually reside in soil, water or air. For surface waters, one of the major worldwide sources of pollution is sewage discharge (Walker et al., 1997). PAHs and PCAs are usually released from chemical production activities or the combustion of fuels or waste materials. Mixtures of PAHs and PCAs are relatively insoluble in water. As a result, these compounds are often detected in soils or sediments affected by industrial discharges. In biological receptors, the PAHs and PCAs often partition into adipose tissues. Industrial wastes containing mixtures of PAHs and PCAs may be discharged into a wastewater treatment system or directly into surface waters. If discharged into the wastewater treatment system PAH and PCA mixtures will be altered with release into the environment. Prior to discharge from a wastewater treatment plant, sewage must have acceptable levels of chemical oxygen demand (COD) and biochemical oxygen demand (BOD). The amount of oxygen required to completely chemically oxidize one liter of sewage is the COD. The amount of dissolved oxygen used by microorganisms to oxidize the organic matter in one liter of sewage is the BOD. If the COD and BOD levels are higher than agreed limits, the sewage discharge could cause a substantial reduction in the water oxygen levels and have serious consequences for aquatic organisms (Walker et al., 1997).

The compound composition and concentration in effluent from industrial facilities is defined largely by the industrial processes that generated the waste. For example, heavy metals are generally associated with mining and smelting operations, many organic chemicals with the chemical industry, radionuclides with atomic power stations, and chlorophenols and fungicides with pulp mills (Walker et al., 1997). The

petroleum industry has also been responsible for the deliberate and accidental discharge of oil into the marine environment. Although oil tanker disasters can create an immediate and obvious release of hydrocarbons to the environment, the contribution from non-point sources such as urban runoff is still much greater than releases of oil from normal tanker operations and industrial and municipal activities (Walker et al., 1997).

Surface waters can also be contaminated by runoff from agricultural fields or the direct application of biocides to control plants or insects. Most industrial contaminants are relatively insoluble in water and must be bound to particulate matter to facilitate transport by air and water currents. Particulate transport may also be a concern for aerial application of pesticides where there is a risk of the chemicals drifting into surface waters where they may be highly toxic to aquatic organisms (Walker et al., 1997).

Soil is the receptacle for much of the waste disposed of in the United States and abroad. As with surface waters, soil may become contaminated through accidental and/or deliberate disposal of waste. Landfills are a large source of complex mixture contaminants and may contain acutely toxic compounds as well as genotoxic compounds (Schrab et al., 1993). The use of sewage sludge as fertilizer for agricultural crops is another source of pollution. Non-point sources of pollution such as urban and agricultural runoff add heavy metals, detergents, phosphates and nitrates to the environment. As rainfall runs off of agricultural fields into surface waters, high nutrient input can cause eutrophication of water bodies. The outbreak of harmful algal blooms and their associated toxins has been attributed to excessive nutrient input into waterways (Paerl et al., 2001). Surface soils may also become contaminated due to deposition of smoke and dust from combustion sources. Moreover, rain, snow and dust particles may transport pollutants from the air to surface soils. Surface soils could be impacted from flooding of rivers and seas. The widespread release of contaminants was recently observed in New Orleans after flooding caused by Hurricane Katrina (USEPA, 2005b).

PAHs and PCAs have been detected in every compartment of the global ecosystem. These chemicals are transported by adhering to particulate matter or through

association with droplets, particles or gasses. Chemicals in the gaseous phase may be transported for long distances. Particulate matter is also transported through the atmosphere, although larger particles have a tendency to be deposited in a relatively short distance. Once deposited on surface soils, complex mixtures attached to particulate matter may remain on the soil surface, be degraded by chemical, biological or photodegradation, or may be transported by wind or water erosion. Industrial and domestic stacks used to vent particulate and gaseous emissions contribute largely to atmospheric pollution (Walker et al., 1997). The combustion of coal, petroleum and other fossil fuels results in the release of PAHs, carbon dioxide, sulphur dioxide, nitrogen oxides, hydrogen fluoride and chlorofluorocarbons (CFCs). Drift of pesticides from domestic and agricultural activities are another source of atmospheric pollution (Walker et al., 1997).

The Center for Disease Control (CDC) reports that in the United States, there were more than 36,000 events involving release of hazardous substances reported to the Hazardous Substances Emergency Events Surveillance (HSEES) between January 2001 and March 2005 (CDC, 2005). One hundred and seven of these events were associated with improper disposal (CDC, 2005). It is difficult to accurately define the concentration of environmental exposures which can lead to adverse effects in humans and wildlife. For example, laboratory studies have clearly demonstrated that endocrine disrupting chemicals (such as xenoestrogens) have the ability to cause developmental, tumorigenic and reproductive effects (Degen and Bolt, 2000). However, the risks associated with exposure to concentrations of xenoestrogens in the environmental are more difficult to quantify (Brucker-Davis et al., 2001; Degen and Bolt, 2000). Natural endocrine disrupting compounds can be found in certain plant foods including soybeans, potatoes, garlic, coffee, parsley, and pomegranates (Colborn et al., 1997). The relative concentration of naturally occurring estrogenic compounds in the diet is generally assumed to be higher than the concentration of synthetic estrogens (Safe, 1995; Safe, 2000). Sufficient data are not available to determine the impact of combined exposure to natural and xenoestrogens at low doses in human and wildlife populations. As another

example, the American Chemical Society (ACS) estimates that more than 1.3 million new human cancer cases will be diagnosed in 2005 (ACS, 2005). It has also been estimated that more than one-half million Americans will die from cancer in 2005 (ACS, 2005). Studies of the factors influencing the risk of human cancer clearly established a link between genetic change and malignant progression (McMahon, 1994; Solomon et al., 1991). These genetic changes may be caused by a variety of factors including exposure to genotoxic environmental contaminants. Although rodent studies have been used to identify the carcinogenic potential of a broad range of industrial chemicals, the number of chemicals that are considered established human carcinogens remains relatively small (USEPA, 2005i). The components of PAH and PCA mixtures may be genotoxic, immunotoxic, and toxic to a range of organ systems. However, the association between exposure to chemicals released from hazardous waste disposal activities and adverse health effects in human and ecological receptors has not been clearly established.

The research conducted for this dissertation has focused on two issues. First, can chemical concentrations in environmental media be used to predict body burden of PAH or PCA mixtures; and, second, can biomarkers of exposure, such as DNA adducts or damage to DNA (as measured by flow-cytometry) be used to predict adverse health effects in ecological receptors. The following text summarizes the methodology used for human health risk assessment (HHRA) and ecological risk assessment (ERA) and how these methods attempt to quantify risk based on the concentration of environmental contaminants.

1.2 Risk Assessment Guidelines for Superfund

On December 11, 1980, Congress passed the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) also known as Superfund. This act created a tax on the chemical and petroleum industries that was used to raise billions of dollars for assessment and remediation at uncontrolled or abandoned hazardous waste sites (USEPA, 2005g). A USEPA report released in 2004 indicates that over the next 30

years as many as 350,000 sites will require remediation under the current regulations (USEPA, 2005h). The present Superfund National Priorities List (NPL) includes 1,244 sites (USEPA, 2005d). These NPL sites are selected based on their potential threat to public health. The studies described in this dissertation utilized samples collected from four different NPL sites. Each of the sites used in this research is considered to have the potential to affect both human and ecological health. The process of estimating the human and ecological risk associated with a specific site is an important first step. The quantitative risk assessment provides a means to rank different sites, as well as to establish appropriate concentrations of major contaminants in environmental media following remediation. Risk characterization is a part of the overall process that also includes risk management and risk communication. These activities are important to ensure that the public is aware of health threats; and, to identify appropriate technologies for site remediation.

The accuracy of complex mixture risk assessment is limited due to several factors. First, limited data are available to accurately predict uptake of complex mixtures. Second, many of the components of a mixture may be unidentified or may have limited toxicity characterization. Finally, little is known about the impact of chemical interactions on the metabolism or toxicity of mixtures (Cizmas et al., 2004a; Donnelly et al., 1995). This research utilized chemical and biological test methods to investigate the uptake and genotoxicity of complex mixtures, with specific focus on mixtures of PAHs and PCAs. These classes of chemicals are ubiquitous at hazardous waste sites.

In addition to the complexity of certain environmental mixtures, human and ecological populations are also highly variable. Variations in genetic characteristics, as well as variations in lifestyle and environmental exposures, produce a large amount of uncertainty in estimating exposure and risk. In addition, variations in the dose and duration of exposure from ingestion, inhalation or dermal exposure to environmental mixtures make it difficult to accurately define intake values for a population. Thus, it becomes even more difficult to develop an accurate characterization of the risk

associated with a chemically contaminated site. Risk assessment is the initial step in ranking contaminated sites and selecting appropriate remedial procedures. Current methods used for assessing the risk of complex mixtures generally assume additive interactions for the components of the mixture.

1.2.1 Human Health Risk Assessment

Both human and wildlife receptors may be exposed to contaminants in the ecosystem. Human populations may both contribute to, and be affected by contaminant releases to the ecosystem. Although both human and ecological receptors may be exposed to contaminant releases from hazardous waste sites, the composition and concentration of these exposures are likely to be very different. Thus, state and federal regulators generally use a separate methodology to estimate the human health and ecological risk. The EPA Risk Assessment Guidance for Superfund (RAGS) (USEPA, 1989) describes a 4-step approach for estimating human health risk (Fig. 1).
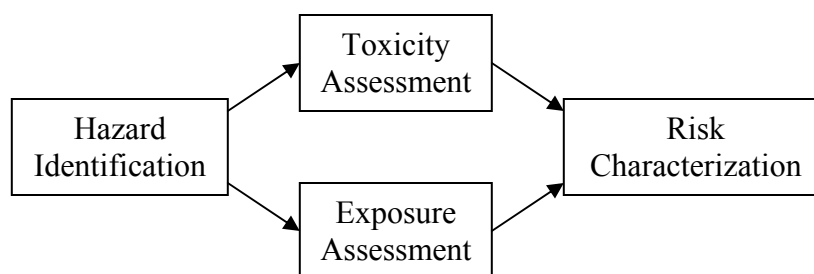
Fig. 1. Four primary steps included in the quantitative evaluation of human health risk for chemically contaminated sites (modified from Asante-Duah 2002).

The initial step in the quantitative risk assessment process is hazard identification. This involves gathering background data regarding contaminants and contaminated media and selecting the contaminants of potential concern (COPCs). This is usually accomplished by comparing the concentration of specific contaminants in

various environmental media with a risk based concentration (RBC). The contaminants of potential concern are selected from those contaminants that are present in environmental media at concentrations that are greater than the risk based concentration. The contaminants of potential concern generally represent those 10 to 15 chemicals that are anticipated to represent the greatest threat to human health. The second step in the assessment of human health risk is the toxicity assessment. For the toxicity assessment, qualitative and quantitative toxicological profiles for each contaminants of potential concern are compiled (Asante-Duah, 2002). Using the EPAs Integrated Risk Information System (IRIS), toxicity values are selected for each contaminant of potential concern. This may include a reference dose (RfD) for non-cancer effects; and, a cancer slope factor (CSF) for carcinogenic effects. The exposure assessment is generally performed at the same time as the toxicity assessment. Knowledge of site conditions and potential usage is employed to identify all potential and future pathways of exposure to site contaminants. For each completed exposure pathway, a cumulative daily intake (CDI) is developed for all contaminants of potential concern detected in each media (i.e., air, soil, surface water, groundwater and sediment). The cumulative daily intake is the product of the contaminant concentration in a specific media, times the ingestion rate, the exposure frequency and exposure duration. This value is then divided by an averaging time (exposure duration times 365 days) and body weight (70kg) to develop an estimate of the daily intake for each contaminant of potential concern. The exposure assessment may also utilize the risk paradigm (Fig. 2) to assemble appropriate information regarding contaminant composition and concentration, affected environmental media, contaminant transportation in the environment, characteristics of the receptor, and the potential adverse health effects associated with a specific dose and duration of exposure. The nature of an adverse health effect associated with a contaminated site is dependent on the route, as well as the dose and duration of exposure. The most common exposure pathways include ingestion, inhalation, or dermal contact with a contaminant of concern. Human populations may also be exposed to contaminants through the ingestion of contaminated plants or animals. The exposure

assessment develops an estimate of intake through various routes of exposure. In addition, exposure estimates are designed to take into consideration potential sensitive groups within a population (i.e. children, elderly, pregnant women).
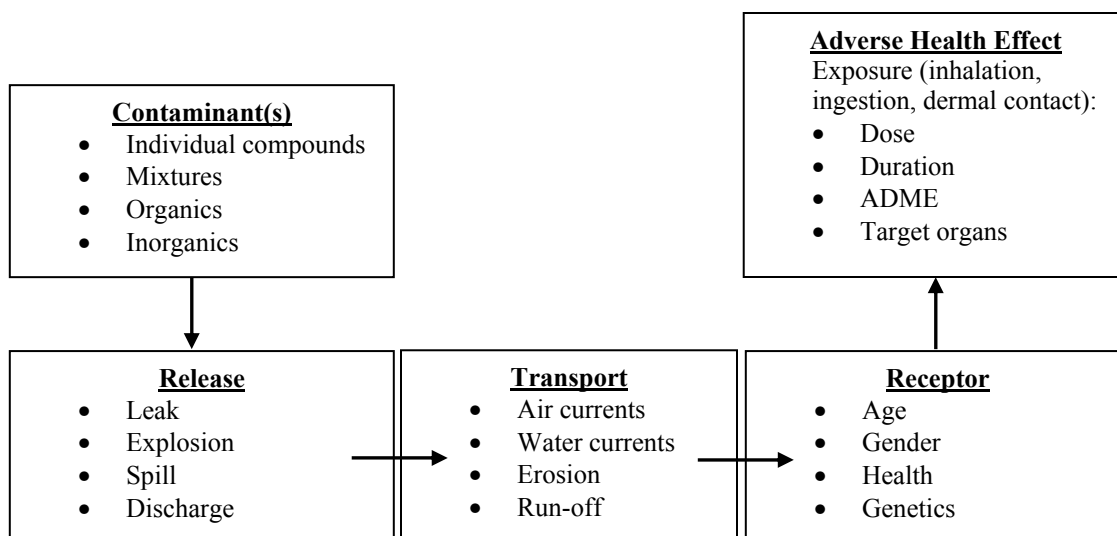
Fig. 2. Model used to identify information required to develop a qualitative estimate of risk (ADME = Absorption, Distribution, Metabolism, and Excretion).

Once information is available from the toxicity and exposure assessments, a quantitative estimate of risk can then be determined. The risk characterization generates a point estimate of the potential for adverse human health effects based on carcinogenic, non-carcinogenic, and/or radiological risks for each exposure scenario (Asante-Duah, 2002). It is also important at this stage to present the uncertainty associated with the various steps in the risk characterization. Uncertainty may result from a limited environmental characterization at a site, or from uncertainties regarding the specific dose and duration of exposure. However, a major source of uncertainty in the risk characterization is associated with the toxicity assessment. Toxicity values (RfDs and CSFs) are available for a limited number of chemicals. Experimental toxicity data is translated into reference doses for non-carcinogens or cancer slope factors for

carcinogens by dividing the no-observed-adverse-effect-level (NOAEL) or the lowest-observed-adverse-effect-level (LOAEL) by various uncertainty factors (UFs). Adding to this uncertainty is the fact that minimal information exists to describe the potential implications of the interactions of the components of complex mixtures on the overall risk of adverse health effects. To estimate non-cancer risk, the cumulative daily intake is divided by the reference dose; while, the estimated lifetime cancer risk is the product of the cumulative daily intake times the cancer slope factor. Generally, if a reference dose or cancer slope factor are not available for a specific contaminant of potential concern a compound of similar toxicity (i.e. a surrogate) is used to provide a reference dose or a cancer slope factor.

For those chemicals which lack a toxicity value, Quantitative Structure Toxicity Relationships (QSTRs) and Quantitative Structure Activity Relationships (QSARs) may be used as a tool to provide a relative toxicity value (a QSAR may be described as a QSTR if the activity being measured is toxicity (Siraki et al., 2004)). In a weight-of-evidence approach these methods can be used in combination with the results of epidemiological studies, animal bioassays and short-term tests to assess contaminant effects (Asante-Duah, 2002). Information describing the structure and physicochemical properties of contaminants of concern such as water solubility (S_w), octanol-water partition coefficient (K_{ow}), liquid vapor pressure ($P_{v,L}$), and Henry's law constants (H_c) is useful for predicting contaminant fate and transport in the environment (Abraham et al., 2005; Dimitriou-Christidis et al., 2003). Physiochemical properties when coupled with pharmacokinetic information (absorption, distribution, metabolism and excretion) are useful for making predictions regarding the fate and persistence of a specific contaminant in biological systems. The use of predictive models may reduce dependence on site-specific information regarding transport and degradation of contaminants (Walker, 2003). Whereas for toxicity assessment, the use of predictive models may reduce dependence on animal studies to obtain toxicity values (Hofer et al., 2004). However, models are only as reliable as the data used to calibrate the model. The utility of models is limited by the criteria used in the assessment process including

the method the model employs for its predictions (Tunkel et al., 2005). With the use of appropriate guidelines for developing and applying QSARs, these models may be implemented for regulatory purposes, and used under limited circumstances (Tunkel et al., 2005; Walker et al., 2003).

The use of models to assess the risk of complex mixtures at contaminated sites is susceptible to numerous sources of error. Models should include methods to account for potential additive, synergistic, or antagonistic interactions of mixture components. Most risk assessment methods assume that mixture components will produce additive interactions. Recommended models for mixtures of chemicals such as PAHs assume strict additivity. These models estimate toxicity using toxic equivalency factors (TEFs) comparing carcinogenic potency of the chemical of interest to the model carcinogen benzo[a]pyrene. Another source of uncertainty in mixtures risk assessment is the chemical analysis. For many mixtures, more than 30% of the components cannot be accurately quantified. Thus, models generally will only use input parameters for those chemicals that have been quantified (Altenburger et al., 2003). QSTR models do, however, provide a tool for developing a preliminary understanding of potential mixture interactions and molecular mechanisms of toxicity (Altenburger et al., 2003). These models may also help link biological effects with chemical analyses for use in designing and interpreting mixture toxicity studies (Altenburger et al., 2003). This applies to both human health and ecological risk assessments. Models are primarily used to extrapolate existing data to make predictions for outcomes under a variety of conditions. The current study was designed to investigate the utility of a range of biological test systems and markers of exposure and early biological effects in ecological receptors. These data were compared with the results of chemical analysis to determine if mixture interactions or unidentified components contributed significantly to the toxic effects of complex mixtures.

Short-term acute and chronic bioassays generally provide qualitative information regarding the risk associated with exposure to complex mixtures. In order to establish primary remediation goals (PRGs), the risk assessment process requires more

quantitative information. The studies conducted in this research focus on the utility of bioassays for providing qualitative data regarding complex mixture toxicity. Studies were conducted to measure the genotoxicity of complex mixtures extracted from environmental media following bacterial degradation. The extracts from these media were analyzed with a battery of microbial genotoxicity bioassays. Data were also obtained from animal studies to investigate the interactions of PCBs and PAHs. This research was done to determine if the concentration of the model carcinogen benzo(a)pyrene could be used to predict the genotoxicity of sediment extracts containing mixtures of PCBs and PAHs. Finally, field studies were conducted to measure genotoxicity in ecological receptors. It is anticipated that the data generated from these studies will be used to identify sources of uncertainty in the quantitative risk assessment process. Reducing the uncertainty in the quantitative risk characterization of complex mixtures will improve the accuracy of both ecological and human health risk assessments.

As part of one of the described studies, the site investigated was undergoing bioremediation. Research has demonstrated that the initial products of biodegradation may be more genotoxic than the parent compounds (Garcia, 2001; Sverdrup et al., 2002b; White and Claxton, 2004). Alterations in genotoxicity following bacterial degradation may result from a variety of factors. In most cases, the products of aerobic degradation are oxidized derivatives of the parent compound(s). These compounds should be more water soluble than the parent compounds. Increased solubility may also result in an increased rate of uptake or absorption of environmental mixtures. Increased toxicity may also be a product of alterations in the rate and extent of metabolism as influenced by mixture components. Alterations in metabolism may enhance or inhibit the overall genotoxicity of the components of a complex mixture. Finally, degradation may result in the removal of the low molecular weight, and less toxic chemicals, with the resulting residual compounds expressing a greater level of genotoxicity than the original mixture. The research conducted for this dissertation addresses several of these issues regarding risk assessment of complex mixtures. The research was performed to identify

sources of uncertainty in both the ecological and human health portions of risk characterization.

1.2.2 Ecological Risk Assessment

Risk characterizations at Superfund sites may consider impacts of environmental contaminants on ecological or human receptors. The ecological risk assessment is designed to develop an evaluation of the potential impacts of environmental contaminants on ecological health. Ecological risk assessments may consider sensitive or endangered species, or may investigate potential impacts on entire ecosystems. The ecological risk assessment follows a similar model as the human health risk assessment. The two primary analytical elements of an ecological risk assessment (Fig. 3) include the characterization of effects and characterization of exposure (USEPA, 1998). During the problem formulation, information is gathered about the source of contaminants, potential ecological effects, stressors, and specific receptors and ecosystem characteristics at the site. The assessment endpoints are also determined. This information is used to generate a conceptual site model and an analysis plan that is implemented in the analysis phase of the ecological risk assessment (USEPA, 1998).

The analysis phase of the ecological risk assessment begins by determining the strengths and limitations of data on exposure, effects, and ecosystem and receptor characteristics (USEPA, 1998). The data are then analyzed to help describe the type of actual or potential exposures and the ecological responses as outlined in the conceptual site model. The exposure and ecological effects are generally not mutually exclusive. For example, when a contaminant enters the environment, exposure to one organism could lead to a cascade of additional exposures and secondary effects. Additional exposures are often through the food web and effects of contaminants can be seen at the level of community organization. From the analysis phase an exposure profile and a stressor response profile are produced (USEPA, 1998). This information is used to help characterize the ecological risk.
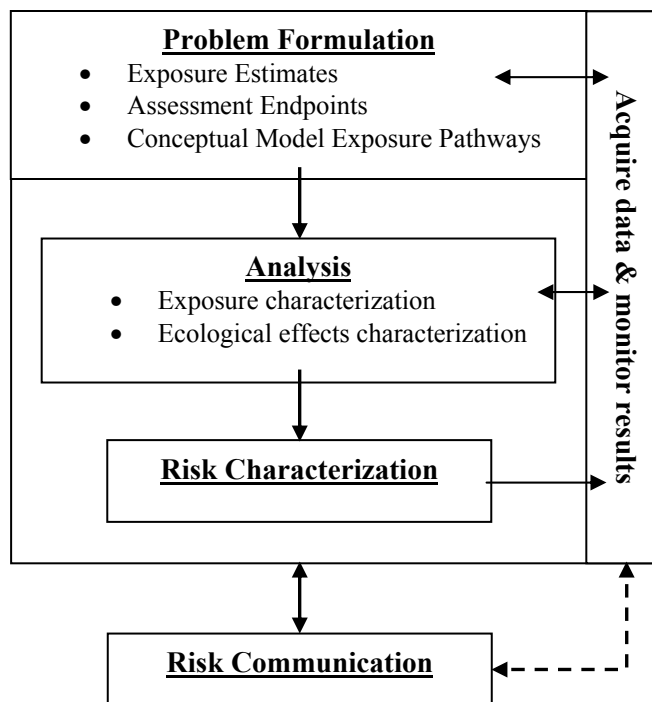
Fig. 3. The basic ecological risk assessment framework (modified from USEPA, 1998).

The risk characterization process of the ecological risk assessment compiles the data from the site analysis and addresses assumptions, uncertainties, and strengths and limitations (USEPA, 1998). The end product is an estimation of potential effects of site contaminants on ecological receptors. This information can then be communicated to stakeholders and any other interested parties. Emphasis should be made on the fact that the ecological risk assessment process is iterative. Throughout the various stages of the process, new questions could arise which require reevaluation of existing data or the collection and analysis of new data. Ecosystems are inherently complex. Whereas the human health risk assessment deals with a single species and generally a limited habitat area, the ecological risk assessment must consider a multitude of species and often includes multiple habitats. Thus, it is important that the analytical phase and risk communication portions of the ecological risk assessment promote an understanding of these complexities (USEPA, 1998).

One of the challenges of ecological risk assessment is understanding the effects of site contaminants on species abundance, diversity and interactions. Community ecotoxicologists are faced with the dilemma of separating the effects of anthropogenic disturbance (such as chemical contamination) from natural community variability (Clements and Newman, 2002). Since humans have been manipulating the composition and structure of ecosystems for nearly 10,000 years (Hessburg and Agee, 2003), establishing what is normal or natural community structure and DNA variation in environments untouched by humans is virtually impossible. It is possible to make comparisons by investigating community structure and DNA variation in relatively pristine populations and in populations in contaminated environments. However, this assumes that: (1) a pristine environment can be identified; and, (2) the community structure in that environment is similar to the site in question.

At any given point in time, natural communities are likely to be exposed to multiple anthropogenic stressors. In order to understand community responses, the relative importance of each stressor must be identified (Clements and Newman, 2002). Even the effect on one entity in an ecosystem can become stressors on other ecological entities. For example, if pesticides reduce earthworm survival, changes in earthworm population densities could be an assessment endpoint (USEPA, 1998). Although, if earthworm densities decline, this could act as a secondary stressor to worm-eating birds that suffer from lowered food supply. A secondary effect of the pesticides could be starvation of young birds. The assessment endpoint might be translated as bird fledgling success. However, other stressors may also affect this assessment endpoint. For example, bird fledgling success could be influenced by the availability of other food sources, nest site quality and nest site competition. These variables in addition to earthworm density and pesticide residue in earthworms (and other food sources) would be valuable measurements for assessing ecological risk (USEPA, 1998).

Predicting the ecological risk from multiple chemical, physical and biological stressors requires knowledge of the interactions of these stressors. Consideration of ecological processes operating at larger spatial scales is necessary when performing

ecological risk assessments for areas with multiple stressors (USEPA, 1998). In terms of chemical stressors, it is also important to understand the bioavailability of the contaminants of concern (COCs). For both the human health and ecological risk assessments, determining fate and transport of contaminants requires an understanding of the structure and physicochemical properties of contaminants of concern including water solubility (S_w), octanol-water partition coefficient (K_{ow}), liquid vapor pressure ($P_{v,L}$), and Henry's law constants (H_c) (Abraham et al., 2005; Dimitriou-Christidis et al., 2003). These properties coupled with toxicity information (absorption, distribution, metabolism and excretion) may provide information that can be used to predict a contaminant's fate and persistence in biological systems. Two classes of environmental mixtures that have been found to impact the health of ecological receptors are the PAHs and PCBs. PCBs with four or more chlorines tend to have high K_{ow} values and high bioconcentration factors (BCFs) due to their lipophillic nature. Thus, the more highly chlorinated PCBs are likely to partition to lipids in exposed populations. PAHs with three or more aromatic rings are usually the components of greatest ecological concern as these compounds are more persistent in the environment and include the carcinogenic PAHs.

Due to the inherent complexity of ecosystems, conceptual site models contain a certain level of uncertainty. Sources of uncertainty include a lack of knowledge about ecosystem functions, failure to identify spatial and temporal parameters, overlooking secondary effects or omitting stressors (USEPA, 1998). It is important to describe the areas of uncertainty and the data limitations to enhance the understanding of the ecological risk assessment. Information regarding the genotoxity and biomarkers of exposure is often lacking for ecological receptors at a Superfund site. Measurements of genotoxicity will be useful when weighing the evidence of ecological risk from complex mixture exposure. Measures of genetic damage may be combined with data from acute and chronic *ex-situ* toxicity tests, chemical analysis of biota and environmental media, and population diversity records to assess ecological risk.

Mixtures of environmental genotoxins have the potential to adversely affect organisms at both the molecular and population levels (Bickham et al., 2000; Shugart et al., 2003). Ecological receptors may come in contact with environmental contamination through ingestion of contaminated food or water, inhalation, and dermal exposure. Following exposure to a genotoxin, the chemical may enter into the organism's cells (depending on its size, charge and other physicochemical properties (such as its water solubility, (K_{ow})) by passive or active transport. During this process it may bind to certain receptors which in turn induce the formation of certain metabolizing enzymes (P450's) or binding proteins (metallothionine). These enzymes may increase the rate of metabolism of the chemical into its genotoxic form. In most cases, the product of Phase I (oxidative) metabolism is the reactive metabolite that is capable of binding with DNA. Some of the physical DNA modifications include the dimerization of pyrimidine bases by UV-B light or breakage of phosphodiester linkages from free radical formation (Shugart, 2000). Chemically, DNA can be altered by the covalent attachment of bulky adducts, modification of existing bases, loss of damaged bases or unstable adducts, breakage of phosphodiester linkages from free radicals and formation of abasic sites, incorrect postreplication, and improper DNA repair (Shugart, 2000). If the initial damage to the DNA is not repaired by DNA repair mechanisms or removed by cell apoptosis (programmed cell death), the DNA mutation could promote itself during cell division. If certain cell growth factors are affected by the mutation, this could result in the cell's inability to regulate replication and cells carrying the mutation might begin to proliferate. If left unchecked by neighboring cells and the body's immune responses, this proliferation could lead to tumor formation. The formation of malignant tumors in mammals is often fatal. However, exposure to chemicals which damage DNA may also lead to reduced reproductive capabilities, birth defects, and modifications to the genetic integrity of a population.

The genotype of an organism largely defines its ability to metabolize and eliminate xenobiotics. One of the major enzyme systems active in the initial stages of metabolism are the Phase I Cytochrome P450 (CYP) metabolizing enzymes. These

enzymes function to oxidize xenobiotics into more hydrophilic polar compounds (usually anions) which are more susceptible to Phase II metabolism. The products of Phase II conjugation generally can be more readily excreted in the urine and the feces (via bile). Other proteins such as metallothionine might also be expressed which bind metals so that they can be more easily excreted or stored. Exposure to a genotoxin has the potential to disrupt energy allocation in the organism. Because the genotoxin is not part of the normal biochemistry of the organism, the organism must expend energy to metabolize the intruding molecule and/or repair the damage the molecule has done to cellular function (including DNA damage). Thus, if an ecological receptor is exposed to elevated concentrations of a xenobiotic, more energy may be required to support the metabolic defenses of the organism, leaving less energy for reproduction.

When a multicellular, sexually reproducing organism is exposed to a xenobiotic there are numerous ways that the toxin can effect the genetic organization of the individual. These effects are generally divided into somatic effects and reproductive effects (germ cells, gametes). If we think of this in terms of energy acquisition, an organism can use the energy it consumes for somatic maintenance, reproduction and defense. From birth to reproductive age, an organism generally allocates more energy to growth and defense (i.e. its goal is to survive and reproduce so that its genes can be passed to the next generation). According to the general principle of allocation, during reproductive age, the organism will allocate more energy to reproduction and less to somatic maintenance (Sibly and Calow, 1986). Following peak reproductive age, the organism begins to senesce, gradually decreasing energy allocation to all areas. Cancer is often a disease of senescence because the organism has decreased energy allocated toward somatic maintenance and repair. As a result, genetic and cellular damage is more likely to occur and less likely to be repaired. Throughout the organism's life there is generally a balance between the energy acquired (its energy budget) and the cost of allocating that energy for different uses (i.e. somatic maintenance, defense, and reproduction). However, during the life of an organism, environmental impacts, including exposure to genotoxins, could considerably alter this budget and decrease the

overall fitness of the individual. One model that could be used to describe how an organism adjusts its energy budget following exposure to a genotoxin would be a cost-benefit model. This type of model might be applied not only to the individual in terms of how the cost of exposure effects fitness, but also to the population level in terms of the potential loss of genetic variability caused by that individual's decreased fecundity.

A recent study using the crustacean, *Daphnia magna*, observed that exposure to toxins (chlorinated pesticides and heavy metals) decreased the organism's ability to forage for food (De Coen and Janssen, 2003). Because the organism was not able to acquire the energy it needed, it increased the production of digestive enzymes to try to make more efficient use of the meals it had already ingested. Exposed individuals also began to feed off their lipid reserves. After 96 hrs. of exposure to the toxins, there was evidence that the Daphnia switched from aerobic metabolism to anaerobic metabolism. During this switch, more oxidative damage occurred as measured by DNA strand breaks. Using a mathematical model, the author's were able to correlate DNA damage with population effects such as Daphnia length, mean brood size and intrinsic rate of natural increase (De Coen and Janssen, 2003).

In general, migrating ecological populations will only receive acute, or short term exposure to contaminants; while, ecological populations that are less mobile would be exposed chronically to contaminants. Both types of exposure could result in similar population effects (increased mortality and potential population bottlenecks; also described as "unnatural selection" (Bickham, 1998)). Although, populations might be able to recover more rapidly from acute exposures once the stressor is removed. If a population is exposed to a toxic contaminant, those individuals within the population that are most susceptible to the effects of the contaminant could be selected against (i.e. they would die). Individuals with resistant genotypes would remain, although in most cases their numbers would be small. The reduction in numbers results in a population bottleneck. With fewer individuals contributing to the gene pool, deleterious recessive alleles could increase in frequency as a result of inbreeding. Further selection could decrease the expression of these deleterious genes, again decreasing genetic variability.

A population that has genetically adapted to a contaminated environment could evolve (where the unit of selection is the individual, the unit of evolution is the gene and evolution is defined as change in gene frequency). This decrease in heterogeneity could be detrimental to the population during stochastic events such as disease, parasitism, seasonal and temporal changes, etc. When looking at populations exposed to toxins they could exhibit either increased genetic variability as a result of increased mutation rate, or decreased genetic variability as a result of population bottlenecking. Effects of decreased genetic variability could be masked by immigration of individuals from unexposed areas contributing to the gene pool. In the absence of immigration, the population size might begin to increase through reproduction, but heterogeneity might continue to decline. If the mutation load continues to increase, this could result in a mutational meltdown and species extinction (Bickham et al., 2000).

In addition to genotoxic affects, some compounds such as PCBs may disrupt endocrine function or also have teratogenic affects on a developing fetus. In the case of teratogens, the timing of exposure is critical for different stages of development. Certain compounds could disrupt the function of natural hormones, altering the normal formation of the reproductive organs of the fetus. Neurotoxic compounds may disrupt the normal formation of the brain and central nervous system. The resulting offspring (if they are not naturally aborted) may lack the ability to reproduce, or to develop normal adaptive behaviors. The decrease in neurological function could disrupt the offspring's ability to obtain food, secure matings, and (in the case of social animals such as humans and honey bees) contribute to social organization. These teratogenic effects could not only decrease fecundity in the parents, but could also decrease fecundity in their offspring. Often these less "fit" individuals are selected against. They either do not make it to reproductive age or they get eliminated by predators or disease. However, diminished reproductive output caused by contaminant exposure at the individual level has the potential to reduce the number of individuals within a population.

Anthropogenic endocrine disrupting chemicals include some of the chlorinated pesticides such as DDT (also heptachlor, dieldrin and aldrin) and the classes of

chemicals known as dioxins and PCBs. These compounds have high K_{ow} values and, thus tend to partition to lipid stores within the body. Because these compounds are not readily metabolized and excreted, they have the ability to bioaccumulate and biomagnify through the food chain. Research conducted on the Great Lakes showed that PCB levels in the herring gull (a top predator) were 25 million times the concentration found in the surrounding water and sediments (Colborn et al., 1997). Predators at the highest level of the food chain tend to accumulate the greatest concentrations of chlorinated hydrocarbons. Chlorinated organics have been detected in a broad range of organisms at great distances from the source of production in industrialized countries. For example, animals in the remote arctic region including polar bears and seals carry heavy body burdens in their fat layers. Steller sea lions have some of the highest body burdens ever measured (Bickham, 1998). Beached whales in Puget Sound could be considered hazardous waste sites because of their heavy contaminant loads. Of particular concern is the mobilization of these compounds in lactating females. In order to increase offspring survival, mammalian breast milk is high in fat and nutrients. Unfortunately, bound to that fat are also these lipophilic endocrine-disrupting molecules which are fed to the offspring at levels which could be highly detrimental to their development. Children born to mothers who consumed an average of 2-3 meals of Great Lakes fish/month (high levels of PCBs) up to six years prior to conception were found to exhibit signs of neurological impairment as measured by both motor skills and cognitive function (Jacobson and Jacobson, 2003; Jacobson et al., 1990). Additional studies showed that women who had consumed moderate levels of Lake Ontario fish (equal to about 40lbs. of fish over a lifetime) bore children with decreased abilities to handle stress, and increased aggression (Colborn et al., 1997; Daly et al., 1996). These results are somewhat controversial and difficult to quantify. However, these studies do suggest that elevated exposures in ecological receptors may result in adverse health outcomes in a human population.

Research is needed to improve our understanding of the toxicity and potential for adverse effects in human and ecological populations exposed to complex environmental

mixtures. The three independent projects performed in this research provide information on the genotoxic potential of mixtures as affected by biodegradation, the interaction of chlorinated and polycyclic hydrocarbons, and the ecological effects in organisms exposed to these compounds. The first project focused on the effects of biodegradation on genotoxicity of contaminated groundwater and surface soils. All complex mixtures are subject to some form of degradation (i.e. photo-chemical degradation or biodegradation) once they are released into the environment. Microbial degradation is one of the most common methods employed for the remediation of soils or sediments contaminated with petroleum or wood preserving waste (Wilson and Jones, 1993). During the degradation process intermediate metabolites form such as phenols, arene oxides, azaarenes, and dihydrodiols many of which have been classified as mutagenic, carcinogenic and teratogenic (Bleeker et al., 2002; Brooks et al., 1998; Brown et al., 1985; Shuttleworth and Cerniglia, 1995; Wilson and Jones, 1993). While these specific intermediates were not directly measured, the complex mixture of PAHs extracted from the soils and groundwater (including parent, intermediate and daughter products) were evaluated for their potential genotoxicity. The risk to both human and ecological health is minimal at this site since the contaminated groundwater and soils have been contained and the property fenced. However, prior to future land use it should be determined if the soils and groundwater have been adequately remediated to minimize adverse human health and ecological effects including genotoxicity.

Animal studies conducted using the extracts of sediments contaminated with PCBs and PAHs can be used to investigate the potential interactions of these chemical mixtures. Benthic organisms in contaminated sediments may be at the greatest risk to exposure to contaminants. However, contaminants in sediments may also be transferred to aquatic species higher in the food web and may also affect humans through ingestion of fish that came into contact with contaminants in sediments. Although the tests used in this research focused on measuring damage to DNA, mixtures of PAHs and PCBs could also impact the immune system, the functioning of the endocrine system, and reproductive capabilities. To support an ecological risk assessment, data are needed to

investigate reproductive effects in aquatic organisms and wildlife following exposure to these sediment mixtures.

The research described in Chapter IV of this dissertation provides the results from *in situ* measurements of exposure and genotoxicity. Data were obtained from environmental media and ecological receptors to investigate potential exposures and biomarkers of effects. The results from these studies were provided to regulators as a supplement to the standard ecological risk assessment. One of the challenging aspects of this research was selecting species that were abundant on site and at corresponding reference sites, and which ones could be successfully trapped. An initial survey was conducted by using site and sound to identify species in the area. Several rounds of trapping were done to determine which species could be successfully trapped to yield an adequate sample size. Seasonal variability was also a factor in terms of species activity. For example, the amphibians were most abundant during their breeding season in the warmer spring and summer months and were more likely caught after rainfall. The small mammals, however, were more likely to frequent traps during the colder, arid months when food was less abundant.

Temporal changes have also been shown to influence contaminant migration. During the warmer and wetter months, some organic compounds are more likely to volatilize with the heat. Rainfall can help to mobilize contaminants in water, soils and sediments, pushing them further into the environment from their source. Moreover, the uptake of contaminants into the food web by primary consumers can vary seasonally. Breeding individuals may also be more susceptible to contaminant exposure as they increase energy acquisition and build up lipid stores (increasing body burden of the lipophilic compounds) for reproduction and brood care. In general, environmental factors including temperature, rainfall, humidity, windspeed and wind direction will have a significant impact on the dose and duration of exposure on humans and other organisms.

1.3 Contaminants of Concern

Two of the most common classes of chemicals detected in the environment include the PCAs and PAHs. The physical, chemical and toxicological properties of the compounds within each of these classes of chemicals are appreciably different. Generally, the low molecular weight, unsubstituted compounds are more soluble in water and more readily degraded; while, the higher molecular weight, or more highly chlorinated compounds are more persistent but less likely to migrate through soil. Thus, if these contaminants are spilled onto a surface soil, the low molecular weight compounds may be leached into groundwater, but tend to degrade in the environment more rapidly. Whereas the higher molecular weight chemicals are less likely to be leached into groundwater, they are far more persistent in a surface soil.

1.3.1 Complex Mixtures

Complex chemical mixtures may be any mixture of organic or inorganic chemicals that includes more than two components. Most complex environmental mixtures consist of several hundred chemicals. In many cases, less than half of the chemicals in complex environmental mixtures can be quantified. This is one reason bioassays provide a useful tool to measure the toxicity of complex mixtures. The research described in this dissertation has focused on complex mixtures of PCAs and PAHs. These chemicals are common contaminants of wood preserving sites and a broad range of industrial sites or areas contaminated with runoff from petrochemical processing areas. Although there are several hundred PCAs and PAHs, toxicity data are available for a small number of these compounds. Thus, this research has employed a battery of biological tests to facilitate an improved understanding of the potential interactions of the components of complex mixtures.

The risk to human or ecological health associated with exposure to a specific mixture of chemicals will be influenced by both the bioavailability and toxicity of the components of the mixture. Complex chemical mixtures may induce a variety of synergistic, antagonistic or inhibitory interactions. There are at least three potential

mechanisms that can alter the toxicity of a complex mixture. Transport across cellular membranes is a critical first step in the production of a toxic effect. In most cases, this transport occurs due to passive diffusion. A mixture of chemicals may inhibit transport (if the components are insoluble), or if the mixture includes solvents may result in an increased rate of transport. Components of a mixture can enhance the activity of Phase I or Phase II enzymes; or, in some cases chemicals can inhibit or deplete enzyme levels resulting in a reduction of chemical activation. In addition, the components of a chemical mixture may compete for binding sites on critical macromolecules within a cell. Depending on the strength and duration of the bond, this competition may result in an increased or decreased toxicity in comparison with exposure to a single compound.

One complex mixture for which a significant amount of animal and human data exists is cigarette smoke. Approximately 4,800 compounds have been identified in tobacco smoke, although it has also been suggested that the actual number of chemicals might approach 100,000 (Green and Rodgman, 1996; Rodgman et al., 2000; Wright, June 1956). Among these chemicals there are irritants, enzyme inducers, carcinogens and promoters. The irritants include chemicals such as acrolein, ammonia and formaldehyde. These compounds can damage cells and increase cell permeability to the other chemicals in the cigarette mixture. There are also percutaneous penetration enhancers such as glycerol and *n*-tetradecane that act by increasing the transdermal delivery of compounds in the cigarettes (Smith et al., 2004). The enzyme inducers are represented by nicotine and the low molecular weight PAH's. Phase I enzyme activity can be increased following exposure to these chemicals. Ingredients such as BaP, vinyl chloride, nickel, benzene, and other PAH's help to initiate carcinogenesis. PAH's and catechol enhance damage to tumor suppressor genes and oncogenes leading to abnormal cell proliferation, thereby promoting cancer.

The primary goal of the research conducted for this dissertation was to investigate the uptake and potential genotoxic effects of complex chemical mixtures. Complex chemical mixtures are released into the environment from a variety of sources including hazardous waste sites. These mixtures may enter the environment as

combustion by-products, and as a result of industrial activities. In addition to cigarettes and cigarette smoke condensate, cooked foods are also an example of complex chemical mixtures. Components of chemical mixtures and their metabolites may be genotoxic to wildlife and humans leading to cancer and heritable gene mutations. Chemical analysis alone does not always provide the most comprehensive view of the fate and toxicity of these mixtures in biological systems. Current risk estimates assume that chemical mixtures will produce primarily additive effects. While this approach is designed to be protective of human and ecological health, it may not accurately reflect the other chemical interactions including potentiation, synergism, and antagonism. The use of microbial genotoxicity bioassays to screen complex mixtures provides a simple inexpensive tool to predict potential carcinogenicity. These methods coupled with chemical analysis provide the framework for understanding the possible toxic interactions of complex mixtures. In addition, *in vivo* experiments or *in situ* research can be used to provide information on the genotoxic potential of these mixtures in higher organisms. A testing approach that integrates chemical analysis with *in vitro* and *in vivo* assays provides more accurate information from which to estimate uptake and risk associated with exposure to complex mixtures.

PAH mixtures represent one of the most common sources of exposure for human and ecological populations. Cell culture studies determined that binary and ternary mixtures of anthracene, chrysene and benzo[a]pyrene (BaP) inhibited the metabolism of BaP, and were capable of reducing BaP nephrotoxicity (Falahatpisheh *et al*. 2004). Therefore, more data is needed to help characterize mixture toxicity. This could be done by using QSARs and QSTRs (as described in section 1.2.1 above), through the use of reconstituted mixtures of known chemical concentration (as was done in the Falahatpisheh *et al*. 2004 study), or by testing extracts of actual field samples. The approach taken for this dissertation was to test actual field samples. Although it is not possible to identify all mixture components in the field samples, the data are more realistic in the sense that no compound is left out in the analysis. However, with this approach comes difficulty in data interpretation. For example, extracts of sediment may

induce an increase in genetic damage compared to the control. Chemical analysis confirms that there are elevated levels of carcinogenic compounds in the mixture. Unfortunately, without knowing all of the mixture components, one cannot correlate genetic damage directly to those carcinogens measured. There could be other unidentified carcinogens contributing to the damage as well as genotoxic metabolites not measured in the chemical analysis.

1.3.2 Polycyclic Aromatic Hydrocarbons (PAHs)

The combustion of almost any type of organic material results in the production of PAHs. Complex mixtures of PAHs may contain hundreds to thousands of compounds (Cizmas et al., 2004b; Giger and Blumer, 1974; Nestler, 1974; USEPA, 2005f). PAHs are characterized as having two or more fused benzene rings in linear, cluster or angular arrangements (Wilson & Jones, 1993) (See Fig. 4 for representative PAHs). Seven PAHs, including the model carcinogenic PAH benzo[a]pyrene (BaP), have been classified by the United States Environmental Protection Agency (U.S. EPA) as probable human carcinogens (U.S. EPA, 2004a). BAP is considered a "model" carcinogen because its toxicity has been well-characterized including the ability of its metabolite benzo[a]pyrene-7,8,9,10-diol epoxide (BPDE) to bind to DNA. BaP is metabolized by a series of enzymes including cytochrome P450 (CYP) into the ultimate carcinogen benzo[a]pyrene-7,8,9,10-diol epoxide. It has been clearly demonstrated that this bay region diol epoxide, is capable of binding with the N-2 of guanine in DNA (Denissenko et al., 1996). If this binding results in the formation of a bulky adduct in the P53 tumor suppressor gene and the adduct is not removed, mutations might occur during replication or during transcription which could effect the functioning of this gene. Mutations in the P53 tumor suppressor gene reduce the ability of certain multicellular organisms to control cellular growth and may result in increased cell proliferation and the progression of tumor formation. Research suggests that the binding of BPDE to the P53 gene exhibits a direct etiological link between a chemical carcinogen and cancer (Denissenko et al., 1996). BaP is also the most studied of the PAHs and often selected as a surrogate

for other PAH compounds. When performing risk assessments, toxic equivalency factors (TEFs) are used as a way to measure the toxicity of a chemical based on how structurally similar that compound is compared to a reference chemical. For PAHs, this reference chemical is BaP.

One of the most common complex PAH mixtures at chemically contaminated sites is wood preserving waste. Depending on the nature of the treatment process, most wood-preserving waste contains creosote which is the oily by-product of making coke from bituminous coal. Creosote is routinely used to treat lumber used for railroad ties and telephone poles. Coal tar creosote is a complex mixture containing several hundred to possibly thousands of chemicals, only a few of which are present in amounts greater than 1% (Melber et al., 2004). Other wood treatment methods utilize diesel fuel and pentachlorophenol, or copper, chrome and arsenic. Typical wood preserving mixtures contain 85% PAHs, 2-10% phenolics and 5% O-, N-, and S- heterocyclics (Bedient et al., 1984; Nestler, 1974). Creosote alone is composed of six major classes of compounds: aromatic hydrocarbons, including PAHs and alkylated PAHs (which can constitute up to 90% of creosote); tar acids/phenolics; tar bases/nitrogen containing heterocycles; aromatic amines; sulfur-containing heterocycles; and oxygen-containing heterocycles, including dibenzofurans (Melber et al., 2004).

PAHs tend to be hydrophobic and bind to particles in the water column which sink to the bottom of lakes and rivers. Low molecular weight PAHs may evaporate from the soil and water and cling to dust particles in the air. The binding of PAHs (as well as other chemicals) to particulate matter is often expressed as the organic carbon partition coefficient (K_{oc}). This is the concentration of a select chemical (such as BaP) in organic carbon versus the concentration of that chemical in water. Compounds with small K_{oc} values are less strongly adsorbed to soils and sediments and tend to move more freely

31

Naphthalene · Fluorene · Phenanthrene · Anthracene · Acenaphthylene · Acenaphthene · Pyrene · Fluoranthene · **Chrysene** · **Benz[a]anthracene** · **Benzo[b]fluoranthene** · **Benzo[k]fluoranthene** · **Benzo[a]pyrene** · **Dibenz[a,h]anthracene** · **Indeno[1,2,3-c,d]pyrene**
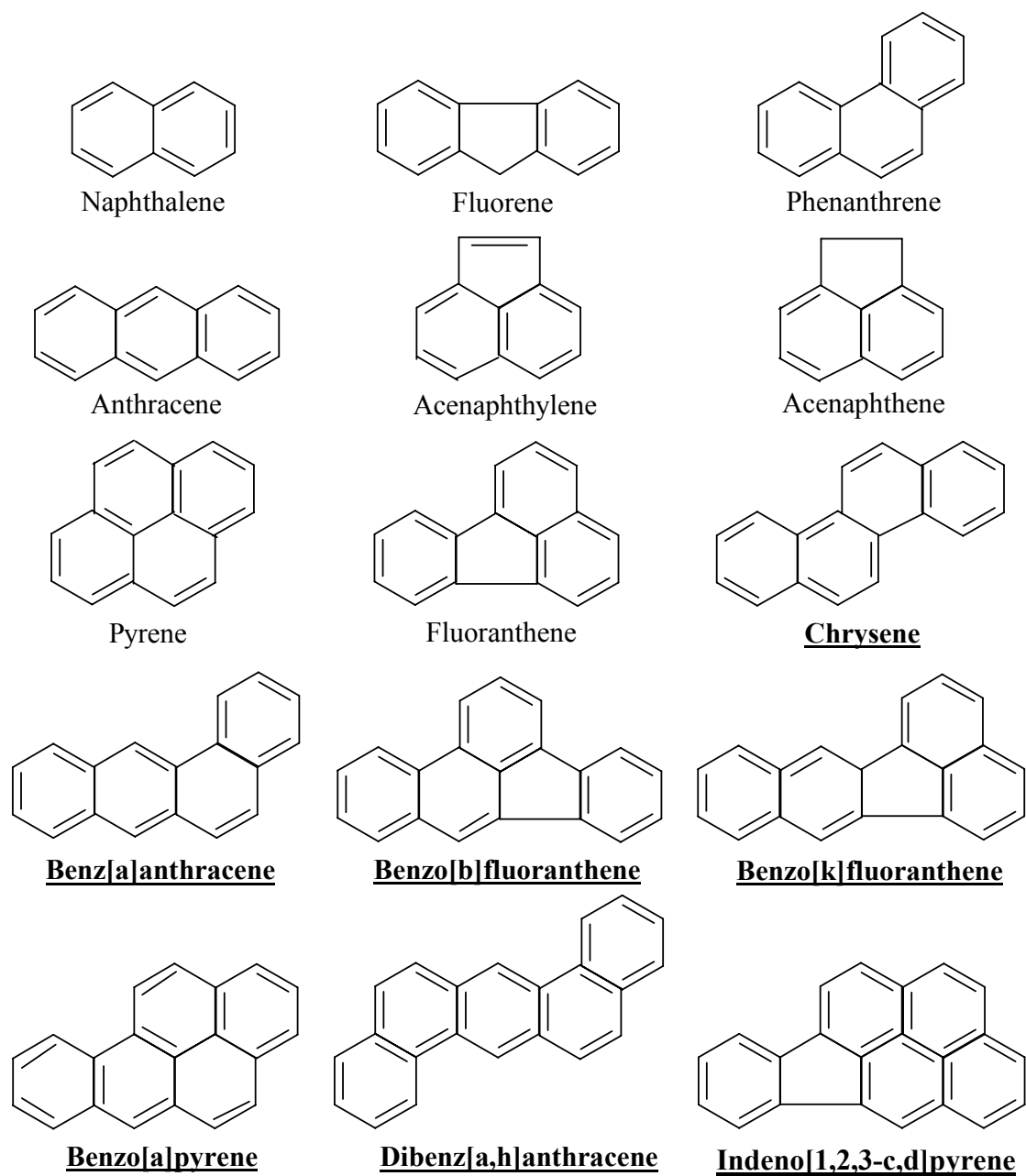
Fig. 4. Representative PAHs. Ones shown in bold and underlined are class B2 carcinogens per the U.S. EPA IRIS classification system (USEPA, 2005i).

with water. In general, PAHs are hydrophobic and have low aqueous solubilities. K_{oc} values of PAHs are variable depending on their molecular size (Koudadoust et al., 2005). Because PAHs tend to be strongly adsorbed to soil particles (especially clays (Luthy et al., 1997)), desorption of PAHs from soil particles has been identified as a key component in their biodegradation. For the lower molecular weight PAHs such as naphthalene, volatilization is important for degradation. Abiotic mechanisms may account for 20% of PAH reduction in bioremediation (DeMarini et al., 1990). However, biotic mechanisms tend to be responsible for the removal of PAHs containing three or more rings (DeMarini et al., 1990). Of the biotic mechanisms, there can be a diverse amount of bacteria in soils contaminated with wood preserving wastes. Research by Mueller et al. found that many of these bacteria are capable of degrading a broad range of PAHs in soil (1991). Those bacteria in the Sphingomonas (formerly Pseudomonas) genus showed the most extensive degradation of 4- and 5-ring PAHs in creosote (Mueller et al., 1991). It was determined that degradation capabilities are associated more with members of specific taxa than with the type of soils from which the bacteria are isolated (Mueller et al., 1991). Futhermore, aerobic conditions are more favorable than anaerobic conditions for PAH degradation (particularly if higher molecular weight PAHs are present) at contaminated sites (Sharak Genthner et al., 1997).

PAHs are often grouped according to molecular weight (MW) with the low molecular weight PAHs (LPAHs) (128 to 203 gram/mole) exemplified by compounds such as naphthalene, anthracene, fluorene, and pyrene and the high molecular weight PAHs (HPAHs) (228 to 278 gram/mole) represented by compounds including benzo[a]anthracene, benzo[a]pyrene, chrysene and indeno[1,2,3-c,d]pyrene. Biodegradation, discussed in more detail below is the most important process in LPAH transformation and degradation (Neff, 1979). HPAHs tend to be more resistant to biodegradation than the LPAHs due to their low solubility, and high affinity for soil/sediment organic carbon. It is the HPAHs that are also known for their carcinogenic potential.

Similar to PCBs, PAHs may bioaccumulate and reach higher levels in plants and animals than in soil and water (ATSDR, 1996). Mixtures of PAHs have been detected in surface waters, plants, soils, sediments and air. Studies conducted with creosote have shown that aquatic invertebrates and fish are particularly prone to the uptake of PAHs. Fish tend to metabolize creosote-derived PAHs better than aquatic invertebrates. PAH profiles in insects and crayfish are often close to sediment concentrations (Melber et al., 2004). In fish, PAH bioconcentration factors (BCFs) from exposure to creosote-contaminated sediments have been estimated to range from 0.3 to 73,000 (Melber et al., 2004). Although some PAHs degrade rapidly in aerobic environments, they tend to persist longer in oxygen-poor environments such as aquatic sediments (Neff, 1985). Due to the chemical structure of PAHs, they readily absorb sunlight, are sensitive to the effects of UV radiation, and may degrade by photolysis (Huang et al., 1995; Arfsten et al., 1996).

Human exposures to PAH mixtures such as wood preserving chemicals could result in irritation or lesions of the skin and eyes (Melber et al., 2004). Exposures may be accompanied by general symptoms including weakness, headaches, vertigo, nausea and vomiting. Workers exposed to creosote might also experience photosensitization of the skin (Melber et al., 2004). PAH exposures (primarily occupational) have been linked to several types of cancers such as scrotal and skin cancers (Carlsten et al., 2005; Gallo, 2001; Melber et al., 2004). Epidemiological studies also suggest that there is an increased risk for bladder cancer, lung cancer, and brain tumors from creosote exposure (Melber et al., 2004). However, these epidemiological studies were based on qualitative estimates of exposure instead of actual measurements (Melber et al., 2004). Extensive research has been done to determine the risk of several types of cancers in tobacco smokers (see Sasco et al., 2004 for review). In laboratory animals, PAHs have been shown to cause lung, stomach and skin cancers via inhalation, ingestion, and dermal exposures respectively (ATSDR, 1996). Animal exposures to PAHs in controlled laboratory and field experiments have been shown to cause reductions in growth rates (Meador et al., 2005; Melber et al., 2004) and impair reproduction (Donnelly et al.,

1990b; Hombach-Klonisch et al., 2005; Melber et al., 2004; Sverdrup et al., 2002a; Sverdrup et al., 2001). Additional studies have suggested that wildlife exposure to PAH contaminated environments could lead to an increased risk of cancer (Martineau et al., 1994; Pinkney et al., 2001).

1.3.3 Polychlorinated Aromatics (PCAs)

PCAs inclue a broad range of compounds such as the polychlorinated dibenzofurans (PCDFs), diphenylethers (PCDEs), biphenyls (PCBs) and dibenzo-*p*-dioxins (PCDDs) (see Fig. 5 for representative PCAs). These compounds are industrial chemicals and by-products and are also ubiquitous environmental contaminants (Safe, 1990). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic PCDD and has been assigned a Toxic Equivalency Factor (TEF) of 1. The TEFs for all other dioxin-like compounds are established based on a comparison of their activity relative to TCDD (Safe, 1990). Included among the PCAs is also the compound pentachlorophenol (PCP). Technical grade PCP often contains PCDFs and PCDDs.

1.3.3.1 Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are a group of synthetic organochlorine chemicals that were widely used in industry for diverse purposes such as organic diluents, wax extenders, cutting oils, flame retardants, plasticizers, heat transfer fluids and dielectric fluids for capacitors and transformers (Safe, 1989) and for products such as cereal boxes and bread wrappers. They were commercialized as congener mixtures (Aroclors, Clophens, Kanechlors, etc.) depending on percent chlorination (Pereg et al., 2001). It has been estimated that as much as 1.4 billion pounds (700,000 tons) of PCBs were produced by the Monsanto Company between 1930 and 1970 in the United States (Safe, 1989).

During the manufacture, use and disposal of PCBs, and from accidental spills, leaks or fires in PCB-containing products, these chemicals made their way into the environment. By the late 1960's PCBs were actually detected in the environment, and

rapidly became ubiquitous contaminants in the global ecosystem (Safe, 1989). Due to the stability and lipophilicity of certain PCB congeners, these chemicals were able to persist, bioaccumulate and bio-magnify through the food chain (Pereg et al., 2001). Carried by ocean currents PCBs found their way into the remote areas of the world including the tissue of Arctic polar bears, ringed seals, and whales, far from their point of
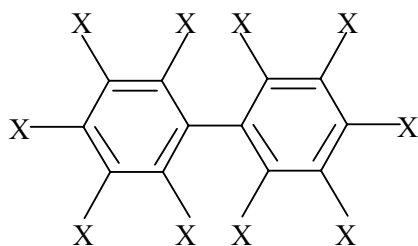
Polychlorinated biphenyl (X = H or Cl)
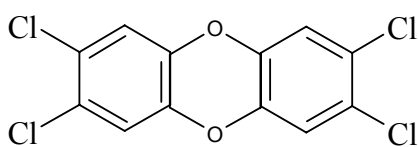
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
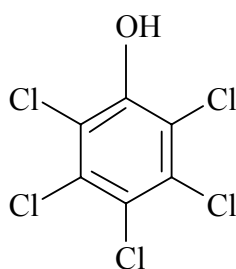
Pentachlorophenol

Fig. 5. Representative polychlorinated aromatics (PCAs).

origin in industrial nations (Bickham, 1997). They have been detected in air, water, soil, sediments, and wildlife, and also human blood, adipose tissue and milk (Safe, 1989). 'Open' uses of PCBs were discontinued in the 1970's, however, 'closed' uses of PCBs

as fluids in transformers and capacitors were not discontinued until 1978 (Safe, 1989). Despite the discontinued use of PCBs in the 1970s, the structure of these chemicals renders them capable of persisting in the environment for centuries (Bickham, 1997).

Regarding the chemical structure of PCBs, two benzene rings of hydrogen and carbon atoms comprise a biphenyl molecule. This molecule is highly flammable. However, by substituting the hydrogen atoms for chlorine, the molecule becomes flame-resistant. The chlorinated biphenyls are a group of chemicals in which the chlorine replaces hydrogen. Those molecules containing more than 1 chlorine atom are named polychlorinated biphenyls. This flame-resistant characteristic of PCBs made them ideal for use with electrical products as flame retardants and electrical insulators. The biphenyl core may be substituted with 1-10 chlorine atoms, giving 209 possible congeners that may be formed (Pereg et al., 2001). There are lower chlorinated PCBs exemplified by Aroclor 1221 (21% Cl weight; 1.5 Cl/biphenyl) and higher chlorinated PCBs typified by Aroclor 1260 (60% Cl weight; 6.30 Cl/biphenyl) (Safe, 1989). The lower and higher chlorinated PCBs exhibit different physical and chemical properties. The higher chlorinated (those containing 5 or 6 Cl groups/biphenyl), laterally substituted, coplanar PCBs, particularly PCB 77, 126, and 169 are the most toxic PCBs known (Safe, 1984).

PCBs are metabolized into hydroxyl-PCBs and may be further conjugated with glutathione or transformed into reactive catechol and quinone products (McLean et al., 1996). The toxic effects of these chemicals appear to be mediated by binding to the aryl hydrocarbon receptor (Ah-R) / aromatic receptor nuclear transporter (ARNT) heterodimer (Klaassen, 2001). This complex enters the nucleus, attaches to the dioxin response element (DRE) and directs transcription of key enzymes (Klaassen, 2001). Through the use of *in vitro* ^{32}P-postlabeling techniques, it has been demonstrated that the lower-chlorinated PCB congeners have the ability to bind DNA (Oakley et al., 1996). Although, *in vivo* covalent binding of PCBs to DNA remains unclear (Whysner et al., 1998).

Human exposure to PCBs is primarily through ingestion of contaminated foods with high fat content such as meat, fish, poultry and dairy products (Schilderman et al., 2000). The adverse health effects in humans were first seen in populations from Japan and Taiwan that were exposed to rice cooking oil contaminated with PCBs, polychlorinated dibenzofurans, and polychlorinated quaterphenyls (Schilderman et al., 2000; Klaassen, 2001). One of the acute PCB exposure effects seen in these populations was chloracne. This type of acne may be distinguished from other acne forms by the progressive degeneration of sebaceous units and the keratinization of sebaceous gland cells (Klaassen, 2001). Those working with PCBs may also be occupationally exposed to these chemicals. Acute exposures may cause other skin diseases in addition to chloracne including allergic contact dermatitis, chemical burns, and irritant dermatitis (Klaassen, 2001). It has been postulated that these contaminants may affect a variety of organs, however, epidemiologic studies have failed to demonstrate an organ-specific illness in humans (Klaassen, 2001). Although, due to the endocrine disrupting ability of certain PCBs, they may interfere with multiple biological functions, exerting strong androgenic, estrogenic and antiestrogenic effects, which may adversely affect human reproduction (Hond et al., 2002). One of the most consistent effects of PCB exposure (primarily in animals) is immunotoxicity (Klaassen, 2001). One study investigated the rise of allergic diseases in industrialized countries and found elevated levels of placental organochlorine compounds and immunoglobulin E (IgE) in cord serum in humans living in industrial regions (Reichrtová et al., 1999). The elevated IgE could signal higher allergic sensitization in these exposed populations (Reichrtová et al., 1999).

Developmental and hormonal effects related to intakes of PCBs during pregnancy are a definite risk to women who are pregnant during exposure. These chemicals have the ability to cross the placenta and expose the fetus to the body burden of the mother (Winneke et al., 2002). PCBs may act through several different pathways, lowering thyroid hormone levels during fetal development, causing body weight and auditory deficits, and affecting neurobehavioral development (Winneke et al., 2002; Klaassen, 2001). The endocrine disrupting function of PCBs is largely undemonstrated

in humans, however, the underlying studies strongly support the potential for such effects (Rogan & Ragan, 2003).

Chronic exposure to PCBs may have reproductive affects in humans, as well as carcinogenic and immunotoxic effects. There have also been reports of higher cases of atopic eczema in industrial regions exposed to high levels of organochlorines (Reichrtová et al., 1999). Most humans carry a significant body burden of PCBs in their adipose tissues (between 0.1-1.0ppm) (Safe, 1989). *In vivo* and *in vitro* animal studies show that PCBs are capable of forming DNA adducts. It appears that the lower molecular weight compounds have a greater ability to bind DNA. The more toxic, highly chlorinated biphenyls are poorly metabolized and exhibit low DNA binding (Safe, 1989). Occupational studies in humans suggest that some PCB-exposed workers may have an increased risk of cancer, but there are other studies that show no increased risk of cancer in workers exposed to PCBs (Safe, 1989). Additional epidemiological studies are needed to clarify the carcinogenic effects of chronic PCB exposure in humans.

In animal studies, PCB mixtures induce monooxygenase activity, particularly aryl hydrocarbon hydroxylase (AHH), and ethoxyresorufin *O*-deethylase (EROD). The levels of these enzymes can be measured as biomarkers of exposure. EROD activity is an accurate indicator of CYP1A1 induction, and previous work with PCB congeners and commercial mixtures shows that the induction of hepatic microsomal AHH and EROD activities is one of the most sensitive indicators of exposure to an aryl hydrocarbon (Ah) receptor agonist (Safe, 1984). Immunotoxicity of PCBs may also be measured by antibody response. Some of the first general toxicity tests of PCBs indicated lymphoid organ atrophy and the reduction of circulating lymphocytes (Klaassen, 2001). A number of animal studies in rhesus monkeys, rabbits, mice, and guinea pigs have measured antibody response following PCB exposure (Klaassen, 2001).

PCB biochemical and toxic effects are highly dependent on the gender, species, age, and strain of animal used as well as on the composition of the PCB mixture (Harris et al., 1993). PCBs have been shown to be complete rodent carcinogens through oral

exposure (Mayes et al., 1998). The higher chlorinated PCBs may also act as promoters of carcinogenesis in rodents exposed to a variety of initiators (Safe, 1989). However, their ability to promote carcinogenesis does remain controversial (Safe, 1989). Recent studies suggest that PCBs may accumulate in the liver, kidney and lung of mice following intraperitoneal injection (Pereg et al., 2001). Using sub-cellular fractionation of these tissues, it was discovered that the majority of PCB compounds were found in cytosols and organellar pellets, with fewer amounts found in the nuclear pellets and microsomes (Pereg et al., 2001). There was no significant binding of the test compounds to DNA in the mice (Pereg et al., 2001). There were, however, high concentrations of PCB metabolites bound to protein in the liver which may suggest that that the proteins are acting as scavengers for PCB-derived reactive metabolites *in vivo* (Pereg et al., 2001). More studies are warranted to determine the carcinogen promoting effects of PCBs.

There are numerous wild and domestic animal studies looking at PCB exposures. For example, in 1999 there was a food contamination incident in Belgium where a mixture of PCBs was combined with recycled fats used in the production of animal feed. Chickens fed the contaminated feed showed signs of chick edema disease as well as a marked drop in egg production, reduced signs of egg hatchability, and increased mortality of chicks (Bernard & Fierens, 2002). PCBs can also reach high concentrations in wildlife, particularly Arctic predators such as polar bears and killer whales which have large amounts of blubber or fat and are at the top of the food chain (i.e. ingest the higher concentrations of PCBs). Pregnant or nursing sea lions transfer much of the contaminated fat to their offspring, exposing the young to higher levels of PCBs than the adult population. This occurs at a critical time in their development when they are most prone to the endocrine-disrupting effects of these chemicals (Bickham, 1997). The reproductive and immune toxicity of PCBs may have significant effects on population size and genetic variability in vulnerable species.

Although PCBs are ubiquitous contaminants, scientists are just beginning to understand the mechanism of PCB toxicity in humans and wildlife. Studies indicate the

ability of PCBs to be both direct-acting carcinogens, carcinogen promoters, and even anti-carcinogens (Safe, 1989). These varying effects could be due in part to the use of different animal models, as well as genetic polymorphisms in the populations being studied. Further studies are needed to elucidate the role of PCBs in affecting ecological and human health.

1.3.3.2 Pentachlorophenol (PCP)

Pentachlorophenol (PCP) is used as a pesticide and wood preservative. It is often applied in conjunction with diesel fuel to pressure-treat lumber. This substance has been found in at least 313 of the 1,585 National Priorities List sites. According to the U.S. EPA, as of 2002, approximately 11 million pounds of PCP were produced (U.S. EPA, 2005d). Humans and wildlife are usually exposed to technical grade PCP which often contains toxic impurities such as PCDDs and PCDFs (ATSDR, 2001). PCP is listed as a probable human carcinogen based on rodent studies. Oral exposure to PCP in mice showed an increased incidence of liver and adrenal medulla tumors in the males, and vascular tumors in the females (U.S. EPA, 2004a). In rats exposed to PCP by gavage, pigmentation of the liver and kidneys was observed. Liver tumors, pheochromocytomas and hemangiosarcomas were formed in female mice following oral exposure to PCP. Liver tumors and pheochromocytomas were formed in male mice following oral exposure to PCP. The hemangiosarcomas observed in the female mice are of particular concern because these types of tumors are "morphologically related to known fatal human cancers that are induced by xenobiotics" (U.S. EPA, 2004a).

1.4 Toxicity Test Methods

Risk characterization based on chemical analysis alone may not accurately predict the adverse effects of exposure to environmental contaminants. Chemical analysis may not be capable of identifying all of the components of a mixture. In addition, chemical analysis is unable to account for potential synergistic or antagonistic interactions of the components of a complex mixture. Thus, to reduce the uncertainty

associated with risk assessment of complex mixtures, it may be useful to employ a battery of toxicity test methods. Standard risk assessment procedures assume additive interactions. While this is generally accepted as the most accurate method for risk assessment, it is possible that this assumption could over or underestimate the actual risk to an exposed receptor. Using toxicity test methods to assist in risk characterization provides more accurate information from which to estimate risk than when either chemical analysis or toxicity testing are used alone. Toxicity tests may include *in vitro*, *in vivo*, and/or *in situ* bioassays. *In vitro* bioassays include microbial or mammalian cells in culture and are useful for investigating the mechanisms of mixture interaction. *In vivo* bioassays provide additional information with regards to the pharmacokinetic interactions of mixture components. *In situ* measurements provide the most relevant data for risk characterization. However, interpretation of *in situ* bioassays may be complicated by population variability or a broad range of confounding factors (i.e., diet, other exogenous exposures, etc).

1.4.1 *In Vitro* Bioassays

A number of *in vitro* microbial bioassays may be used to investigate the genotoxic potential of chemical mixtures including the Salmonella/microsome assay, the *B.subtilus* DNA repair assay, the *E. coli* prophage induction assay, and the *Aspergillus nidulans* chromosome assay. These are only 4 of more 50 microbial bioassays that have been described in the literature. In addition, a number of bioassays using mammalian cells in culture have been developed. For example, the micronucleus test may be used to look for the presence of micronuclei (formed during cell division from either chromosomes lagging in anaphase or from chromosome fragments) following *in vitro* treated cell cultures (Kirsch-Volders et al., 1997); receptor binding assays may be employed to measure the binding affinity of a substance to a hormone receptor (Baker et al., 1999); the measurement of gap junction intercellular communication (GJIC) in various cell lines is useful for studying changes in cell to cell communication following exposure to certain xenobiotics (Salameh and Dhein, 2005); and the use of microarrays

allows researchers to look at gene expression following chemical exposure (Gant and Zhang, 2005). Although microbial bioassays are a quick and inexpensive means of investigating the carcinogenic potential of chemical mixtures, each system has inherent limitations. For example, the Salmonella/microsome assay ranges from being roughly 90% (McCann et al., 1975b) to 77% (Zieger, 1998) accurate for detecting animal carcinogens as bacterial mutagens, but is less accurate at detecting carcinogenic metals and organochlorines (McCann et al., 1975b; Rossman et al., 1984; Zeiger and Tennant, 1986). While the *E. coli* prophage induction assay is less sensitive to PAHs, it has been found to be sensitive to carcinogenic organochlorine compounds (Houk and DeMarini, 1987). Thus, for testing mixtures of PAHs and PCAs, the most accurate results may be obtained when using both of these microbial bioassays.

1.4.1.1 Salmonella/Microsome Assay

The Salmonella/microsome assay was developed by Dr. Bruce N. Ames and validated in a study of 300 chemicals as a method for detecting animal carcinogens as bacterial mutagens (Ames et al., 1973a; Ames et al., 1973b; Maron and Ames, 1983; McCann et al., 1975b). The assay is a short-term bacterial reverse mutation assay that uses histidine dependent strains of *Salmonella typhimurium*. When studying histidine mutants of Salmonella, scientists found that some mutants contained base-pair substitutions and others had deletions of one or more bases (frameshift mutants). It was later discovered that these mutant strains could revert to wild-type (histidine-independence) in the presence of a mutagen and could be used to characterize and identify mutagenic chemicals (Mortelmans and Zeiger, 2000). Because bacteria lack the ability to metabolize xenobiotics using cytochromes P450, the inclusion of an exogenous mammalian metabolic activation system made the assay highly useful (Ames et al., 1973b). Certain carcinogenic chemicals such as the PAHs are biologically inactive unless they are metabolized to active forms. In humans and other mammals the cytochrome P450 system is present primarily in the liver and to a lesser extent in the lungs and kidneys. This system is capable of transforming a large portion of these

chemicals into DNA-reactive electrophilic forms. Some of these intermediate metabolites are potent mutagens in the Salmonella/microsome assay. To test the mutagenic potential of these indirect-acting mutagens, a rodent metabolic activation system (generally a 9000 x g supernatant fraction of rat liver homogenate (S9 microsomal fraction)) may be used in the presence of NADP and cofactors for NADPH-supported oxidation (Maron and Ames, 1983). Animals used to make the S9 fractions are often pretreated with a mixed-function oxidase inducer (Aroclor 1254) to increase the level of metabolizing enzymes in the final fraction. Other inducers such as phenobarbital, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) may also be used. However, research suggests that the type of induction is important for the metabolism of mutagenic chemicals and complex mixtures and can considerably alter the response in the Salmonella/microsome assay (Markiewicz et al., 1996). Aroclor 1254 induction is commonly used and is effective at helping to screen for the mutagenic potential of binary and complex mixtures (Markiewicz et al., 1996).

The tester strains used in the Salmonella/microsome assay have been manipulated to enhance their sensitivity towards genotoxic compounds. For example, the *Salmonella typhimurim* strain TA98 used in this research has the following characteristics and is capable of detecting the mutagenic potential of compounds that induce frameshift mutations (Ames et al., 1973b):

- *HisD3052* mutation – eliminates enzyme metabolizing histidinol to histidine, results in inability to grow in absence of histidine; this mutation is a -1 frameshift mutation which affects the reading frame of a nearby repetitive –C-G-C-G-C-G-C-G sequence (Isono and Yourno, 1974);
- *Bio, chlD, uvr*B and *gal* deletions – deletion mutations through the *uvrB-bio* genes increases strain sensitivity to chemicals producing DNA damage that is repaired by excision repair (deletion of the biotin gene makes the bacteria biotin dependent);

- Plasmid *pkm101* - enhances chemical and UV-induced mutagenesis through an increase in the error prone recombinational DNA repair pathway (this plasmid renders the strain ampicillin resistant); and,

- *rfa* mutation – defective lipopolysaccharide membrane increasing permeability of bacteria and sensitivity to higher molecular weight (bulky) compounds.

In addition to these mutations, the bacteria also have a normal background level of spontaneous revertants (i.e. a certain number revert back to the ability to produce histidine on their own and form colonies). For TA98, this number ranges between 15-50 spontaneous revertant colonies per plate. In the presence of a known mutagen, the bacteria may also respond in a predictable manner. For TA98, a dose of 25 µg of 2-nitrofluorene without metabolic activation (-S9) results in the formation of more than 1,000 revertant colonies per plate; whereas a dose of 10 µg of BaP with metabolic activation (+S9) results in the formation of approximately 300 revertant colonies per plate.

To date, the Salmonella/microsome assay has been used to test a broad range of pure chemical compounds and complex mixtures (DeMarini, 1998; Mortelmans and Zeiger, 2000). It is often used as a screening tool to detect the mutagenic potential of new chemicals and drugs. Data from this assay is often presented to regulatory agencies for acceptance or registration of chemicals including biocides and drugs (Mortelmans and Zeiger, 2000). In 1975, McCann et al. published a paper describing the use of the Salmonella/microsome assay to detect carcinogenicity in 300 chemicals. The compounds tested included the following chemical classes: aromatic amines, alkyl halides, polycyclic aromatics, esters, epoxides and carbamates, nitro aromatics and heterocycles, aliphatics, nitrosamines, fungal toxins and antibiotics, cigarette smoke condensate (complex mixture), azo dyes and diazo compounds. Of the chemicals tested, there was a high correlation (90%, 156/174) between carcinogenicity and mutagenicity. Few non-carcinogens showed any degree of mutagenicity. The cigarette smoke condensate, a complex mixture containing PAHs, showed 18200 revertants/cigarette using the strain TA1538 (contains the same histidine mutation as TA98, which later

replaced TA1538 as a more sensitive indicator for some mutagens) (McCann et al., 1975b). As part of the U.S National Toxicology Program, numerous chemicals (301) (Ashby and Tennant, 1988; Ashby et al., 1989; Tennant and Ashby, 1991) have been tested in the Salmonella/microsome assay. These tests were performed using predictions of carcinogenicity based on structural alerts. The research revealed that some predictions of carcinogenicity disagreed with the Salmonella results. The majority of disagreements were for structural alerts for non-mutagens. It was suggested that a complementary assay be used when screening for potential genotoxic carcinogens. In general, however, the correlation between structural alerts and mutagenicity to Salmonella was predicted to be greater than 90% (Ashby et al., 1989). It is possible that some of the compounds tested fall into the category of non-genotoxic carcinogens (Weisburger and Williams, 1981) which act through epigenetic effects (such as DNA methylation).

Several complex chemical mixtures have also been tested using the Salmonella/microsome assay. This test has been successfully used to screen for the mutagenic potential of complex environmental mixtures such as wood preserving wastes (Barbee et al., 1996; Brooks et al., 1998; Cizmas et al., 2004a; Donnelly et al., 1987; Donnelly et al., 1995; Hughes et al., 1998), manufactured gas plant residues (Cizmas et al., 2004b; Randerath et al., 1999), sewage sludges (Donnelly et al., 1990b), agricultural soils (Brown et al., 1985) and other complex industrial wastes (DeMarini et al., 1989; White and Claxton, 2004). This assay has also been employed to investigate the mutagenic potential of binary mixtures of chemicals including BaP with PCP (Donnelly et al., 1990a; Markiewicz et al., 1996), BaP with 2-nitro-3,7,8-trichlorodibenzo-p-dioxin (2NTCDD) (Donnelly et al., 1990a), 4-nitro-4'-chlorobiphenyl with 6-nitro-4,2',3',4',5'-pentachlorobiphenyl, 4-nitrobenzo-p-dioxin with 4-nitro-2,3,8-trichlorodibenzo-p-dioxin, and benzo[a]pyrene with either nitropentachlorobiphenyl or nitrotrichlorodibenzo-p-dioxin (Donnelly et al., 1988).

1.4.1.2 *E. coli* Prophage Induction Assay

Although the Salmonella/microsome assay has been used extensively for screening complex mixtures for mutagenic potential, there are certain classes of chemicals such as the chlorinated organics that are not detected well by this test (McCann et al., 1975b; Zeiger and Tennant, 1986). The *E. coli* prophage induction assay was developed to help detect the mutagenic potential of chlorinated organics (Houk and DeMarini, 1987). It was modeled as a complement to the Salmonella/microsome assay and, thus also employs the used of S9 fractions to induce metabolic activity. The assay was built from previous test methods which measured the prophage lambda induction in *Escherichia coli*.

In organisms, replication error rates are normally kept low through error avoidance mechanisms. However, there are some stress conditions that are capable of lowering the efficiency of DNA replication (Humayun, 1998). The SOS response in *E. coli* to DNA damage is an example of one of these stress responses. There are more than thirty proteins involved in the SOS response (Courcelle et al., 2001; Fernandez De Henestrosa et al., 2000). Two proteins are particularly important. The LexA protein acts by inhibiting the expression of SOS genes under normal conditions. In the event of DNA damage, the RecA protein inactivates the LexA repressor (Friedman et al., 2005; Sassanfar and Roberts, 1990). DNA damage resulting from chemical exposure or radiation can alter genetic information and cause the bacteria to go into SOS response.

A prophage is a latent bacterial virus (i.e. it does not cause disruption of the bacterial cell) covalently integrated into its bacterial host's chromosome. If the bacterium goes into the SOS response, proteins (such as RecA) within the bacterium inhibit the phage repressor and the phage goes from being in a latent stage to a lysogenic stage, begins to reproduce within the bacterium and causes the bacterium to lyse (releasing the phage). The prophage lambda is one manifestation of the SOS response in *E. coli* and may be evoked by a wide range of chemical classes. The *E. coli* prophage induction assay involves the use of a lambda lysogen strain (WP$_2\lambda$) of *E. coli* as well as the use of the indicator *E. coli* strain TH008. During the microsuspension assay the

lysogen strain is exposed to the test chemical(s). If the chemical(s) have the ability to induce the SOS response in the bacteria by causing DNA damage, the cells will lyse and release phage into the media. Following incubation the lambda phage concentration is determined by exposing the indicator strain to the microsuspension mixture. Because the indicator strain is not resistant to the lambda phage, infected cells die and form plaques which can then be counted as a measurement of phage concentration and consequent mutagenic potential of the test compound(s).

Several chemical compounds including complex industrial wastes have been tested using the *E. coli* prophage induction assay (Houk and DeMarini, 1987; Houk and DeMarini, 1988). For example, the method was employed with chlorophenols (including pentachlorophenol). It was found that of the 19 isomers tested, the ones with one or no chlorine atoms *ortho* to the OH group were the most potent and those with two chlorine atoms *ortho* to the OH group were the least potent (DeMarini et al., 1990). These isomers were not mutagenic in Salmonella. It was hypothesized that the metabolism of certain isomers and subsequent formation of free radicals could cause DNA strand breaks resulting in prophage induction (DeMarini et al., 1990).

An additional twenty-eight chlorinated organics were tested which had been previously tested in the Salmonella/microsome assay by Ashby and Tennant (1988). The prophage induction assay had somewhat higher specificity than Salmonella (70% vs 50%), and detected six carcinogens that were not detected in Salmonella (DeMarini and Brooks, 1992). As in the previous study with the chlorophenol isomers, it was suggested that the *E. coli* prophage induction assay is useful for detecting some genotoxic carcinogens that induce certain types of DNA damage (such as the formation of free radicals) that do not revert the standard Salmonella tester strains (DeMarini and Brooks, 1992). Subsequent research delved further into the mechanism of prophage induction and found that whether the chemicals were poisons of the DNA gyrase subunit A, or intercalated in between the DNA bases and also formed reactive-oxygen species, the same type of damage was produced, i.e. DNA strand breaks (DeMarini and Lawrence, 1992).

Similar to the Salmonella/microsome assay, the *E. coli* prophage induction assay has been used to screen for the mutagenic potential of complex environmental mixtures including wood preserving wastes (Cizmas et al., 2003; Cizmas et al., 2004a; Donnelly et al., 1995) and other complex industrial chemical mixtures (DeMarini et al., 1989; Houk and DeMarini, 1988). It has generally been used in conjunction with the Salmonella/microsome assay in order to more thoroughly characterize the mutagenic potential of these wastes.

1.4.2 *In Vivo* ^{32}P-Postlabeling of DNA Adducts

DNA adducts are formed when electrophilic chemicals or their metabolites covalently bind to DNA. These chemicals can be either endogenous or exogenous in origin. Of the endogenous adducts there are Type I compounds that may be functionally important or have a protective role, and Type II compounds that form oxidative lesions. DNA adducts are one of the most sensitive biomarkers that can be measured following exposure to carcinogens. If adducts are not repaired, gene mutations can develop during cell division. Accumulation of mutations can lead to cancer. Correlations between the levels of DNA adducts and tumorigenesis have been demonstrated (Poirier and Beland, 1994; Poirier et al., 1995). For example, a reduction in DNA adduct quantities using chemopreventive agents reduces tumor formation (Breinholt et al., 1995; Dashwood et al., 1998; Egner et al., 2003).

DNA is a polymer composed of monomer units called nucleotides. Each nucleotide is comprised of a nitrogen containing base attached to a 5-carbon sugar (deoxyribose) and a phosphate group. The nitrogen-containing base is either a purine (Adenine or Guanine) or a pyrimidine (Cytosine or Thymine). These nucleotides form the backbone of DNA and 2 complementary strands bind together to form DNA's double helix molecular structure. This helix is formed by non-covalent hydrogen bonding between complementary base pairs (ex. Adenine and Thymine are connected by 2 hydrogen bonds, and Guanine and Cytosine are connected by 3 hydrogen bonds).

An adduct is formed when an electrophilic chemical is able to bind to a nucleotide. For example, the PAH, benzo[a]pyrene is metabolized into its reactive form, the bay region 7,8 diol-9,10 epoxide which has a high binding affinity for guanine. Oxidative damage may also cause DNA adduct formation. It has been shown that newborn mice have low levels of antioxidants in their systems and are less able to handle xenobiotics which cause oxidative damage (Zhou et al., 2004). Recent research has demonstrated that adduct levels from tumor tissues are lower than adduct levels in tissues adjacent to the tumor (Gyorffy et al., 2004). There are 3 hypotheses for this: 1) The tumor grows so rapidly that adducts are diluted, 2) The structure of DNA is changed and as a result has a low affinity for adducts, and 3) Phase II enzymes are upregulated in tumor cells and are better able to detoxify xenobiotics (Dr. Zhou, personal communication).

^{32}P postlabeling is able to detect $1/10^9$ adducts/nucleotide. This particular method is easy to employ because only 1-5µg of DNA are required. Human tissues such as blood from control and treatment populations can be extracted for DNA and the DNA ^{32}P postlabeled for adducts. Tissues from animal studies including tumor cells may also be analyzed using this method. *In vitro* studies with DNA (generally DNA from skin or lung is used because these tissues naturally have low levels of endogenous adducts) may be conducted by exposing healthy DNA to xenobiotics (with or without microsomes) and then by measuring the DNA for adduct formation.

1.4.3 *In Situ* Techniques

There are numerous ways to test for toxicity *in situ* via the use of biomarkers of exposure. Some of the methods directly measure genetic damage (such as quantifying DNA strand breaks, and looking at cytogenetic effects and mutations). Whereas, other methods look at gene expression as measured by protein induction (such as allozymes). Below is a list of several biomarkers which can be used to identify the effects of environmental exposure in humans and wildlife.

- MICRONUCLEUS TEST: As mentioned above, this test looks at the number of cells containing chromatid fragments that have lagged during anaphase and have not been incorporated into either daughter nuclei after cell division (Schmid, 1976). These fragments form their own small nuclei. The number of cells containing these fragments (micronuclei) is compared to the total number of cells counted (roughly 1,000 cells are counted (Matson et al., 2005b)).

- COMET: This assay measures DNA strand breakage in individual cells (Singh et al., 1988). Cells are imbedded in agarose, lysed, and then run through electrophoresis using alkaline conditions. Fragmented DNA migrates away from the nuclei of the cells and toward the anode. The image is stained and the amount of fragmentation can be quantified. The further the DNA migrates, the greater the number of strand breaks (Fairbairn et al., 1995).

- EROD: Ethoxy-resorufin-O-deethylase (EROD) can be measured as a biomarker of P450 induction (Klotz et al., 1984). This is an enzyme that mediates the activity of CYP1A1 and is a parameter for aryl hydrocarbon receptor activation. Measurement of EROD is particularly useful for looking at the effects of PCB and dioxin exposures.

- SEQUENCE DIVERSITY: There are a number of methods available for looking at sequence diversity in genomes such as RAPD and the isolation and characterization of microsatellites (Bickham et al., 2000). The RAPD (randomly amplified polymorphic DNA) technique uses one (10 base pair) primer to produce DNA fragments that are analyzed using gel electrophoresis. The bands are dyed by ethydium bromide for visualization and banding patterns can be compared (similar to DNA fingerprints (Bickham et al., 2000)). This method (Theodorakis and Shugart, 1997) was used in a study with mosquitofish from radionuclide-contaminated sites to investigate genetic effects from contaminant exposure (Shugart and Theodorakis, 1998). The variability of the bands among individuals collected within the contaminated environment was compared to the band pattern seen in individuals collected from a control environment.

Homologous bands were seen in *Gambusia affinis* and *Gambusia holbrooki* exposed to radioactivity at two separate sites suggesting that these bands have an adaptive significance for exposure to radioactivity (Shugart and Theodorakis, 1998).

Microsatellites are non-coding regions of the DNA that tend to evolve rapidly and are highly polymorphic. These regions can be sequenced and the lengths of the regions compared between individuals within or among populations to determine genetic variability. This method is not specific to a particular type of contaminant. It is a population genetic marker that can be used to assess the potential genetic effects seen in a population as a result of contaminant exposure (Ellegren et al., 1997) or to other stochastic events.

- MICROARRAYS: Toxicogenomics is an emerging field that integrates the tools from genomics and bioinformatics to investigate genes expressed as a result of chemical exposure (Gant and Zhang, 2005). The tool used to perform these investigations is the microarray. The microarray is a number of target genes arranged on a substrate in an array formation. The array is capable of simultaneously gathering information on the expression and alteration of thousands to tens of thousands of genes in a biological system in response to xenobiotics (Cheung et al., 1999).

- LYSOSOME STABILITY: The lysosome is a cytoplasmic particle within plant and animal cells. It contains hydrolytic enzymes and is bound by a single unit membrane that acts as a barrier between the enzymes within the lysosome and the cell cytoplasm. If the membrane stability of the lysosome is compromised, the enzymes will leak into the cytoplasm and rupture the cell. Certain metals and organic pollutants are known to alter lysosomal structure (Koehler et al., 2002; Moore et al., 1984; Viarengo et al., 1985). The destabilization of the lysosome membrane can be measured in response to PAH (redox cycling/reactive oxygen species formation), heavy metal and organochlorine exposures and their interactions (Broeg et al., 2005).

- Measuring blood & urine levels of indictor proteins or metabolites: Looking at proteins and metabolites of certain xenobiotics in the blood and urine can be indicators of contaminant exposure. For example, cotinine is a major metabolite of nicotine and can be measured in human populations to determine smoking status.

It should be mentioned that toxicity tests used in risk assessment generally look at changes in survival, growth and reproduction. The biomarkers of exposure described above are considered sublethal endpoints and may not directly translate to a physiological effect in an individual that disrupts survival, growth or reproduction. For example, some species carry many copies of the same gene. Following exposure some of the copies may be affected, while others remain perfectly functional. As a result, the organism may express certain biomarkers of effect, but exhibit no alteration in survival, growth or reproduction.

1.4.3.1 Flow Cytometry

The primary *in situ* method used for this dissertation (Chapter IV) was flow cytometry. DNA that has been abnormally processed during cell division may potentiate irreversible cellular events (Shugart, 2003). One of the ways to measure this damage is by looking at chromosomal aberrations. During the cell cycle, when chromosomes are damaged, acentric fragments of the chromosomes may lag behind at anaphase. These fragments do not become part of either daughter nuclei, but instead form their own small nucleus. These are called micronuclei. The difference in DNA content among cells following chromosome fragmentation or rearrangement after cell division can be measured using flow cytometry (Shugart, 2003). This method has been shown to detect chromosome damage in a number of species exposed to complex mixtures of environmental contaminants (Bickham et al., 1988; Bickham et al., 1992; Bickham et al., 1994; George et al., 1991; Lamb et al., 1991; Matson et al., 2004; Matson et al., 2005a; Matson et al., 2005b; McBee and Bickham, 1988; Theodorakis et al., 2001). Moreover,

flow cytometry data tend to correlate well with petroleum products and specifically with PAHs (Bickham et al., 1998a; Custer et al., 2000).

1.5 Objectives and Specific Aims

The goal of these studies was to improve the accuracy of information employed to characterize the human or ecological health impact associated with exposure to complex chemical mixtures. While the research has focused on the toxic effects of mixtures isolated from Superfund sites, the data are applicable to any population exposure to mixtures. More specifically, a series of research experiments have been conducted to utilize biological testing to investigate genotoxic interactions of complex chemical mixtures. In addition, the results from biological analysis have been used to determine if chemical analysis accurately predicts the toxicity of complex mixtures.

The specific aims of this research include:

1. Monitor changes in genotoxicity and chemical concentration in complex mixtures undergoing bioremediation.

Hypothesis 1: Genotoxicity of wood preserving waste contaminated soil extracts as measured in the Salmonella/microsome assay will be correlated with levels of benzo[a]pyrene (BaP) and levels of carcinogenic PAHs in the soil extracts.

Hypothesis 2: Genotoxicity of contaminated groundwater extracts as measured in the *E.coli* prophage induction assay will be correlated with levels of pentachlorophenol (PCP) in groundwater extracts; genotoxicity will not be measured well in the Salmonella microsome assay due to PCP cytotoxicity.

Hypothesis 3: Bioremediation results in a reduction of contaminant concentration in soil and groundwater and a corresponding reduction in genotoxicity over time.

2. Investigate the genotoxic interactions of complex mixtures isolated from sediments using DNA ^{32}P-postlabeling *in vivo*.

Hypothesis 1: Genotoxicity of PAH and PCB contaminated sediment extracts as measured in the Salmonella microsome assay will be correlated with levels of

PAHs in the sediments extracts; genotoxicity as measured by the *E.coli* prophage induction assay will be correlated with levels of PCBs in sediment extracts.

<u>Hypothesis 2</u>: PAH & PCB contaminated sediment extracts will cause DNA adduct formation in a 24hr. topical exposure study using ICR female mice; adduct levels will correspond with concentration of carcinogenic PAHs.

<u>Hypothesis 3</u>: Co-administration of BaP and sediment extracts will result in increased levels of DNA adduct formation *in vivo*.

3. Examine the uptake and genotoxicity of complex mixtures *in situ*.

<u>Hypothesis 1</u>: Fish and amphibians collected from a contaminated area will have increased levels of genetic damage compared to reference fish and amphibians (as measured by flow cytometry).

<u>Hypothesis 2</u>: Genetic damage in fish and amphibians will correspond with contaminant concentrations detected in environmental media (sediments, surface water).

<u>Hypothesis 3</u>: Tissue contaminant concentrations in wildlife exposed to wood preserving waste *in situ* will correspond with measurements of genotoxicity.

CHAPTER II

GENOTOXICITY OF COMPLEX CHEMICAL MIXTURES IN SOIL

AFTER BIOREMEDIATION

2.1 Introduction

In 2005 the United Stated Environmental Protection Agency made public its Toxics Release Inventory report for 2003 (USEPA, 2005a). Over 4 billion pounds of chemicals were released into the environment from more than 23,000 U.S. facilities in 2003 (USEPA, 2005a). The release of toxic chemicals has been an adverse consequence of industrialization and industrial production. Although many of these wastes can be treated, reused and recycled, certain land disposal activities have contributed to surface soil and subterranean aquifer contamination. Contaminants such as wood preserving wastes (WPW) contain hazardous constituents that are genotoxic. These wastes are also generally composed of complex mixtures of chemicals. Usually only a fraction of the components of a complex mixture from hazardous waste sites are identified and even fewer are quantifiable. Additionally, exposure and toxicity data are extremely limited. Short-term microbial bioassays, used in this study in conjunction with chemical analysis, are a more practical means of determining parameters of toxicity for complex mixtures than chemical analysis alone.

All complex mixtures are subject to some form of degradation (i.e. photo-chemical degradation or biodegradation) once they are released into the environment. Microbial degradation is one of the most common methods employed for the remediation of soils or sediments contaminated with petroleum or WPW (Wilson and Jones, 1993). During the degradation process intermediate metabolites form such as phenols, arene oxides, azaarenes, and dihydrodiols many of which have been classified as mutagenic, carcinogenic and teratogenic (Bleeker et al., 2002; Brooks et al., 1998; Brown et al., 1985; Shuttleworth and Cerniglia, 1995; Wilson and Jones, 1993). Remediation of the more refractory genotoxic compounds found in WPW often shows increased mutagenicity that remains for prolonged periods (White and Claxton, 2004).

The goal of this study was to determine whether measured chemical endpoints alone accurately reflect the genotoxic risk posed by exposure to a mixture of chemicals. One of the most difficult questions considered during the remediation of contaminated waste sites is "how clean is clean?" While biological degradation may be effective at reducing contaminant concentrations, little is know about the toxicity or genotoxicity of the products of degradation. *In vitro* bacterial mutagenicity tests were used to investigate the genotoxic risk of soil and groundwater collected from a Superfund site. At this site, both soil and groundwater have been contaminated with chemicals from wood-preserving waste. Creosote, is the oily by-product of making coke from bituminous coal and contains hundreds of compounds (USEPA, 2005f), over 85% of which are PAHs (Mueller et al., 1991). When mixed with other types of wood preservatives such as PCP and released into the environment, this complex mixture of wood preserving waste can contaminate soils and leach into aquifers. Contaminants of concern at the site include carcinogenic PAHs and PCP. The PAHs are a class of chemicals characterized as having two or more benzene rings. The higher molecular weight PAHs tend to be of most concern due to their carcinogenic abilities. There are seven PAHs that are currently listed as probable human carcinogens by the U.S. EPA (USEPA, 2005i). PCP is also listed as a priority pollutant. It is found in at least 313 of the 1,585 National Priorities List sites (USEPA, 2005h).

2.2 Materials and Methods

2.2.1 Site History

The site investigated is a former wood preserving facility located in a relatively flat intermontane valley in the northwestern United States. This site has been contaminated with wood preserving waste including creosote and PCP. Chemicals were mixed with diesel fuel to extend their usage (USEPA, 2004c). During the wood treatment process, uncontrolled releases of creosote and PCP at several locations on site resulted from either disposal in unlined pits or spills. In 1979, shortly after private wells

were installed, residents near the site began to complain of a creosote smell in their drinking water (USEPA, 2004c). In 1981 the USEPA confirmed contamination of the drinking water (USEPA, 2004c). In 1983 the site was placed on the National Priorities List (NPL) of Superfund Sites, and in 1985 the Principal Responsible Party (PRP) agreed to provide an alternate water source to those people with contaminated wells (i.e. they were put onto municipal drinking water). Full-scale soil and groundwater remediation began in 1991 (USEPA, 2004c).

The valley where the site is located is bordered by mountains. Deposits of both alluvial and glacial sediments and erosional material from the mountains characterize the complex geology at the site. Due to the complex stratigraphic system, contaminants have not been distributed uniformly in the subsurface. Consequently, locating underground contamination and predicting contaminant migration patterns is difficult (USEPA, 2005c). In order to address the hazard posed by contamination of the upper aquifer, an *in situ* bioremediation system for the upper aquifer was designed and constructed. A pilot-scale test was initially performed which consisted of injecting oxygenated water and nutrients into the aquifer to promote microbial degradation of PCP and PAHs (USEPA, 2005c). Results from this test indicated that this was a viable remedial technique for the contaminated aquifer. Figure 6 shows a map of the site including the bioreactor treatment building that was constructed to treat the groundwater, and also shows the soil treatment areas.

The bioreactor treatment building houses an oil/water separator (the current one was installed in 1999) which removes the non-aqueous phase liquids (NAPL) from the extracted groundwater (the two extraction wells are close to 75 feet deep; since 1997, well 1 has been the principal extraction well (Fig. 6)). Once the NAPL is removed, the dissolved phase of the contaminants is biodegraded in a series of bioreactors (Fig. 7). The bioreactors are two 10,000 gallon tanks filled with polyethylene media to support microbial growth. The bioreactor system was originally inoculated with an indigenous group of bacteria isolated from the extracted groundwater. Ammonium polyphosphate and urea ammonium nitrate are added to the process water prior to entering the first
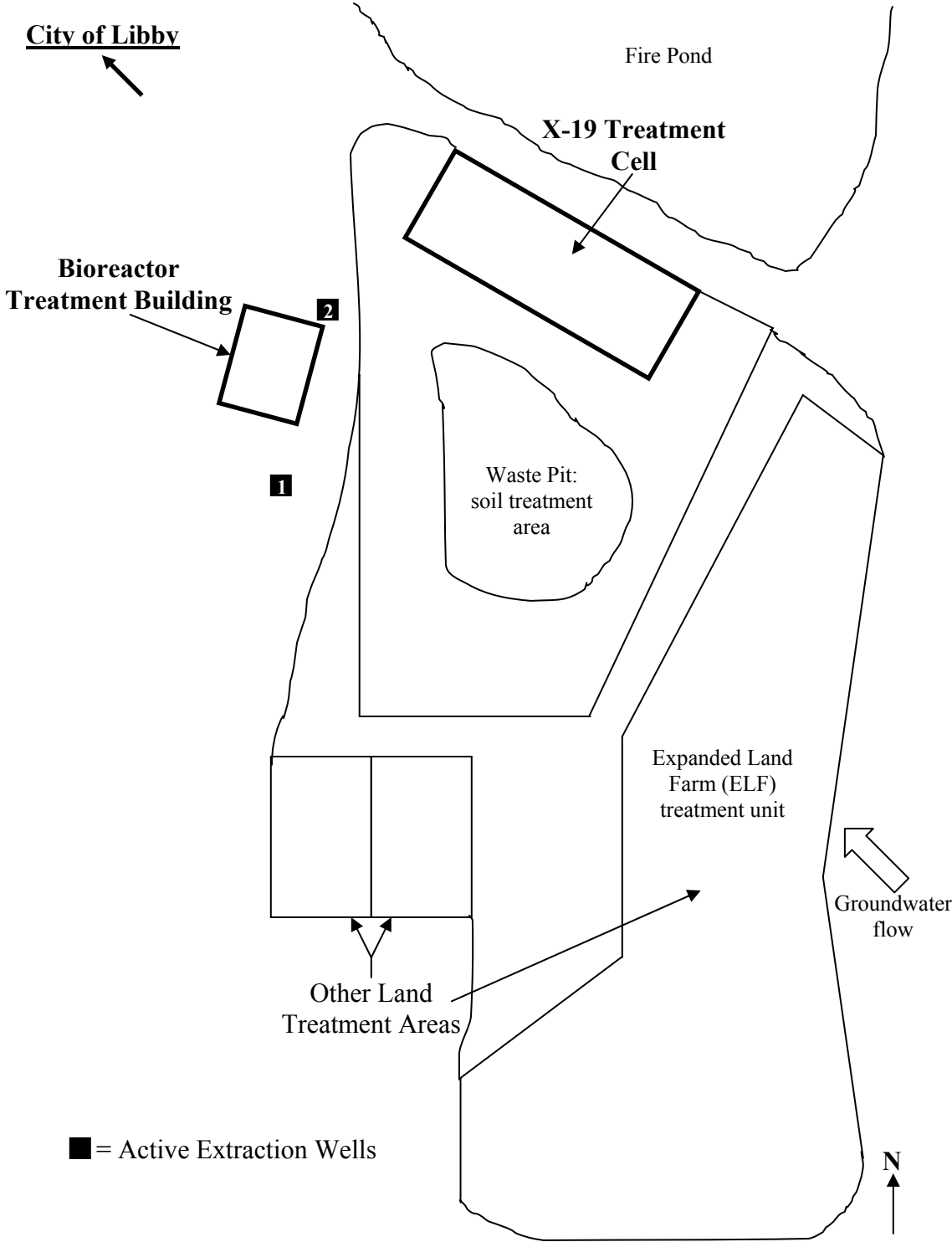
Fig. 6. Site map of land treatment units and bioreactor facility (modified from USEPA, 2005b).
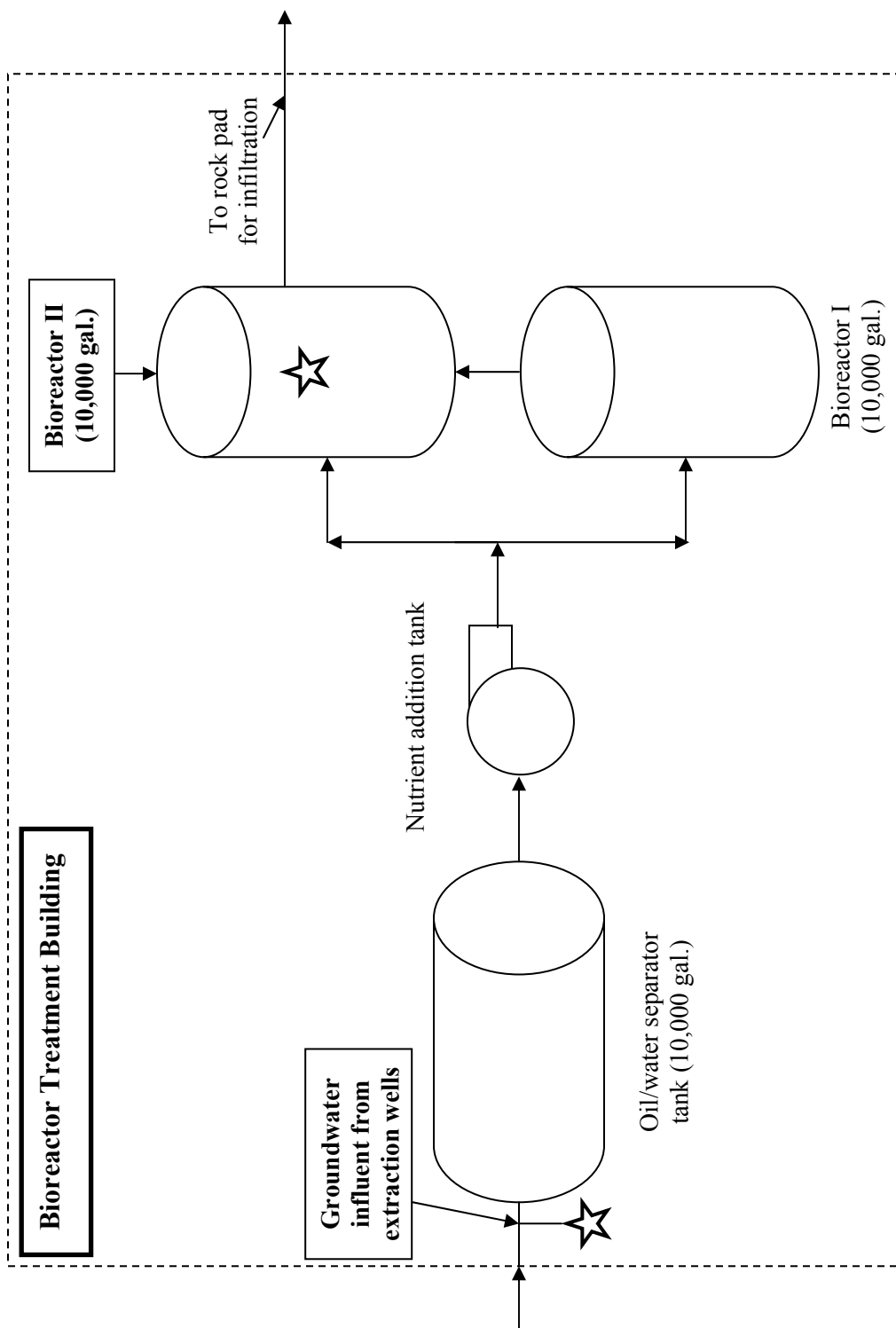
Fig. 7. Bioreactor treatment system schematic. Stars indicate sampling points (groundwater influent, and directly from Bioreactor II).

bioreactor as liquid nutrients for the bio-film bacteria. The bioreactors are maintained at a 22ºC temperature and supplied with dissolved oxygen to stimulate microbial activity (USEPA, 2005c). The final effluent is then discharged to a rock percolation pad. Recovered oil (primarily the dense NAPLs) from the oil/water separator is shipped off-site to be recycled and reused by another wood treating facility (USEPA, 2005c). Since 1989 more than 19,000 gallons of WPW have been recovered from the subsurface (USEPA, 2005c). The bioreactors have been reported to maintain ≥ 80% efficiency rating for the removal of PAHs and PCP from the groundwater since operations began (USEPA, 2005c). The PAHs are primarily degraded in the first bioreactor. However, PCP requires degradation by both the first and second bioreactors. This suggests that the PAHs are more amenable to biological breakdown than PCP under the existing remedial system (USEPA, 2005c). The current target clean-up goals for total PAHs (tPAHs), carcinogenic PAHs (cPAHs), and PCP are 400 ng/L, 40 ng/L and 1000 ng/L respectively (USEPA, 1986; USEPA, 2005c). It should be noted that the target clean-up goals for PCP were changed from 1050000 ng/L in 1988 to 1000 ng/L in 1994 (USEPA, 2005c).

Contaminated soils have also been excavated and are being biologically treated at areas onsite. Soils were first treated in the waste pit area (see Fig. 6). This involved a closed-loop, bacteria rich groundwater injection and extraction system in the waste pit to remove and degrade contaminants adsorbed to the soils. These soils are further degraded biologically after transfer to the land treatment areas (Fig. 6). The land treatment units are maintained by weekly tilling and periodic irrigation to maintain soil moisture. In 1998 all of the soils in the waste pit (except 3000 cubic yards of highly contaminated soil) were placed on the expanded land farm treatment unit (ELF). The waste pit was backfilled with clean soil and rock. The remaining 3000 cubic yards of soil was placed on top of the backfill area and a proprietary amendment called X-19 was added. The soil from this X-19 cell was then covered with a plastic membrane. The contaminant levels in the X-19 cell soils have decreased dramatically since 2000. As a result, the U.S. EPA approved transfer of these soils to the ELF treatment unit (USEPA, 2005c). To date approximately 15,400 cubic yards of contaminated soil have been

treated to target clean-up goals specified in the record of decision (ROD) (USEPA, 1986; USEPA, 2005c). It is expected that the remaining soils in the ELF will take several years to remediate. The current remediation goals for cPAHs, and PCP are 88,000 ng/g and 36,000 ng/g respectively (USEPA, 1986; USEPA, 2005c).

2.2.2 Soil Collection

Surface soil samples (6 inch depth) were collected from one land treatment unit called the X-19 cell over 3 years. Sampling locations were based on a grid pattern laid out over the X-19. Twelve samples were collected at the X-19 once a year using pre-cleaned stainless steel trowels and stored in 16oz. glass I-chem certified sampling jars (VWR Scientific). After collection, the soil was shipped to Texas A&M University following chain of custody protocols and stored at 4ºC prior to extraction.

2.2.3 Soil Extraction

Soil extractions were performed on either a Tecator-Soxtec apparatus (Perstorp Analytical, Silver Spring, MD) or on a Dionex (Dionex Corp., Sunnyvale, CA) Model 200 Accelerated Solvent Extractor (ASE) using a 1:1 ratio of hexane and acetone (USEPA, 1996b). Approximately 30 g of soil from each sample was oven dried at 60°C for 16 hours. Ten g ($\pm$1 g) of the dried soil was placed into pre-weighed thimbles for extraction. Following extraction, samples were transferred to pre-weighed sterile culture tubes with teflon-lined caps and dried under a stream of nitrogen. An aliquot of each sample was saved for the Salmonella/microsome assay, the *E.coli* prophage induction assay, and chemical analysis and stored at 4°C.

2.2.4 Ground Water Collection

Organic compounds in contaminated ground water were collected using 2.5cm x 50cm glass columns (Biorad) packed with resin. The bottom half of each column was packed with 80cm^3 of XAD-2 resin (Supelco) and the top half was packed with 80 cm^3 of XAD-7 resin. The XAD-2 is a nonpolar polystyrene resin that is useful in sensitive

analytical procedures and identification of organic contaminants. The XAD-7 is an acrylic ester resin with an intermediate polarity that is capable of adsorbing phenols and other hydrophobic materials from water. Water was collected at two different locations at the facility. The first set of 3-4 columns was set up parallel to one another at the groundwater inlet prior to the oil-water separator. The second set of 3-4 columns was set up to collect the bioreactor effluent from bioreactor II (Fig. 7). Anywhere between 12 g to 63 gallons of water was allowed to flow through the columns for each sample. This variability was due to changes in flow rate as the resin adsorbed the contaminants as well as field conditions (power outages, cracked columns, loose connectors, etc.). Duplicate composite samples were taken from each location. Columns were shipped back to Texas A&M University on ice following chain of custody protocols.

2.2.5 Ground Water Extraction

Organic compounds absorbed on the XAD column were first eluted with 150mL of distilled water, followed by 150mL of acetone. Following the acetone, 300mL of methylene chloride (CH_2Cl_2) was added to each column and the samples collected in pre-cleaned beakers. The majority of solvent was evaporated and the samples transferred to pre-weighed glass culture tubes. Aliquots were taken for the Salmonella/microsome assay, the *E.coli* prophage induction assay, and chemical analysis. The samples were then dried completely and the extract weight determined. The tubes were sealed with Teflon-lined caps and stored at 4°C until analysis.

2.2.6 Salmonella/Microsome Assay

The Salmonella/microsome assay (Ames et al., 1975; Maron and Ames, 1983) was used to evaluate the mutagenic potential of the complex PAH mixture from the X-19 (2002 & 2003, see Gomez 2002 for X-19 Salmonella data from 2001) and groundwater samples (2001-2003). Extracts were tested in the presence and absence of metabolic activation in the standard plate incorporation assay (Maron and Ames, 1983). The S9 supernatant of homogenized Aroclor 1254 induced Sprague-Dawley rat liver was

obtained from Molecular Toxicology, Inc. (Boone, NC). Positive direct-acting (2-nitrofluorene (2NF): 25 µg/plate), positive indirect-acting (benzo-a-pyrene (BaP): 10 µg/plate) and negative solvent (dimethylsulfoxide (DMSO)) controls were included in each test to ensure consistency in TA98 sensitivity and S9 mix activity.

Soil extracts were resuspended in DMSO and tested on duplicate plates in two independent experiments at five dose levels (1.0, 0.5, 0.25, 0.1, 0.05 mg/plate). The plate incorporation assay includes approximately 1-2 x 10^8 cells, 50 µL of sample extract, and 0.5 mL of sodium phosphate buffer (minus S9) or 0.5 mL of S9 mix (plus S9) poured onto a minimal glucose agar plate. Plates were then incubated at 37°C for 72 hours. Revertant colonies were counted on an Artek Model 880 automated colony counter (Dynatech Laboratories, Inc.). An extract was considered mutagenic if the average response at a minimum of two dose levels was greater than twice the average response for the corresponding negative solvent control (Chu et al., 1981). Weighted activity of the X-19 samples was calculated using the following equation:

(net revertants/mg extract)(mg extract/dry g soil) = net revertants/dry g soil

Water samples were converted to ng/L by taking the total residue weight (mg) divided by the total volume of water collected (reported as liters). This value was then multiplied by the ng analyte / mg residue.

2.2.7 *E. coli* **Prophage Induction Assay**

The *E. coli* prophage induction assay was used to screen for the mutagenic potential of the chlorinated compounds from the X-19 mixtures (DeMarini and Brooks, 1992; DeMarini et al., 1990) (2001-2003) and from the groundwater samples (2001-2003). This bioassay was chosen to obtain information about mutagenicity of the chlorinated compounds which the Salmonella/microsome assay might not be able to detect (Houk & DeMarini 1987). The tester strain *E. coli* lysogen WP2sλ (*lon$_{11}$,sulA$_1$, trpE$_{65}$, uvrA$_{155}$, lamB$^+$*), and indicator strain TH008 (streptomycin^r) was provided by V. S. Houk (USEPA, Research Triangle Park, NC). The surface soil and groundwater sample extracts were each dissolved at 20 mg/mL in acetone. The bioassay includes both

a microsuspension assay and a plaque formation assay. The microsuspension assay exposes mid-log phase WP2sλ (~2 x 10^6 cells) to sample extracts. This was done in a 96-well microtiter plate (Corning) and samples were tested both with and without metabolic activation ($\pm$ S9). Controls including a positive direct-acting chemical (2NF), a positive indirect-acting chemical (2-aminoanthracene (2AA)), a negative solvent (acetone), and media (supplemented VBMM) were tested along with the sample extract to ensure consistency in induced *E. coli* DNA damage and in S9 mix activity. The final concentrations in rows A to H of the microtiter plate were 2000, 1000, 500, 250, 125, 62.5, 31.25 and 15.625 µg/mL respectively. The contents of the plate were mixed using a vibrating shaker, covered with mylar and 2 layers of plastic wrap (Saran Wrap, Dow Chemical Co.), and incubated overnight (approximately 16 hours) at 37°C.

Following incubation, the microtiter plate wells were scored for turbidity, with turbid wells indicating cell growth and clear wells indicating cytotoxicity and/or growth inhibition. At least five wells adjacent to a clear well were sampled to determine the concentration of lambda phage. Fifty µL samples from the selected wells were diluted 1:100 in 5 mL VBMM. Next, 100 µL of the diluted phage and 200 µL of the log-phase indicator cells (TH008) were added to 2.5 mL top agar (0.65 Bacto agar and 10 mM MgSO$_4$). Overnight cultures of TH008 bacteria were grown in an incubating shaker until a concentration of ~2 x 10^8 cells/mL was achieved. After addition of the TH008 cells to the top agar tubes, the tubes were poured onto plates containing tryptone media (10g Bacto tryptone, 5 g NaCl, and 12 g Bacto agar per L of glass-distilled, deionized water and supplemented with streptomycin sulfate (100 g/mL) to select against the lysogen). Plates were inverted and incubated overnight at 37°C. After 24hrs., the plates were quantified by counting the number of plaques on each plate by hand on a Darkfield Quebec colony counter. All samples were tested in duplicate in at least two independent experiments. The induced plaque-forming units (PFUs)/plate was calculated by subtracting the average PFUs produced by the acetone control from the average PFUs produced by the treatment at the same concentration (DeMarini et al., 1990). A sample extract was considered positive if the sample induced a 3-fold or greater increase in

PFUs/plate and also induced a concentration-related increase (DeMarini and Brooks, 1992). If an extract was positive at only one concentration, or if a 3-fold or greater induction occurred but failed to show a concentration-related increase, the result was scored as a weak positive (w[+]) (DeMarini et al., 1990). Samples that did not induce a response three times greater than the acetone control at any dose were considered negative. Samples that did not induce a consistent response in two independent experiments were scored as a $\pm$.

2.2.8 Chemical Analysis

Organic chemical analysis of PAHs and PCP was performed using a modified 8270C United States Environmental Protection Agency (USEPA) standard method (USEPA, 1996c). Analysis was conducted on a Hewlett-Packard 5890 Series II gas chromatograph with a 5972 mass selective detector in selected ion monitoring mode. A 60m x 0.25mm ID x 0.25mm film thickness column (Agilent Technologies, Palo Alto, CA) was used. The injection port was maintained at 300ºC and the transfer line at 280ºC. The temperature program was as follows: 60ºC for 6 minutes, increased at 12ºC/minute to 180ºC and then increased at 6ºC/minute to 310º and held for 11 minutes for a total run time of 47 minutes. Data were reported as ng/mg dry extract for both the water and surface soil samples. The surface soil concentrations were converted to ng/dry g soil using the following equation:

(ng analyte/mg dry extract) (mg dry extract/dry g soil) = ng analyte/dry g soil

Table 1 shows a list of the target PAH analytes including the seven carcinogenic PAHs described in the record of decision for the contaminated site (USEPA, 1986).

2.3 Results

2.3.1 X-19 Surface Soils

Table 2 is a summary of the biological response and chemical analysis data from each of the X-19 soil samples collected over three years (N=12 for all data except for 2002 where chemical analysis was performed on an N=11). In general, the samples tested positive in the Salmonella/microsome assay (+S9) all three years and exhibited a clear mutagenic response with increased concentration (Fig. 8). Only sample 1 in 2001 was weakly positive minus S9 (data not shown). In 2003, the samples on average had a positive mutagenic response (+S9). However, this response was lower than the average response from 2002 (Fig. 8). The Salmonella/microsome assay is effective at detecting the mutagenicity of PAHs. When comparing the carcinogenic PAHs (cPAHs) with the Salmonella/microsome assay, the response was variable. Sample extracts showed a lower average concentration of cPAHs from 2001 to 2002 (101000 ng/dry g soil to 52100 ng/dry g soil respectively) and about a 20-fold increase in average cPAH concentration from 2002 to 2003 (52100 ng/dry g soil to 1070000 ng/dry g soil respectively) (Fig. 9). Based on these values, a stronger mutagenic response would be expected in the Salmonella/microsome assay between 2002 and 2003. Instead, the opposite was seen with fewer average revertants/plate at each dose level in 2002 compared to 2003 (Fig. 8). This is likely due to cytotoxicity from both the elevated PAH concentrations and PCP concentrations in the samples collected in 2003. Cytotoxicity is evident in the slightly lower average revertants/plate shown at the 2.5 mg/plate concentration compared to the 1.0 mg/plate concentration in 2003 (Fig. 8). When looking at the weighted activity of the X-19 soil samples in the Salmonella microsome assay, the net revertants/dry g soil did show a decline in mutagenicity over time (Fig. 10).

Table 1. List of total target PAH analytes screened in the X-19 surface soil and groundwater chemical analysis.

Naphthalene	Acenaphthylene	C1-Phenanthrene/Anthracenes	C1-Fluoranthenes/Pyrenes	C2-Chrysenes
C1-Naphthalenes	Acenaphthene	C2-Phenanthrene/Anthracenes	C2-Fluoranthenes/Pyrenes	C3-Chrysenes
C2-Naphthalenes	Dibenzofuran	C3-Phenanthrene/Anthracenes	C3-Fluoranthenes/Pyrenes	C4-Chrysenes
C3-Naphthalenes	Fluorene	C4-Phenanthrene/Anthracenes	Naphthobenzothiophene	**Benzo(b)fluoranthene**
C4-Naphthalenes	C1-Fluorenes	Dibenzothiophene	C1-Naphthobenzothiophenes	**Benzo(k)fluoranthene**
Benzothiophene	C2-Fluorenes	C1-Dibenzothiophenes	C2-Naphthobenzothiophenes	Benzo(e)pyrene
C1-Benzothiophenes	C3-Fluorenes	C2-Dibenzothiophenes	C3-Naphthobenzothiophenes	**Benzo(a)pyrene**
C2-Benzothiophenes	Carbazole	C3-Dibenzothiophenes	**Benz(a)anthracene**	Perylene
C3-Benzothiophenes	Anthracene	Fluoranthene	**Chrysene**	**Indeno(1,2,3-c,d)pyrene**
Biphenyl	Phenanthrene	Pyrene	C1-Chrysenes	**Dibenzo(a,h)anthracene**
				Benzo(g,h,i)perylene

Bold/underline denotes the carcinogenic PAHs per the U.S. EPAs IRIS database (USEPA, 2005i)

The *E. coli* prophage induction assay is effective at screening for the mutagenic potential of chlorinated compounds. The results from this assay using the X-19 surface soil extracts are also variable from year to year. Fig. 11 shows the three concentrations consistently tested in the *E. coli* prophage induction assay over the three year period for all sample extracts tested with metabolic activation (+S9). In 2001, the samples on average did not show a mutagenic response (Fig. 11). However, the 2001 samples did show a consistent elevated fold increase with increasing sample concentration (Fig. 11). In 2002, half of the samples had a weak positive response (Table 2) and on average were weakly positive (Fig. 11). Despite half the samples in 2003 also having a weak mutagenic response (Table 2), the samples on average were consistently mutagenic at all three concentration levels reported (Fig. 11). These findings are in keeping with the chemical concentrations of PCP in the samples. Average PCP concentrations increased from 2001 to 2003 (Fig. 9) and mutagenic response in the *E. coli* prophage induction assay also increased from 2001 to 2003 (Fig. 11). However, the PCP concentrations roughly doubled from year to year and the mutagenic response did not reflect this trend. This suggests that mixture interactions are affecting the mutagenic potential of these samples. Without metabolic activation (-S9), sample 6 in 2001, sample 23 in 2002, and samples 26-29 in 2003 were weakly positive in the *E. coli* prophage induction assay; all other samples were negative or produced an inconsistent ($\pm$) response (data not shown). It would seem that from this response that the samples contain some direct-acting mutagens. The majority of samples in 2001 exceeded the target remediation levels (clean-up goals) for cPAHs and PCP; only one sample in 2002 exceeded the remediation goal for cPAHs whereas all samples were above the goal for PCP; all samples in 2003 were above remediation goals for cPAHs and PCP (Table 2).

Table 2. Summary of X-19 data from 2001-2003 including microbial bioassay results and chemical analysis.

Sample Number	Year Collected	Biological Response				Chemical Analysis			
		[a]Salmonella (+S9)		[b]E. coli (+S9)		tPAHs	cPAHs	BaP	PCP
		Mean revertants/plate ± SD*, 0.5mg/plate conc.	Total Response	PFU/plate, 250 µg/mL conc. (2 experiments)	Total Response	ng/dry g soil	ng/dry g soil	ng/dry g soil	ng/dry g soil
1	2001	109 ± 5	+	68/32	±	366000	71000	15200	181000
2	"	88 ± 4	+	59/54	w+	772000	143000	21100	325000
3	"	92 ± 11	+	66/75	+	595000	112000	17500	238000
4	"	124 ± 7	+	55/128	w+	382000	91300	17400	236000
5	"	98 ± 4	+	6/80	±	449000	98800	20200	260000
6	"	103 ± 4	+	65/188	+	737000	10800	17200	276000
7	"	91 ± 6	+	29/36	-	NA			
8	"	115 ± 8	+	155/58	+	774000	138000	21500	330000
9	"	107 ± 7	+	78/139	+	540000	80700	15000	186000
10	"	102 ± 4	+	36/73	±	549000	89800	17700	210000
11	"	114 ± 7	+	50/60	±	619000	94500	16800	182000
12	"	95 ± 8	+	22/55	w+	525000	87200	17400	169000
AVERAGES		103 ± 6	+	63/82	±	573000	101000	17900	236000

* Gomez 2002 was consulted for the Salmonella biological response for 2001 X-19 samples, **data were reported as mean revertants ± SD and not ± SEM.**

Table 2 continued.

Sample Number	Year Collected	Biological Response				Chemical Analysis			
		ᵃSalmonella (+S9)		ᵇE. coli (+S9)		tPAHs	cPAHs	BaP	PCP
		Mean revertants/plate ±SEM, 0.5mg/plate conc.	Total Response	PFU/plate, 250 µg/mL conc. (2 experiments)	Total Response	ng/dry g soil	ng/dry g soil	ng/dry g soil	ng/dry g soil
13	2002	78±5	+	136/122	w⁺	165000	39600	10400	568000
14	"	83±9	+	134/115	+	281000	64700	15500	800000
15	"	102±11	+	71/79	w⁺	205000	47800	11700	654000
16	"	93±7	+	143/88	w⁺	209000	52700	13700	604000
17	"	92±4	+	128/80	w⁺	162000	42700	10700	577000
18	"	93±7	+	116/65	w⁺	295000	71800	16700	682000
19	"	106±7	+	90/95	+	218000	60100	14900	602000
20	"	85±9	±	172/70	w⁺	312000	91500	23800	747000
21	"	91±8	+	84/75	+	24000	7410	1850	41600
22	"	87±4	+	123/62	+	365000	77400	20200	800000
23	"	99±5	+	223/136	+	172000	36700	9310	474000
24	"	87±5	+	83/100	+	118000	33200	8820	359000
AVERAGES		164±7	+	125/91	±	210000	52100	13100	576000

Table 2 continued.

| | | **Biological Response** | | | | **Chemical Analysis** | | | |
| | | **^aSalmonella (+S9)** | | **^bE. coli (+S9)** | | | | | |

Sample Number	Year Collected	Mean revertants/plate ± SEM, 0.5mg/plate conc.	Total Response	PFU/plate, 250 µg/mL conc. (2 experiments)	Total Response	tPAHs ng/dry g soil	cPAHs ng/dry g soil	BaP ng/dry g soil	PCP ng/dry g soil
25	2003	73 ± 9	-	157/76	w⁺	1450000	**282000**	65900	**462000**
26	"	65 ± 8	-	159/30	+	1200000	**747000**	176000	**1390000**
27	"	69 ± 11	±	116/151	w⁺	1610000	**937000**	203000	**1530000**
28	"	80 ± 5	+	134/111	w⁺	2170000	**963000**	200000	**1750000**
29	"	82 ± 6	±	177/157	w⁺	3310000	**1500000**	317000	**1540000**
30	"	71 ± 6	-	146/154	w⁺	2720000	**1340000**	242000	**2100000**
31	"	82 ± 9	+	56/72	+	1770000	**1060000**	237000	**2070000**
32	"	81 ± 4	+	31/52	w⁺	1930000	**964000**	228000	**1440000**
33	"	93 ± 7	+	83/71	+	3730000	**1250000**	239000	**969000**
34	"	103 ± 5	+	54/51	+	1720000	**703000**	148000	**1340000**
35	"	110 ± 10	+	44/93	+	2690000	**1380000**	301000	**2000000**
36	"	95 ± 3	+	46/34	+	842000	**1720000**	410000	**2080000**
AVERAGES		*84 ± 7*	*±*	*100/88*	*±*	*2090000*	*1070000*	*230000*	*1560000*

^{a} + = # revertants/plate $\geq$ 2x's the solvent control for 2 consecutive doses in 2 independent experiments; w$^+$ = # revertants/plate $\geq$ 2x's the solvent control for 1 dose or non-consecutive doses in 2 independent experiments; - = # revertants/plate < 2x's solvent control at all doses in 2 independent experiments; $\pm$ = sample failed to induce a consistent positive or negative response in 2 independent experiments.

^{b} + = PFUs/plate $\geq$ 3x's the solvent control for 2 consecutive doses in 2 independent experiments; w$^+$ = PFUs/plate $\geq$ 3x's the solvent control for 1 dose or non-consecutive doses in 2 independent experiments; - = PFUs/plate < 3x's the solvent control for all doses in 2 independent experiments; $\pm$ = sample failed to induce a consistent positive or negative response in 2 independent experiments.

Bold denotes soil concentrations that are above the record of decision concentrations for cPAHs (88000 ng/g) and PCP (36000 ng/g).
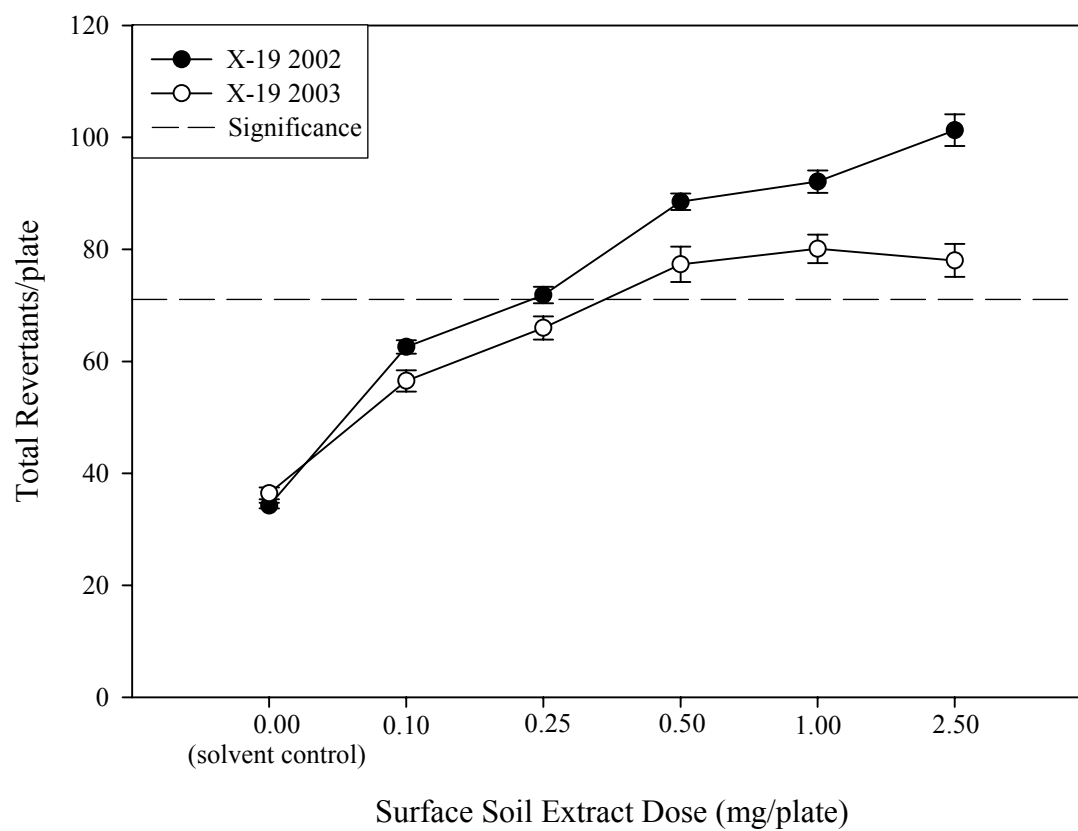
Fig. 8. Microbial mutagenicity as measured in *S. typhimurium* strain TA98 with metabolic activation, of extracts of X-19 surface soils (N=12/year) over two years. The averaged data show a positive mutagenic response ($\geq$ 71 total revertants/plate) for these samples (dashed line). Data are presented as mean total revertants/plate $\pm$ SEM.
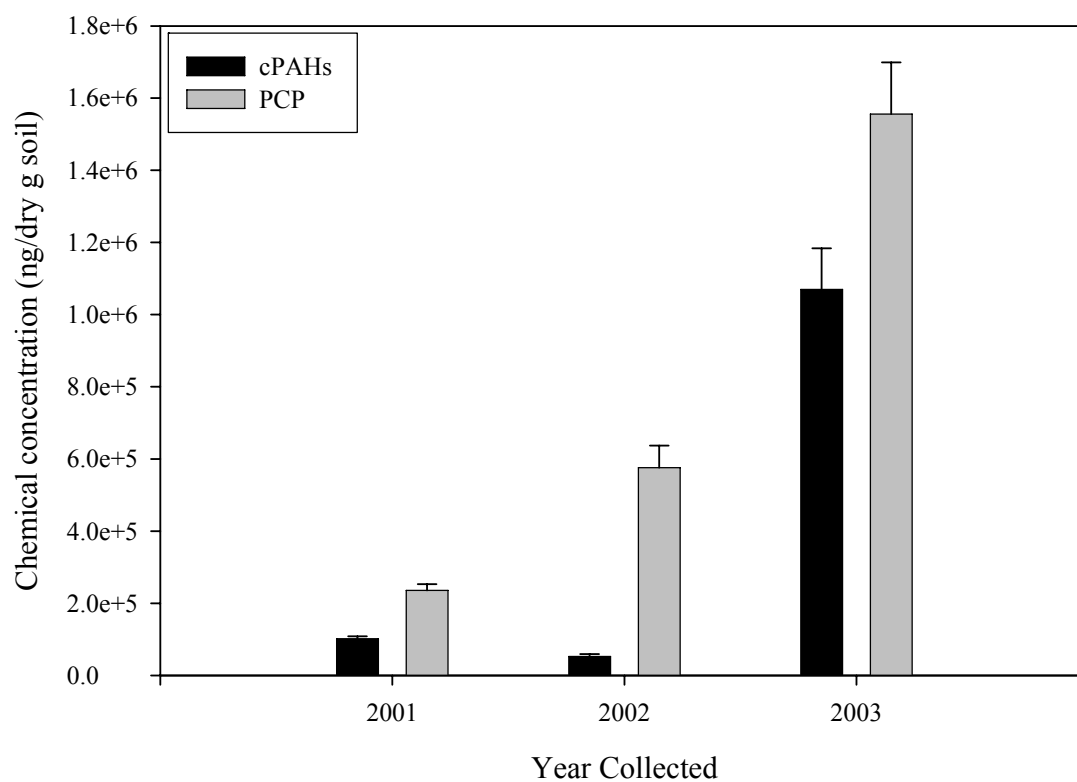
Fig. 9. Mean concentration of carcinogenic PAHs (cPAHs) and PCP in X-19 surface soil samples collected over three years (2001 N=11, 2002 and 2003 N=12). Data are presented as mean $\pm$ SEM

Fig. 10. Weighted activity of X-19 surface soil samples collected from 2001-2003. Net revertants were calculated by subtracting the total revertants for the solvent control from the total revertants for each sample. Weighted activity was calculated by taking the average net revertants/1 mg residue x the average mg residue/g soil for each year (N=12 for each year). Data are presented as mean $\pm$ SEM.

Fig. 11. Microbial mutagenicity as measured in the *E. coli* prophage induction assay with metabolic activation, of X-19 surface soil extracts over three years. Fold increase = # plaque forming units (PFUs) per sample / # PFUs for the solvent control. A fold increase of ≥ 3 (dashed line) for two or more doses was considered a positive mutagenic response. Data are presented as the mean fold increase $\pm$ SEM.

2.3.2 Water Samples

Water samples were collected from two locations in the bioreactor treatment facility onsite. Table 3 shows a summary of the biological response and chemical analysis for the duplicate samples collected from the groundwater influent (GW) and the samples collected from the bioreactor effluent (BE) over three years. The groundwater samples showed an average decline in mutagenic response (+S9) as tested in the

Table 3. Summary of contaminated water data from 2001-2003 including microbial bioassay results and chemical analysis (GW= groundwater, BE = bioreactor effluent).

Sample Location & Number		Year Collected	Biological Response				Chemical Analysis			
			[a]Salmonella (+S9)		[b]E. coli (+S9)					
			Mean revertants/plate ± SEM, 1 mg/plate conc.	Total Response	PFU/plate, 125 µg/mL conc. (2 experiments)	Total Response	tPAHs ng/L	cPAHs ng/L	BaP ng/L	PCP ng/L
GW	1	2001	49 ± 3	±	248/723	+	16000000	321000	47000	5450000
	2		71 ± 8	±	107/140	+	12100000	208000	21000	1810000
	3	2002	80 ± 9	w$^+$	535/721	+	1170000	21100	1200	697000
	4		59 ± 13	±	427/805	+	1150000	24300	2000	647000
	5	2003	44 ± 9	-	398/256	+	1430000	16200	1900	976000
	6		55 ± 3	-	1076/534	+	1260000	20200	2300	770000
BE	1	2001	76 ± 13	±	104/59	±	170000	6210	800	59600
	2		53 ± 5	±	99/25	±	4390000	92400	13000	2360000
	3	2002	64 ± 4	-	38/74	±	52000	390	80	42300
	4		78 ± 4	-	56/56	±	64000	360	60	5400
	5	2003	54 ± 5	-	175/123	+	66000	130	20	63000
	6		52 ± 7	-	876/898	+	2210000	41400	4600	1450000

[a] + = # revertants/plate ≥ 2x's the solvent control for 2 consecutive doses in 2 independent experiments; w$^+$ = # revertants/plate ≥ 2x's the solvent control for 1 dose or non-consecutive doses in 2 independent experiments; - = # revertants/plate < 2x's solvent control at all doses in 2 independent experiments; ± = sample failed to induce a consistent positive or negative response in 2 independent experiments.

[b] + = PFUs/plate ≥ 3x's the solvent control for 2 consecutive doses in 2 independent experiments; w$^+$ = PFUs/plate ≥ 3x's the solvent control for 1 dose or non-consecutive doses in 2 independent experiments; - = PFUs/plate < 3x's the solvent control for all doses in 2 independent experiments; ± = sample failed to induce a consistent positive or negative response in 2 independent experiments.

**Bold** denotes water concentrations that are above the record of decision concentrations for tPAHs (400 ng/L), cPAHs (40 ng/L) and PCP (1000 ng/L).

Fig. 12. Microbial mutagenicity as measured in *S. typhimurium* strain TA98 with metabolic activation, of extracts of groundwater collected from a contaminated aquifer over a three year period. The averaged data show only a weak positive mutagenic response ($\geq$ 76 total revertants/plate) for these samples. Data are presented as mean total revertants/plate $\pm$ SEM.

Salmonella/microsome assay from 2001 to 2003 (Fig. 12). Revertants/plate increased with increasing sample concentration from 0.1 to 0.5 mg/plate (Fig. 12). At concentrations 1.0 and 2.5 mg/plate, cytotoxicity was observed. The decrease in average mutagenicity of the samples over three years was consistent with a decline in cPAHs in these samples from 2001 to 2003 (Fig. 13). Only sample GW-3 in 2002 was weakly positive without metabolic activation. All other groundwater samples were negative without metabolic activation (data not shown). The weighted activity of the groundwater samples also decreased from 2001 to 2003 (Fig. 14)

Results from the *E. coli* prophage induction assay showed a consistent positive mutagenic response for the groundwater samples all three years (+S9) (Table 3 and Fig. 15). Average PCP concentrations were highly variable from year to year as was the response in the *E. coli* prophage induction assay (Fig. 13 and Fig. 15). Samples GW-1 was weakly mutagenic in this assay without metabolic activation; all other samples showed either a negative or inconsistent ($\pm$) response without S9 added (data not shown).
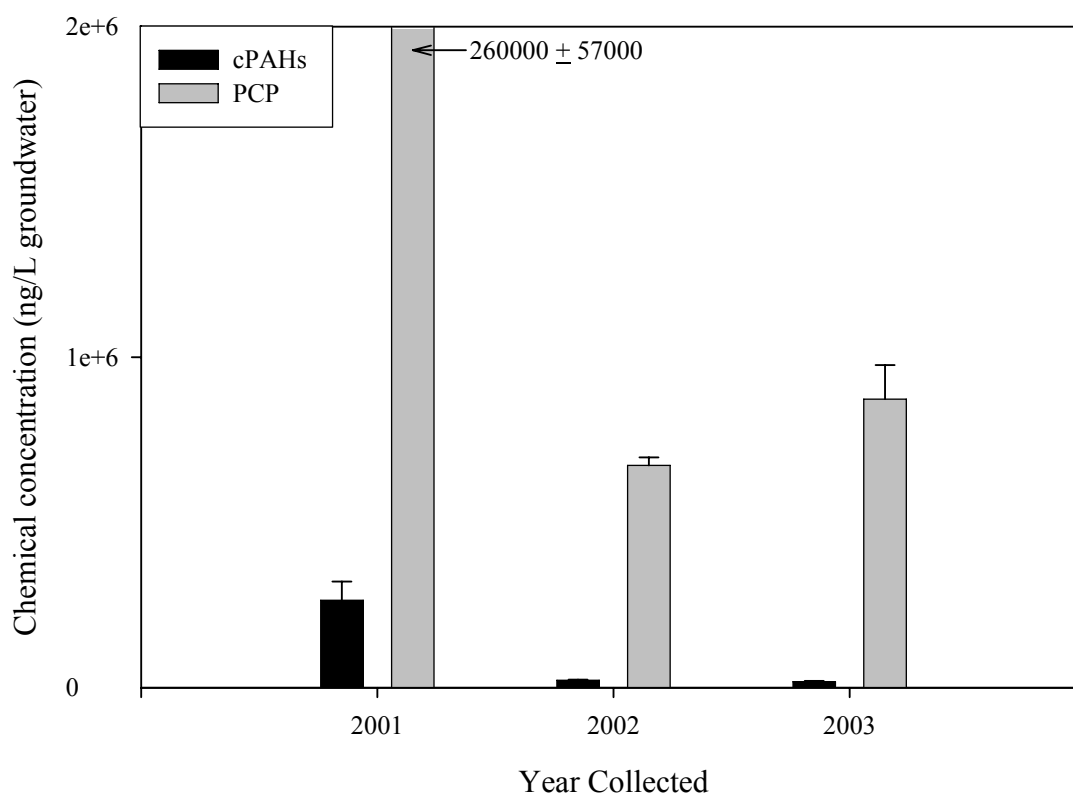
Fig. 13. Mean concentration of carcinogenic PAHs (cPAHs) and PCP in groundwater samples collected from a contaminated aquifer over three years. Data are presented as mean $\pm$ SEM.
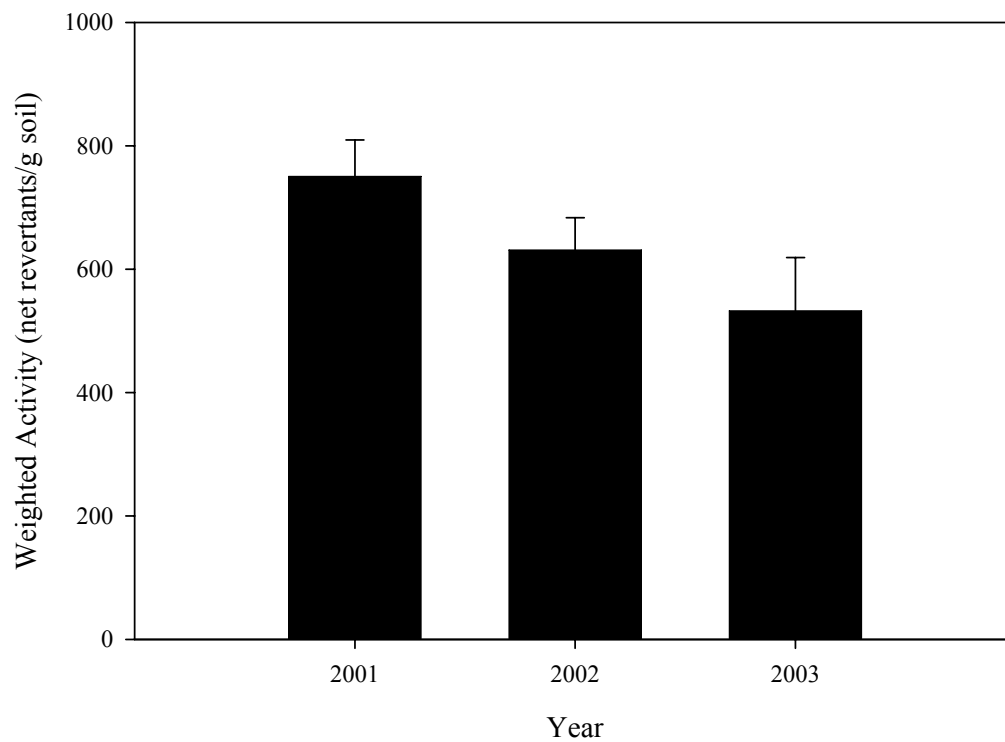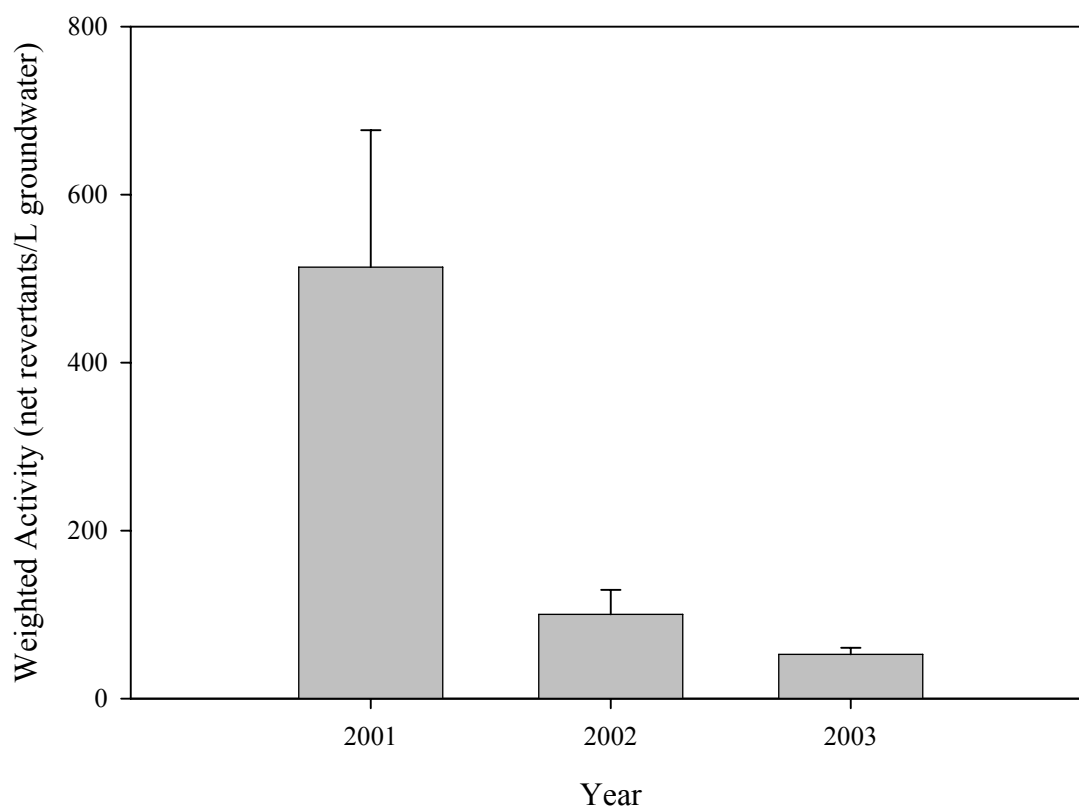
Fig. 14. Weighted activity of groundwater samples collected from 2001-2003. Net revertants were calculated by subtracting the total revertants for the solvent control from the total revertants for each sample. Weighted activity was calculated by taking the average net revertants/1 mg residue x the average mg residue/L water for each year (N=2 for each year). Data are presented as mean $\pm$ SEM.
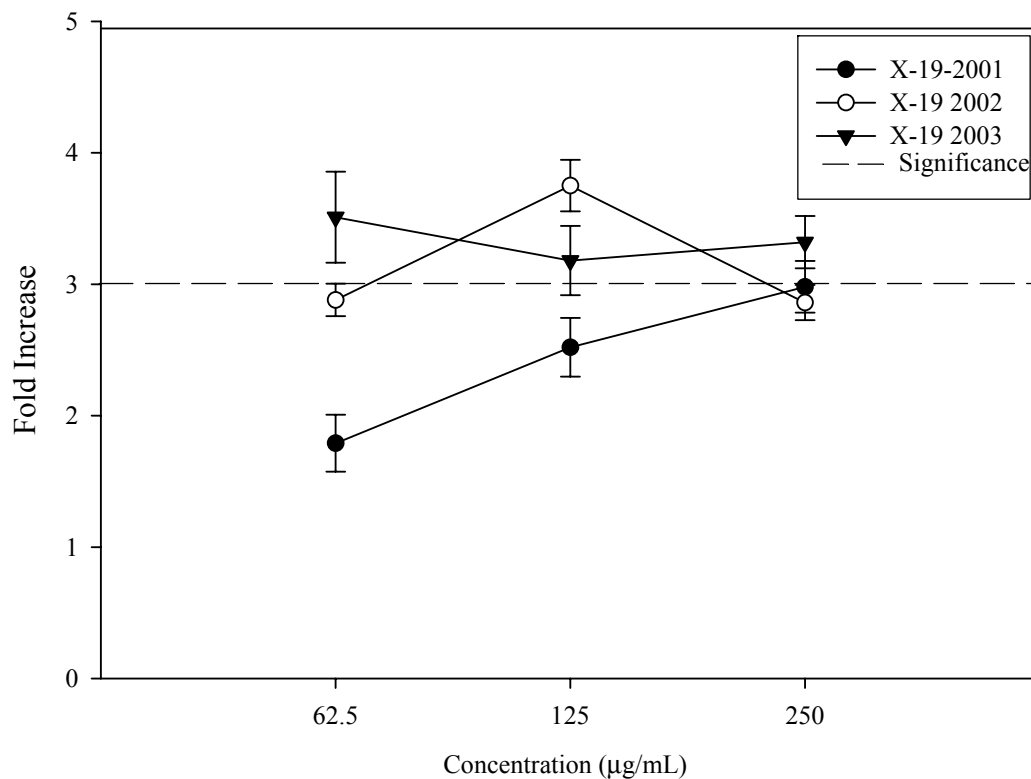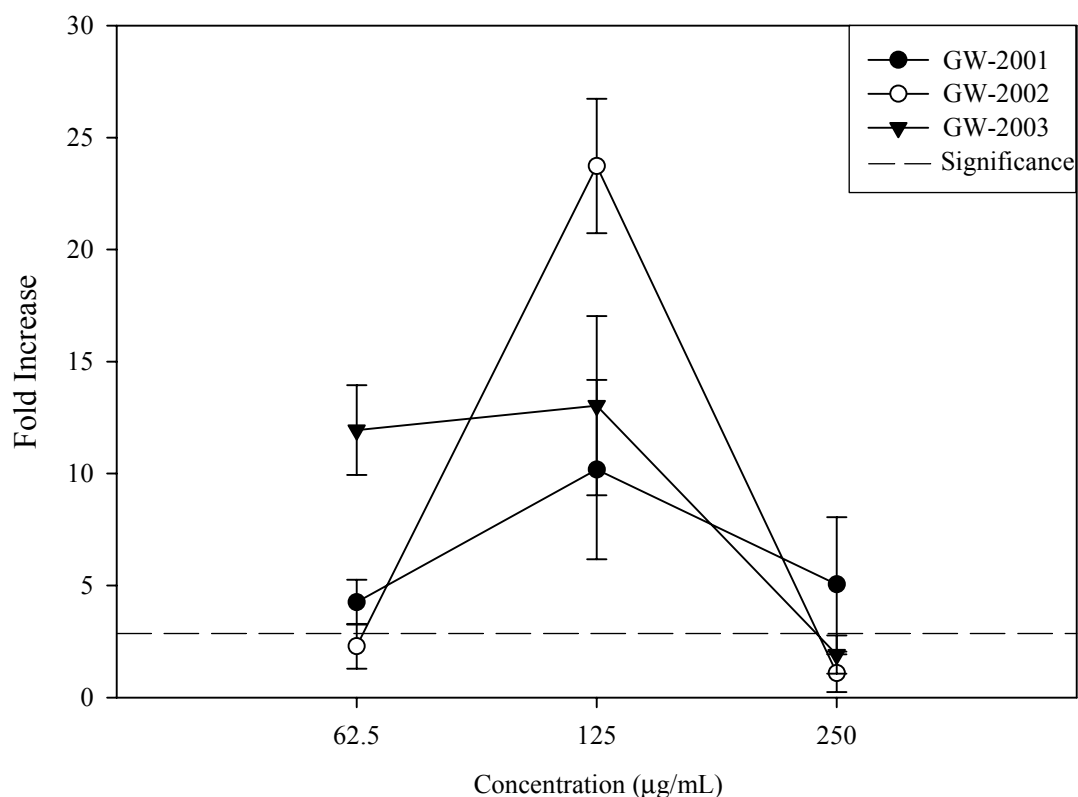
Fig. 15. Microbial mutagenicity as measured in the *E. coli* prophage induction assay with metabolic activation, of extracts of groundwater collected from a contaminated aquifer over a three year period. The fold increase = # plaque forming units (PFUs) per sample / # PFUs for the solvent control. A fold increase of ≥ 3 (dashed line) for two or more doses is considered a positive mutagenic response. Data are presented as the mean fold increase ± SEM.

With the exception of BE 2 and BE 6, the water samples collected from the bioreactor effluent were lower in chemical concentration compared to the groundwater samples (Table 3). However, all water samples exceeded the remediation goals for tPAHs, cPAHs and PCP (Table 3). Chemical concentration varied from year to year with 2001 showing the most elevated levels of all chemicals including cPAHs and PCP (Table 3, Fig. 16). The bioreactor effluent samples were not mutagenic in the Salmonella/microsome assay with or without metabolic activation (Fig. 17 shows the

+S9 data). Weighted activity of the bioreactor effluent samples showed a decline in mutagenicity from 2001 to 2003 (Fig. 18).

In the *E. coli* prophage induction assay these samples did show a mutagenic response plus S9. In 2001 and 2002 the samples were weakly positive (showing a 3-fold increase at one dose) (Fig. 19). In 2001 samples were on average slightly more mutagenic compared to samples from 2002. In 2003 samples induced an average fold increase as high as 12 and no lower than 7 (Fig. 19). The mutagenic response in these water samples was consistent with concentrations of PCP which were highest in 2001 (possibly cytotoxic) compared to 2002 and elevated again in 2003 (Fig. 16). Only samples BE 1 and BE 2 from 2001 showed a weak mutagenic response without metabolic activation in the *E. coli* prophage induction assay. Samples from subsequent years either induced a variable ($\pm$) or negative response in *E. coli* without S9.

2.4 Discussion

The site investigated for this research was a former wood preserving facility that historically used both creosote and PCP to treat wood products. The contaminants, collectively called wood preserving waste, found onsite primarily include PAHs and PCP. These chemicals are known to be hazardous to human and ecological health due to their persistent, bioaccumulative and toxic effects (USEPA, 2005j). The goal of this study was to investigate if chemical analysis of the contaminants of concern was predictive of genotoxic potential. Surface soil samples were collected onsite from a land treatment unit called the X-19 cell over three years. Groundwater samples were also collected from both the extracted groundwater influent and from the bioreactor effluent located within the bioreactor facility onsite over three years. Samples were extracted for target PAHs and PCP and were also tested for genotoxicity in two *in vitro* microbial bioassays: the Salmonella/microsome assay and the *E. coli* prophage induction assay. Results from the microbial bioassays were then compared to chemical concentrations.
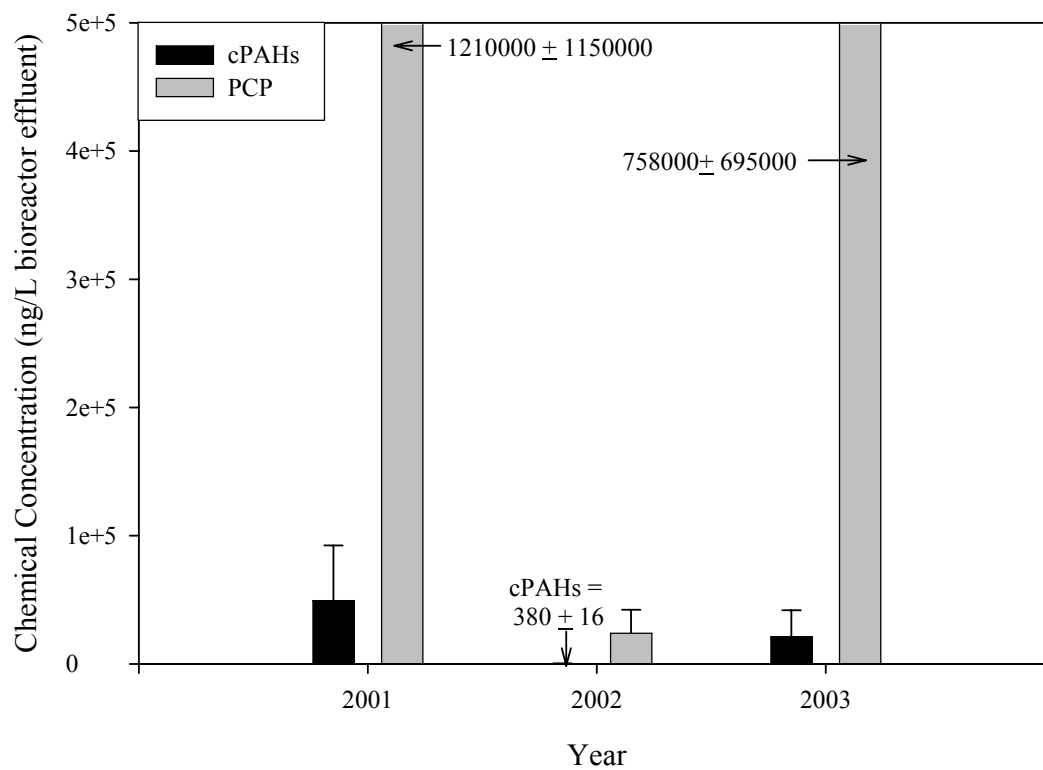
Fig. 16. Mean concentration of carcinogenic PAHs (cPAHs) and PCP in bioreactor effluent water samples collected over three years. Data are presented as mean $\pm$ SEM.

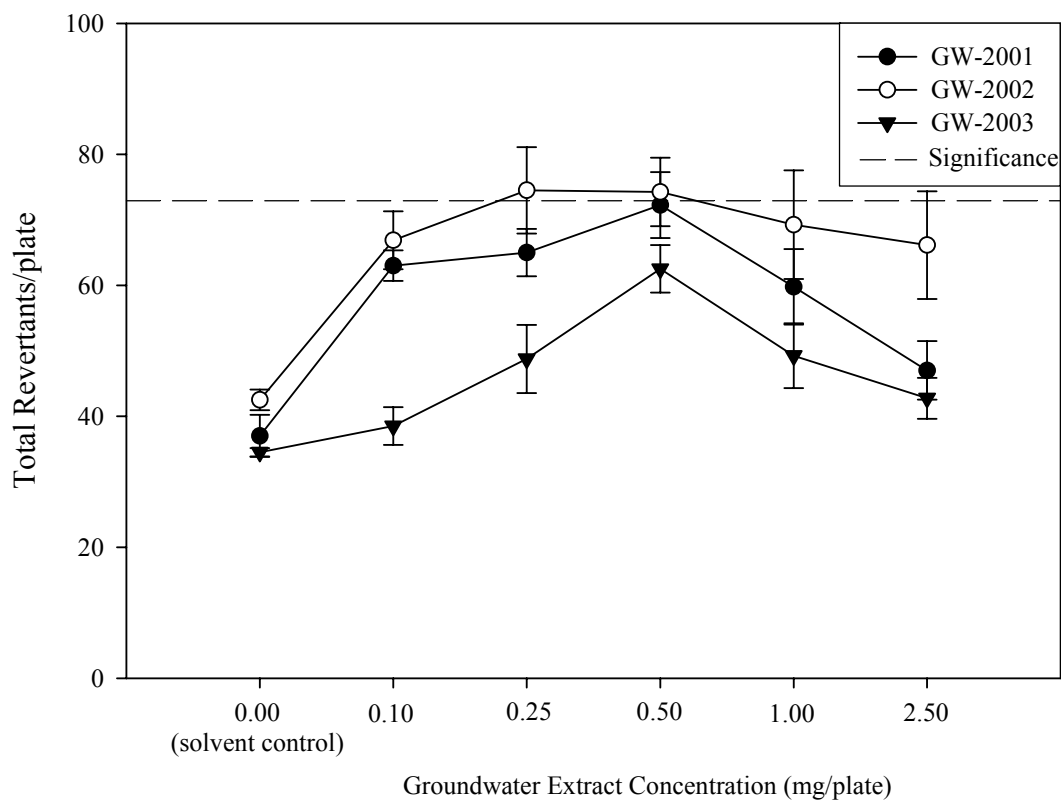Bioreactor Effluent Concentration (mg/plate)
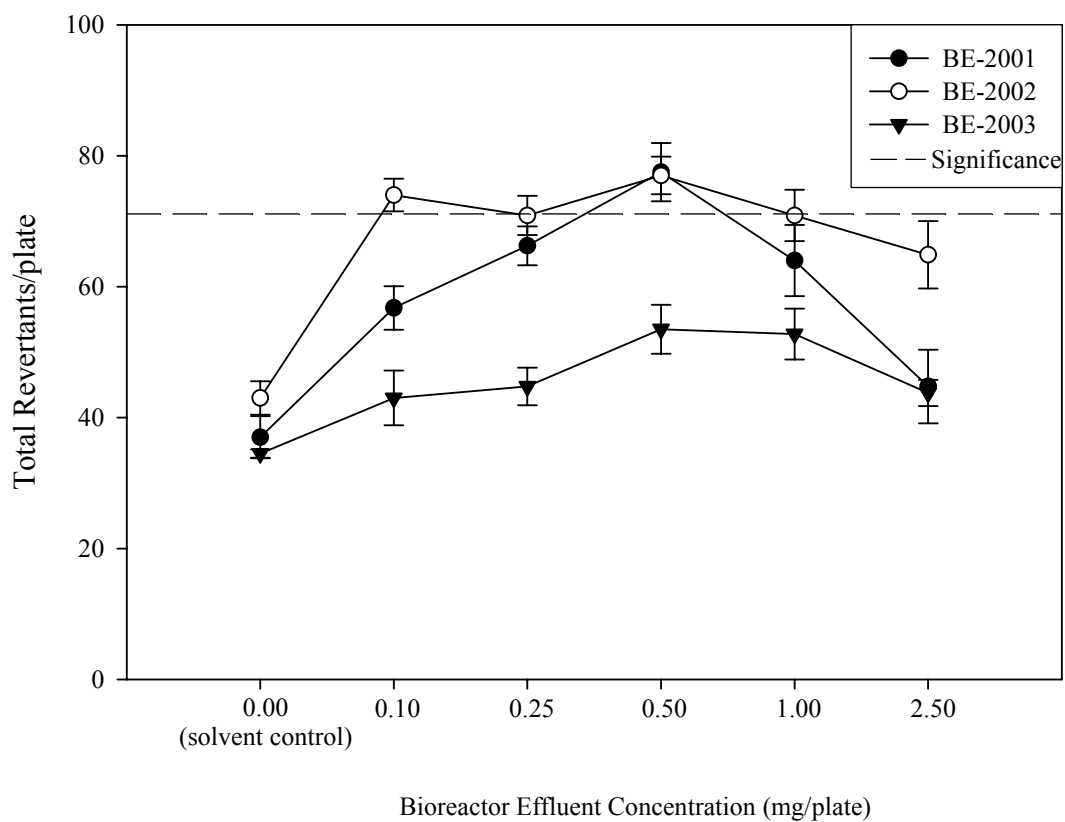
Fig. 17. Microbial mutagenicity as measured in *S. typhimurium* strain TA98 with metabolic activation, of extracts of bioreactor effluent collected from the bioreactor facility over a three year period. The averaged data show a negative mutagenic response ($\geq$ 76 total revertants/plate) for these samples. Data are presented as mean total revertants/plate $\pm$ SEM.
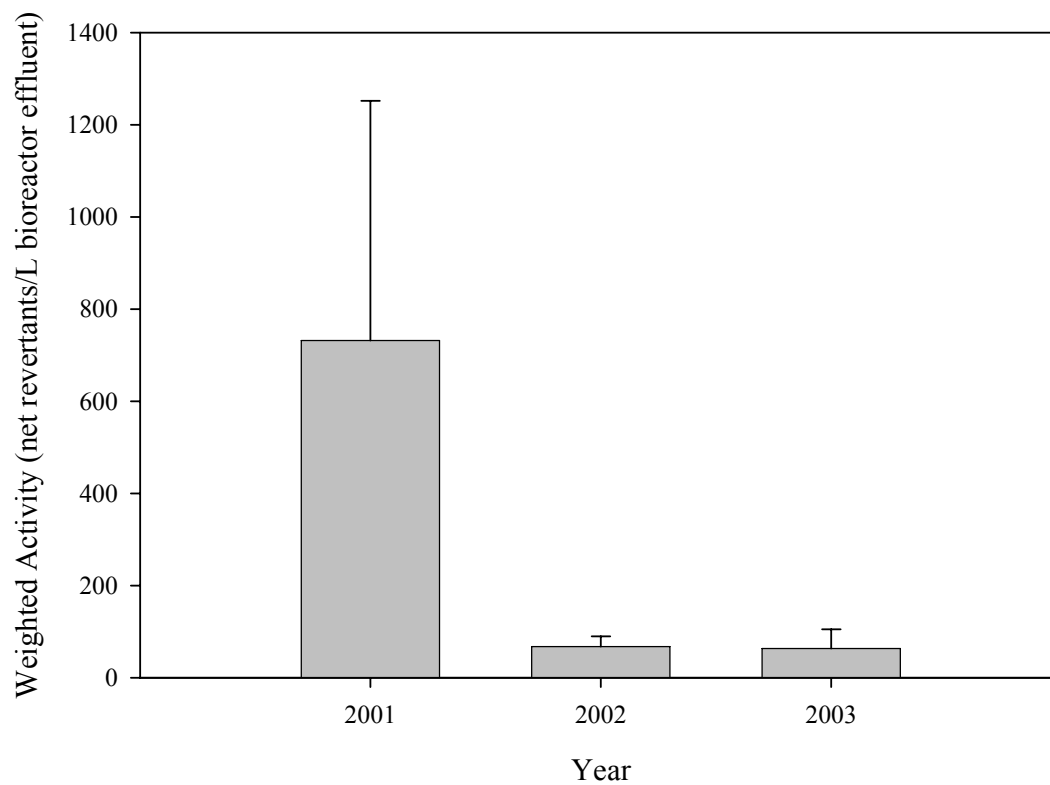
Fig. 18. Weighted activity of bioreactor effluent samples collected from 2001-2003. Net revertants were calculated by subtracting the total revertants for the solvent control from the total revertants for each sample. Weighted activity was calculated by taking the average net revertants/1 mg residue x the average mg residue/L water for each year (N=2 for each year). Data are presented as mean $\pm$ SEM.
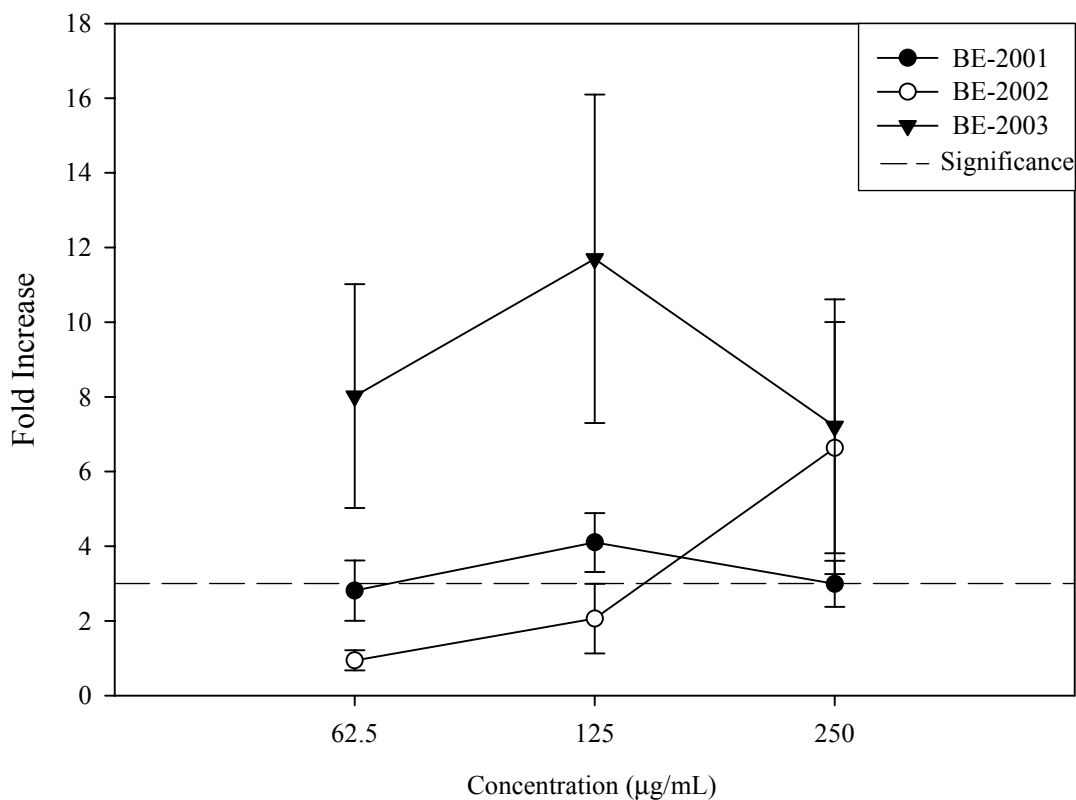
Fig. 19. Microbial mutagenicity as measured in the *E. coli* prophage induction assay with metabolic activation, of extracts of bioreactor effluent collected from the bioreactor facility over three years. old increase = # plaque forming units (PFUs) per sample / # PFUs for the solvent control. A fold increase of ≥ 3 (dashed line) for two or more doses is considered a positive mutagenic response. Data are presented as the mean fold increase $\pm$ SEM.

Overall, due to the heterogeneity of the soil and water samples, it is difficult to make comparisons among the data sets shown here. The sampling times represent only four days out of the year and do not reflect seasonal variability or alterations in the treatment processes such as cycling of waste in the bioreactor facility. The concentrations of carcinogenic PAHs did not correspond well with Salmonella mutagenicity in the X-19 surface soil samples over three years. When tested in the *E. coli* prophage induction assay the soil samples did show a mutagenic relationship with corresponding levels of

PCP, however the fold increase was not consistent with PCP concentrations in the samples. Average chemical concentrations actually increased from 2001 to 2003.

The groundwater samples did show a mutagenic response in Salmonella that corresponded with cPAH concentrations. The mutagenic response in the *E. coli* prophage induction assay was highly variable for the groundwater and did not correspond with PCP concentrations at the doses reported. The bioreactor effluent water samples showed lower chemical concentrations than the groundwater influent. This was to be expected and was indicative of the effective microbial remedial technique being used onsite. There was no mutagenic response from the bioreactor samples in the Salmonella/microsome assay. The samples did induce mutagenic responses that corresponded with PCP concentrations. It is known that the first bioreactor is capable of degrading 70-80% of the PAHs in the contaminated water, but only about 10% of the PCP. The second bioreactor (from which the reported samples were taken) is capable of degrading the remaining PAHs and most (70-80%) of the PCP. However, because PCP is resistant to degradation, it is possible that in 2003 a new cycle of waste had been introduced to the second bioreactor and had not sufficiently degraded the contaminants at the time of sampling.

Previous research at this site (1996-1999) indicated that during the first stages of remediation, genotoxicity of treated soils were unchanged over time despite reduced contaminant levels (Garcia, 2001). Contaminant levels in the groundwater (as measured at the same sampling locations as this study from 1998-1999) were reduced to within target levels and the genotoxicity as measured by the *E. coli* prophage induction assay was eliminated (Garcia, 2001). The X-19 cell was also tested in 2000 for genotoxicity using the Salmonella/microsome assay and showed a positive response (Gomez, 2002). Surface soil samples were generally more genotoxic than subsurface samples collected from the X-19 (Gomez, 2002).

In addition to the high variability of the samples, historical information indicates that background soils have increased mutagenic potential despite negligible levels of PAHs (Garcia, 2001). Data from these samples suggest that compounds not identified

by GC/MS can cause a mutagenic response. Current chemical analysis methods are able to detect only a fraction of the compounds in the environment. This also holds true for hazardous waste sites that contain complex chemical mixtures. There may be a large number of chemical components contributing to mixture toxicity. Interactions such as synergism, additivity, potentiation or inhibition could influence the bioavailability and genotoxicity of complex mixtures. Moreover, degradation processes (biological or physical) may produce more toxic or more soluble end products. The results indicate that a combined testing protocol using both biological and chemical methods provides a more comprehensive view of the fate of genotoxic compounds in soil treatment systems than chemical analysis alone.

CHAPTER III

GENOTOXICITY OF SEDIMENTS CONTAINING MIXTURES OF PCBs
AND PAHs

3.1 Introduction

Complex chemical mixtures containing PCBs and PAHs are ubiquitous
environmental contaminants. Complex mixtures have been detected in environmental
media including sediments, biota, air, surface waters, and industrial effluents (Eisler,
1987; Erickson, 1997; Kannan et al., 2000; Neff, 1979; USEPA, 1980). PCB and PAH
mixtures are known to have adverse affects in aquatic organisms (Akcha et al., 2003;
Harvey et al., 1997; Pinkney et al., 2001; Schenke et al., 2000; van den Hurk et al., 2002;
van Schanke et al., 2000) and in laboratory animals (Alvares and Kappas, 1977; Deml et
al., 1983; Safe, 1989). Certain congeners of PCBs and PAHs may interact with the aryl-
hydrocarbon receptor (Ahr) and have dioxin-like toxic effects (Safe, 1990; Villeneuve et
al., 1998). These effects may include tumor promotion (Schwarz and Appel, 2005),
neurobehavioral problems (Kuriyama et al., 2003; Kuriyama and Chahoud, 2004;
Schantz et al., 1989) and decreased immune system function (Clemons et al., 1999;
Hutchinson et al., 2003; Hutchinson et al., 1999). However, there may exist both Ahr-
active and Ahr-inactive congeners within these mixtures and their interactions or
potential effects may differ depending upon the bioassay used (Villeneuve et al., 2001).
Despite the strong conservation of the AhR-dependent pathway, there is also
considerable inter- and intraspecies differences in the toxicological responses to AhR
ligands (Ema et al., 1994; Poland and Glover, 1990; Poland et al., 1994).) These
differences are likely due to genetic polymorphisms in the AhR (Schwarz and Appel,
2005).

PCBs are known to be potent inducers of phase I and phase II drug-metabolizing
enzymes and may act as co-carcinogens, or anticarcinogens (Safe, 1989). The genotoxic
potential of PCBs depends largely upon the level of chlorination of the molecules. The
higher molecular weight PCBs tend to be poorly metabolized and exhibit low binding to
DNA (Safe, 1989). However, it is these higher molecular weight PCBs that may act as

strong promoters when treated with an initiator such as the PAH benzo[a]pyrene (BaP) (Safe, 1989). With respect to PAHs, complex mixtures of these chemicals might contain hundreds to thousands of different compounds (Cizmas et al., 2004b). Seven PAHs, including the representative carcinogenic PAH benzo[a]pyrene (BaP), have been classified by the United States Environmental Protection Agency (USEPA) as probable human carcinogens (USEPA, 2005i).

Environmental media contaminated with complex mixtures of both PCBs and PAHs may exhibit numerous chemical interactions that affect the genotoxicity of these compounds both *in vitro* and *in vivo*. These interactions could include inhibition, potentiation, additivity, synergism and antagonism. Evidence suggests that PCBs are potent enzyme inducers (Safe, 1989; van den Hurk et al., 2002) which might enhance or inhibit stages in PAH metabolism leading to additive or synergistic toxic effects. The purpose of this study was to investigate the genotoxicity of mixtures extracted from estuarine river sediments contaminated with both PCBs and PAHs in order to test whether differences in genotoxicity could be explained by differences in the sediment concentrations, particularly for PCBs. In addition to chemical analysis of sediment and water at each sampling site, a battery of three genotoxicity bioassays were implemented to better characterize the potential of PCB/PAH mixtures to cause DNA damage. Each extracted sample was analyzed for *in vitro* genotoxicity using the Salmonella/microsome assay (Ames et al., 1975; Maron and Ames, 1983) to screen for PAH genotoxicity, and the *E. coli* prophage induction assay (DeMarini and Brooks, 1992; DeMarini et al., 1990) to screen for chlorinated compound genotoxicity. Two different dermal exposure studies using ICR female mice were conducted to examine the *in vivo* genotoxicity of the sediment extracts using the ^{32}P-postlabeling assay (Reddy and Randerath, 1986).

Aquatic organisms are exposed to contaminated sediments both directly through ingestion, and indirectly via the food web. The PCBs and some of the priority pollutant PAHs are relatively insoluble in water and tend to partition between the pore water and sediment depending on their organic carbon partition coefficients. Because of their persistence and ability to accumulate in estuarine sediments, these contaminants may be

bioavailable to commercially important species such as the native salmon (Missildine et al., 2005). Ingestion of fish is a potential pathway of contaminant exposure for humans. As a result, PAH and PCB-contaminated sites are a major concern for managers and regulatory agencies. Understanding the potential genotoxicity and role of PCBs in complex mixtures in sediment extracts, is a first step in understanding similar potential effects within food webs at contaminated sites.

3.2 Materials and Methods

3.2.1 Site History

The site chosen for the collection of sediments used in this research is the lower 6-mile portion of a river located in the Pacific Northwest. Heavily industrialized upland areas surround this segment of the river. Historical and current industrial use of these areas includes cargo handling and storage; marine construction; boat manufacturing; marina operations; paper and metals fabrication and recycling; food processing; and airplane parts manufacturing. Contaminants may have entered the river by a number of different routes including spillage during product shipping and handling, direct disposal or discharge, contaminated ground water discharge, surface water runoff, storm water discharge, or contaminated soil erosion (Windward, 2003). The results from previous studies in this area have shown that sediments are contaminated with PCBs and PAHs, as well as arsenic, mercury, pesticides and organotins.

3.2.2 Sample Collection, Extraction and Chemical Analysis

Surface sediment samples were collected from a boat in April 2003 using a petite ponar grab (WILDCO, Buffalo, New York, part #1728-G30). Sampling locations were selected based upon previous measurements (Windward 2003) that identified known areas of elevated PCB levels. A total of 5 sediment samples were collected from the contaminated site at locations that were separated by approximately 15m. One reference sample (sediment background) was collected approximately 1 mile up-river of the

contaminated site. Sediments were brought to the water surface, homogenized with a pre-washed stainless steel spoon in a pre-washed stainless steel bowl, and transferred to I-chem certified sampling jars (VWR, catalogue # IR221-1000). Samples were shipped on ice overnight following chain-of-custody protocols to the laboratory at Texas A&M University, where they were stored at 4ºC until extraction.

Each sediment sample was oven-dried overnight at 60ºC. Samples were then ground in a mortar and pestle and passed through a #20 (850μm) sieve. Approximately 10g of dried sample were weighed for extraction. Dried sediments were extracted using a 1:1 mixture of hexane:acetone in a Dionex (Dionex Corp., Sunnyvale, CA) Model 200 Accelerated Solvent Extractor (ASE). Samples were analyzed for PAHs and other semivolatile organic compounds (SVOCs) using USEPA method 8270C (USEPA, 1997) and for total PCBs and PCB homologs following USEPA method 680 (Stevens et al., 1985). Sediment collected from station 3 was extracted once for the microbial bioassays (sample 3A). Chemical analysis indicated that sample 3A had the highest concentration of PCBs. Therefore, the sediment was extracted again (sample 3B) to yield enough residue for the second *in vivo* experiment. Sample 3A and 3B were chemically analyzed separately.

3.2.3 *In Vitro* Bioassays

3.2.3.1 Salmonella/Microsome Assay

The Salmonella/microsome assay was employed to evaluate the mutagenic potential of the complex PCB/PAH mixtures extracted from the river sediments (except for sample 3B which was only tested in the second *in vivo* experiment). This particular bioassay was chosen because it has routinely been used to detect genotoxicity of hazardous waste (Houk and DeMarini, 1988) and has been shown to be as much as 90% effective as detecting the mutagenic potential of PAHs (McCann et al., 1975a). Sediment extracts were tested in the presence and absence of metabolic activation ($\pm$ S9) in the standard plate incorporation assay (Maron and Ames, 1983) using the *Salmonella*

typhimurium (*S. typhimurium*) TA98 tester strain (kindly provided by Dr. B.N. Ames, University of California at Berkley). TA98 was selected because previous research indicates that this strain is sensitive to the types of PAHs found in the sediment samples (Randerath et al., 1999). S9 supernatant of homogenized Aroclor 1254 induced Sprague-Dawley rat liver was obtained from Molecular Toxicology, Inc. (Boone, NC). A 1 mL volume of 20% S9 mix contained 0.2 mL S9 fraction and 0.8 mL cofactor supplement (1.41 mg glucose-6-phosphate, 3.06 g NADP, 0.02 mL of a solution containing 1.65 M KCl and 0.4 M $MgCl_2$, 0.5 mL of 0.2 M sodium phosphate buffer (pH 7.4).

Positive direct-acting (2-nitrofluorene (2NF): 25 μg/plate), positive indirect-acting (benzo-a-pyrene (BaP): 10 μg/plate) and negative solvent (dimethylsulfoxide (DMSO)) controls were included in each test to ensure consistency in TA98 sensitivity and S9 mix activity. Sediment extracts were resuspended in DMSO and tested on duplicate plates in two independent experiments at five dose levels (1.0, 0.5, 0.25, 0.1, 0.05 mg/plate). Following the plate incorporation assay, plates were stored inverted at 37°C for 72hrs. prior to analysis. Revertant colonies were counted on an Artek Model 880 automated colony counter (Dynatech Laboratories, Inc.).

Salmonella/microsome assay data were analyzed using the modified two-fold rule of Chu *et al*. (1981). A mutagenic response was considered positive if the mean number of revertant colonies was greater than two times the concurrent solvent control for two consecutive doses. A response was considered weakly positive if the number of revertant colonies was greater than two times the concurrent solvent control for only one dose.

3.2.3.2 *E. coli* **Prophage Induction Assay**

The *E. coli* prophage induction assay (DeMarini and Brooks, 1992; DeMarini et al., 1990) was also employed to screen for the mutagenic potential of all the sediment extracts (except for extract 3B). This bioassay was used because previous studies have indicated that it is sensitive to chlorinated hydrocarbons that may be inactive in the

Salmonella/microsome assay (Houk and DeMarini, 1987; Houk and DeMarini, 1988).

The tester strain *E. coli* lysogen WP2sλ (*lon$_{11}$, sulA$_1$, trpE$_{65}$, uvrA$_{155}$, lamB$^+$*), and

indicator strain TH008 (streptomycin^r) were provided by V. S. Houk (USEPA, Research

Triangle Park, NC). The bioassay included both a microsuspension assay and a plaque

formation assay. The microsuspension assay exposed mid-log phase WP2sλ (~2 x 10^6

cells) to sample extracts resuspended in acetone. This was done in a 96-well microtiter

plate (Corning). Samples were tested both with and without metabolic activation ($\pm$ S9).

Controls including a positive direct-acting chemical (2NF), a positive indirect-acting

chemical (2-aminoanthracene (2AA)), a negative solvent (acetone), and media

(supplemented VBMM) were tested along with the sample extracts to ensure consistency

in induced *E. coli* DNA damage and in S9 mix activity. The contents of the plate were

mixed using a vibrating shaker, covered with mylar and 2 layers of plastic wrap (Saran

Wrap, Dow Chemical Co.), and incubated overnight (approximately 16 hours) at 37°C.

Following incubation, the microtiter wells were scored for turbidity, with turbid

wells indicating cell growth and clear wells indicating cytotoxicity and/or growth

inhibition. At least five wells adjacent to a clear well were sampled to determine the

concentration of lambda phage. Fifty µL samples from the selected wells were diluted

1:100 in 5 mL VBMM. One hundred µL of the diluted phage and 200 µL of the log-

phase indicator cells (TH008) were added to 2.5 mL top agar (0.65 Bacto agar and 10

mM MgSO$_4$). Overnight cultures of TH008 bacteria were grown in an incubating shaker

until a concentration of ~2 x 10^8 cells/mL was achieved. After addition of the TH008

cells to the top agar tubes, the tubes were poured onto plates containing tryptone media

(10g Bacto tryptone, 5 g NaCl, and 12 g Bacto agar per L of glass-distilled, deionized

water and supplemented with streptomycin sulfate (100 g/mL) to select against the

lysogen). Plates were inverted and incubated overnight at 37°C. After 24hrs., the plates

were quantified by counting the number of plaques on each plate by hand on a Darkfield

Quebec colony counter. All samples were tested in duplicate in at least two independent

experiments. A sample extract was considered positive if the average number of

induced plaque-forming units (PFUs) at two doses was three times the average number

of induced plaques for the corresponding acetone well. If an extract induced a three-fold increase in PFUs at only one dose, the result was scored as a weak positive. Samples that did not induce a response three times greater than the acetone control at any dose were considered negative.

3.2.4 *In Vivo* Bioassays

3.2.4.1 Animal Treatment

Two experiments were conducted using ICR female mice. The first experiment used sediment extracts from three river stations to compare across stations. The second experiment used sediment 3B (a second extract from Station 3) coupled with BaP to compare to BaP administered alone. Institutional guidelines were followed for animal care and use in all experiments. For the first animal experiment, 5 groups of mice containing 4 mice each were treated topically with sediment extracts from samples 2, 4, and 5 (Table 1). A patch of hair (approximately 4cm^2) was shaved on each mouse 3 days before treatment and then approximately 2hrs. prior to treatment. Sediment extracts were suspended in methylene chloride and 150μL of control or treatment chemical was applied to the shaved area of each mouse using a glass capillary micropipette. Two control groups were selected, 1) methylene chloride, and 2) sediment background (BKG) extract at 3 mg/mouse. Five treatment groups were used including, 1) sediment extract #2 at 3 mg/mouse, 2) sediment extract #5 at 3 mg/mouse, 3) sediment extract #4 at 3 mg/mouse, 4) sediment extract #4 at 1.2 mg/mouse, and 5) sediment extract #4 at 0.48 mg/mouse. Sediment extract #4 was selected for measurement of a dose response because it was the sample that yielded the largest mass of residue in the extraction procedure. Animals were exposed to the treatments for 24hrs. and then sacrificed using carbon dioxide (followed by cervical dislocation). The liver, lungs, and skin were removed and stored at -80ºC until DNA isolation.

For the second *in vivo* experiment 9 groups of 4 mice each were used including one methylene chloride control group. The third sediment sample was re-extracted

(#3B) for this experiment because it had the highest levels of PCBs and PAHs (Table 4). The extracted sample was resuspended in methylene chloride. The 8 treatment groups were as follows, 1) sediment extract at 3 mg/mouse, 2) sediment extract at 1.2 mg/mouse 3) BaP at 100nmol, 150μL/mouse, 4) BaP at 10 nmol, 150μL/mouse, 5) sediment extract at 3 mg/mouse + BaP at 100nmol, co-administered, 6) sediment extract at 1.2 mg/mouse + BaP at 100nmol, co-administered, 7) sediment extract at 3.0 mg/mouse + BaP at 10nmol, co-administered, 8) sediment extract at 1.2 mg/mouse + BaP at 10nmol, co-administered. Mice were treated topically, sacrificed after 24hrs. of exposure, and the tissues processed as described above.

3.2.4.2 ^{32}P-Postlabeling Assay

The ^{32}P-postlabeling assay using nuclease P1 enrichment is currently the most sensitive method for detecting and quantifying DNA adducts. The formation of DNA adducts, even at low levels, is an important process in carcinogenesis. DNA was extracted from select tissues and ^{32}P-postlabeled following the method of Reddy and Randerath (1986). Adducted radioactive nucleotides were separated by multidirectional anion-exchange TLC using polyethyleneimine (PEI)-cellulose sheets. To remove any remaining radioactive impurities and orthophosphate, thin layer chromatography (TLC) was performed using 2.3 M NaH_2PO_4, pH 5.75. Labeled products were then contact-transferred to fresh PEI-cellulose sheets and resolved by two-dimensional TLC. The first dimension employed 3.82M lithium formate + 6.75M urea, pH 3.35. The second dimension was developed with 0.72M NaH_2PO_4+ 0.4M TRIS + 7.65M Urea, pH 8.2. Radioactivity was determined using an Instant Imager (Packard Instrument, Downers Grove, IL). Imaging was accomplished by using a template to isolate identified spots on each map. Counts per minute (CPM) for each adduct were determined. Adduct levels were quantified as mean relative adduct labeling (RAL) values $\pm$ SEM using the following equation: RAL = sample count rate/(DNA-P x specific activity $_{ATP}$).

3.2.4.3 Statistics

Statistical analyses were performed using SPSS v.13.0 software (SPSS, Inc., Chicago, IL). RAL values for both *in vivo* experiments were tested for normality using the Shapiro-Wilk test, and for equal variance using Levene's test for equal variances. For the first animal experiment, sediment extracts at the 3.0mg/mouse dose were compared to the sediment background at 3.0mg/mouse dose and the methylene chloride solvent control using contrast testing. Sediment extract 4 was further analyzed to compare RAL values at the three different doses using a one-way ANOVA. The primary focus of the second animal experiment was to compare BaP alone to BaP plus two different doses of sediment. Polynomial contrast testing was used to test for a linear response seen in BaP alone plus BaP plus sediment. For all tests, $p < 0.05$ was considered significant.

3.3 Results

3.3.1 Chemical Analysis and *In Vitro* Biossays

In order to understand the toxicity of the sediment extracts, it was necessary to determine PCB and PAH composition in each sample. Chemical analysis confirmed the presence of elevated concentrations of PCBs and PAHs in all of the sediment extracts from sampling stations in the industrialized area (Table 4). The five stations in the contaminated area exhibited almost 10-fold variation in PCB concentration. Sample 3B had the highest levels of PCBs (125,000 ng/mg sediment extract) and sample 4 had the lowest levels of PCBs (800 ng/mg sediment extract) within the contaminated area (Table 4). The extract of sediment from the background area had relatively low levels of PCBs (60 ng/mg sediment extract) (Table 4). All samples had higher levels of PCBs compared to PAHs. The sediment extracts were dominated by the penta, hexa, and hepta chlorinated homologues (Fig. 20). With regards to the PAHs, sample 3B had the

Table 4. Summary of *in vitro* bioassay and chemical analysis data. Information includes biological response from both the Salmonella/microsome assay and the *E.coli* prophage induction assay as well as total PAHs (tPAHs), carcinogenic PAHs (cPAHs), benzo[a]pyrene (BaP), and total PCB (tPCBs) amounts for samples collected from 5 sites within an industrialized zone and one site from a reference area (BKG). NA = Not Analyzed.

Summary of Sediment Samples from 2003						
Sample Number	Biological Response		Chemical Analysis			
	Salmonella +S9 *	*E.coli* +S9 **	tPAHs ng/mg	cPAHs ng/mg	BaP ng/mg	tPCBs ng/mg
1	(+ -)	(-)	220	40	4	1000
2	(+ -)	(-)	490	90	8	3000
3A	(+)	(-)	400	70	6	7000
3B	NA	NA	6,300	1,300	160	125,000
4	(-)	(-)	270	50	4	800
5	(-)	(-)	1000	150	20	1500
Sediment Background	(-)	(-)	300	40	4	60

* (+) = # revertants/plate $\geq$ 2x's the solvent control for 2 consecutive doses in 2 independent experiments.

(+ -) = # revertants/plate $\geq$ 2x's the solvent control for 1 dose in 2 independent experiments

(-) = # revertants/plate < 2x's solvent control at all doses in 2 independent experiments.

** (-) = # plaques/plate NOT < 3x's the solvent control for all doses in 2 independent experiments.
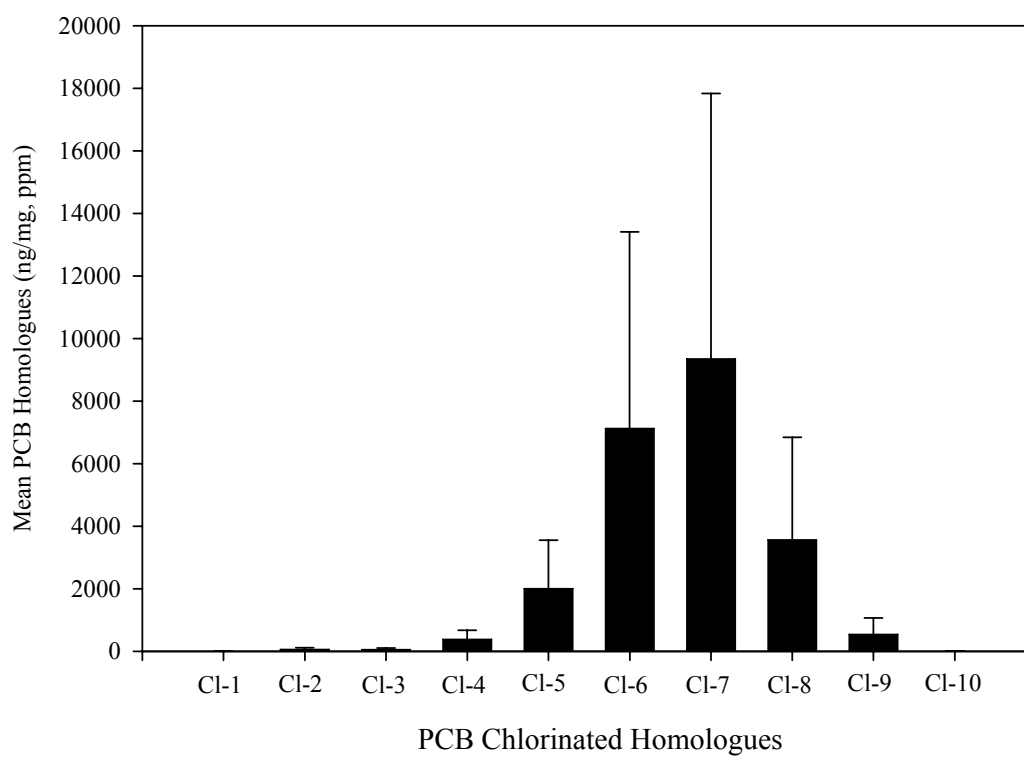
Fig. 20. Average concentration of selected PCB congeners in sediment extracts (N=6). Statistical interval = SE.

highest levels of total PAHs (6,300 ng/mg sediment extract), total carcinogenic PAHs (cPAHs) (1,300 ng/mg sediment extract) and total benzo[a]pyrene (160 ng/mg sediment extract) (Table 4).

Based on the presence of PAHs in the samples, it was hypothesized that the sediment extracts would cause no response without metabolic activation and a positive response with metabolic activation in the Salmonella/microsome assay (a test 90% effective at detecting the mutagenicity of PAHs). Of the samples used in this test, sample 5 had the highest levels of total PAHs (1,000 ng/mg sediment extract) and total carcinogenic PAHs (cPAHs) (150 ng/mg sediment extract) (Table 4). In contrast, sample 1 had the lowest levels of total PAHs and cPAHs (see Table 4). As predicted, all tested samples were negative in the Salmonella/microsome assay without metabolic activation (-S9). This response is consistent with other complex PAH mixtures that have been tested in this laboratory (Cizmas et al., 2004a). However, contrary to our hypothesis, only sample 3A exhibited a positive mutagenic response in the Salmonella/microsome assay with metabolic activation (+S9). Samples 1 and 2 had a weak positive response +S9 (Fig. 21). Sample 5 did not induce a positive response.

With the presence of PCBs in the samples, it was predicted that all of the samples would be negative without metabolic activation and positive with metabolic activation using the *E. coli* prophage induction assay (a test sensitive to the detection of mutagenic chlorinated compounds). Sample 3A had the highest concentration of PCBs (7,000 ng/mg sediment extract) and was expected to induce the strongest positive response of the samples. However, all tested samples were negative both with and without metabolic activation in the *E. coli* prophage induction assay (Table 4).

3.3.2 *In Vivo* Bioassays

The primary purpose of the first animal experiment was to see if the sediment extracts induced significantly greater levels of adducts than the methylene chloride solvent control or the sediment background. When comparing the *in vivo* genotoxicity of the sediment extracts, sample #5 induced the highest levels of total skin adducts at a

dose level of 3.0 mg/mouse, followed by sample #2 (Fig. 22). Sample #4 induced the lowest adduct level for the contaminated sediments (Fig. 22). Sample #4 also induced a near-linear dose response curve, with the frequency of total skin adducts and spot #4 adducts increasing with increasing dose per treatment group (Fig. 23). Although these findings are consistent with the levels of total PAHs found in each sample, cPAH concentrations in the sediment extracts varied by approximately four-fold, whereas DNA adducts varied by less than 30% (Table 4 & Fig. 22). This suggests that the capacity of
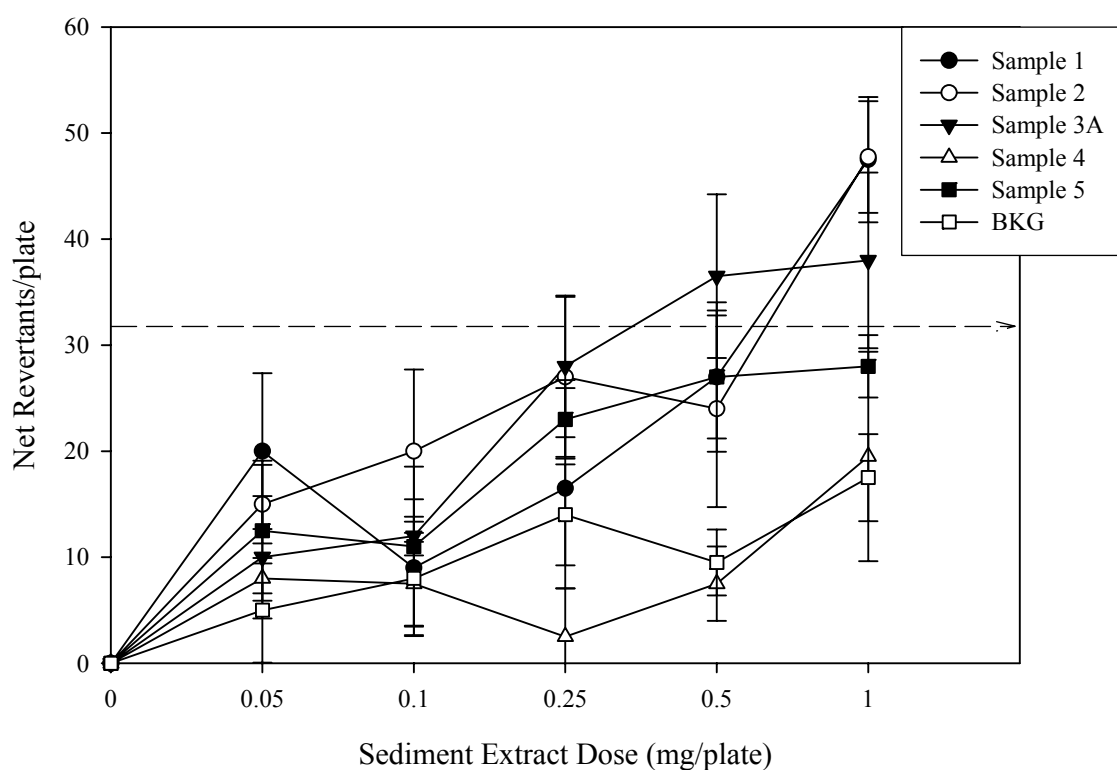
Fig. 21. Microbial mutagenicity, as measured in *S. typhimurium* strain TA98 with metabolic activation, of extracts of sediment samples collected from five stations in an industrialized area and a reference station (BKG). Dashed line represents concurrent solvent control value. Data are presented as mean $\pm$ SEM.

the PAHs to bind with DNA may have been saturated. Of these three samples, sample #5 had the highest levels of PAHs and cPAHs (Table 4) and highest level of skin adducts (Fig. 22), whereas, sample #4 had the lowest levels of PAHs and cPAHs (Table 4) and induced the lowest levels of skin adducts (Fig. 22). The adduct data from this experiment were normally distributed ($p=0.173$) and had equal variances ($p=0.772$). The mean adduct levels were significantly higher in the groups administered 3.0mg/mouse of contaminated sediment extracts compared to the mean adduct levels induced in the methylene chloride control group ($p=0.001$) (Fig. 22). The contaminated sediment extracts also induced a significantly higher level of mean adducts at the 3.0mg/mouse dose compared to the sediment background extract at 3.0mg/mouse dose ($p=0.004$) (Fig. 22). When analyzing the dose response data from sample #4, the data were normally distributed ($p=0.062$), with equal variances ($p=0.090$). There was a significant difference in the level of adducts induced at varying doses of sample #4 ($p=0.048$) (Fig. 23). Representative skin DNA adduct profiles are shown in Fig. 24.

Based on results from the first experiment, it was apparent that the sediment extracts had the ability to form DNA adducts at levels consistently greater than the controls. Due to the dominant concentrations of PCBs in the sediments, understanding how these levels could be affecting PAH genotoxicity was pursued. In the environment, humans and other animals are most commonly exposed to complex mixtures versus pure compounds. What is not well understood is how the chemicals within these complex mixtures interact with one another. For the second animal experiment it was hypothesized that the elevated levels of PCBs in the sediment extracts would enhance PAH genotoxicity as measured by the induction of DNA adducts. Skin, lung and liver DNA from treated and control animals were extracted and ^{32}P-postlabeled following 24hr. topical exposure to varied concentrations of BaP and sediment extract. The highest frequency of bulky DNA adducts were observed in skin from animals treated with BaP at 100 nmol (Fig. 25). The administration of 100 nmol BaP + sediment extract induced higher levels of DNA adducts than were observed in mice treated with 10 nmol BaP + sediment extract. Mice treated with 3.0 mg sediment extract +100 nmol BaP

Fig. 22. Skin DNA adducts in female ICR (CD-1) mice after topical treatment with a methylene chloride control (150 uL/mouse), an extract of background sediment (Sed. BKG.), and extracts of contaminated sediment. Mouse groups for Sed. BKG, Sample # 2, Sample # 4, and Sample # 5 were treated with 3.0mg/mouse extract. *Total adduct levels were significantly higher than the control and Sed. BKG ($p \leq 0.05$). Data are presented as mean $\pm$ SEM.
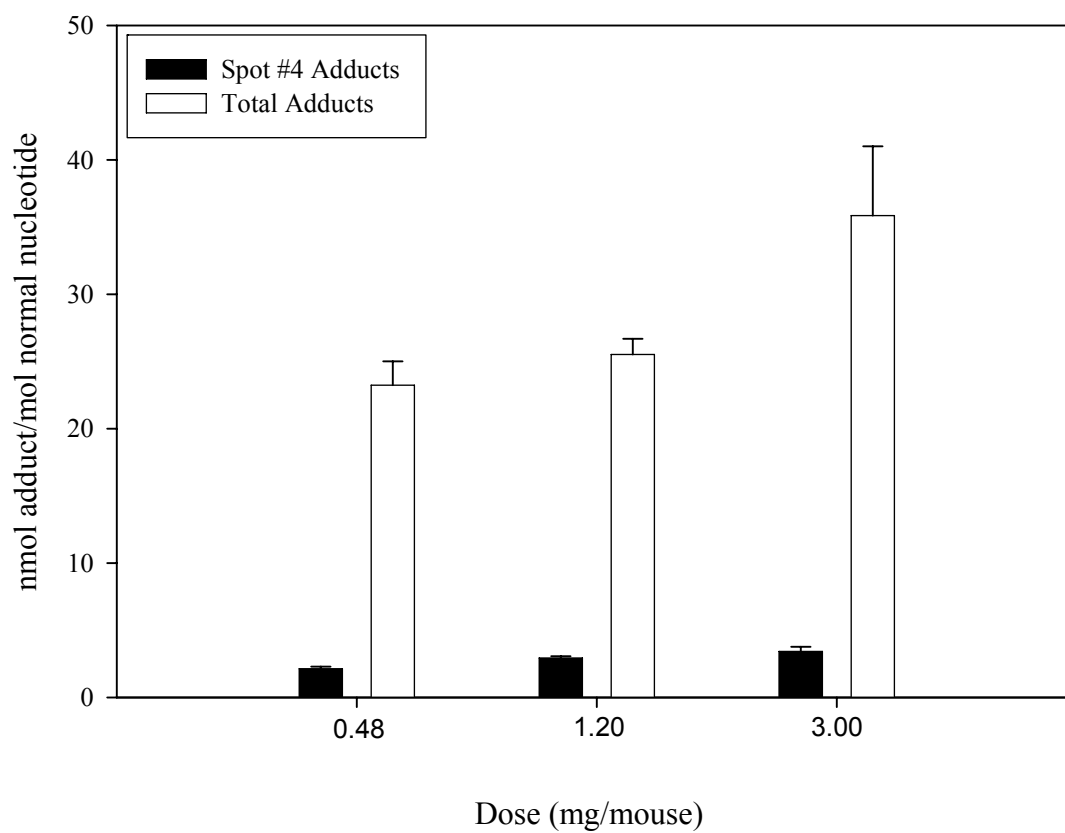
Fig. 23. Skin DNA adducts in female ICR (CD-1) mice after topical treatment with sediment extract sample # 4 at varying doses. Data are shown as mean $\pm$ SEM.
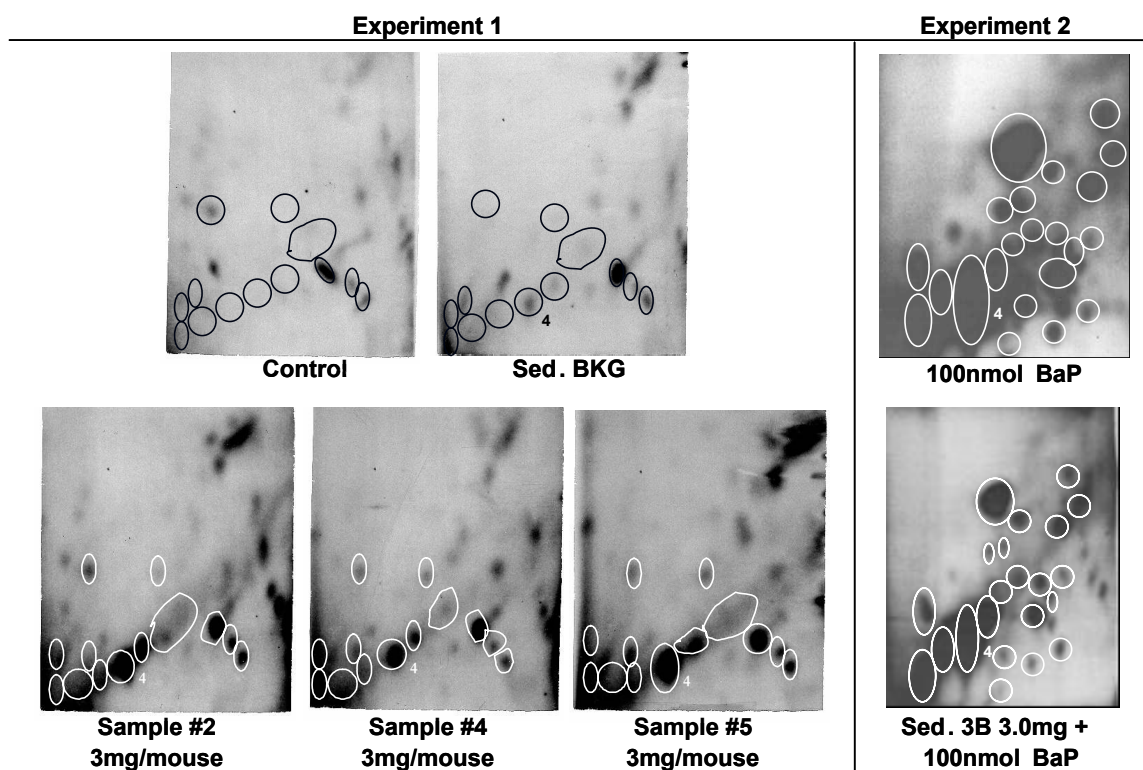
Fig. 24. Representative DNA adduct profiles from skin tissue of female ICR mice treated for 24hrs with topical applications of methylene chloride solvent control and sediment extracts (Experiment 1) and sediment 3B extract + BaP (Experiment 2). Spot 4 is indicated by the number 4 on select maps.

exhibited a reduced frequency of DNA adducts in comparison with animals treated with BaP alone. The inhibitory effects of the sediment extracts on BaP-induced DNA adducts exhibited a dose-response relationship. The treatment group receiving 3.0 mg sediment extract + BaP at 100 nmol induced a RAL of less than 200×10^{-9}, whereas a RAL of more than 300×10^{-9} was observed in mice receiving the 1.2 mg sediment extract + BaP (Fig. 25). Total adduct data in the BaP at 100nmol groups were normally distributed and had equal variances. There was a significant inhibitory effect seen in BaP at 100nmol + increasing sediment concentration ($p<0.001$) (Fig. 25). Spot #4 data

in the BaP at 100nmol groups were normally distributed. However, the test for equal variances was violated (p=0.022). Despite variances not being equal, a polynomial contrast was still an appropriate test. There was also a significant inhibitory effect seen in the induction of Spot #4 adducts from BaP at 100nmol alone compared to BaP at 100nmol + increasing sediment concentration (p<0.001) (Fig. 25). A small amount of endogenous skin adducts were observed in the methylene chloride control group. Adduct levels in liver and lung samples were comparable to background adduct levels (data not shown). Representative skin DNA adduct profiles are shown in Fig. 24.

3.4 Discussion

The extracts of sediment samples collected from a river located in a heavily industrialized area were found to contain elevated levels of PCBs and PAHs. Although the sources of PAHs are anticipated to be more numerous than PCBs, the PCB levels in sediment extracts were generally two to ten times higher than PAH concentrations. When the sediment extracts were tested for genotoxicity in the Salmonella/microsome assay, the *E. coli* prophage induction assay, and the DNA adduct ^{32}P-postlabeling assay, the samples from contaminated areas exhibited relatively low levels of genotoxicity. Genotoxicity of the PAHs in sediment extracts appeared to be measured most accurately using ^{32}P-postlabeling of DNA adducts in mouse skin (treated topically with sediment extracts).

The role that PCBs play in the genotoxicity of complex mixtures containing both PCBs and PAHs remains unclear. This role may be largely dependant on the level of chlorination of the PCB constituents, and the dose of the PCB extract. Of the samples tested in the Salmonella/microsome assay, sample #3A was the only sample that produced a positive response. The extract of this sediment also had the highest levels of PCBs (Fig. 20). The Salmonella/microsome assay is roughly 90% effective at detecting the carcinogenicity of PAHs (McCann et al., 1975a). Considering the fact that sample #3A had only moderate levels of PAHs (Fig. 20), it is possible that the elevated levels of PCBs in this sample enhanced the genotoxicity of the PAHs. Although PCBs are not
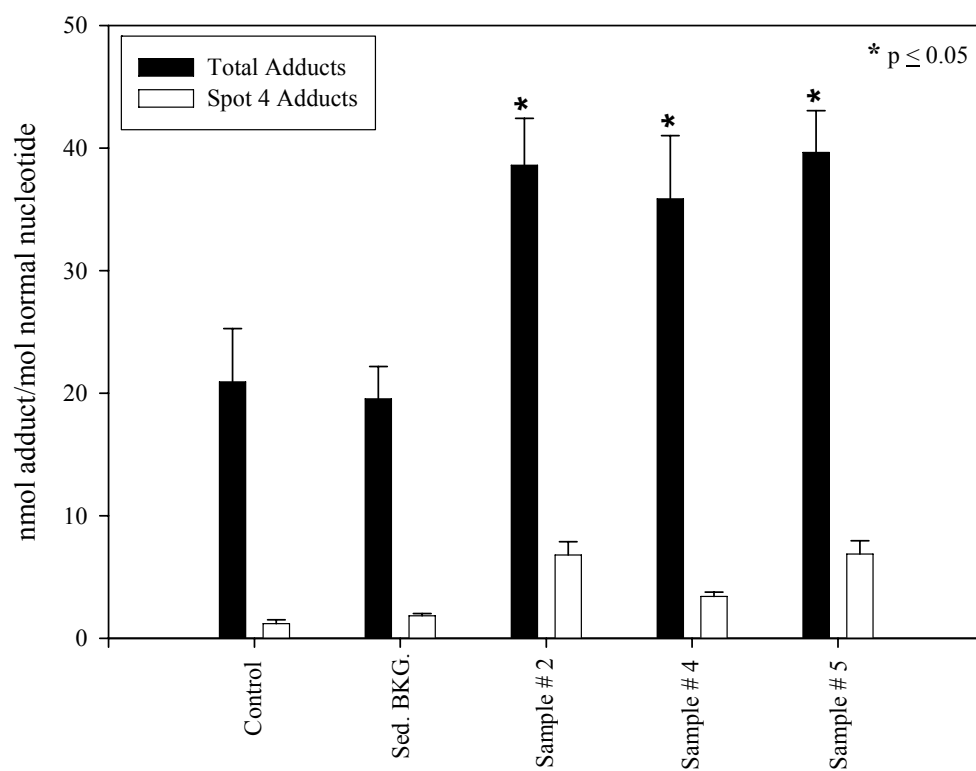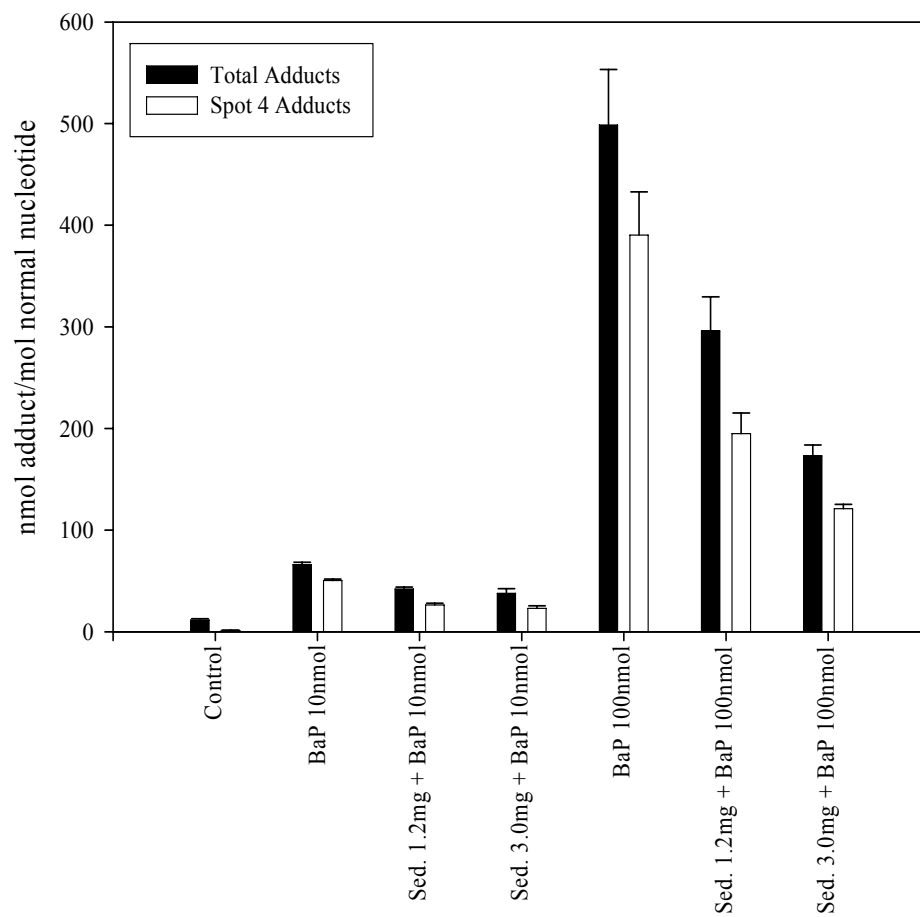
Fig. 25. Skin DNA adducts in female ICR (CD-1) mice after topical treatment with methylene chloride solvent control, extracts of contaminated sediment sample # 3B and BaP. Data presented as mean $\pm$ SEM.

generally mutagenic in bacterial systems (Safe, 1989), the *E. coli* prophage induction assay has been shown to be sensitive to the lower chlorinated phenols (DeMarini and Brooks, 1992; DeMarini et al., 1990). The sediment extracts used in this study were found to contain primarily the more highly chlorinated biphenyl congeners. The higher molecular weight PCBs tend to be poorly metabolized and show a low binding affinity for DNA (Safe, 1989). These characteristics may have produced the negative response observed in the *E. coli* prophage induction assay.

Dermal application of sediment extracts demonstrates that these complex chemical mixtures are capable of inducing DNA adduct formation in ICR female mice. PAHs are known to have the ability to form DNA adducts as demonstrated by the carcinogen, BaP (Denissenko et al., 1996; Marston et al., 2001; Melendez-Colon et al., 1999). Animal studies indicate that the lower chlorinated PCBs may be metabolically activated into electrophilic quinoid species which can bind to DNA (Oakley et al., 1996; Pereg et al., 2002; Schilderman et al., 2000). PCBs may also act as potent inhibitors of glucuronosyltransferase, a Phase II enzyme important in the detoxification pathway for phenolic metabolites of PAHs (van den Hurk et al., 2002). However, other studies using oral doses of PCBs in mice indicate that there is no significant DNA adduct formation as measured using the ^{32}P-postlabeling assay (Whysner et al., 1998). Recent studies suggest that elevated concentrations of PCBs may induce cell apoptosis (Sanchez-Alonso et al., 2004), thereby reducing the concentration of viable cells and ultimately total levels of DNA adducts. Data from the current study indicate that DNA adduct formation was more consistent with PAH concentrations than with PCB concentrations (Fig. 25). However, when analyzing complex mixtures it is not yet possible to identify all mixture components. Chemical analysis of the adducts is needed to determine which chemicals or class of chemicals are attributed to the response seen in the ^{32}P-postlabeling assay.

Studies were also conducted to investigate the interaction of PCBs and PAHs in sediment extracts. The genotoxic effect of complex chemical mixtures may be altered by impacts on absorption, distribution, metabolism and binding with critical

macromolecules. The sediment extracts consistently and significantly lowered BaP adduct levels. The results indicate spot #4 adducts were higher at a sediment dose of 1.2 mg/mouse than at a sediment dose of 3.0 mg/mouse. The battery of *in vitro* and *in vivo* bioassays used in the present study indicate that the mixture of PCBs and PAHs extracted from sediments were weakly genotoxic. In addition, co-administration of sediment extracts with BaP produced a significant inhibition of DNA adduct formation in mice. Thus, for the mixtures and doses examined in the current study, the primary interaction was inhibition.

CHAPTER IV

GENOTOXICITY STUDIES ON ECOLOGICAL RECEPTORS AT

HAZARDOUS WASTE SITES

4.1 Introduction

Genetic ecotoxicology is the study of pollutant-induced genetic changes in organisms (Depledge, 1994). There is a broad range of potential biochemical and molecular effects following exposure to certain xenobiotics including alterations to DNA (Landis and Yu, 1995). One of the alterations to DNA that can be measured in exposed organisms is chromosomal breakage. Unlike the formation of DNA adducts and other subtle molecular changes, chromosome breaks cannot be repaired and are an indicator of permanent genetic damage in organisms. These types of breaks have the potential to cause reduced fitness in the affected organism and can be a useful tool for assessing ecosystem health at the individual and population levels (Bickham et al., 2000; Shugart, 2003). Although we are just beginning to understand the normal variation and damage to DNA seen in unpolluted environments, exposure to certain environmental contaminants such as complex mixtures of PAHs have been shown to be genotoxic to animals in controlled laboratory experiments (Bickham et al., 1998b; Incardona et al., 2004; Maria et al., 2003; Peterson and Bain, 2004; Winter et al., 2004) and through *in situ* field studies (Gauthier et al., 2004; Maria et al., 2004; Matson et al., 2005b; Winter et al., 2004; Wirgin and Waldman, 1998).

PAHs are primarily produced from the incomplete combustion of organic compounds such as coal, crude oil and natural gas used for industrial and domestic purposes. The PAH mixture investigated for this research is WPW which is largely composed of creosote and often PCP and diesel fuel. Creosote is obtained from the distillation of coal tar at high temperatures. Coal tar itself contains hundreds of organic compounds. In creosote, over 100 chemicals have been identified (USEPA, 2005f), more than 85% of which are PAHs (Mueller et al., 1991). In the United States, creosote has been found in at least 33 of the 1,430 national priorities list sites identified by the U.S. EPA (ATSDR, 2004). The compounds in creosote of most concern to human and

ecological health are the PAHs. Seven PAHs found in WPW, including benzo(a)pyrene (BaP), have been classified by the U.S. EPA as probable human carcinogens (USEPA, 2005i). There are 17 priority pollutant PAHs found in WPW that are ecologically threatening due to their ubiquity and/or persistence in the environment (Table 5). PCP has also been used historically as a wood preservative and is often found mixed with creosote in WPW contaminated environments. According to the U.S. EPA, as of 2002, approximately 11 million pounds of PCP were produced (USEPA, 2005e). Humans and wildlife are usually exposed to technical grade PCP which often contains toxic impurities such as polychlorinated dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs) (ATSDR, 2001). Like the 17 PAHs, PCP is also listed as a priority pollutant because it is persistent, bioaccumulative and toxic (USEPA, 2005j).

Exposure to WPW has the potential to adversely affect wildlife that is in intimate contact with the contaminated environment. At both contaminated sites in this research, the aquatic areas at Site-1 and wetland at Site-2 are most impacted by the contaminants. Species that are particularly sensitive to the pollution of aquatic environments are fish and amphibians. Studies suggest that fish and amphibians exhibit adverse effects from both PAHs (Bickham et al., 1998b; Eisler, 1987; Gagne et al., 1995; Malins et al., 1988; Matson et al., 2005b; Monson et al., 1999; Wirgin and Waldman, 1998) and PCP (Farah et al., 2003; Maenpaa et al., 2004; Schuytema et al., 1993; Vaal et al., 1997). As a result, they may be used as sentinel species when investigating ecological risk at WPW contaminated sites. Due to the interspecies variability in contaminant toxicokinetics, and in food web dynamics it is important to have a multi-species approach to discern the genetic effects of contaminants across taxa (Bihari and Fafandel, 2004). The amphibians (*Acris crepitans* and *Bufo valiceps*) and fish (*Gambusia affinis* and *Lepomis microlophus*) were chosen for this research because of their abundance at both the contaminated sites and at associated references.

One of the potential endpoints that can be measured in exposed species is chromosome damage using the flow cytometric method (FCM). FCM is useful for detecting sublethal, genetic damage from PAHs, which can be both mutagenic and

Table 5. List of total target PAH analytes screened in the media and biota chemical analysis.

Naphthalene	_Acenaphthylene_	C1-Phenanthrene/Anthracenes	C1-Fluoranthenes/Pyrenes	C2-Chrysenes
C1-Naphthalenes	_Acenaphthene_	C2-Phenanthrene/Anthracenes	C2-Fluoranthenes/Pyrenes	C3-Chrysenes
C2-Naphthalenes	_Dibenzofuran_	C3-Phenanthrene/Anthracenes	C3-Fluoranthenes/Pyrenes	C4-Chrysenes
C3-Naphthalenes	_Fluorene_	C4-Phenanthrene/Anthracenes	Naphthobenzothiophene	**_Benzo(b)fluoranthene_**
C4-Naphthalenes	C1-Fluorenes	Dibenzothiophene	C1-Naphthobenzothiophenes	**_Benzo(k)fluoranthene_**
Benzothiophene	C2-Fluorenes	C1-Dibenzothiophenes	C2-Naphthobenzothiophenes	Benzo(e)pyrene
C1-Benzothiophenes	C3-Fluorenes	C2-Dibenzothiophenes	C3-Naphthobenzothiophenes	**_Benzo(a)pyrene_**
C2-Benzothiophenes	Carbazole	C3-Dibenzothiophenes	**_Benz(a)anthracene_**	Perylene
C3-Benzothiophenes	_Anthracene_	_Fluoranthene_	**_Chrysene_**	**_Indeno(1,2,3-c,d)pyrene_**
Biphenyl	_Phenanthrene_	_Pyrene_	C1-Chrysenes	**_Dibenzo(a,h)anthracene_**
				Benzo(g,h,i)perylene

Bold denotes carcinogenic PAHs (cPAHs).
Underline denotes 17 priority pollutant PAHs (ppPAHs), this includes the 15 most common PAHs as well as naphthalene and dibenzofuran which are also listed as priority chemicals (USEPA, 2005j).
Bold/underline denotes PAHs that are both carcinogenic and priority pollutants.

clastogenic (Custer *et al*. 2000). This method has been shown to detect chromosome damage in a number of species exposed to complex mixtures of environmental contaminants (Bickham et al., 1988; Bickham et al., 1992; Bickham et al., 1994; George et al., 1991; Lamb et al., 1991; Matson et al., 2004; Matson et al., 2005a; Matson et al., 2005b; McBee and Bickham, 1988; Theodorakis et al., 2001). Moreover, flow cytometry data tend to correlate well with petroleum product concentrations in animal tissues and specifically with PAHs (Bickham et al., 1998a; Custer et al., 2000). A recent paper by Matson et al. demonstrated a correlation between chromosome damage in turtles and three-ring PAHs using FCM (Matson et al., 2005a).

Currently in ecological risk assessment, chemical analysis is combined with *ex-situ* toxicity tests and species surveys to determine environmental risk. Conducting *ex-situ* toxicity tests is valuable for determining the sensitivity of species or comparing chemical toxicity, but there is a lack of ecological realism in controlled laboratory environments (Preston and Shackelford, 2002). Laboratory tests where the data generated are extrapolated to ecosystems often involve considerable uncertainty (Preston and Shackelford, 2002). For example, the test organisms might not be representative of indigenous species (La Point and Waller, 2000), the toxicity tests are used with single compounds and not complex mixtures found in the contaminated environment, natural physical/chemical variation found in the environment is often overlooked (Preston et al., 2001), and risk estimates generally assume additive effects from multiple stressors when antagonistic or synergistic interactions are common (Folt et al., 1999). Moreover, methods such as genotoxity testing are rarely employed in the risk assessment process primarily because the results cannot easily be translated into remedial goals for the contaminated site.

However, in the field, researchers are often faced with multiple anthropogenic and natural stressors contributing to ecosystem health. For example, toxic anthropogenic chemicals and natural variability in total suspended solids or dissolved oxygen can stress aquatic environments (Preston and Shackelford, 2002). With multiple stressors, it is a challenge in field studies to create a link between stressor and effect (Preston and

Shackelford, 2002). Despite these challenges, conducting *in-situ* tests is valuable because effects seen in the field are a result of the net effects of stressors and can be directly observed (Preston and Shackelford, 2002).

This study was designed as a collaborative effort between risk assessors and university researchers to incorporate genotoxicity testing into ecological risk assessment in a weight-of-evidence approach. The primary goal was to generate data regarding biomarkers of exposure in multiple ecological receptors. For those areas where elevated levels of contamination have been observed in environmental media or biota samples, the flow cytometry data provides an alternative metric to determine if exposed receptors are experiencing increased levels of genetic damage. A combined testing protocol that integrates *in-situ* and *ex-situ* data can be used as a supplement to chemical analysis and may help to strengthen the risk assessment process.

4.1.1 Site Histories

Samples were collected from two former wood preserving facilities in the United States. Site-1 was active until 1993 (USEPA, 2004a). While it was active, the facility used coal-tar creosote for wood treatment operations and disposed of waste into four unlined ponds. During the initial removal action these ponds were drained and the water treated. The remaining sludge from the ponds was removed and stored on-site in a fenced waste cell. This temporary waste cell was completed in 1995, the contaminants capped and the area seeded (USEPA, 2004a). However, there are some areas on site that were not targeted during the removal action and still contain visible product. One of these areas is on the west side of the property along the bank of a creek. This unnamed creek feeds into a larger creek which is used for sport fishing (USEPA, 2004a). There is also an on-site pond that was not drained during the removal action, and is a current habitat for aquatic organisms.

Site-2 used both creosote and PCP to pressure treat lumber, and operated from approximately 1946 through 1986 (USEPA, 2004b). Waste was drained into a ditch on the east side of the facility. The ditch flows southeast until it reaches a culvert which

drains the ditch into an unnamed wetland area. This wetland ultimately flows into a creek which feeds into a lake used for sport fishing. A benthic macroinvertebrates study was conducted in 1985 which concluded that the adverse effects to biological communities was confined to the wetland area and that the impact of the site discharge was insignificant in the creek located 0.5 miles east of the site. Similar to Site-1, a temporary waste cell was created to cap the contaminants on-site. However, the drainage ditch and wetland areas were not treated during the removal action. Currently, PAHs, PCP and dioxin/furans have been detected in the drainage ditch and in the wetland area (USEPA, 2004b).

4.2 Materials and Methods

All animals were collected using a scientific collecting permit from Texas Parks and Wildlife and an approved animal use protocol on file at Texas A&M University. A map of collecting locations for Site-1 is presented in Fig. 26. Site-2 collecting locations are presented in Fig. 27. Table 6 provides site information and codes for each of the seven reference locations.

4.2.1 Research Organisms

The species used in this research were selected based on their abundance at the contaminated aquatic sites and at adjacent reference sites. Two scouting trips were done to look for species prevalent in the contaminated areas using site identification for captured animals and also sound identification for the amphibians (their calls could be identified at night).

The mosquitofish (*Gambusia affinis*) is native to east Texas and belongs to the family Poeciliidae. Poeciliidae are livebearing fish that give birth to live young after internal fertilization (Boschung et al., 1983). Female Gambusia exhibit a conspicuous black spot on their abdomens during the reproductive period (Boschung et al., 1983). Adult females are generally larger than adult males. Males also have a modified anal fin used for reproduction. For these reasons, it was easy to distinguish adult males and
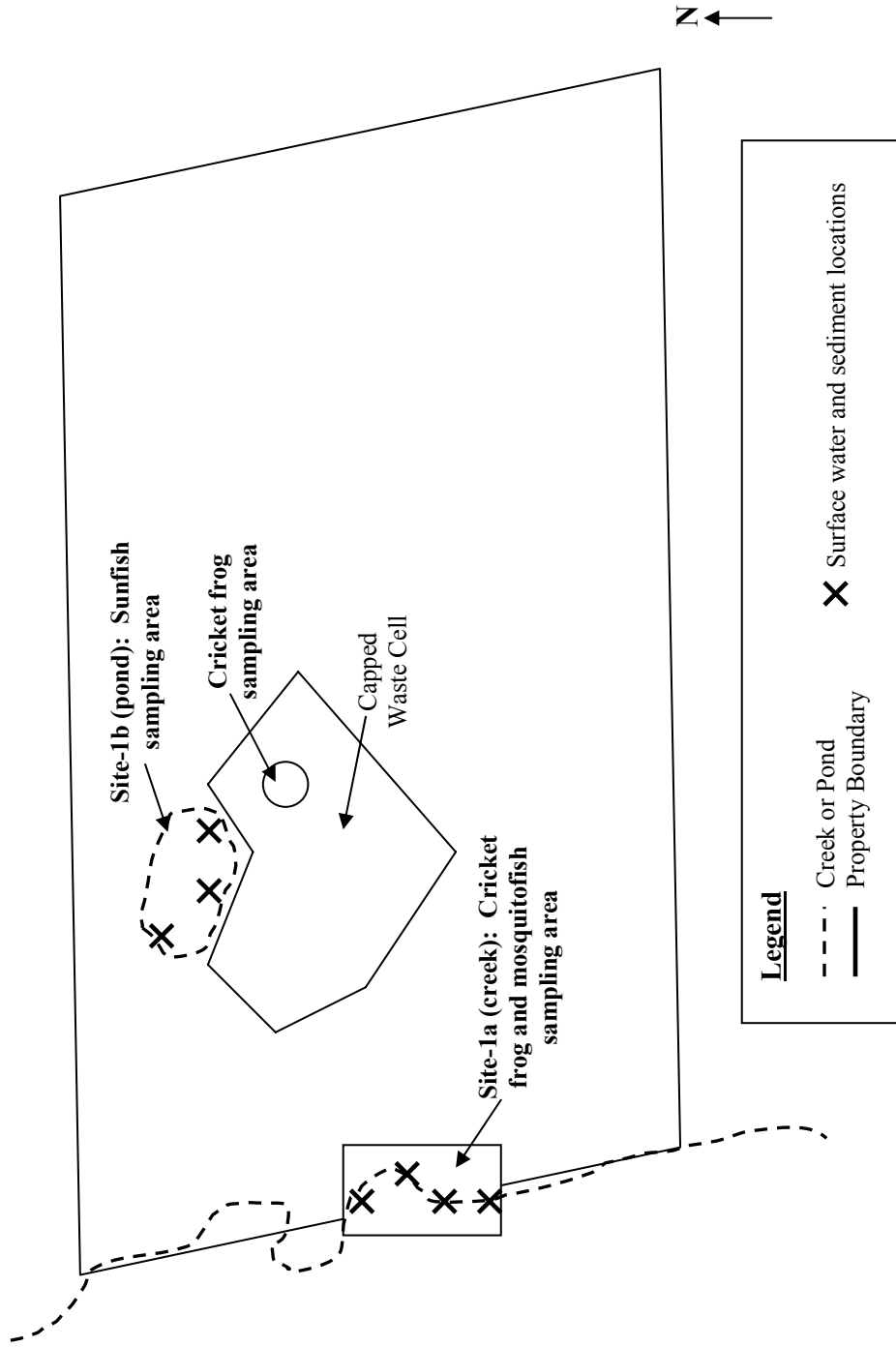
Fig. 26. Aerial view of Site-1 showing sampling locations for sunfish, mosquitofish, cricket frogs, and surface water and sediment samples (map not drawn to scale).
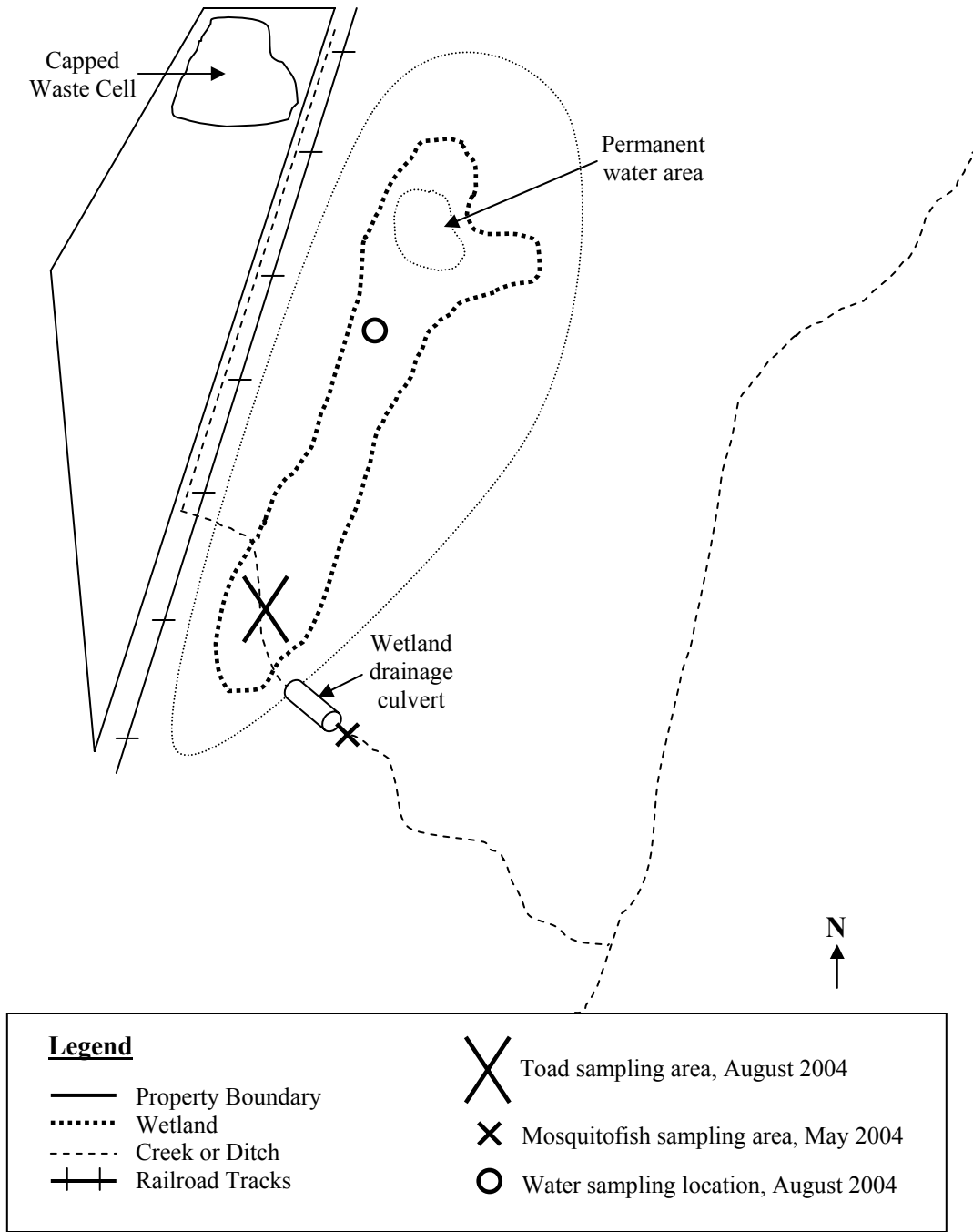
Fig. 27. Aerial view of Site-2 showing sampling locations for toad, mosquitofish and surface water samples (map not drawn to scale).

Table 6. Location codes for the seven reference sites sampled for biota and media.

Location Code	Location Description
Ref.-1	Rural pond on private property in Bryan, TX (Brazos Co.).
Ref.-2a	Martin Dies Jr. State Park (Jasper Co.), slough area on north side of hwy. 190. Off first walking trail past Nature Center.
Ref.-2b	Martin Dies Jr. State Park, boat ramp on north side of hwy. 190.
Ref.-2c	Martin Dies Jr. State Park, Gum Slough on south side of hwy. 190.
Ref.-2d	Martin Dies Jr. State Park, canoe launch at Gum Slough bridge on south side of hwy. 190.
Ref.-3	Pond on private property in College Station, TX (Brazos Co.)
Ref.-4	Austin's Colony Park, wetland area along footpath in Bryan, TX (Brazos Co.).

females during collection. This species is ubiquitous and can be found in areas ranging from lakes to drainage ditches. Because they are known to feed on mosquito larvae, in some areas of the United States they were introduced as mosquito control. However, they are generalist predators and will also feed on other small insects, zooplankton, and detritus. In areas where they have been introduced, they compete with native fish species and are often considered a pest. Fish in general can be exposed to contaminats through ingestion of contaminated food, and via contaminant transport across the gills. Due to Gambusia's presense in the contaminated intermittent creek at Site-1 (and abundance across east Texas) and to their generalist feeding habits this species was ideal for biomarker research and enough individuals could be captured for an adequate sample size year-round.

The redear sunfish (*Lepomis microlophus*) belongs to the family Centrarchidae. Sunfish are a popular group of sport fish and have been widely introduced to many areas of North America. The redear sunfish prefers clear quiet pools in warm streams, ponds, lakes and reservoirs with adequate vegetation and cover (Boschung et al., 1983). The fish tend to nest in colonies where the males guard the eggs and the hatchlings. They

feed on snails, insect larvae and aquatic crustaceans, using their specialized jaws for crushing hard-shelled prey. Because they are predatory, the redear sunfish have the potential to bioaccumulate contaminants in their bodies. As sport fish, they may also transfer these contaminats to the humans that eat them.

Of the amphibians used in this research, the Northern cricket frog (*Acris crepitans*) is in the treefrog family (Hylidae). However, unlike its arboreal relatives the Northern cricket frog leads a terrestrial existence and is adept at leaping. They prefer slow moving creeks with sunny banks as well as shallow, sunny ponds with plenty of vegetation in the water or on shore (Behler and King, 1995) (similar to both Site-1a and Site-1b respectively). After hatching, the tadpoles feed on detritus and zooplankton. The adults feed on small terrestrial insects and spiders. Due to their biphasic nature, amphibians are vulnerable to both aquatic and terrestrial contamination. They may be exposed to environmental contaminants through ingestion, inhalation and absorption through their permeable skin. Habitat destruction and environmental contamination have been implicated in the decline of amphibian populations across North America.

The Gulf Coast toad (*Bufo valiceps*) is found along the Gulf of Mexico from southern Mississippi, through east Texas and south into Mexico (Behler and King, 1995). It belongs to the toad family Bufonidae. This family is characterized by having large parotoid glands located on each side of the neck that secrete a white viscous poison when the toad is attacked. The species prefers humid habitats ranging from roadside ditches to the barrier beaches of the Gulf of Mexico (Behler and King, 1995). It has even been located in city storm sewers (Behler and King, 1995). Adults are commonly found during twilight under streetlights catching insects. Breeding occurs during the spring and summer in both permanent and temporary ponds (sometimes shallow puddles of water after rain). As with the cricket frog described above, the Gulf Coast toad can be exposed to environmental contaminants through ingestion of contaminated food, inhalation of chemicals in the air, and absorption through their permeable skin.

4.2.2 Site-1 Sample Collection

  Mosquitofish (*Gambusia affinis*) were collected using hand-held dip nets from the creek (Site-1a) and associated references during May 2004 (N=1 composite sample for both Site-1a and Ref.-2a), August 2004 (Site-1a N=10, Ref.-1 N=22), February 2005 (Site-1a N=18, Ref.-1 N=23, Ref.-2b N=14,), and May 2005 (Site-1a N=26, Ref.-2b N=19, Ref.-2c N=19). Approximately 1-4 μL of caudal vein blood was collected from individual fish using a microhematocrit capillary tube (Fisher Scientific, 22-362566) and stored in 25 μL of citrate buffer (EcoGen, 1999) in cryogenic vials (Fisher Scientific, 09-761-71) (no blood was collected from fish in May 2004). All blood samples and fish carcasses were frozen on liquid nitrogen until they could be stored at -80ºC.

  Sunfish (*Lepomis microlophus*) were collected in May 2005 from the Site-1 pond (Site-1b) and associated references. The fish were divided into adults and juveniles (Site-1b adults N=4, Site-1b juveniles N=12, Ref.-2b adults N=4. Ref.-2c juveniles N=12). The juveniles were caught using dip-nets, whereas the adults were caught using baited traps and fishing poles. As with the *G. affinis* samples, caudal vein blood was collected from the sunfish and stored in 25μL of citrate buffer. Blood samples and carcasses were frozen on liquid nitrogen and then stored at -80ºC prior to analysis.

  Cricket frogs (*Acris crepitans*) were collected in August 2004 using hand-held dip nets from along the creek bank, on top of the waste cell adjacent to the pond, and from associated references (Site-1 N=18, Ref.-2d N=10, Ref.-3 N=8). Animals were sacrificed, placed in cryogenic vials, frozen whole on liquid nitrogen and then stored at -80ºC before dissection.

  Environmental media including co-located surface water samples were collected from the Site-1 creek and Ref.-2a in August 2004, and from the creek, pond and Ref.-2b and Ref.-2c in May 2005. Sediment samples from the creek and Ref.-2b and Ref.-2c were collected in May 2005. Surface waters were collected by hand using 1 L amber glass I-chem certified sampling bottles (VWR Scientific). Sediments were also collected by hand using pre-cleaned stainless steel trowels and stored in 16oz. glass I-

chem certified sampling jars (VWR Scientific). After collection, the water and sediments were transported on ice and stored at 4ºC prior to extraction.

4.2.3 Site-2 Sample Collection

In May 2004 there was considerable flooding in the wetland at Site-2. During the flooding mosquitofish were collected from a culvert that drains the wetland. It is assumed that these fish came from the northern part of the wetland where there is permanent standing water (see Fig. 27). This northern part of the wetland is not known to be heavily impacted by the contaminants. A composite sample of 10 fish was taken for chemical analysis only.

Gulf Coast toads (*Bufo valiceps*) were collected in August 2004 using hand-held dip nets from the southern tip of the wetland at Site-2 and from a reference wetland (Site-2 N=27 , Ref.-4 N=14). Specimens were frozen on liquid nitrogen and then stored at -80ºC prior to analysis. Surface water samples were collected in August 2004 using 1 L I-chem certified sampling bottles (VWR Scientific). The U.S. EPA remedial investigation was consulted for both surface water and sediment chemical analysis (CH2M Hill, 2005).

4.2.4 Flow Cytometry

Flow cytometry was used to detect chromosome damage and DNA changes in the collected specimens following the methods of Vindelov and Christensen (1994). All samples were randomized before processing to avoid experimental bias. For the amphibians, a small portion of the livers was excised for analysis while the carcasses were still largely frozen. The remainders of the carcasses were stored again at -80ºC until contaminant screening. Due to the small amount (1-4μL) of blood collected from the mosquitofish and sunfish, the entire 25 μL blood+citrate buffer sample from each fish was used in the analysis. Samples were quickly thawed and added to a trypsin/detergent solution for digestion. Trypsin inhibitor solution and RNase were added after 10 min. to stop the reaction and to degrade the RNA (which can also be

stained by the propidium iodide). The solution was then filtered through a 30 μm nylon mesh and 375 μL of propidium iodide (PI) was added. The samples were kept on ice for 15 min. and then analyzed on a Coulter Epics Elite flow cytometer (Beckman Coulter Inc., Fullerton, CA). Cells were illuminated with a laser (Coherent, Santa Clara, CA, USA) at 514 nm and 500 MW of power to excite the PI, and fluorescent emission was measured. Cells were gated on forward scatter, side scatter and the ratio of peak to integrated fluorescence. From each sample, 10,000 nuclei which satisfied all gating parameters were measured and intercellular variation in DNA content was reported as either half peak coefficient of variation (HPCV) (for fish and frogs) or full peak coefficient of variation (FPCV) (for toads). Samples that did not yield 10,000 nuclei within a 4-min. run were excluded from the statistical analyses.

4.2.5 Statistics

All FCM data were compared using SPSS ver. 11.0.1 software (SPSS Inc., Chicago, IL). Reference and experimental samples were compared by testing the samples for normality (Shapiro Wilk test for normality) and equal variance (Levene's test for equal variances). ANOVA and Bonferroni corrected post hoc comparisons were used to evaluate differences between Site-1 and reference mosquitofish and cricket frogs. A one-way ANOVA was used to compare the sunfish data from Site-1. A student's t-test was performed on the toad data from Site-2. For all tests, $p \leq 0.05$ was considered significant.

4.2.6 Chemical Extraction

4.2.6.1 Tissues

Cricket frogs from August 2004 were grouped according to high and low HPCV values. Mosquitofish from May 2005 were grouped according to high, medium and low HPCV values. Mosquitofish from May 2004, August 2004 and February 2005 were randomly composited for each sampling location. Toads were grouped according high,

medium and low FPCV values. Composite tissues were ground in a blender and dried with hydromatrix (Varian Inc., Palo Alto, CA, part #198003). The samples were extracted using a Dionex Model 200 Accelerated Solvent Extractor (ASE) (USEPA, 1996b) using pesticide-grade methylene chloride (VWR, BJ300-4). Tissue sample extracts were then run through silica gel columns (Resprep, Bellefonte, PA, part #24038) and eluted with 1:1 methylene chloride:pentane. The extracts were concentrated to 3 mL in a water bath (60ºC), re-suspended in methylene chloride, and processed through HPLC to minimize matrix interference. All samples were then analyzed using Gas Chromatography/Mass Spectrometry (GC/MS) for PAHs, PCP, and semi-volatile organics (USEPA, 1996c).

4.2.6.2 Surface Waters

In order to enhance extraction of the PCP, all samples were adjusted to pH 3 with hydrochloric acid (HCl). Separatory funnel liquid-liquid extraction using pesticide-grade methylene chloride (VWR, BJ300-4) was performed following the methods outlined in U.S. EPA method 3510C (USEPA, 1996a).

4.2.6.3 Sediments

Sediment extractions were performed using a Dionex (Dionex Corp., Sunnyvale, CA) Model 200 Accelerated Solvent Extractor (ASE) using a 1:1 ratio of hexane and acetone (U.S. EPA Method 3545, U.S. EPA 1996). Approximately 30 g of sediment from each sample was oven dried at 60°C for 16 hours. Ten g (±1 g) of the dried sediment was weighed for extraction. Following extraction, samples were transferred to pre-weighed sterile culture tubes with teflon-lined caps, dried under a stream of nitrogen and stored at 4°C until chemical analysis.

4.2.7 Chemical Analysis

Samples were analyzed for PAHs, PCP and other semivolatile organic compounds (SVOCs) using USEPA method 8270C (USEPA 1997). Analysis was

conducted on a Hewlett-Packard 5890 Series II gas chromatograph with a 5972 mass selective detector in selected ion monitoring mode. A 60m x 0.25mm ID x 0.25mm film thickness column (Agilent Technologies, Palo Alto, CA) was used. The injection port is maintained at 300ºC and the transfer line at 280ºC. The temperature program was as follows: 60ºC for 6 minutes, increased at 12ºC/minute to 180ºC and then increased at 6ºC/minute to 310º and held for 11 minutes for a total run time of 47 minutes. Table 5 shows a list of the total PAH analytes screened and highlights those carcinogenic PAHs, and priority pollutant PAHs described throughout the results.

4.3 Results

4.3.1 Mosquitofish (*Gambusia affinis*)

In May 2004, one composite sample each of mosquitofish from Site-1a, Site-2, and Ref.-2a were analyzed for priority pollutant PAHs. Data from the chemical analysis revealed elevated levels of PAHs in fish collected from Site-1a and from fish collected at the wetland drainage culvert from Site-2 (Fig. 28). There was not a large difference between the contaminated wetland at Site-2 and the reference site in terms of total PAHs (Fig. 28). However, when looking only at the carcinogenic PAHs (cPAHs), there was about 3 times the amount of cPAHs at the contaminated wetland at Site-2 compared to the reference site (21 ng/wet g vs. 8 ng/wet g respectively). Tissue chemical analysis from August 2004, February 2005, and May 2005 samples also revealed elevated levels of total PAHs in mosquitofish from Site-1a compared to the references (Fig. 29).

The maximum tissue concentrations of priority pollutant PAHs were seen in the composite sample collected in May 2004 during a flooding event (See Fig. 30 for relative water levels during mosquitofish sampling events). At this time the fish were evenly distributed throughout the sampled creek area at Site-1a. Looking at the maximum surface water concentrations taken from Site-1a in May 2005 (a year later), eight of the priority pollutant PAHs are above the screening level criteria for fish in surface waters (Table 7). During the time the surface water sample in Table 7 was

taken, the creek water level at Site-1a was low and there were free standing pools of water (Fig. 30). The pool from which this surface water was sampled yielded no organisms, confirming the high toxicity of the water for aquatic life. The data suggest that seasonal variability in water levels at Site-1a considerably affect fish exposure. Exposure may be high for fish during flooding events when the contaminants are more mobile in the water column. During dry periods, fish isolated to the water pools with highest contaminant concentrations would die. Whereas, fish isolated in cleaner pools of water (such as those fish collected in May 2005) would have increased survivability and decreased contaminant body burden. Table 8 shows the variability in the co-located water samples collected from the reference sites and Site-1a in August 2004 and May 2005. In May 2004 there was a definite contamination gradient seen as samples were collected from areas with the highest concentration (Site-1a-1) where there was no life, to pools with lower concentrations (Site-1a-3 & 4) from which fish were taken (Table 8).

In August 2004, the mosquitofish FCM data from Site-1a and Ref.-1 were not normally distributed. Using a non-parametric test there was a significant difference in FPVCs between the sites (Mann-Whitney U, $p = 0.021$). It was decided to repeat sampling at the same locations, increase the sample numbers and add another reference for comparison. In February 2005, HPCV data from Site-1a, Ref.-1, and Ref.-2b were normally distributed. Using a one-way ANOVA, there was a significant difference between the sampling sites ($p = 0.005$). A Bonferroni corrected post-hoc comparison was used to compare the 3 different sites. There was a significant difference between HPCV values from Site-1a and Ref.-2b ($p = 0.004$) (Fig. 31). The contaminated site showed significantly elevated levels of genetic damage compared to Ref.-2b. There was no significant difference between Site-1a and Ref.-1 ($p = 0.591$) (Fig. 31).
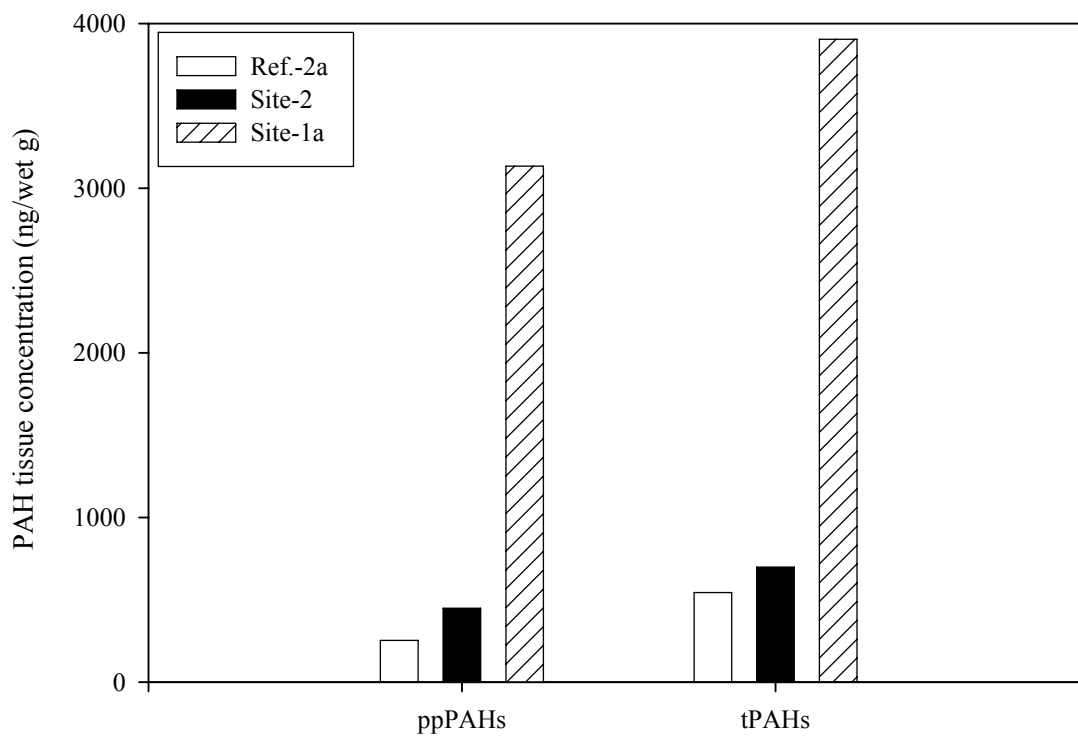
Fig. 28. PAHs detected in extracts of *Gambusia affinis* (mosquitofish) collected from a reference location (Ref.-2a), and 2 contaminated sites in May 2004 (Site-1a and Site-2). <u>One</u> composite sample was created for each site by compositing tissue from 10 fish from each site (ppPAHs = priority pollutant PAHs, tPAHs = total PAHs).
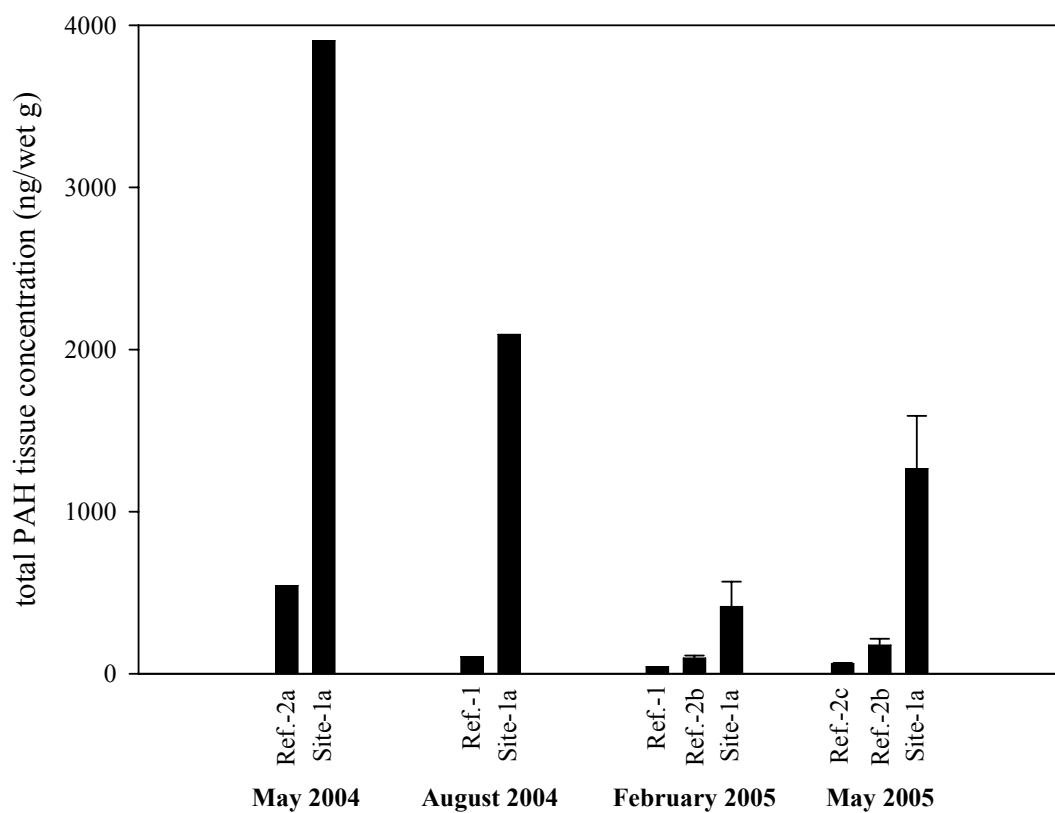
Fig. 29. Total PAH tissue concentrations (ng/wet g) detected in whole body composite samples of mosquitofish collected from Site-1 creek (Site-1a) and corresponding references (for May 2004 and August 2004, N=1 composite sample for each site; for all other sampling dates, N=3 composite samples for each site). Data are presented as mean $\pm$ SEM.
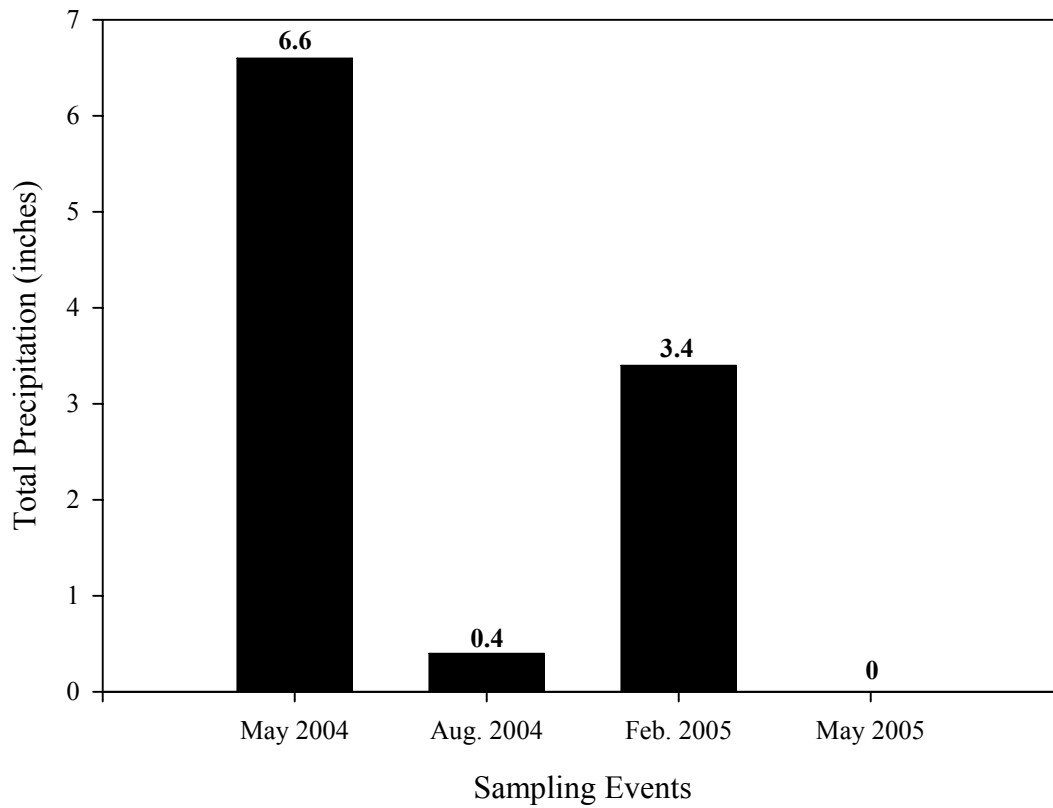
Fig. 30. Total precipitation associated with each mosquitofish (*Gambusia affinis*) sampling event from Site-1a including the days in which samples were collected plus 7 days (1 week) prior to sample collection.

Table 7. Priority pollutant chemicals (PAHs and PCP) in surface water and corresponding screening level criteria for fish at Site-1a. The composite mosquitofish sample collected in May 2004 represents the maximum tissue concentration of priority pollutant PAHs from Site-1a.

[a]Chemical Class	Chemical	[b]Maximum Concentration (surf. water) ng/L	[c]Screening Level Criteria ng/L	Maximum Tissue Concentration ng/wet g
L(mw) PAH	**Naphthalene**	301	250000	147
L(mw) PAH	**Acenaphthylene**	4000	5800	7
L(mw) PAH	**Acenaphthene**	290	2300	675
L(mw) PAH	**Anthracene**	*10300*	300	21
L(mw) PAH	**Fluorene**	2320	11000	543
L(mw) PAH	**Dibenzofuran**	238	NSL	511
L(mw) PAH	**Phenanthrene**	*32700*	30000	954
H(mw) PAH	**Fluoranthene**	*58300*	6160	174
H(mw) PAH	**Pyrene**	*45600*	7000	72
H(mw) PAH	**Benz(a)anthracene**	19000	34600	4
H(mw) PAH	**Chrysene**	*31300*	7000	6
H(mw) PAH	**Benzo(b)fluoranthene**	*36200*	27	4
H(mw) PAH	**Benzo(k)fluoranthene**	*11600*	27	1
H(mw) PAH	**Benzo(a)pyrene**	*13300*	14	12
H(mw) PAH	**Indeno(1,2,3-c,d)pyrene**	*4830*	27	1
H(mw) PAH	**Dibenzo(a,h)anthracene**	1250	5000	0.3
H(mw) PAH	**Benzo(g,h,i)perylene**	3330	7640	1
Organochlorine	**Pentachlorophenol**	201	3133	NA

[a]L(mw) PAH = low molecular weight ($<$ 200 atomic mass units) PAH, H(mw) PAH = high molecular weight ($>$ 200 atomic mass units).

[b]***Bold italics*** denote tissue concentrations that are above screening level criteria for fish.

[c]Screening level criteria for surface waters were derived from sources detailed in the remedial investigation (CH2M Hill, 2005),

NSL = no screening level.

NA = Not available.

Table 8. Summary chemical analysis of co-located surface water samples collected from Site-1a (creek) and corresponding references during August 2004 and May 2005 mosquitofish sampling events. Data were averaged for locations with more than 1 sample collected. For averaged samples, data are presented as mean ng/L $\pm$ SEM.

Sample date & location	N	tPCP ng/L	tBaP ng/L	cPAHs ng/L	ppPAHs ng/L	tPAHs ng/L
Aug. 2004						
Ref.-1	2	997 $\pm$ 794	11 $\pm$ 0.05	191 $\pm$ 35	4710 $\pm$ 2720	7600 $\pm$ 4070
Ref.-2a	1	5800	50	965	8330	15300
Ref.-2b	1	3800	10	135	4460	7170
Site-1a-1	1	80	86	714	3650	5880
Site-1a-2	1	160	1140	8280	16170	24400
Site-1a-3	1	50	800	6080	15600	22700
May 2005						
Ref.-2b	1	19	1	44	713	1210
Ref.-2c	1	24	9	131	2910	4740
Site-1a-1	1	201	13300	117000	275000	555000
Site-1a-2	1	59	2390	38400	121000	187000
Site-1a-3	1	38	303	3940	6530	10600
Site-1a-4	1	71	23	383	1410	2280

tPCP = total pentachlorophenol, tBaP = total benzo(a)pyrene, cPAHs = total carcinogenic polycyclic aromatic hydrocarbons (N=7), ppPAHs = total priority pollutant PAHs (N=17), and tPAHs = total PAHs.
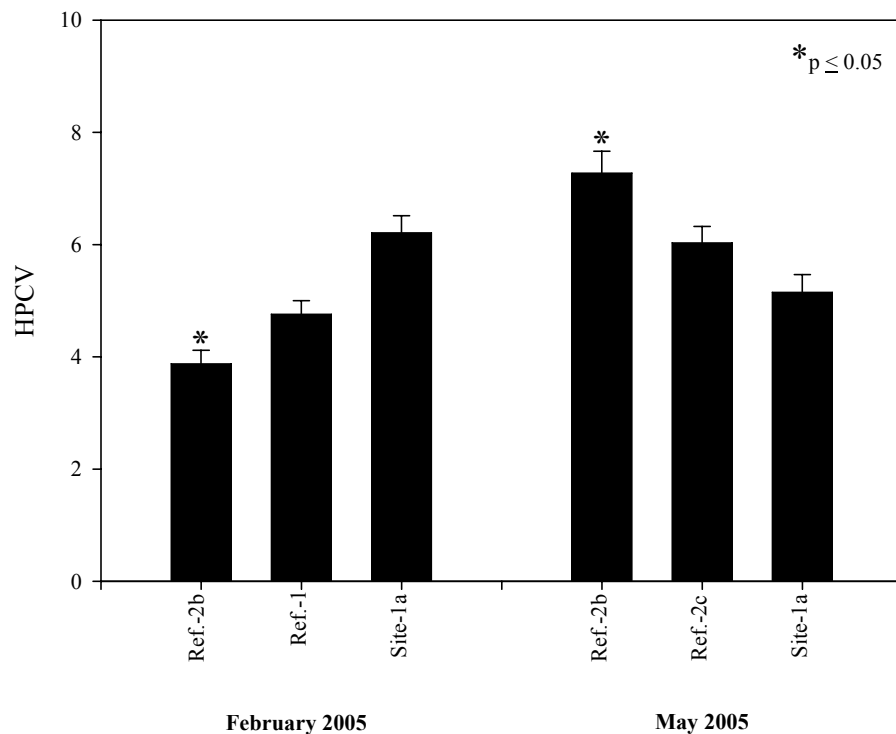
Fig. 31. HPCV flow cytometry data from mosquitofish collected from Site-1a and corresponding references. Data are presented as mean HPCV ± SEM. *Reference HPCV means were significantly different from Site-1a HPCV means (p ≤ 0.05).

The final sampling event was conducted in May 2005. Ref.-1 was deleted from the sample design in favor of using Ref.-2b and Ref.-2c located within the same state park. Using HPCV values, the data were not normally distributed (*p* < 0.05). Following a fractional rank transformation of the data, a non-parametric ANOVA was used to compare the sites. There was a significant difference between the sampling sites (*p* = 0.047). Using a Bonferroni corrected post-hoc comparison, there was a significant difference in HPCV values between the Site-1a and Ref.-2b (*p* = 0.043). However, contradictory to the second sampling event, Ref.-2b had significantly elevated levels of genetic damage compared to the contaminated site (Fig. 31). There was neither a significant difference between Site-1a and Ref.-2c nor between the two reference sites.

Following flow cytometry analysis, the individual fish from May 2005 were equally grouped according to low, medium, and high HPCV values for contaminant screening. This was done to compare levels of genetic damage with whole body burdens of priority pollutants. Although low contaminant levels were seen in the low HPCV group from Site-1a and high contaminant levels seen in the high HPCV group from Site-1a, the medium HPCV group showed higher levels of total PAHs than did the high HPCV group (Fig. 32).
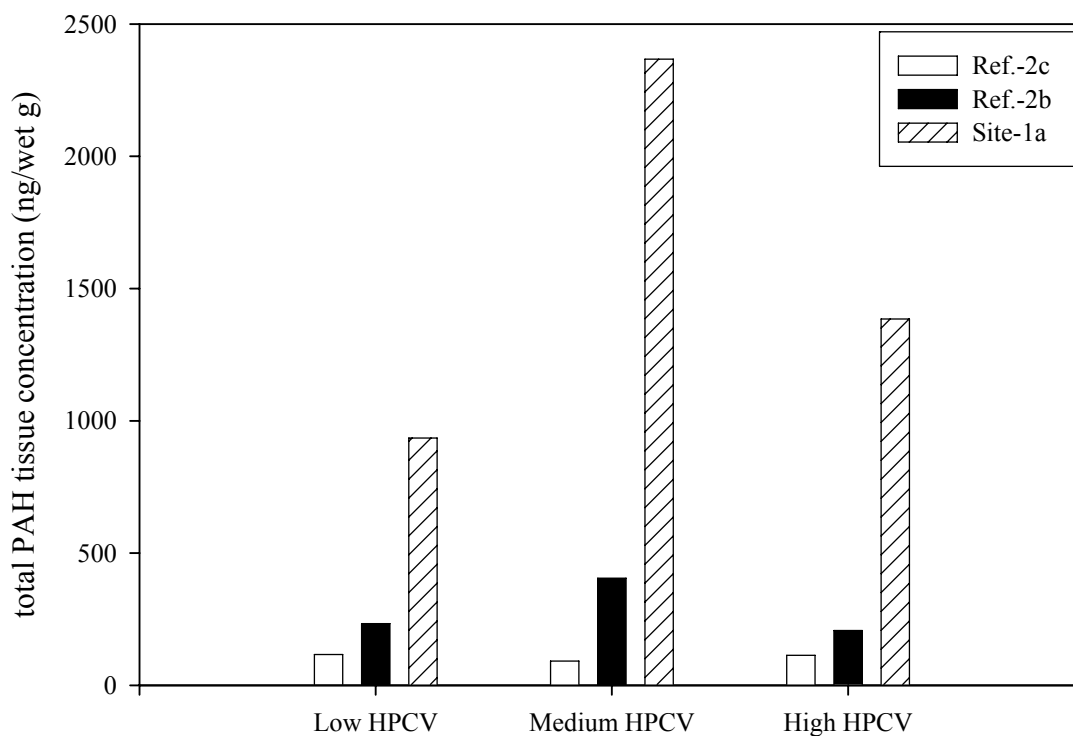
Fig. 32. Total PAH concentrations in mosquitofish tissues grouped according to low, medium and high HPCV values for Site-1a and two corresponding references sampled in May 2005 (N=1 composite tissue for each HPCV group).

4.3.2 Redear Sunfish (*Lepomis microlophus*)

For the juvenile redear sunfish collected from Site-1b, HPCV values from blood samples were normally distributed and equal variances were assumed based on results of the Levene's test for equal variances. There was no significant difference in HPCV values between Site-1b and Ref.-2c ($p = 0.158$) using a one-way ANOVA (Fig. 33). Adult sunfish were also sampled from Site-1b. The data were normally distributed and equal variances were assumed. However, using a one-way ANOVA there was no significant difference between Site-1b and Ref.-2b ($p = 0.051$) (data not shown).

Tissue analysis was not performed for this species. However, only benzo(b)fluoranthene was above the surface water screening level criteria for fish (Table 9). Surface water chemical analysis showed that the water chemistry between Site1-b and Ref.-2c was not very different with the reference showing higher levels of contamination than Site-1b (Table 10). Ref.-2c had over three times the amount of PAHs compared to Ref.-2b. Ref.-2b was from a large reservoir whereas Ref.-2c was from a slough area that was beginning to dry up (might have been concentrating the chemicals).
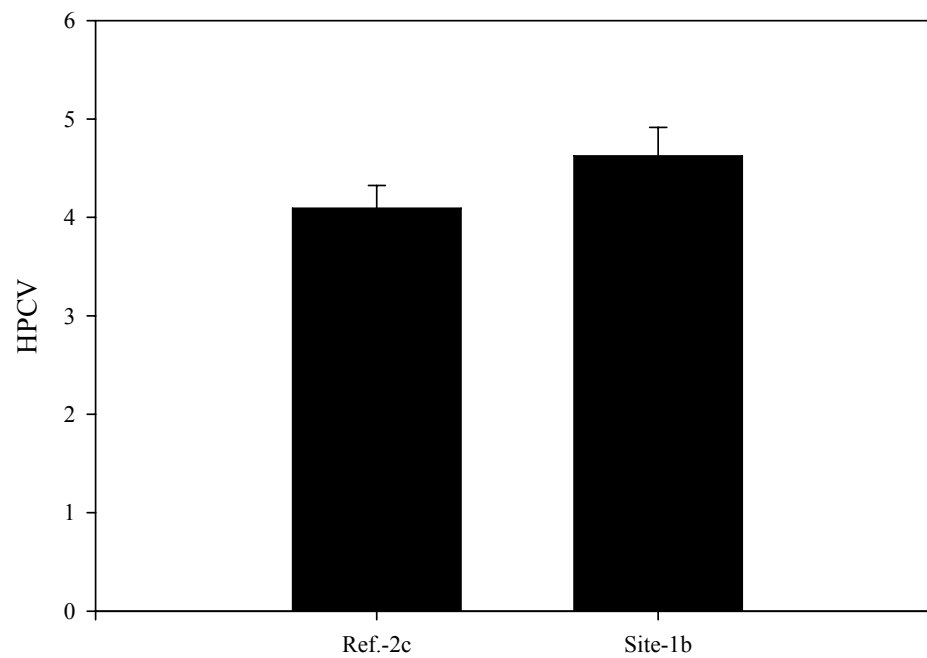
Fig. 33. HPCV flow cytometry data from juvenile sunfish (*Lepomis microlophus*) collected from Site-1b and corresponding reference in May 2005. There was no significant difference between the two locations. Data are presented as mean HPCV $\pm$ SEM.

Table 9. Priority pollutant chemicals (PAHs and PCP) in surface water and corresponding screening level criteria for fish at Site-1b.

Chemical	[a]Maximum Concentration (surf. water) ng/L	[b]Screening Level Criteria ng/L
Naphthalene	1790	250000
Acenaphthylene	33	5800
Acenaphthene	287	2300
Anthracene	248	300
Fluorene	143	11000
Dibenzofuran	173	NSL
Phenanthrene	248	30000
Fluoranthene	228	6160
Pyrene	111	7000
Benz(a)anthracene	17	34600
Chrysene	44	7000
Benzo(b)fluoranthene	*48*	27
Benzo(k)fluoranthene	17	27
Benzo(a)pyrene	13	14
Indeno(1,2,3-c,d)pyrene	5	27
Dibenzo(a,h)anthracene	0	5000
Benzo(g,h,i)perylene	4	7640
Pentachlorophenol	10	3130

[a]*Bold italics* denote tissue concentrations that are above screening level criteria for fish.
[b]Screening level criteria for surface waters were derived from sources detailed in the remedial investigation (CH2MHill, 2005). NSL = No screening level.

Table 10. Summary chemical analysis of co-located surface water samples collected from Site-1b (pond) and corresponding references during May 2005 redear sunfish sampling events. Ref.-2b is the location where reference adult sunfish were collected and Ref.-2c is where reference juvenile sunfish were collected. Data were averaged for locations with more than 1 sample collected. For averaged samples, data are presented as mean ng/L $\pm$ SEM.

Sample date & location	N	tPCP ng/L	tBaP ng/L	cPAHs ng/L	ppPAHs ng/L	tPAHs ng/L
May 2005						
Ref.-2b (adults)	1	19	1.2	44	713	1210
Ref.-2c (juveniles)	1	24	9	131	2912	4739
Site-1b	3	21 ± 6	7 ± 3	93 ± 28	2160 ± 522	3250 ± 660

tPCP = total pentachlorophenol, tBaP = total benzo(a)pyrene, cPAHs = total carcinogenic polycyclic aromatic hydrocarbons (N=7), ppPAHs = total priority pollutant PAHs (N=17), and tPAHs = total PAHs

4.3.3 Cricket Frogs (*Acris crepitans*)

Initially, the specimens collected in August 2004 along the banks of Site-1a and from the top of the waste cell near Site-1b were grouped separately during FCM analysis. However, statistically there was no difference between these groups (student's t-test, $p=0.95$) and they were combined as one site in the final analysis. HPCV values were normally distributed. Using an ANOVA, there was a significant difference between sites ($p=0.036$). A Bonferroni corrected post-hoc comparison was used to compare differences among sites. There was a significant difference in HPCV values between Site-1 and Ref.-3 ($p = 0.05$) with Site-1 showing elevated levels of genetic damage compared to the reference (Fig. 34). However, there was not a significant difference between Site-1 and the Ref.-2d ($p = 1.00$).
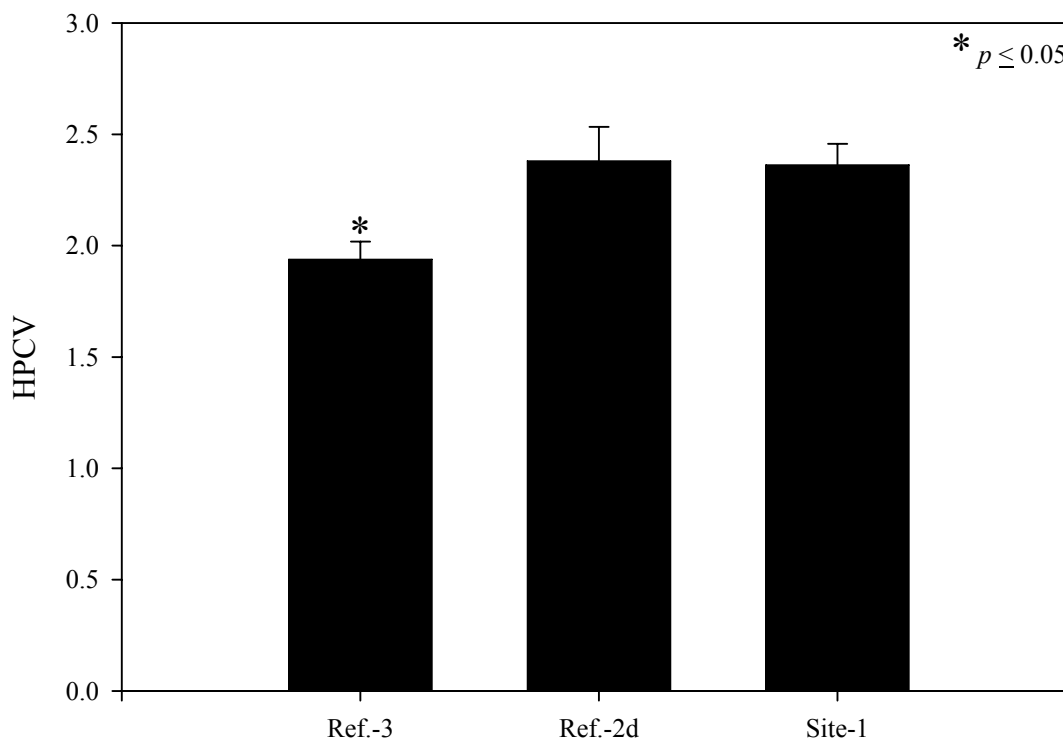
Fig. 34. HPCV flow cytometry data from cricket frogs (*Acris crepitans*) collected from Site-1 and corresponding references. Data are presented as mean HPCV + SEM. *Ref.-3 HPCV mean was significantly different from Site-1 HPCV mean ($p \leq 0.05$).

Following flow cytometry analysis, individual frogs were equally grouped according to low and high HPCVs for chemical analysis. Levels of genetic damage corresponded well with total PAH concentrations in the pooled tissues (Fig. 35).

Co-located surface water samples were taken from Site-1 and associated references. Table 11 shows the maximum priority pollutant PAH concentrations found in surface waters collected from Site-1a and Site-1b and the corresponding lowest observed effect concentrations and hazard quotients for amphibians. At Site-1a, the levels of PAHs found in the surface water exceeded a hazard index of one (HI = 20) (Table 11). A hazard index above one indicates that adverse effects in amphibians from

total PAH exposure is likely. However, Site-1b had a hazard index of less than one (HI =0.23) (Table 11). The tissue sample that yielded the highest level of PAHs did not surpass the toxicity reference value (TRV) for benzo(a)pyrene (10,200 ng/wet g) which was used as a surrogate for PAHs in the remedial investigation (CH2M Hill, 2005) (Table 11). Table 12 shows PAH chemistry from the co-located surface water samples collected in August 2004 during the time of cricket frog sampling. Two samples from Site-1a had roughly double the amount of total PAHs compared to Ref.-3. Samples from Site-1a also had approximately fifty times the amount of BaP compared to Ref.-3 (Table 12).
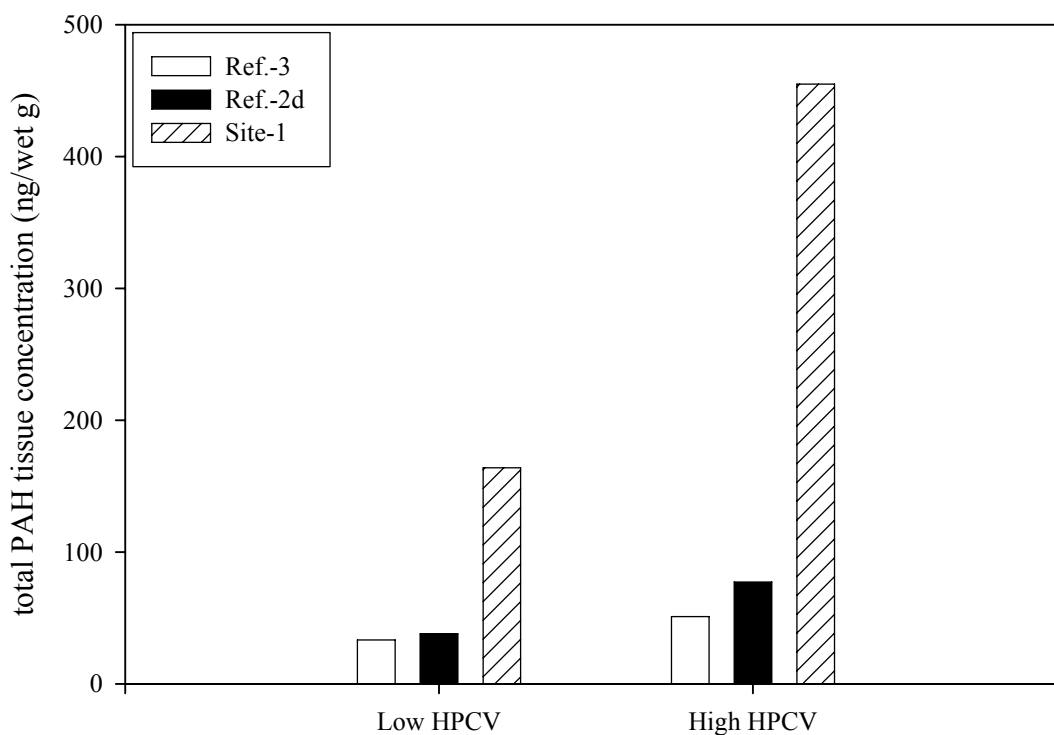
Fig. 35. Total PAH concentrations in cricket frog (*Acris crepitans*) tissues grouped according to low and high HPCV values for Site-1 and two corresponding references sampled in August 2004 (N=1 composite tissue for each HPCV group).

Table 11. Priority pollutant chemicals (PAHs and PCP) in surface water and corresponding LOEC HQ values for amphibians at Site-1a & Site-1b. The composite cricket frog sample with the maximum tissue concentrations of priority pollutant PAHs from Site-1 is also shown.

Chemical	Maximum Conc. Site-1a (surf. water) ng/L	Maximum Conc. Site-1b (surf. water) ng/L	Species (used to derive LOEC) (CH2M Hill, 2005)	Receptor Life Stage	Normalized LOEC ng/L (CH2M Hill, 2005)	[a]LOEC HQ Site-1a	[a]LOEC HQ Site-1b	Maximum Tissue Conc. ng/wet g
Naphthalene	301	1790	*Xenopus laevis*	Tadpole	210000	0.0014	0.0085	10
Acenaphthylene	4000	33	*Xenopus laevis*	Tadpole	210000	0.0190	0.0002	1
Acenaphthene	290	287	*Xenopus laevis*	Tadpole	210000	0.0014	0.0014	49
Anthracene	10300	248	*Rana pipiens*	Embryo	2500	4.1200	.09920	2
Fluorene	2320	143	*Xenopus laevis*	Tadpole	210000	0.0111	0.0007	29
Phenanthrene	32700	248	*Xenopus laevis*	Tadpole	210000	0.1557	0.0012	144
Dibenzofuran	238	173	NA	NA	NA	NA	NA	25
Fluoranthene	58300	228	*Rana pipiens*	Tadpole	9000	6.4778	0.0253	74
Pyrene	45600	111	*Rana pipiens*	Tadpole	14000	3.2571	0.0079	50
Benz(a)anthracene	19000	17	*Pleurodeles waltl*	Embryo	50000	0.3800	0.0003	7
Chrysene	31300	44	*Pleurodeles waltl*	Embryo	50000	0.6260	0.0009	33
Benzo(b)fluoranthene	36200	48	*Pleurodeles waltl*	Embryo	50000	0.7240	0.0010	5
Benzo(k)fluoranthene	11600	17	*Pleurodeles waltl*	Embryo	50000	0.2320	0.0003	2
Benzo(a)pyrene	13300	13	*Pleurodeles waltl*	Embryo	50000	2.6600	0.0003	0.4
Indeno(1,2,3-c,d)pyrene	4830	5	*Pleurodeles waltl*	Embryo	50000	0.0966	0.0001	1
Dibenzo(a,h)anthracene	1250	ND	*Pleurodeles waltl*	Embryo	50000	0.0250	ND	0.2
Benzo(g,h,i)perylene	3330	4	*Pleurodeles waltl*	Embryo	50000	0.0666	0.0001	0.5
Pentachlorophenol	201	10	*Bufo bufo japonicus*	Tadpole	125	1.608	0.0824	NA
					[b]**HI =**	**20**	**0.23**	

[a]LOEC HQ = lowest observed effect concentration hazard quotient; formula = maximum surface water concentration ng/L / normalized LOEC ng/L.
[b]HI = Hazard Index (HI = $HQ_1 + HQ_2 \ldots HQ_{16}$ for each priority pollutant listed).
NA = Not available, ND = Not Detected

Table 12. Summary chemical analysis of co-located surface water samples collected from Site-1a (creek) and corresponding references during August 2004 cricket frog sampling events. Data were averaged for locations with more than 1 sample collected. For averaged samples, data are presented as mean ng/L $\pm$ SEM.

Sample date & location	N	tPCP ng/L	tBaP ng/L	cPAHs ng/L	ppPAHs ng/L	tPAHs ng/L
Aug. 2004						
Ref.-2d	1	1440	2	84	2590	4540
Ref.-3	3	776 $\pm$ 337	21 $\pm$ 7	528 $\pm$ 114	6740 $\pm$ 1580	10400 $\pm$ 1970
Site-1a-1	1	80	86	714	3650	5880
Site-1a-2	1	160	1140	8280	16170	24380
Site-1a-3	1	53	828	6080	15570	22720

tPCP = total pentachlorophenol, tBaP = total benzo(a)pyrene, cPAHs = total carcinogenic polycyclic aromatic hydrocarbons (N=7), ppPAHs = total priority pollutant PAHs (N=17), and tPAHs = total PAHs.

4.3.4 Gulf Coast Toads (*Bufo valiceps*) and Site-2 Media

      Juvenile Gulf Coast toads were collected from Site-2 in August 2004. Full Peak Coefficients of Variation (FPCV) were calculated as an estimate of intra-individual genome size variability, a biomarker of chromosomal damage. FPCV values were normally distributed. Using a t-test the FPCV values from Site-2 specimens were significantly elevated compared to FPCV values from Ref.-4 specimens ($p < 0.001$) (Fig. 36).
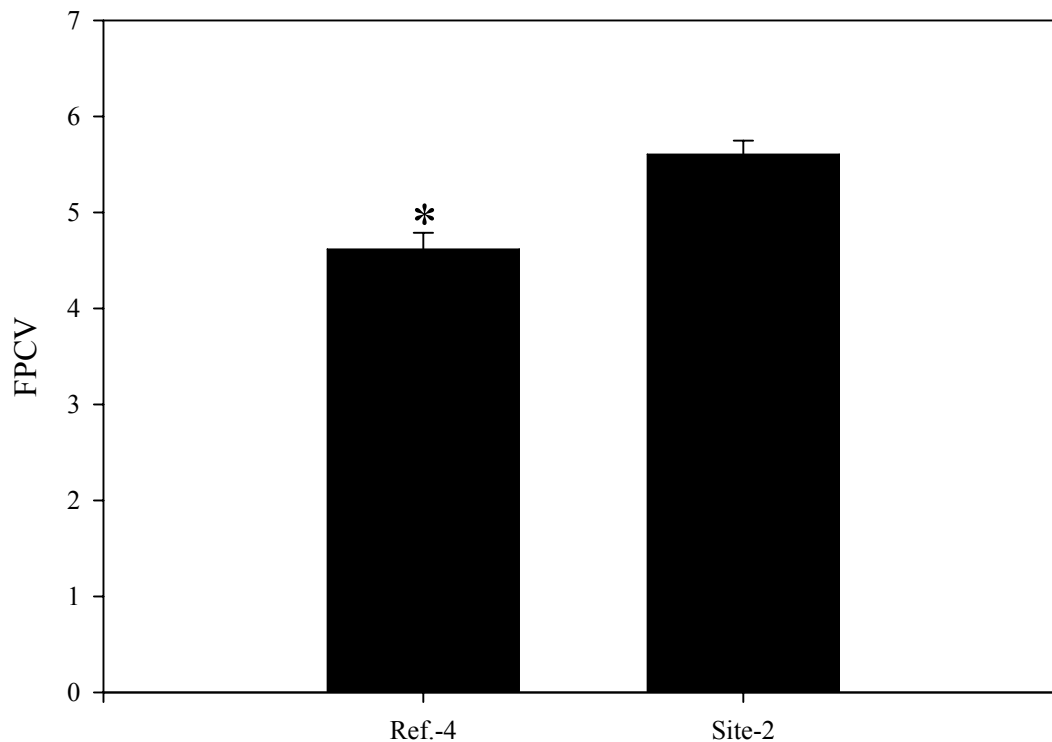
Fig. 36. FPCV flow cytometry data from juvenile Gulf Coast toads (*Bufo valiceps*) and Ref.-4. Data are presented as mean HPCV $\pm$ SEM. *Reference HPCV means were significantly lower than Site-2 HPCV means ($p < 0.001$).

Tissue contaminant analysis was variable for the contaminated site samples and the reference and did not correspond well with FPCV groupings. The medium FPCV sample had the highest levels of PAHs (236 ng/wet g) and the high FPCV sample had the lowest levels of PAHs (124 ng/wet g) (Fig. 37).
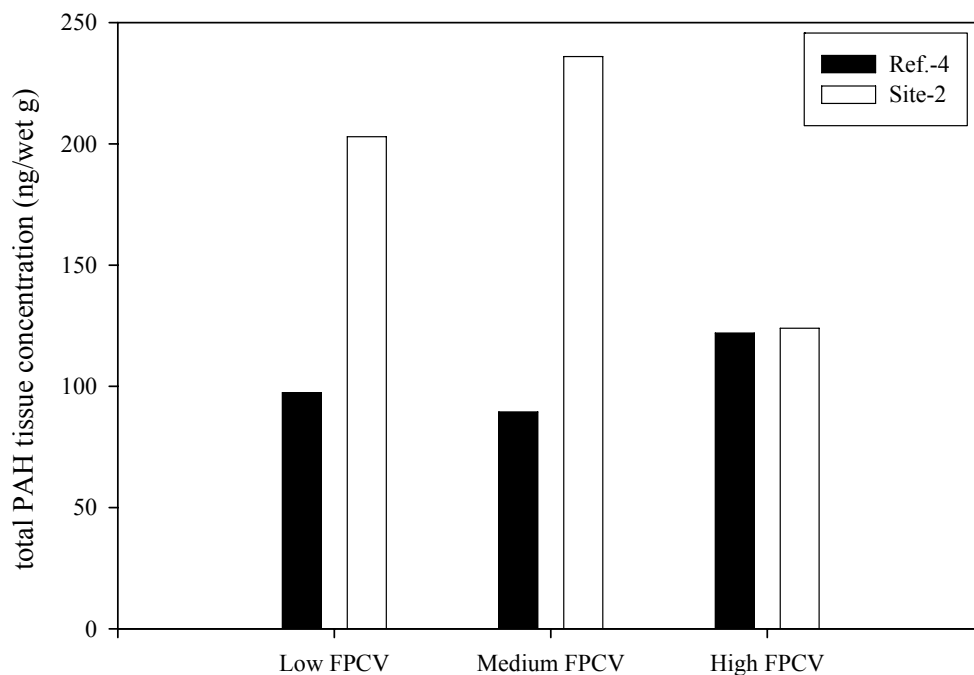
Fig. 37. Total PAH concentrations in juvenile Gulf Coast toad (*Bufo valiceps*) tissues grouped according to low, medium and high FPCV values for Site-2 and Ref.-4 sampled in August 2004 (N=1 composite tissue for each FPCV group).

Table 13. Summary chemical analysis of co-located surface water samples collected from Site-2 and corresponding reference during August 2004 Gulf coast toad sampling events. Data were averaged for locations with more than 1 sample collected. For averaged samples, data are presented as mean ng/L $\pm$ SEM.

Sample date & location	N	tPCP ng/L	tBaP ng/L	cPAHs ng/L	ppPAHs ng/L	tPAHs ng/L
Aug. 2004						
Ref.-4	3	962 $\pm$ 693	180 $\pm$ 157	2920 $\pm$ 2420	11300 $\pm$ 6550	17900 $\pm$ 10200
Site-2	2	250 $\pm$ 129	64 $\pm$ 47	531 $\pm$ 360	3120 $\pm$ 154	4660 $\pm$ 841

tPCP = total pentachlorophenol, tBaP = total benzo(a)pyrene, cPAHs = total carcinogenic polycyclic aromatic hydrocarbons (N=7), ppPAHs = total priority pollutant PAHs (N=17), and tPAHs = total PAHs.

Co-located water samples at Site-2 were collected in August 2004 from puddles about 300 ft. north of where toads were collected and away from the contaminant zone. This was the first surface water encountered. The toad's natal pools had presumably evaporated by the time of specimen collection. Water from the reference was collected from a creek in the wetland area and as with Site-2, may not be indicative of toad surface water exposure from their natal puddles. As shown in Table 13, surface water from the reference was more contaminated with PAHs and PCP than the surface water from Site-2. However, surface water samples collected from the forested wetland during the remedial investigation contained levels of PAHs above the screening values (CH2M Hill, 2005). Of the 17 priority pollutant PAHs found in the surface water, the low molecular weight PAHs (L(mw)PAHs) were below the screening levels. Fluoranthene, benzo(b)fluoranthene, benzo(k)fluoranthene, indeno(1,2,3-c,d)pyrene and BaP were the high molecular weight PAHs (L(mw)PAHs) above screening levels in the surface water (CH2M Hill, 2005). Surface waters also contained PCP and dioxins above screening levels (CH2M Hill, 2005). Sediment chemistry from the remedial investigation indicated that all surface sediment samples (0-1 ft. deep) within the toad sampling area were above screening values for BaP, total PAHs and PCP (CH2M Hill, 2005). In addition to PCP and the H(mw)PAHs, dioxins and zinc were also among the contaminants of concern for vertebrates (deer mice, mink , armadillo and American woodcock) in the wetland (CH2M Hill, 2005). Since the sediments in the wetland are often exposed during dry spells, adult amphibians are likely to be in close contact with the contaminated sediments and surface waters.

4.3.5 Sediment Site-1

Sediments were collected from Site-1a, Site-1b, Ref.-2b and Ref.-2c in May 2005. Table 14 shows the priority pollutant chemicals detected in the sediments and their corresponding screening level criteria for the benthic communities at both Site-1a and Site-1b. In general, sediments collected from both Site-1a and 1b had elevated levels of PAHs compared to the reference samples (Table 15). Comparing those

samples with the highest concentrations of PAHs to screening level criteria for benthic organisms, it is apparent that PAHs in the sediments exceed the screening level criteria at both Site-1a and Site-1b. Site-specific toxicity reference values were derived for benthic invertebrates using *ex-situ* toxicity tests. All sediment samples exceeded a hazard index of 1 for the PAHs tested (CH2M Hill, 2005).

4.4 Discussion

Over the course of one year, two hazardous waste sites contaminated with wood preserving waste were sampled for biota and environmental media. Of the biota samples, mosquitofish (*Gambusia affinis*), redear sunfish (*Lepomis microlophus*), and cricket frogs (*Acris crepitans*) were obtained from Site-1 and Gulf Coast toads (*Bufo valiceps*) were collected from Site-2. Although the results varied among species, mosquitofish and frogs collected from Site-1 and toads collected from Site-2 exhibited increased markers of genotoxic damage as measured by FCM. Markers of genetic damage were consistent with increased concentrations of PAHs in tissues compared to reference animals, despite the fact that tissue levels were far below the lowest observed effect concentrations reported in the remedial investigation (CH2M Hill, 2005). Surface water and sediment sample PAH concentrations exceeded screening level criteria for fish, amphibians, and benthic organisms at Site-1a. Sediment samples from Site-1b exceeded screening level criteria for the benthic community. Surface water and sediment samples exceeded PAH screening level criteria for vertebrates at Site-2. Genetic damage in the mosquitofish from Site-1a was variable and could have been due to several factors including (but not limited to): contamination at the reference site, fluctuating water levels in Site-1a altering fish exposure, changes in water temperatures between February and May, and adaptation by the fish to the contaminants. Although mosquitofish collected in May 2005 showed elevated levels of PAHs in their tissues compared to the reference fish, levels of genetic damage at the contaminated site were actually lower in May than the references. One of the challenges in performing ecological risk assessments is finding appropriate references. Due to the high variability

Table 14. Priority pollutant chemicals (PAHs and PCP) in sediments collected in May 2005 and corresponding screening level criteria for benthic communities at Site-1a and Site-1b.

Chemical	[a]Maximum Concentration Site-1a ng/dry g sed.	[a]Maximum Concentration Site-1b ng/dry g sed.	[b]Screening Level Criteria ng/dry g sediment
Acenaphthylene	*1490*	*87*	6
Acenaphthene	*39000*	*102*	7
Anthracene	*47500*	*738*	57
Fluorene	*143000*	*508*	77
Dibenzofuran	*110000*	*203*	NSL
Phenanthrene	*214000*	*2550*	204
Fluoranthene	*169000*	*2100*	423
Pyrene	*114000*	*1720*	195
Benz(a)anthracene	*83100*	*975*	108
Chrysene	*62500*	*1350*	166
Benzo(b)fluoranthene	*51100*	*1730*	6
Benzo(k)fluoranthene	*20700*	*562*	240
Benzo(a)pyrene	*7410*	*644*	150
Indeno(1,2,3-c,d)pyrene	*6900*	*465*	200
Dibenzo(a,h)anthracene	*2000*	*108*	33
Benzo(g,h,i)perylene	*3190*	*290*	170
Total PAH	*1550000*	*20300*	12200
Pentachlorophenol	0	71	--

[a]Bold italics denote sediment concentrations that are above screening level criteria for the benthic community.
[b]Screening level criteria for sediments were derived from sources detailed in the remedial investigation (CH2M Hill, 2005).
NSL = No Screening Level

Table 15. Summary chemical analysis of co-located sediment samples collected from Site-1a (creek), Site-1b (pond) and corresponding references during May 2005 sampling events. Data were averaged for locations with more than 1 sample collected. For averaged samples, data are presented as mean ng/dry g sediment $\pm$ SEM.

Sample date & location	N	tPCP ng/dry g sediment	tBaP ng/dry g sediment	cPAHs ng/dry g sediment	ppPAHs ng/dry g sediment	tPAHs ng/dry g sediment
May 2005						
Ref.-2b	1	34	7	70	1090	1630
Ref.-2c	1	7	16	1090	2880	4160
Ref.-3	3	24 $\pm$ 10	5 $\pm$ 0.4	47 $\pm$ 7	548 $\pm$ 261	828 $\pm$ 315
Site-1a-1	1	3	521	6320	10500	17200
Site-1a-2	1	0	12500	110000	330005	519000
Site-1a-3	1	0	7410	234000	1080000	1550000
Site-1a-4	1	61	84	648	4750	6440
Site-1b	3	46 $\pm$ 21	342 $\pm$ 173	3890 $\pm$ 1660	9400 $\pm$ 4230	13500 $\pm$ 6040

tPCP = total pentachlorophenol, tBaP = total benzo(a)pyrene, cPAHs = total carcinogenic polycyclic aromatic hydrocarbons (N=7), ppPAHs = total priority pollutant PAHs (N=17), and tPAHs = total PAHs.

inherent in ecosystems, references close to the study sites could be considerably different and may also be contaminated. Reference 2 (a-d) in this study has several mercury advisory signs posted throughout the park. It is known that mercury has the ability to cause chromosome damage (De Flora et al., 1994; Zucker et al., 1990) and may have contributed to elevated genetic damage in the fish and amphibians collected from areas within Ref.-2. In addition to the mercury problem at the reference location, there was also considerable boat traffic during the May 2005 sampling event. This lead to the consideration that elevated levels of genetic damage seen in the reference fish from Ref.-2b could be attributed to gasoline pollution from boat traffic. However, the surface water and sediment analysis did not confirm greater levels of PAHs at Ref.-2 compared to Site-1.

Moreover, with Site-1a in particular, the water levels in the creek appear to correspond to increased levels of genetic damage. When water levels were at their highest in May 2004, tissue body burden in mosquitofish was also at its highest. In February 2005, water levels were higher than during May 2005 which might help to explain the increased levels of genetic damage seen in the fish in February compared to May. Although total PAH tissue body burden in the May 2005 mosquitofish was greater than the February 2005 fish, it is possible that the fish collected in May 2005 had had longer to adjust to the contaminants in their isolated pool. Studies have shown that exposed animals may exhibit increased genetic damage immediately after exposure to a mutagen followed by recovery and sometimes lower genetic damage compared to controls (Bickham et al., 1994). It has also been demonstrated that fish collected from PAH contaminated environments have developed a tolerance for the contaminants as measured by refractory response to CYP1A induction (Meyer et al., 2002), and upregulated antioxidant defenses (Bacanskas et al., 2004).

Bacanskas et al. studied oxidative stress in killifish from a creosote polluted environment and showed that not only were antioxidant defenses upregulated in the fish from the contaminated environment compared to controls, but also that there were sex specific and temperature specific differences in the parameters measured. Females

showed greater glutathione peroxidase (a protective enzyme) activity in May which could have been indicative of increased uptake of contaminants during spawning. The researchers also hypothesized that certain antioxidant parameters could increase with increasing water temperatures. In other words, as the water temperature increases, biological functions in fish including metabolic rate and enzyme activities may also rise (Bacanskas et al., 2004). The mosquitofish collected for this research were primarily females. When specimens were collected in February 2005 water temperatures would have been colder, and females would not have been spawning. Therefore, not only would their metabolisms be slower, their uptake of contaminants might also be low. In May the water temperatures would have been warmer increasing enzyme function; and females would have been spawning which might have increased their body burden of contaminants. However, the genotoxicity of increased PAH body burden in May could have been offset by an increase in protective enzymes. Warmer temperatures, reproductive status and increased exposure from flooding would all help to explain the high body burden in fish collected in May 2004 as well as the differences in genetic damage in fish from February 2005 and May 2005. It is important to consider both environmental conditions during the time of collection as well as reproductive status of the target species when investigating potential causes of genotoxicity.

With regard to the amphibians, frogs and toads are unique in the sense of being biphasic. As tadpoles they live in the water and feed on detritus. As adults both frogs and toads feed on insects and other organisms. They can be highly susceptible to contaminants and are exposed via dietary routes and from dermal absorption through their permeable skin. Frogs and toads are an essential link in the food chain in terms of being predators themselves and also being preyed upon by larger aquatic and terrestrial predators such as fish, snakes, foxes, raccoons and hawks found at the sites investigated. Changes in their individual fitness due to contaminant exposure could have effects at the population levels and disrupt ecosystem balance at these sites.

The results of genotoxicity testing, *ex-situ* toxicity tests and chemical analysis indicate that ecological receptors are impacted by residual contaminants at both Site-1

and Site-2 former wood preserving facilities. Although genotoxicity data were not used to generate remediation goals, the FCM method coupled with chemical analysis demonstrates that genotoxicity tests can be used as lines of evidence in ecological risk assessment provided they are considered early in the process and specifically designed to be part of the remedial investigation. This collaborative effort may help improve the risk assessment process and enhance our understanding of the impact that certain xenobiotics have on genetic stability in contaminated ecosystems.

CHAPTER V
SUMMARY AND CONCLUSIONS

5.1 Summary

Hazardous chemicals are released into the environment from a broad range of sources including industrial activities, combustion of fossil fuels, and uncontrolled hazardous waste sites. This research was conducted to provide information that could be used to improve our understanding of the potential adverse human or ecological health impact associated with exposure to complex chemical mixtures. Although the research focused on the toxicity of mixtures isolated from Superfund sites, the data are applicable to any population exposure to mixtures. Mixture toxicity was measured using a battery of *in vitro, in vivo,* and *in situ* bioassays. Data were collected from four Superfund sites including: (1) environmental media from an active bioremediation facility treating wood-preserving waste contaminated soil and water; (2) contaminated sediments from an industrial waterway; and (3) wildlife tissues and environmental media from two former wood-preserving sites for an ecological risk characterization. The projects show a progression from the use of *in vitro* to *in vivo* to *in situ* work with complex mixtures, and demonstrate the utility of each approach in measuring genotoxicity.

5.1.1 Genotoxicity of Complex Chemical Mixtures in Soil After Bioremediation

The genotoxicity of soil and water samples were compared with the results of chemical analysis from a site using bioremediation to treat contaminated environmental media. Bioremediation is one of the most cost-effective technologies for treatment of contaminated soils and groundwater. While limited information exists to define the genotoxicity of complex environmental mixtures, even less is known regarding the potential of the products of biodegradation to produce adverse health effects. Samples were collected to monitor the genotoxicity of complex mixtures extracted from soil and groundwater samples from a site where creosote and pentachlorophenol were used for wood treatment. Twelve surface soil samples were collected annually for three years

from a land treatment unit. Ground water samples were collected using resin-packed columns from an oil-water separator and as effluent from a bioreactor tank. PAHs and pentachlorophenol in the soil and groundwater extracts were quantified using gas chromatography-mass spectrometry (GC-MS). Genotoxicity was measured using both the *Salmonella*/microsome assay and the *E. coli* prophage induction assay.

Chemical concentrations were highly variable in both the surface soil and ground water extractions as was the mutagenic response in these samples. The concentration of carcinogenic PAHs or BaP did not correlate with genotoxicity in either bioassay. These data suggest that mixture interactions have altered the microbial mutagenicity of the PAHs. In addition, genotoxicity as measured using the *E.coli* prophage induction assay did not coincide with PCP concentrations in the surface soils or groundwater.

Results from the five year review of this site have indicated that surface soil and groundwater concentrations of contaminants have successfully reached remediation goals for PAHs and PCP in older treatment units (USEPA, 2005c). The results of the current study indicate that overall total PAH and carcinogenic PAH concentrations in surface soils were reduced during the years samples have been collected. However, genotoxicity, as measured in microbial bioassays did not exhibit a corresponding reduction over time. A range of contaminant interactions could account for the lack of change in soil genotoxicity. Microbial bioassays may not accurately reflect the concentration of genotoxic compounds in soil extracts if high concentrations of cytotoxic compounds reduce survival of microbes. In addition, competition for metabolizing enzymes in a mixture may reduce activation of genotoxic compounds. Finally, diffusion of the genotoxic compounds into the cell may be inhibited by insoluble compounds in a complex mixture. In most cases, biodegradation initially results in a reduction of the low molecular weight, and non-genotoxic, components of a complex mixture. During the time which samples were collected for the present study, chemical interactions may have enhanced or inhibited expression of genotoxic compounds in soil extracts. Because wood preserving wastes may potentially contain thousands to tens of thousands of compounds it is not yet possible (or economical) to screen for all of the mixture

components. However, biological analysis of mixture extracts provides information regarding potential component interactions. The current study found that a combined testing protocol using both biological and chemical analysis provides a more comprehensive view of the fate of contaminants in bioremediation systems than either method alone.

5.1.2 Genotoxicity of Sediments Containing Mixtures of PCBs and PAHs

The potential interactions of mixtures of PAHs and PCBs were investigated in a whole animal study using the extracts of sediments collected from an industrialized river. Contaminants in sediments may be transferred to benthic and other aquatic species as well as to terrestrial species (including humans) in the food web. Complex chemical mixtures in sediment represent a significant challenge in terms of both chemical composition and toxicological effects.

Sediment samples were collected from a reference station and five locations along a contaminated tidal, estuarine river segment. Chemical analysis identified high molecular weight PCBs as the major contaminant. Total PCB concentrations were generally at least 4-5 times greater than total PAH concentrations. Sediment extracts were also analyzed *in vitro* using microbial genotoxicity bioassays and *in vivo* using the ^{32}P- postlabeling assay to detect levels of DNA adducts in female ICR mice. The extracts were either negative or weakly positive (for one sample) in the microbial bioassays. Bioassay results did not correspond with concentrations of either PAHs or PCBs. Extracts from contaminated sediments applied to the skin of mice were found to induce a significant increase in DNA adducts when compared to the results obtained with background sediments. DNA adduct levels in tissue corresponded with PAH and not with PCB concentrations. When the sediment extract with the highest level of PCBs was topically co-administered with benzo[a]pyrene (BaP) to female ICR mice, the complex mixtures extracted from the sediment inhibited the formation of adducts including spot 4 adducts (previously identified as the BPDE adduct). At a concentration of 3.0mg/mouse, the sediment extract produced a 3-fold reduction in BaP adducts.

These data suggest that chemical interactions may have reduced the potential genotoxicity of the complex mixtures extracted from sediments. The overall genotoxic effect seen with these sediment mixtures was inhibition.

5.1.3 Genotoxicity Studies on Ecological Receptors at Hazardous Waste Sites

Data were also obtained to measure the genotoxic potential of contaminants in ecological receptors. Flow cytometry studies conducted on four species were used in a weight-of-evidence approach to assess ecological risk at two former wood treatment facilities contaminated with wood preserving waste. Mosquitofish (*Gambusia affinis*), cricket frogs (*Acris crepitans*), redear sunfish (*Lepomis microlophus*), and Gulf Coast toads (*Bufo valiceps*) were collected from contaminated areas in former wood preserving facilities as well as from several reference stations. Whole body chemical analysis of polycyclic aromatic hydrocarbons (PAHs) was performed on pooled tissue samples grouped according to high and low CVs recorded in flow cytometry analysis. Co-located surface water and sediment samples were also analyzed for PAHs and pentachlorophenol (PCP). Media chemistry results were compared to literature based screening values.

Using non-parametric ANOVA and Bonferroni corrected *post hoc* comparisons, there was a significant difference in half-peak coefficients of variation (HPCVs) between *Gambusia* ($p = 0.004$) and *Acris* ($p = 0.05$) from select references and Site-1. There was also a significant difference in full-peak coefficients of variation (FPCV) values between *Bufo* ($p<0.001$) from the reference and Site-2 (student's t-test). Surface waters and sediments exceeded PAH screening values at both Site-1 and Site-2. All lines of evidence indicate that PAHs at the sites are genotoxic to mosquitofish and amphibians. The results from the analysis of ecological receptors at wood-preserving sites indicate that fish and amphibians collected from a contaminated area will have increased levels of genetic damage compared to reference fish and amphibians (as measured by flow cytometry); and, genetic damage in fish and amphibians will correspond with contaminant concentrations detected in environmental media (sediments, surface water).

However, tissue concentrations were below screening level criteria for fish and amphibians and contaminant body burden did not correspond well with CV grouping for the mosquitofish from Site-1 or for the toads collected from Site-2. HPCV groupings did coincide with PAH body burden in cricket frogs from Site-1. The overall results are similar to results determined for the benthic community in the same water bodies based upon a weight-of-evidence approach that included media chemistry analysis, *ex-situ* toxicity tests, species surveys, and analysis of crayfish tissue relative to literature screening values (CH2MHill, 2005). The genotoxicity data were integrated into a weight-of-evidence ecological risk assessment performed by the U.S. EPA for both sites.

5.2 Conclusions

To investigate the potential risk associated with exposure to complex environmental mixtures, the current study has coupled chemical analysis with a battery of *in vitro*, *in vivo*, and *in situ* techniques. While the *in vitro* techniques (Salmonella/microsome assay and *E. coli* prophage induction assay) are useful for screening for mutagenic potential, additional bioassays such as mammalian cell culture might offer more target-specific information to discern mixture toxicity. The ^{32}P-postabeling assay used *in vivo* and the flow cytometry method used *in situ* were sensitive to the mixtures of concern and effective biomarkers of PAH exposures. For the PAH and PCB mixtures studied in this research, additional biomarkers that may have provided valuable data include the ethoxyresorufin-O-deethylase (EROD) assay, lysosome stability, or disruptions in endocrine function. These biomarkers might help to detect the epigenetic effects that PCBs have within complex environmental mixtures.

Although the focus of risk assessment is on hazardous waste sites that directly threaten human health, those sites that threaten ecological health are also of concern. While aquatic toxicity bioassays and chemical analysis provide valuable information to describe existing site characteristics, data are also needed to define the potential genotoxic risk of site contaminants on wildlife. In order to establish primary remediation goals (PRGs), the risk assessment process requires more quantitative

information. This research has used a battery of *in vitro* genotoxicity bioassays to provide qualitative data regarding complex mixture toxicity. More quantitative data regarding mixture genotoxicity was obtained from the *in vivo* and *in situ* bioassays. While genetic damage is considered a sublethal effect, exposure to chemicals which damage DNA may lead to reduced reproductive capabilities, birth defects, and modifications to the genetic integrity of a population. An integrated approach, combining *in situ* and *ex-situ* methods to characterize genotoxicity should be considered in both the human and ecological risk assessment process.

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