The Development and Application of an Antibody-based Biosensor for the Detection of the Petroleum-derived Compounds

Candace Rae Spier
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The Development and Application of an Antibody-based Biosensor for the Detection of Petroleum-derived Compounds

A dissertation presented to
The faculty of the School of Marine Science
The College of William & Mary in Virginia

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy

by
Candace Rae Spier
2011
This dissertation is submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

Candace R. Spier

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In loving memory of Zedia Mae Fludd, may she never be forgotten.
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ABSTRACT

Petroleum is one of the most important natural resources, but can also be problematic to environmental and human health. Petroleum is comprised of thousands of compounds, including polycyclic aromatic hydrocarbons (PAHs) and heterocycles, some of which are toxic and/or carcinogenic. Traditional analytical methods for environmental monitoring of low-level PAHs are time-consuming, labor-intensive, and often laboratory-bound. Efforts to achieve timely, sensitive, and accurate analysis of PAHs in the field have become a priority for environmental research and monitoring. Antibody-based biosensors are presently being developed for environmental analysis. Anti-PAH antibody molecules can be coupled with electronic transducers to provide new biosensor technology for the rapid determination and quantification of PAHs. Although PAHs are not immunogenic on their own, advances in immunology have provided the means to develop antibodies to PAHs.

Thiophenes, a defined subset of aromatic heterocycles, were selected as the target molecules for antibody development. Characterization of a monoclonal antibody (mAb) to dibenzothiophene revealed specificity for 3 to 5-ring PAHs and heterocycles. Therefore, the goals of antibody development were focused on developing additional antibodies to 2-ring PAHs and to alkylated PAHs. Characterization of antibodies to these novel targets revealed unexpected insights into antibody induction and specificity; namely suitable hapten sizes for small hydrophobic molecule recognition should be larger than one benzene ring, derivatization of the hapten target in immunogen synthesis must preserve structural characteristics, the utility of heterologous assay formats can improve antibody inhibition, and high antibody titers can result in limited assay sensitivity.

The anti-dibenzothiophene mAb 7B2.3 was employed, along with a fluorescence-based transducer, for the generation of a new biosensor for PAHs. The biosensor was utilized in a variety of different applications to determine dissolved PAH concentrations including: 1) sampling groundwater at a former wood-treatment (creosote) facility, 2) analyzing estuarine water during the dredging of PAH-contaminated sediments, revealing a plume of PAHs emanating from the dredge site, 3) frequent monitoring of phenanthrene (a 3-ring PAH) concentrations during a laboratory toxicological dosing study, and 4) monitoring PAH concentrations in stormwater runoff into both a retention pond and a river near a roadway.

Overall, these applications demonstrated the utility of this biosensor for rapid analysis of PAHs in a variety of aqueous environments. The biosensor was operated on-site for both the estuarine and groundwater monitoring trials. The biosensor could process samples, produce quantitative measurements, and regenerate itself in approximately 10 minutes. Sample volumes of 400 µl could be used with little to no sample pretreatment. Most importantly, PAHs could be quantified down to 0.3 µg/l in the field using the sensor platform. These results were validated with conventional gas chromatography-mass spectrometry and high performance liquid chromatography analytical methods. This system shows great promise as a field instrument for the rapid monitoring of PAH pollution.
The Development and Application of an Antibody-based Biosensor for the Detection of Petroleum-derived Compounds
INTRODUCTION

Petroleum

_Petroleum as a pollutant_

Petroleum is one of the most important natural resources. It is extensively used for fuel, plastics, fertilizers, and artificial fibers in clothing, in addition to other common products. However, it becomes problematic when it enters the environment resulting in deleterious effects on ecosystem and human health. Although some of the petroleum that enters the aquatic environment is from natural seepages, the more detrimental inputs are from extraction, transportation, and consumption of petroleum (NRC 2003). These petroleum inputs include, but are not limited to, land-based runoff, recreational vessel discharge, operational discharges, spills, pipeline leaks, atmospheric deposition, and aircraft dumping (NRC 2003). The potential for accidental release into the aquatic environment is magnified in areas where transportation, storage, or use is centered near waterways. If and when spills occur, there is a need for immediate and sensitive water quality assessments to better understand the potential harm to sensitive aquatic habitats.

_Petroleum composition, characteristics, and fate_

Petroleum is formed from the ancient remains of marine plant and animal life under extreme heat and pressure in an anaerobic environment. Depending on the composition of the organic material, varying proportions of aliphatic or aromatic hydrocarbons will be formed. The third most abundant element in petroleum is sulfur, which can account for 0.05 to 13.9% of the total weight depending upon the source of the petroleum (Kropp and Fedorak 1998).
The polycyclic aromatic hydrocarbons (PAHs) can be divided into two groups based on their physical and chemical characteristics. For my purposes, the low molecular weight PAHs include the 2- to 3-ring structures while the high molecular weight PAHs are those with 4- to 5-rings (Figure 1). Generally speaking, as the molecular weight increases, PAH aqueous solubility decreases, as does their susceptibility to vaporization (Neff 1979). Furthermore, alkyl substitutions on the aromatic ring results in an overall decrease in PAH solubility. Solubility is enhanced three- to four-fold by a rise in temperatures from 5 to 30°C, and by dissolved and colloidal organic fractions which incorporate PAHs into micelles (Neff 1979). Vapor pressure characteristics influence the persistence of PAHs in the aquatic environment, with low molecular weight PAHs being more volatile and high molecular weight PAHs demonstrating insignificant volatilizational loss under all environmental conditions (Moore and Ramamoorthy 1984). As a result of these varying characteristics, PAHs will differ in their behavior, distribution, and biological effects.

Although PAHs are hydrophobic, they are slightly soluble in water, and those that are soluble in water are therefore termed the water-accommodated fraction (WAF). In fact, the aquatic ecosystem is one of the major sinks of PAH contamination (Tao et al. 2003). Due to their hydrophobic nature, PAHs entering the aquatic environment exhibit a high affinity for suspended particulates in the water column and will tend to sorb to these particles (Kayal and Connell 1990, Shi et al. 2007). Because of this partitioning with the sediments, the PAH concentrations in water are usually quite low relative to bottom sediments (Moore and Ramamoorthy 1984). Moreover, because of their hydrophobic nature and high lipophilicity PAHs are known to bioaccumulate in aquatic organisms (Meador et al. 1995). The overall fate of a PAH fraction will highly depend on the temperature, turbulence, depth, and pollution status of the water (NRC 2003). As a result, loss/degradation of a PAH will vary both in time and space.
Figure 1. Structures and names of selected PAHs.
Toxicity from the WAF of petroleum

Despite the lower concentrations in the WAF, it is this bioavailable fraction that is responsible for the aquatic toxicity associated with petroleum (Byrne and Calder 1977, Nicol et al. 1977, Millemann et al. 1984, Neff et al. 2000, Mori et al. 2002, Neff et al. 2005, Rhodes et al. 2005). The low molecular weight PAHs demonstrate significant toxicity, whereas the high molecular weight PAHs are less toxic, but are carcinogenic. As a general trend, toxicity increases as a PAH gains alkyl groups (Neff et al. 2005). Acute toxicity has been seen in a range of species of fish and other aquatic organisms at concentrations much less than 1 ppm (mg/l). The alkylated PAHs found in petroleum are relatively more abundant, tend to persist longer (i.e., do not volatilize, biodegrade, or photooxidize as readily) and bioaccumulate to a greater degree than the non-alkylated compounds (Sauer and Uhler 1994). Moreover, Barron and colleagues (1999) postulate that perhaps it is not only the PAHs, but also the heterocycles (PAH analogs containing sulfur, nitrogen or oxygen) that contribute to the toxicity of the WAF. Similarly, heterocycles are expected to follow the same trends as PAHs with regard to increased toxicity with increased alkylation and a tendency to bioaccumulate and persist. Within the subclass of heterocycles, sulfur-containing analogs, although less studied than PAHs, are the next most abundant compounds and are predominantly arylthiophenes (benzothiophene, dibenzothiophene, naphthobenzothiophene) and their alkyl derivatives. Seymour and coworkers (1997) showed that many of the condensed thiophenes were more soluble than the similarly sized PAHs. Research on the fate of thiophenes suggests that they persist longer than PAHs in aquatic systems (Kropp and Fedorak 1998). Therefore the toxicity, solubility and persistence of polycyclic aromatic heterocycles, especially thiophenes, make them excellent targets when monitoring for petroleum (Figure 2).
Figure 2. The thiophenes targeted for antibody development. These compounds are found in petroleum, in the WAF of petroleum, and demonstrate some acute toxicity.
Current technologies for measuring PAHs

Classical analytical chemistry: laboratory-based methods

There are several analytical methods available for detecting and measuring PAHs in water, sediment, air and biological samples. The earliest and simplest technique is a gravimetric analysis in which a sample is extracted using a non-polar organic solvent (Stenstrom et al. 1986). The extracted fraction is then evaporated and the residue is weighed on a balance. Although this remains a cheap and readily available option for quantifying total extracted organics, very large samples must be used for low level analysis. In addition, the low molecular weight molecules are commonly lost to volatilization and the high molecular weight compounds are often not recovered well in liquid/liquid extractions (Stenstrom et al. 1986). Furthermore, there is a high risk of extracting naturally-derived compounds, or unrelated compounds, with a similar solubility to the PAHs, which makes it inaccurate as a petroleum measuring tool.

PAHs exhibit high absorptivities of UV radiation. Likewise, they exhibit strong fluorescence emission patterns that are specific to aromatic structures (Lee et al. 1981). Beyond quantification, these techniques allow for differentiation among aromatic structures. The disadvantage of these tools is the interference from other similar molecules. However, by acquiring proper standards and known material, these interferences can be accounted for. The more common and acceptable practice is the combination of some form of chromatographic separatory preparation prior to employing these technologies for detection.

Most well-established analytical methods employ gas chromatography (GC, equipped with a mass spectrometer (MS) or flame ionization detector) or high-performance liquid chromatography (HPLC, coupled with an UV detector or a fluorescence detector) (Poster et al. 2006). GC separates complex PAH mixtures via differential partitioning between a mobile gas phase and a stationary liquid phase. The polarity-based affinity of the column for the compounds permits the differential elution of the PAH compounds whereupon a
MS can be employed to speciate the compounds. Employing a similar principle, HPLC pumps a mobile liquid phase at high pressure through a column. Compounds are eluted from the column and are detected by UV or fluorescence.

Federal agencies such as the United States Environmental Protection Agency (US EPA) and National Institute for Occupational Safety and Health routinely use GC-MS and HPLC methods in their protocols for environmental sample analysis (e.g., Methods 8270 and 8310). A complication to this analysis is that environmental samples involve a variety of media (aqueous, solids, sludges, biological samples, or a combination of these). Under such conditions, these methods will be compromised by interfering compounds having PAH-like physical characteristics, however much of this has been eliminated by the use of standards and with the sensitivity of the detection devices (i.e., MS). Thus although these methods may be standard for laboratory analyses of environmental samples, they suffer considerably as an option for more routine monitoring, as the approaches are expensive, labor-intensive, and time-consuming.

Analytical on-site PAH assessment tools

Efforts to improve on the laboratory-bound methodologies have become a priority for environmental research and monitoring (Rogers 1995, Plaza et al. 2000, Rodriguez-Mozaz et al. 2006). The goal of developing new on-site assessment technologies is to reduce the expertise, time, and equipment needed, as well as offering comparable or improved measurements. Other requirements for on-site technologies are to minimize the power requirements and to reduce dangerous waste materials (toxic reagents, halogenated solvents, etc.), produced. Although not all of these characteristics can be obtained with a single tool, the goal is to maximize performance with a minimized input.

For the analysis of PAHs in motor oil in soil, a thin-layer chromatography (TLC) field method has been developed as a screening tool (Newborn and Preston 1991). Following separation by TLC, iodine staining and UV exposure were employed for visualization of UV active material. Although it only has a detection limit of 100 ppm (ppb detection
often required), it is a cost-effective tool for preliminary assessments compared to bringing every sample back to the laboratory for conventional analysis.

The first field portable GC-MS has demonstrated environmental analysis capabilities. The Viking SpectraTrak™ 672 GC-MS has been verified by the Environmental Technology Verification Program created by the US EPA and determined to provide detection limits of about 5 ppm for volatile and semi-volatile organic contaminants (which includes PAH detection) in air samples, and 5 ppb (μg/l) for soil and liquid samples (1997). The system cost and training starts around $150,000, requires a power source during field operation, and the columns are more susceptible to breaking because they must be wound around a smaller cage than normally required in the lab (US EPA 1997). On-scene arson crime investigations made use of these portable GC-MS devices when testing for petroleum-based accelerants in the case of arson (Pert et al. 2006). Though they are not concerned with quantification, the use of GC allows the ability to distinguish between the pyrogenic and petrogenic PAHs.

This ultimately leads to the most recently employed ICx Griffin 400 field portable GC-MS instrument, equipped with a helium gas tank, by the Army Corps of Engineers (Bednar et al. 2009). Solvent extractions of both sediment and water samples collected from the dredging of a PAH-contaminated area were conducted in the field. In addition to the time it took for sample preparation, sample analysis required 21 minutes. The instrument possessed a field method detection limit of 20 ppb (μg/l); however none of the samples assessed in this study were quantifiable in the field, because they were lower than the field method detection limit. The laboratory results confirmed this assessment, whereby the authors suggest that this field method produced no false positives or negatives. Nonetheless, all of these field-based methods still lack speed, ease of use, and sensitivity.

**Overview of immunoassays**

Presently, immunoassays are being developed as tools for environmental monitoring. An
immunoassay is a chemical test based on the use of antibodies, which exhibit molecular specificity and high affinity binding characteristics for a particular target or antigen. Immunoassays allow selective recognition even in complex matrices, due to their selective antibody/antigen interaction. Other factors rendering immunoassays as desirable tools for environmental analysis are their reliability, low cost, speed of analysis, ease of use, portability, and sensitivity (Van Emon and Gerlach 1998). Immunoassays can be faster and cheaper to manufacture and use than traditional techniques, as shown in Table 1 composed by Plaza et al. (2000).

The sensitivity of immunoanalysis is reliant on the antibody’s affinity. As noted by Van Emon and Gerlach (1998), immunoassays have a tendency to report higher analyte concentrations when compared to GC-MS or HPLC. They further suggest that this is the result of the need for fewer procedural steps, resulting in higher analyte recoveries or because of antibody cross-reactivity with similarly structured molecules or derivatives. The most commonly employed immunochemical assay is the Enzyme-Linked ImmunoSorbent Assay (ELISA), which was first described in 1972 by Eva Engvall and Peter Perlman. Though the name suggests it uses enzymes, the actual recognition molecule is an antibody, while the enzyme portion is coupled to the antibody to elicit a colorimetric signal. An ELISA is a technique that allows for the determination of antibodies in a sample. In short, the antigen (analyte) specific to the enzyme-linked antibody is immobilized onto a surface. This surface is then exposed to the sample allowing antibodies to bind to the antigen-coated surface. After the surface is washed, it is immersed in a chromogenic substrate solution resulting in an enzyme-catalyzed reaction of the substrate producing a colorimetric change in direct proportion to bound antibody. The amount of antibody bound to the antigen is determined from the initial rate of reaction, which is proportional to the quantity of enzyme captured.

In this research, for the most part, a ‘competitive inhibition ELISA (cELISA)’ is performed (Figure 3). An antigen specific to the enzyme-linked antibody is immobilized on a surface. This surface is then exposed to the sample in combination with the enzyme-linked antibodies. If an analyte is present in the sample it will compete with the
Table 1. Comparison of traditional and immunoassay techniques for environmental sampling. Taken from Plaza et al. (2000).

<table>
<thead>
<tr>
<th>PROPERTIES</th>
<th>TRADITIONAL TESTING</th>
<th>IMMUNOASSAYS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TURNAROUND TIME</td>
<td>1 - 4 weeks</td>
<td>less than 2 hours</td>
</tr>
<tr>
<td>TEST COST</td>
<td>$65 - $250 per test</td>
<td>$10 - $25 per test</td>
</tr>
<tr>
<td>PORTABILITY</td>
<td>laboratory bound</td>
<td>in-lab or on-site</td>
</tr>
<tr>
<td>ACCURACY</td>
<td>high</td>
<td>high</td>
</tr>
<tr>
<td>RELIABILITY</td>
<td>high</td>
<td>high</td>
</tr>
<tr>
<td>EASE OF USE</td>
<td>difficult</td>
<td>moderate to simple</td>
</tr>
</tbody>
</table>
Antigen-coated surface

Antigen-coated surface

Free antibody bind to the antigen-coated surface generating a measurable signal (e.g., greater refractive shift, reduced light intensity, reduced current, etc.)

Fewer antibodies are free to bind to the antigen-coated surface thereby generating a reduced signal

Figure 3. An illustration of an antibody assay demonstrating competitive inhibition by underivatized PAHs in a sample. The resulting signal is inversely proportional to the PAH concentration. (Antibodies are illustrated as Y-shaped structures.)
immobilized antigen, leaving less antibody molecules available to bind the immobilized antigen. During chromogenic development, the rate of reaction, which is proportional to the quantity of enzyme captured, is inversely proportional to the quantity of soluble antigen (e.g., PAH) in the sample. Examples of commercially available immunoassay test kits are home pregnancy tests, HIV tests, and a few environmental monitoring kits for PAHs. Although PAH-specific immunoassays already exist, it is the goal of this project to improve upon the sensitivity of such systems and to link the antibodies with instrumentation to make them available as a monitoring tool.

**Commercially available immunoassays for PAHs**

Commercially available PAH immunoassays are available through SDIX, Abcam, ExBio, Novus Biologicals, Santa Cruz Biotechnology and Quantix Systems. Although they are not all explicitly for water analysis, some valuable information can be gleaned from their use, as well as, how their assay systems are designed. For instance, the Quantix Systems assay is a disposable plastic analyte detector that has been used on wildlife exposed to oil in seawater (Mazet et al. 1997). The target antigen (PAH) is conjugated to an enzyme, while the anti-PAH antibody was immobilized. This allows for a competitive assay, in which the underivatized PAH in the extracted sample can compete with the PAH-enzyme to bind the immobilized antibody. The unbound PAH-enzymes will be washed away. Subsequently, the chromogenic substrate is introduced and the colored product is quantified using a hand-held refractometer. As the PAH concentration in the sample increases, the color endpoint decreases in intensity.

SDIX has offered a variety of field-deployable immunoassays. For the PAH immunoassay, samples require extraction of the PAH analytes into an aqueous phase before analysis. Chuang et al. (2003) compared the SDIX immunoassay with GC-MS concluding that the ELISA measurements are highly correlative and thus is a suitable broad screening tool for environmental PAH monitoring. Explicitly, the ELISA often provided higher estimates than the GC-MS, which the authors expected because of the
ability of the antibody to cross-react with a number of other PAHs not included in the 19 targeted by the GC-MS method.

In the published literature on PAH immunoassays, typically ELISA kits use one antibody to determine the overall PAH concentration. The concentration is reported as a single compound, frequently benzo[a]pyrene. More precisely, the result is reported as benzo[a]pyrene equivalents. One goal of this study was to develop antibodies to different petroleum targets, lower versus higher molecular weight polycyclic aromatic heterocycles. The majority, if not all of the current PAH immunoassays possess a fair amount of cross-reactivity. For example, Nording and Haglund (2003) evaluated the cross-reactivity of a commercially available antibody induced by phenanthrene. Cross-reactivity with fluorene was 140% while compounds with a sulfur, nitrogen, oxygen, or carbonyl group at the nine position of a fluorene molecule showed cross-reactivity values of 28, 8, 7, and 6%, respectively (Figure 4). They concluded that replacing carbon-7 with other atoms had an influence on the cross-reactivity.

Overview of biosensors
A biosensor is simply a hybrid of biological material capable of molecular recognition coupled to an electronic transducer. More advanced instrumentation, such as biosensors, can enable remote and automated environmental monitoring. A variety of biorecognition elements can be used, such as; enzymes, whole cell receptors, DNA, and antibodies. These are then linked with a transducer, such as an electrochemical, optical, piezoelectrical, or thermal device, which converts the biorecognition event into a quantifiable signal (Nakamura and Karube 2003). Fluorophores or enzymes are often used to augment or generate detectible signals. The transduced signal is then recorded as a digital output, typically onto a computer facilitated by dedicated software (Figure 5). For a summary and discussion of PAH biosensors, see Appendix A.

Ideally, biosensors should operate automatically with a user simply introducing the sample and the sensor producing a digital result. The sample may require user
Figure 4. Structures of tested compounds (black) superimposed on a contour of the phenanthrene molecule (grey), showing the general similarities between molecules, with cross-reactivity values in the brackets. (Figure from Nording and Haglund (2003) with slight modifications).
Figure 5. Schematic of a generalized biosensor. The bioreceptor and the transducer are linked such that the transducer quantifies the biorecognition event occurring between the sample and the bioreceptor. The arrows indicate user interaction, in which a sample may require some manipulation prior to introduction to the biosensor. The resulting data are then compared to a standard curve to provide an estimate of analyte concentration.
manipulation prior to introduction (as shown by an incomplete arrow in Figure 5). The biosensor is responsible for detecting the analytes of interest and translating this biorecognition event into a digital output. The resulting data are then compared to a standard curve to estimate the concentration of analyte in the sample.

The goal in developing effective biosensors is to make the technology user-friendly, portable, sensitive, accurate, reliable, and inexpensive (Van Emon and Gerlach 1998). Biosensors can cost less than traditional analytical techniques, require fewer reagents, provide faster turnaround times and higher sample throughput. On-site technologies can require minimal use of power, less dangerous reagents, and produce less potentially toxic waste. Advances in manufactured materials and miniaturization are facilitating portability and on-site operation of biosensors (Rodriguez-Mozaz et al. 2006).

Unfortunately, the development of many environmental biosensors is still in an immature stage, such that automation and portability are often not described nor accomplished. Portability is often emphasized as an advantage of biosensors, but rarely have the analyses been conducted on-site (Rodriguez-Mozaz et al. 2006). Therefore, it was a goal of this project to develop a biosensor for PAH quantification and to demonstrate its ability for autonomous operation and portability.

Moreover, most PAH immunoassay studies used natural water samples fortified with a single PAH analyte to test their methods, while only a few have used PAH-contaminated water (Barceló et al. 1998, Li et al. 2000, Beloglazova et al. 2008). In these cases, the analysis of contaminated water has fallen short by limiting validation to a sub-set of unsubstituted PAHs (typically the 16 EPA priority pollutants) or to a single PAH.

Immunoassay speed is often limited by the time required for antibody/antigen (i.e., analyte) equilibrium to occur. Incubation typically requires 30 to 60 minutes; however, antibody kinetics are often not considered in order to optimize timing.
Development of antibodies to small molecules

The success of an immunoassay depends heavily on the affinity characteristics of the antibody. Antibodies are formed *in vivo* as an adaptive specific immune response. The use of antibodies has been desirable for the development of diagnostics because of their ease of extraction and purification. Antibodies are generated in response to non-self antigens, such as bacteria, viruses or toxins. There are molecular features among the antigens that direct the immune response to produce antibodies, which include size (10 to 100 kDa), foreignness, and chemical composition. Specifically speaking, polysaccharides (composed of repeating epitopes) alone and regardless of their size are less effective at producing antibodies, whereas proteins (composed of amino acids and having defined conformations) exhibit structural complexity and therefore induce antibody production by the induction of somatic mutational processes (Benjamin et al. 1984). Also intriguing are the cellular processes that antibodies undergo in order to obtain high affinity (antigen-driven selection and affinity maturation), with regards to specificity and selectivity (Kindt et al. 2007).

Antibody recognition characteristics

Strictly speaking antigens are comprised of those molecules and cells that are capable of reacting with antibodies, while immunogens comprise a subset of these molecules which are capable of inducing antibody formation. (Any substances capable of producing an immune response are said to be immunogenic and are called immunogens.) Antigens are referred to as the substances an antibody recognizes, and yet, it is not the whole molecule that antibodies recognize, but only the small portion that can fit within an antibody binding site called the epitope, or antigenic determinant (Pressman and Grossberg 1968). In the 1930’s, Nobel Prize winner, Karl Landsteiner investigated many of the fundamental principles of immunochemistry using an elegant molecular level approach to understanding antibody recognition (Landsteiner 1962). He made extensive use of immunoprecipitation as a method to detect the binding of antibodies to antigens. Through the cross-linking of relatively large antigens and antibodies, precipitates would form. However, at the time, there was insufficient knowledge about large protein
structures, so he turned to the use of small molecules which had a defined chemical composition, yet were not capable of inducing an immune response. He found that these small molecules, haptens, could be covalently attached to a larger molecule (i.e., protein carrier) and thereby induce hapten-specific antibodies. He also noticed that immunoprecipitation of the hapten-carrier conjugates could be inhibited by using only the smaller haptenic monovalent molecules. This was because the haptens lacked the ability to cross-link the bivalent antibodies, which can typically occur with larger molecules that possess multiple antibody-binding epitopes. This led to the eventual realization that antibodies were capable of discriminating subtle differences in the molecular structure of the hapten.

Landsteiner's work was extended by both Linus Pauling and David Pressman to develop the concept of an antibody binding pocket that was both complementary and shape-selective to its hapten (Pressman and Grossberg 1968). Elvin Kabat's studies led him to conclude that antibodies could recognize structures as small as 370 Da (Kabat 1976). Today, a commonly used hapten in immunological studies is trinitrophenyl, a mere 212 Da (Rittenberg and Pratt 1969). Further development came from Michael Heidelberger and Elvin Kabat in the evolution of immunology from a descriptive field into a quantitative chemical discipline. They advocated the precise quantification of antigen-antibody interactions and performed pioneering studies using new physical chemical techniques to characterize antigen molecules.

By exploiting the fact that antibodies reversibly bind haptens by non-covalent interactions, such as hydrogen bonds, Van der Waals forces, or electrostatic forces, quantitative assessments of affinity can be measured. The affinity, or strength of interaction between an antigen binding site on an antibody and a hapten, is described by the following reversible equation:

\[
Ab + H \overset{k_a}{\rightleftharpoons} \overset{k_d}{\leftarrow} Ab-H
\]

\[
K = \frac{k_a}{k_d} = \frac{[Ab-H]}{[Ab][H]}
\]
where \([H]\) is the concentration of free hapten, \([Ab]\) is the concentration of the free antigen binding site, \([Ab-H]\) is the bound hapten concentration, \(k_a\) is the forward (association) rate constant, and \(k_d\) is the reverse (dissociation) rate constant. The ratio of \(k_a/k_d\) is the equilibrium constant \(K\), a measure of affinity, which is the ratio of the concentration of bound Ab-H (antibody-hapten) complex to the concentration of unbound antibody and unbound hapten. Based on this equation, high affinity antibodies strongly interact with the specific antigen, and tend to dissociate slowly; whereas the low affinity antibodies possess high dissociation rate constants, and/or low association rate constants (Steward 1978).

**Affinity maturation and antigen binding site diversity**

For the purposes of making an immunochemical biosensor, high affinity antibodies are desirable. However, the immunogen alone (a combination of the epitope’s structure and the form of the entire immunogen) is not responsible for inducing high affinity antibodies, but rather in the context of affinity maturation. Clonal selection promotes the preservation of high affinity antibody secreting cells initially through continued stimulation of the high affinity clones as the immunogen concentration falls. High affinity B cells will be selected because of their greater ability to bind to low concentrations of antigen. As circulating antigen continues to decline, only those B cells with receptors having the highest affinity will be able to compete for antigen in a process called antigen-driven selection, which continually shifts expression to higher affinity antibodies. Thus, immunizations with a high concentration of antigen will initially produce a larger repertoire of high and low affinity antibodies, while immunizations with a low concentration of antigen will initially select for only the high affinity antibodies.

Each immunogen can initially elicit a large number of antibody secreting B cells. The germline repertoire that encodes the components of the binding sites for these cells undergoes the process of somatic recombination that rearranges the gene sequences, which results in a large variety of binding sites (Tonegawa 1983). Susumu Tonegawa earned a Nobel Prize (1987, nobelprize.org) for the discovery that the ability of B cells to
develop a broad repertoire of antibodies toward a virtually limitless diversity of antigens is governed by the germline diversity of the immunoglobulin gene complex.

Antigen binding sites are best explained within the context of the chemical structure of the antibody molecule which was elucidated by Rodney Porter and Gerald Edelman in the 50s and 60s earning them a Nobel Prize (1972, nobelprize.org). They determined that the monomeric antibody molecule was comprised of 2 heavy polypeptide (H, 50 kDa) chains and 2 light (L, 25 kDa) chains and that these chains were arranged such that there are 2 antigen binding sites located at the ends of the variable regions and connected by a constant, non-antigen binding fragment (Figure 6). Each binding site arises from the arrangement of six distinct contact residues (3 per polypeptide chain) within adjoining framework regions that position these contact residues, or complementarity determining regions (CDRs). Genetically speaking, either chain, H or L, can arise from over a million different arrangements of a few hundred gene segments, thus producing over a million different antibodies (Schroeder 2006). This vast diversity is aided by both chains containing three distinct CDRs that are modified by high mutation rates if antigen-specific T cells are elicited. Ultimately, CDRs lie in close spatial proximity to one another within the binding site and provide a unique three-dimensional structure that is complementary to the antigen (Wu and Kabat 1970, Eisen 2001).

**Immunizations and mAb production**

Typically, antibodies are only synthesized in vivo. With advances in cancer research, methods for the production of immortal B cell lines (hybridomas) have greatly facilitated the large-scale isolation, selection and production of highly specific (monoclonal) antibodies in vitro. From an immunochemical standpoint, an antiserum represents the sum total of antibodies from potentially tens to hundreds of different B cells with different germline rearrangements, each uniquely recognizing specific aspects or even a single epitope (or hapten) (Figure 7). Similar to any immunogen possessing a large variety of epitopes, in a synthesized immunogen, not only will the carrier molecule, the linking structure, as well as the desired hapten be targeted. In addition, this diversity of
Figure 6. A schematic of a classical monomeric antibody molecule illustrating the 2 H and 2 L chains, the variable and constant domains within each, and the terminal antigen binding sites connected by non-antigen constant binding fragments. (Image taken from www.biology.arizona.edu/IMMUNOLOGY/tutorials/antibody/graphics/antibody.gif)
Figure 7. Antibody recognition diversity of the immune response. For my purposes, the desired type of antibody is "Antibody C" that solely recognizes the hapten. "Antibody A" is shown as recognizing the carrier molecule, while "Antibody B" binds the linking arm of the conjugate. (Image adapted from Vanderlaan et al. (1988).)
recognition will lead to cross-reaction with fairly unrelated antigens (Vanderlaan et al. 1988) (Figure 7). These polyclonal collections of antibodies can shift within an individual and differ considerably between individuals. This variability detracts from their utility as a standard and precise analytical tool. Thus the goal of developing monoclonal antibodies (mAbs) to small molecules is to isolate a specific and high affinity B cell, which subsequently produces ‘Antibody C’ (Figure 7), recognizing only the hapten target.

From a technical standpoint, polyclonal antibodies are less expensive and faster to produce, but in the long term considerable expense is saved by the possession of a single, high specificity antibody in virtually unlimited supply. Therefore, the development of mAbs, led by Kohler and Milstein (1975) in the 1970s, has eliminated the variability in molecular recognition that plagued analyses using polyclonal antibodies. Thus mAbs have become the preferred biological recognition molecule of the immunoassay.

Briefly, mAbs are generated by immunizing a mouse with an immunogen, which can be a hapten attached to a carrier protein. Repeated immunizations over prolonged periods allow affinity maturation thus producing higher affinity antibodies targeting the hapten. Then the B cells, harbored within the spleen, are fused with "immortal" myeloma cells (fusion partners). The resulting fused cells are called hybridoma cells (Kohler and Milstein 1975).

The crux of procuring the most specific antibody is the screening of these hybridoma cells while cloning them to insure monoclonality. The process entails the determination of specificity for a particular antigen. The determination of specificity can be accomplished by the employment of a cELISA, via the use of various hapten analogue inhibitors to select the most specific antibody.

Once a single hybridoma line has been selected and cloned, it can be grown in vivo by growth of cells in an ascites form, or in vitro within a culture flask (Harlow and Lane
1988) to produce large quantities of antibody. Purified mAbs become the biological molecular recognition molecule of the biosensor.

**Hapten production and protein conjugation**

When developing a hapten, preservation of important structural features of the target analyte is required. A wide variety of conjugation techniques are available depending on the chemical structure; size, polarity, and, more importantly, the available functional groups of both the carrier molecule and the hapten (Hermanson 1996). Although PAHs do not possess the functional groups needed for conjugation to a protein carrier (such as a free carboxyl), some functional derivatives are commercially available. In cases where suitable derivatives are not available, a variety of different synthetic strategies are available for preparation of functional derivatives. To this end, researchers have successfully conjugated PAH haptens to proteins (see Table 6 in Appendix A for a list of haptens and references). To determine specific recognition of the hapten, different carrier molecules are used for immunizations (i.e., keyhole limpet hemocyanin; KLH) as opposed to screening (i.e., bovine serum albumin; BSA) for reactivity. Figure 8 provides an example of a target analyte, functionalized hapten, and hapten-protein conjugate.

The average number of haptens conjugated per carrier molecule can often be assessed by titrating the remaining functional groups (i.e., free amines) by the use of amine-reactive compounds such as TNBS (2, 4, 6-trinitrobenzene-1-sulfonic acid), which provides a spectroscopic signal proportional to its degree of conjugation (Habeeb 1966). Another technique, matrix-assisted laser desorption ionization-MS (MALDI-MS), can establish the approximate increase in the molecular weight of the carrier protein resulting from the addition of varying numbers of small hapten residues. The MALDI-MS analysis requires a relatively expensive instrument and some expertise to use it, but MALDI-MS has been demonstrated to be a more accurate tool for conjugate characterization because it has no solubility constraints, a high tolerance for impurities, and lacks dependence on hapten composition (Adamczyk et al. 1994). It can be used for BSA conjugate characterization, but not KLH because the BSA protein fraction is smaller and more homogeneous in
Figure 8. Examples of a target PAH analyte/hapten, a functionalized hapten, and hapten-protein conjugate. The amorphous shape of the conjugate represents a protein molecule.
molecular weight. Because MALDI-MS can differentiate between the addition of these approximately 200 Da haptens, the variation among the protein fraction’s molecular weight must also be small.

Sapidyne’s KinExA Inline sensor

The sensor employed in this project was the KinExA Inline sensor manufactured by Sapidyne Instruments (Boise, ID) (Figure 9). The flow cell is a small (~2 mm in diameter) clear tube equipped with a semi-porous membrane. It is positioned directly in front of the laser source and fluorescence detection meter. Polymethylmethacrylate (PMMA; Sapidyne) particles, or beads, are used and are transferred to the flow cell where they cannot pass through the membrane and thus provide the immobilized support. There are a total of fourteen reagent lines; a buffer reservoir, a waste receptacle, a syringe for mixing, an antibody reservoir, and any specific buffers needed for washing, leaving at least seven lines free for samples. Pumps are used to move the fluid within the lines. The operational control is handled on a computer equipped with Inline Sensor software provided and designed by Sapidyne. The collected data is reported as a voltage change from the voltage reading at the start of a run of one sample to the end and referred to as the delta $V (\Delta V)$. A typical biosensor run is essentially similar to a cELISA where the reduction in signal is inversely proportional to the concentration of the target in a sample (Figure 9, bottom right). It has been shown by Bromage et al. (2007a) that the detection limit of the KinExA Inline biosensor is lower than the detection limit determined by ELISA.

Benefits of environmental assessment of PAHs

Each petroleum source has a unique chemical fingerprint and thus authorities have been able to link petroleum releases with specific sources and/or the offenders. In particular, because of the uniqueness of sulfur heterocycles, sulfur fingerprints have been shown to be a valuable aid in petroleum characterization and source correlation (Mackenzie and Hunter 1979). Therefore, it is the goal of this research to generate antibodies specific to the 2- and 3-ring sulfur heterocycles. Furthermore, these antibodies will possess different
Figure 9. The top left picture is the KinExA Inline sensor from Sapidyne Instruments (Boise, ID) with the front panel removed. The middle is a schematic of its fluidics, courtesy of Dr. Bromage. The bottom diagram illustrates the flow cell and how the signal is inversely proportional to the PAH concentration.
cross-reactivity patterns than antibodies previously developed to the larger PAHs. This may also exclude the possibility of cross-reacting with a large majority of similar PAHs as the small size of the analyte will exclude detection of the larger compounds.

It would also be desirable to have a single instrument that could perform multiple immunoassays simultaneously. This could provide a broad-spectrum, yet differential analysis, by incorporating antibodies with specificities to, for example, the higher molecular weight PAHs, and the lower molecular weight heterocycles. Multi-analyte detection has been demonstrated for the KinExA Inline biosensor with high reproducibility (Bromage, personal communication).

If the technology were available, programs could be designed to provide cheap, rapid, and dependable assessments required for clean-up efforts. Physical actions, such as weather, wave, currents, and the addition of dispersants can influence the mixing rates of petroleum into water. Linking the biosensor with devices to estimate flow rates, would make it possible to model the direction and rate of movement of the spill. Because more samples can be collected and analyzed than by laboratory-based methods, a sampling scheme can be used to span the entire exposure area as well as water-column depths. In all, the rapid quantification of petroleum can be used to verify the cleanup activity, determine the necessary actions, and streamline the amount and type of resources needed for such efforts.

Other uses of a field-deployable, rapid petroleum biosensor tool would be to monitor oil wells while drilling is underway. If a leak occurs, it can go unnoticed if the oil drilling is in a remote location (in deep seawater, under an ice sheet, etc.). If petroleum biosensors were in place, the early detection of the leak would allow for faster response times. Faster response times could enable effective salvaging of the leaking resource as well as reducing the amount of oil that enters the environment, both an advantage for the oil industry and the environment. The methods developed to analyze small, hydrophobic molecules could be applied to pesticides, pharmaceuticals, or any other contaminants, which are currently being measured by traditional analytical techniques.
RATIONALE AND OBJECTIVES

The overall goal of this research was to develop a biosensor for the detection of petroleum-derived thiophenes in aquatic environments. In order to accomplish this goal, 1) anti-dibenzothiophene mAbs were generated, 2) the mAbs were integrated into the KinExA Inline sensor format and validated, and 3) the KinExA Inline biosensor was employed in near real-time sampling applications.

To accomplish Objective 1, first, a carboxylic acid derivative of the target contaminant was synthesized and subsequently conjugated with a protein carrier. Immunizations with the conjugated carrier were carried out until a satisfactory response was induced. MAbs were then produced, purified and extensively characterized with respect to affinity and cross-reactivity.

To accomplish Objective 2, the antibodies were evaluated with the sensor instrument in a number of different environmental matrices to determine the sensitivity, accuracy, and precision of the biosensor under realistic conditions. A series of experiments were conducted to determine the effects of salinity, dissolved organic carbon (DOC) concentrations, and natural water sources on the performance of the biosensor.

The biosensor was then evaluated for near real-time sampling to complete Objective 3. This was accomplished in the form of a laboratory-based time-series experiment where the concentration of contaminant decreased over time. Field-based applications were made when potential PAH-contaminated sites were identified and accessible. Validation of the biosensor results was made by comparison of results with those obtained by standard HPLC and/or GC-MS methods.
MATERIALS AND METHODS

All reagents were of analytical grade and purchased from either Fisher Scientific (Pittsburgh, PA, USA), VWR (Bridgeport, NJ, USA), or Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Hapten synthesis and validation

Haptens were synthesized to contain a carboxylic acid to provide a means for conjugation to protein carriers. All haptens, along with their abbreviations, are shown in Figure 10.

*dibenzothiophene-4-acrylic acid* – The two-step synthesis of dibenzothiophene-4-acrylic acid (DBTAA) began with preparing 4-lithiodibenzothiophene via a reaction of dibenzothiophene (98%, Alfa Aesar, Ward Hill, MA, USA) with n-butylithium by the method of Kuehm-Caubère et al. (1996), then this mixture was treated with 3.7 equivalents of anhydrous N,N-dimethylformamide (DMF, Arcos Organics, Morris Plains, NJ, USA) at -70 °C. The resultant mixture was warmed to room temperature and stirred for 12 hours. The reaction was quenched with an excess of diluted HCl, the product was extracted into dichloromethane (DCM; Burdick & Jackson, Muskegon, MI, USA) and the solution was evaporated to dryness. The carbonyl products were isolated by TLC on silica gel using 2,4-dinitrophenylhydrazine for visualization.

The Doebner-Knoevenagel reaction was employed for the preparation of the acrylic acid; this reaction has been reviewed by Johnson (1942). A mixture of the previously synthesized dibenzothiophene-4-carbaldehyde (186.3 mg, 0.87 mmol) and malonic acid (192.5 mg, 1.85 mmol) plus two drops of piperidine in anhydrous pyridine (6 ml) was refluxed for 90 minutes and then poured over a mixture of ice and HCl to yield a
Synthesized haptens

- Dibenzothiophene-4-acrylic acid (DBTAA)
- Thieno-2-ylacrylic acid (2TAA)
- Thieno-3-ylacrylic acid (3TAA)
- 3-Methylbenzo[b]thiophene-2-acetic acid (3MBT2AA)
- Benzothiophene-5-acrylic acid (BT5AA)
- 5-Methyl-2-thiopheneacrylic acid (5M2TAA)
- 2-Methylfluorene-9-ylideneacetic acid (2MF9AA)
- 3-Methylfluorene-9-ylideneacetic acid (3MF9AA)

Commercially available haptens

- 2-Benzothiophene-3-acetic acid (BT3AA)
- Thiophene-2-propionic acid (2TPA)
- 3-Methylbenzo[b]thiophene-2-acetic acid (3MBT2AA)

Gift from Dr. Harris

- Biphenyl-2-yiacrylic acid (2BIPAA)
- Biphenyl-4-yiacrylic acid (4BIPAA)

Figure 10. Structures of all haptens used throughout this investigation. The capitalized abbreviations below the chemical name are used throughout the text. The headings above the groups indicate how the hapten was obtained. The synthesized haptens are described in the text.
crystalline solid, which was then collected by vacuum filtration through filter paper, washed with approximately 20 ml of water and allowed to air dry.

Both compounds were characterized by melting point determinations, proton nuclear magnetic resonance ($^1$H NMR) and GC-MS. The $^1$H NMR studies were carried out on a Varian Mercury VX-400 (Palo Alto, CA, USA) employing samples dissolved in deuterochloroform containing 0.03% (v/v) tetramethylsilane as the internal standard. GC-MS was performed on a Varian 4D GC-MS-MS ion trap spectrometer (Walnut Creek, CA, USA) operated in electron ionization mode over a mass range of 100-500 m/z. The GC was a Varian 3800 and the temperature program was as follows: initial temperature 75°C, hold for 1 minute, 4°C/minute to 350°C and hold for 0.25 minutes.

**benzothiophene-5-acrylic acid** – The synthesis of benzothiophene-5-acrylic acid (BT5AA) employed the Doebner-Knoevenagel reaction as described above for DBTAA with the following modifications. The starting material was 1-benzothiophene-5-carbaldehyde (230 mg, 1.4 mmol; Maybridge, England, 95%). Recrystallization from hot ethanol yielded the product as yellowish brown crystals (170 mg, 59.0% yield). The product was characterized by $^1$H NMR, GC-MS, and melting point determinations.

**thiophen-2-ylacrylic acid** – The syntheses of thiophen-2-ylacrylic acid (2TAA) employed the Doebner-Knoevenagel reaction as described above for DBTAA with the following modifications. The starting material was 2-thiophenecarboxaldehyde (1.0 g, 8.9 mmol; Aldrich, 98%). Recrystallization from hot ethanol yielded the product as yellowish brown crystals (740 mg, 53.3% yield). The final product was characterized by $^1$H NMR, GC-MS, and melting point determinations.

**thiophen-3-ylacrylic acid** – The syntheses of thiophen-3-ylacrylic acid (3TAA) employed the Doebner-Knoevenagel reaction as described above for DBTAA with the following modifications. The starting material was 3-thiophenecarboxaldehyde (290 mg, 2.6 mmol; Aldrich, 98%). Recrystallization from hot ethanol yielded the product as light tan
colored crystals (360 mg, 90.0% yield). The final product was characterized by $^1$H NMR, GC-MS, and melting point determinations.

5-methyl-2-thiopheneacrylic acid – The syntheses of 5-methyl-2-thiopheneacrylic acid (5M2TAA) employed the Doebner-Knoevenagel reaction as described above for DBTAA with the following modifications. The starting material was 5-methyl-2-thiophenecarboxaldehyde (530 mg, 4.2 mmol; Aldrich, 98%). Recrystallization from hot ethanol yielded the product as dark brown colored crystals (410 mg, 58.0% yield). The final product was characterized by $^1$H NMR, GC-MS, and melting point determinations.

2-methylfluorene-9-yldieneacetic acid – In a two-step procedure, first, the generation of 2-methylfluorenone was completed following the procedure of Snyder and Werber (1950) with minor modifications. In a typical procedure, polyphosphoric acid (1.4 g; Arcos Organics, 84%) was warmed in an oil bath prior the addition of 4-methyl-2-biphenylcarboxylic acid (500 mg, 2.4 mmol; Aldrich, 97%). Additional polyphosphoric acid (1.2 g) was then added, the mixture was allowed to cool, and then hydrolyzed by the addition of ice and water. The solution changed from orange to yellow with yellow crystals forming as the final product.

The method described by Goehring (1994) was employed for the synthesis of 2-methylfluorene-9-yldieneacetic acid (2MF9AA). Under a blanket of nitrogen gas, sodium hydride (61 mg, Arcos Organics, 60%) was washed 3 times with hexane and suspended in anhydrous tetrahydrofuran (THF; 1 ml; Arcos Organics, 99.9%). Triethylphosphonoacetate (348 mg; Sigma) was dissolved in THF (500 µl), added slowly to this solution and stirred magnetically. 2-Methylfluorenone (250 mg, 1.3 mmol) was dissolved in THF (1 ml) and added slowly to the reaction. Once the reaction was complete, the mixture partitioned between water and DCM. The DCM fraction was collected. Water was removed from it with anhydrous sodium sulfate and the solution was evaporated leaving a yellow crystalline product. This ester product was then hydrolyzed in 5M NaOH (293 µl), acidified and extracted with ether and water. The aqueous phase was collected and yellow crystals formed following the addition of
concentrated HCl. The solid was collected by vacuum filtration and allowed to air dry over night (150 mg, 0.64 mmol, 48.9% yield). The final product was characterized by $^1$H NMR, GC-MS, and melting point determinations.

3-methylfluorene-9-ylideneacetic acid – In a three-step procedure, first, the generation of the diazonium salt of 2-amino-4-methylbenzophenone was accomplished following the procedure of Lipowitz and Cohen (1965) with modifications. A solution of 2-amino-4-methylbenzophenone (250 mg, 0.001 mol; Aldrich, 99%) and 50% tetrafluoroborate (640 mg, Arcos Organics) in 5.9 ml of ethanol was cooled below 0°C. To this solution, a solution of sodium nitrite (89.6g, 1.29 mmol, Sigma) was added and the mixture was allowed to sit for 30 minutes. Cold ether (23.6 ml) was then added and again the mixture kept cold for 30 minutes giving a precipitate. Filtration and washings with cold ether afforded a white powder of the diazonium fluoroborate salt.

Decomposition of the diazonium fluoroborate into 3-methylfluorenone was completed as described in Lewin and Cohen (1967). To the entire previous white powder product, 100 ml of 0.1 N sulfuric acid was added and the solution was heated at 45°C until the powder dissolved. Cuprous oxide (125.4 mg, 0.88 mmol, Arcos Organics, 97%) was added. The red powder gradually turned blue. The reaction time for the cuprous oxide catalyzed reaction was approximately 2 hours. The product was filtered to remove the copper and extracted three times with DCM. Water was removed from the DCM solution by the addition of anhydrous sodium sulfate and the solution was evaporated leaving a dark amber brown colored thick viscous material (42 mg, 18.3% yield).

Conversion of the previously synthesized 3-methylfluorenone to 3-methylfluorene-9-ylideneacetic acid (3MF9AA) was completed as described above following the method of Goehring (1994). The final product was characterized by $^1$H NMR, GC-MS, and melting point determinations.
**Other haptens employed**

A few commercially available haptens were employed, such as: 3-methylbenzo[b]thiophene-2-acetic acid (3MBT2AA; Alfa Aesar, 97%), 2-benzo[b]thiophen-3-ylacetic acid (BT3AA; Maybridge, England), and 2-thiophene propionic acid (2TPA). Other haptens used were obtained as gifts from Dr. Harris: biphenyl-2-y lacrylic acid (2BIPAA) and biphenyl-4-y lacrylic acid (4BIPAA; commercially available as 4-phenylcinnamic acid, Alfa Aesar, 98%; or in the reduced form of 4-biphenylacetic acid; Aldrich, 98%). All hapten structures, along with their abbreviations, are shown in Figure 10.

**Antigen and immunogen preparation**

*Hapten activation*

The activation of the haptens, the carboxylic acid derivatives of the target molecules, (i.e., DBTAA, BT5AA, 2TAA, 2TPA, 3TAA, 5M2TAA, 2MF9AA, 3MF9AA, 3MBT2AA, BT3AA, 2BIPAA, and 4BIPAA), and their subsequent conjugations to a carrier protein were carried out by the method described by Singh et al. (2004), with minor modifications. Briefly, the hapten was dissolved in anhydrous DMF to a final concentration of 0.3 M along with equimolar amounts of N-hydroxysuccinimide (Pierce, Rockford, IL, USA) and dicyclohexylcarbodiimide (Pierce). The solution was rotated at room temperature until a visual precipitate of dicyclohexylurea had formed, usually between 20 to 60 minutes. The precipitate was removed by centrifugation at 5,000 x g for 5 minutes at room temperature; the supernatant contained the activated hapten.

*Protein conjugation*

Activated derivatized haptens were conjugated to a variety of carrier proteins (e.g., KLH, BSA, ovalbumin (OVA), and thyroglobulin (Thyro)). All of the haptens shown in Figure 10 were conjugated to both KLH and BSA. Typically, the hapten-KLH complex was used for immunizations, while the hapten-BSA complex was employed in the ELISA for
anti-hapten detection. Additionally, 4BIPAA was conjugated to Thyro such that when the 4BIPAA-BSA conjugate was used as the immunogen, the 4BIPAA-Thyro conjugate was used as the screening antigen. Likewise, when the 4BIPAA-Thyro conjugate was used as the immunogen, the 4BIPAA-BSA conjugate was used as the screening antigen. The following haptens were also conjugated to OVA; 2TAA, 2TPA, 3TAA, 5M2TAA, and 4BIPAA.

_Hapten to KLH conjugation_

Hapten-KLH conjugates were produced by first mixing 3.5 μl of the activated derivatized hapten (0.3 M) into 250 μl of DMF and then added to 1ml of KLH (~5 mg/ml, ~2.5 nM in manufacturers’ phosphate buffered saline (PBS), pH 7.4), resulting in approximately a 400:1 ratio of hapten to KLH. The reaction mixture was rotated for 2 hours at room temperature to complete the conjugation. The conjugate was then dialyzed against PBS (1.85 mM NaH₂PO₄, 8.41 mM Na₂HPO₄, 150 mM NaCl, pH 7.4) with three 1 L changes at room temperature over 24 hours. The conjugate was filter-sterilized through 0.22 μm filters and a final protein concentration was determined by bicinchoninic acid protein assay (BCA, Pierce), read on a microtiter plate reader (MTX Lab Systems, Vienna, VA, USA) using a 540 nm filter.

_Hapten to BSA conjugation_

To improve the efficiency of conjugation, varying amounts of DMF solvent were added to two different buffer solutions. In all, six DBTAA-BSA conjugates were made, three in PBS (pH 7.4) and three in borate buffer (50 mM boric acid, pH 9.0). Aliquots (5 ml) of a 20 mg/ml BSA solution, three for each buffer type, were placed in separate tubes. The concentration was adjusted to 10 mg/ml BSA (1.5 μmol) with the addition of the appropriate amount of DMF and/or buffer, such that the final solutions contained approximately 0%, 25% or 50% DMF. Activated DBTAA hapten (50 μl, 15 μmol) was added in aliquots to each BSA solution resulting in a molar ratio of 10:1 of hapten to BSA. If the solution began to turn opaque, indicating protein precipitation, the addition of the activated hapten was terminated. As with the KLH conjugation, the BSA reaction mixtures were rotated, dialyzed, sterile-filtered, and quantified as described above.
The DBTAA-BSA conjugates were analyzed by MALDI-time-of-flight MS, similar to that described elsewhere (Adamczyk et al. 1994), employing a Voyager Elite DE instrument (Perseptive Biosystems, Ramsey, MN, USA) at the Vanderbilt Mass Spectrometry Resource Center operating in the positive ion mode using 3-hydroxypicolinic acid matrix containing ammonium hydrogen citrate (7 mg/ml) to suppress sodium and potassium adducts. The spectra were calibrated using a native BSA (MW 66 kDa). The average number of bound DBTAA molecules (MW 254 Da) was calculated according to the equation: 

\[
\frac{(\text{MW}_{\text{conjugate}} - \text{MW}_{\text{protein}})}{\text{MW}_{\text{hapten}}}.
\]

**Hapten to BSA, OVA, and Thyro conjugation**

Following the results obtained from the above experiment, hapten-BSA, -OVA, and -Thyro conjugates were produced by first mixing 50 µl of the activated hapten (0.3 M) into 100 µl of DMF and then added dropwise to 10 ml of protein (~10 mg/ml, in PBS with 25% DMF), resulting in approximately a 10:1 ratio of hapten to protein. If the solution began to turn opaque, indicating protein precipitation, the addition of the hapten was terminated. As per the KLH conjugation, the reaction mixtures were rotated, dialyzed, sterile-filtered, and quantified as described above.

**Animals and immunization routines**

All animal studies were conducted in accordance with the guidelines established by the College of William & Mary’s Institutional Animal Care and Use Committee. Typically, three to seven (6 week old) female BALB/c mice were immunized by intraperitoneal injection of 100 µl of a 1:1 Freund’s complete adjuvant emulsion containing 50 µg of hapten-carrier. All haptens (DBTAA, BT5AA, 2TAA, 2TPA, 3TAA, 5M2TAA, 2MF9AA, 3MF9AA, 3MBT2AA, BT3AA, 2BIPAA, and 4BIPAA) were employed for immunizations. Typically the carrier protein was KLH, except in the additional experiments where 4BIPAA was conjugated to BSA and Thyro as the carrier protein. Two subsequent immunizations were administered at 4 week intervals with the same conjugate concentration emulsified 1:1 in Freund’s incomplete adjuvant.
**Monoclonal antibody production**

Mice exhibiting the desired serum antibody specificity and selectivity (determined by the screening methods described in the subsequent sections) were given a final booster injection 3 to 5 days prior to the fusion procedure (Harlow and Lane 1988). Briefly, a 10 μg dose of hapten-KLH was diluted with PBS to a total volume of 200 μl, with 100 μl of it administered intravenously, while the other 100 μl was given intraperitoneally. Splenocytes were isolated and fused with SP2/0 myeloma cells using the 50% polyethylene glycol method as described by Harlow and Lane (1988). The resulting cells were distributed into ten 96-well tissue culture plates (Costar, Corning, NY, USA) at 150 μl/well.

After visible colonies had formed, the plates were screened via ELISA using a 96-pin replicator (Fisher) to sample culture supernatant. Additionally, for the DBTAA fusion, a cELISA was employed using dibenzothiophene as the inhibitor. This was performed by pre-loading DBTAA-BSA coated plates with a solution of the inhibitor at 1 μg/ml in 100 μl of PBS prior to the introduction of hybridoma supernatants. Hybridoma supernatants were added to the wells and compared to the wells without inhibition. Those hybridomas from wells demonstrating significant inhibition were selected for clonal expansion.

Positive hybridomas were cloned (3 times) by limiting dilution (Harlow and Lane 1988). Stock mAbs were produced from ascites of pristane-primed male mice injected with the cloned hybridoma (NRC 1999). Ascitic fluid was collected and purified using a Melon® Gel Monoclonal IgG Purification Kit (Pierce). The isotype classification was determined by Mouse Typer® Sub-Isotyping Kit (BioRad, Hercules, CA, USA). MAb protein concentration was determined by BCA and the purity was confirmed by running a 12% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (Harlow and Lane 1988) followed by Silver Staining (Sigma-Aldrich). MAb stocks were stored at 1 mg/ml concentration in PBS with 50% glycerol at -20°C until used.
**Magnetic bead isolation**

A large excess of irrelevant hybridomas are produced during a fusion. In an attempt to procure the few valuable clones, preselection of hapten-specific splenocytes via magnetic panning was performed (Steenbakkers et al. 1993). Paramagnetic beads (Dynal M-450, epoxy activated; Invitrogen) were coated with 4BIPAA-BSA and cell isolation was conducted according to the manufacturer’s method. After the splenocytes from a 4BIPAA-KLH immunized mouse were isolated, the beads were incubated with the cell suspension for 30 minutes at 4°C with occasional tilting and rotation. The solution was subjected to a magnet and the supernatant was drawn off. Both the magnetic bead-bound cells and those cells in the supernatant were fused separately, as described above.

**Antibody characterization**

**ELISA plate preparation**

For sera, supernatant and mAb screening, 96-well microtiter plates (Costar) were coated for 1 hour at room temperature with 100 μl/well of a hapten-BSA conjugate solution typically ranging from 0.1 to 10 μg/ml in sodium carbonate coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). The plates were blocked with 200 μl/well of 0.5% BSA in Tween Tris buffered saline (TTBS; 0.1% Tween 20, 50 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 8.0) for 1 hour at room temperature. The plates were either used immediately, or the blocking solution was removed and the plates stored at -20°C until required.

**Titration screening assays**

Prior to each immunization, the mice were bled from the tail vein for antibody titer determination using an ELISA procedure (Harlow and Lane 1988). All sera were screened against a plate coated with the same hapten-BSA conjugate, as described above. Sera were serially diluted in PBS (as opposed to TTBS, to maintain diluent consistency with that which was required in competitive inhibition assays described later), loaded at
100 µl/well and placed on a rocker for 1 hour at room temperature. The plates were washed with 3 rinses of TTBS. A goat anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (Jackson Immunoresearch, West Grove, PA, USA) was used at 0.25 µg/ml in PBS, loaded at 100 µl/well and placed on a rocker for 1 hour at room temperature. The plates were washed again, developed using a solution containing 7.7 µmol of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Calbiochem, Gibbstown, NJ, USA), 10 µl of 30% H₂O₂ in 10 ml of citrate buffer (10mM citric acid, pH 4.0), and read on a microtiter plate reader using a 405 nm filter. The maximum rate of reaction (mOD/min) was calculated using the Deltasoft JV software (BioMetalics, Princeton, NJ, USA) and determined for each sample. The antibody titer (activity units per ml) was determined by the amount of sera required to generate a response of 50% of the maximum rate.

Hapten-specific titers, as determined by the above ELISA, were compared to protein carrier-specific titers by screening sera from 5M2TAA-, BT3AA-, 4BIPAA-, and 2MF9AA-KLH immunized groups against KLH. Plates were coated with 1 to 5 µg/ml of KLH, following the microtiter plate coating method described above.

For hapten titer screening of the 4BIPAA-BSA group, 10 µg/ml of 4BIPAA-Thyro was coated onto the microtiter plate as described above, except blocked with only TTBS; 0.5% BSA was not added to the blocking solution. Likewise, for carrier protein titer determinations, mice immunized with 4BIPAA-BSA were screened against 5 mg/ml of BSA, and those immunized with 4BIPAA-Thyro were screened against 5 mg/ml of Thyro.

To explore heterologous hapten specificity (i.e., activity towards a hapten not used in the immunization), sera from BT3AA-KLH immunized mice were screened against BT5AA-BSA conjugate.

The addition of thiomersal to the diluent was examined because of its use as a preservative. MAb 7B2.3 was employed in a titration ELISA to assess thiomersal affects.
**Checkerboard assay**

A checkerboard titration was completed in order to establish the optimum working dilutions of the coating antigen and the antibody for ultimately detecting various inhibitors (e.g., PAHs) in solution with the highest sensitivity. Plates were coated and blocked in the same format as described above, except that they were coated with the hapten-protein conjugate using a two-fold dilution series across the plate in duplicate with the first two columns at approximately 2 µg/ml. MAb or sera was then titrated down each column. Based on each titration, the coating concentration was selected that resulted in a quantifiable signal response and its corresponding antibody concentration at about 50% of the maximum signal response.

**Competitive inhibition assays (cELISAs)**

All cELISAs were conducted employing a specified inhibitor at a soluble concentration with serial dilutions of that concentration in PBS* containing 5% methanol to ensure inhibitor solubility. Control wells contained only PBS with 5% methanol, to which the response was normalized. First, coated ELISA plates were loaded with 50 µl/well of each inhibitor concentration. The sera or mAb was then loaded at 2X strength in 50 µl/well such that 1X was the concentration optimally determined above by the checkerboard titration. The plates were washed, incubated with the secondary antibody, washed again, and developed as described above.

The inhibitors chosen for each assay varied depending on the goal of the experiment. For a typical sera screening, the inhibitors chosen were those with a similar structure to that of the hapten, without the carboxyl group and its carbon chain linking arm. For example, for the sera generated to hapten BT3AA, benzothiophene was used as the inhibitor. For the sera generated to hapten 4BIPAA and 2BIPAA, biphenyl was used as the inhibitor. Table 2 provides an illustration of the derivatized haptens employed along with the PAH it was meant to represent.

* TTBS was avoided, because it contains Tween, which interacts with hydrophobic analytes, competing with the antibody and therefore less analyte is available to cause inhibition (Manclús and Montoya 1996).
Table 2. Illustration of structural similarities of derivatized haptens and target analytes.

<table>
<thead>
<tr>
<th>Derivatized hapten(s)</th>
<th>Target analyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>2TAA</td>
<td>thiophene</td>
</tr>
<tr>
<td>3TAA</td>
<td></td>
</tr>
<tr>
<td>2TPA</td>
<td></td>
</tr>
<tr>
<td>5M2TAA</td>
<td>methyl-thiophene</td>
</tr>
<tr>
<td>BT5AA</td>
<td>benzothiophene</td>
</tr>
<tr>
<td>BT3AA</td>
<td></td>
</tr>
<tr>
<td>3MBT2AA</td>
<td>methyl-benzothiophene</td>
</tr>
<tr>
<td>DBTAA</td>
<td>dibenzothiophene</td>
</tr>
<tr>
<td>2BIPAA</td>
<td>biphenyl</td>
</tr>
<tr>
<td>4BIPAA</td>
<td></td>
</tr>
<tr>
<td>2MF9AA</td>
<td>methyl-fluorene</td>
</tr>
<tr>
<td>3MF9AA</td>
<td></td>
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</tbody>
</table>
In one scenario, a cELISA was conducted employing a heterologous hapten assay format. Sera from BT3AA-KLH mice were assessed on a plate coated with BT5AA-BSA and inhibited by benzothiophene. This was compared to the inhibition profile produced when the same sera was assessed on a plate coated with its homologous hapten BT3AA-BSA inhibited by benzothiophene.

Additionally, some sera were analyzed to provide a recognition profile of other PAHs. For example, 4BIPAA-KLH sera were employed in a cELISA with the following inhibitors: pyrene (Aldrich, 99%), naphthalene, phenanthrene (ICN Pharmaceuticals Inc, Plainview, NY, USA), biphenyl, and anthracene (J. T. Baker, Phillipsburg, NJ, USA, 99%).

Specificity of 2MF9AA-KLH sera were examined using the following inhibitors: anthracene, phenanthrene, benzo[a]anthracene (Pfaltz Bauer, Stamford, CT, USA), 9,10-dimethylbenzantracene, 7,12-dimethylbenzantracene, 9,10-dimethylantracene, 3,6-dimethylphenanthrene, 2,3,5-trimethylnaphthalene, 1,4,5-trimethylnaphthalene, 1,3-dimethylnaphthalene, 4-methyl dibenzothiophene and 2-methylphenanthrene.

The initial characterization of the DBTAA mAb, 7B2.3, was conducted using unsubstituted PAHs and heterocyclic inhibitors ranging from 2 to 5 rings: anthracene, benzo[a]pyrene, benzo[a]anthracene, biphenyl, chrysene (Aldrich, 95%), dibenzofuran, dibenzothiophene, fluoranthene (Aldrich, 98%), fluorene (Aldrich, 98%), naphthalene, phenanthrene and pyrene; and the following alkylated PAHs and heterocycle: 9,10-dimethylbenzantracene, 7,12-dimethylbenzantracene, 9,10-dimethylantracene, 3,6-dimethylphenanthrene, 2,3,5-trimethylnaphthalene, 1,4,5-trimethylnaphthalene, 1,3-dimethylnaphthalene, 4-methyl dibenzothiophene and 2-methylphenanthrene.

The stormwater runoff samples (described later), were determined by GC-MS to contain other environmental contaminants, such as, triclosan, nonylphenol, bisphenol-A, and caffeine. As such, these compounds were used as inhibitors of 7B2.3 in a cELISA.
After evaluation of the toxicological study (described later), it was suspected that phenanthrene metabolic products might be detected during biosensor analysis. To assess 7B2.3 specificity, a standard solution containing the putative oxidation product of phenanthrene (1-hydroxy-phenanthrene, Dr. Ehrenstorfer GmbH, Augsburg, Germany, 99%) was evaluated on the biosensor.

**Biosensor development**

Features and principles of the KinExA Inline sensor (Sapidyne) have been described elsewhere (Darling and Brault 2004, Drake et al. 2004, Yu et al. 2005, Bromage et al. 2007a). An attached laptop computer provided instrumental control, graphic signal output, and data storage. The immobilized antigen DBTAA-BSA was coated onto PMMA beads following previously described methods (Bromage et al. 2007b). The specificity of 7B2.3 for 3- to 5-ring PAHs (refer to the mAb inhibition screening section in the Results section or Spier et al. 2009) was coupled to the fluorescent dye, AlexaFluor 647 (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s protocol. An antibody concentration of 0.38 μg/ml resulted in a voltage change of approximately 1 V.

The speed in which an antibody comes to equilibrium with a sample is determined by the antibody’s on and off rates. The $k_{on}$, $k_{off}$, and $K$ for the antibody employed in the biosensor studies that follow, mAb 7B2.3, were determined using the Kinetics Direct methods employing the KinExA®3000 (KinExA®3000 Instrument Manual, Sapidyne) with slight modifications to the manufacturer’s instructions. Essentially, concentrations of antibody and dibenzothiophene were held constant and the amount of time that they are mixed together (i.e., incubated) was varied and then assessed (voltage response recorded).

The automated sample handling program (see Appendix B) was similar to others that have been described (Bromage et al. 2007b). Briefly, for every sample, a new bead pack (~400 μl) coated with antigen was loaded into the flow cell. Then, 400 μl of fluorescently labeled 7B2.3 was pulled into the drawing syringe, followed by 400 μl of
deionized water, a standard, or a sample. This mixture was pushed into and drawn out of a mixing syringe to complete mixing. Half of the solution was discarded to waste and the remaining 400 µl was pushed over a previously loaded bead pack in the flow cell. For fluorescence detection, excitation and detection wavelengths were 620 and 670 nm, respectively. The resulting fluorescence signal was based on competitive exclusion by underivatized PAHs in the sample such that the amount of 7B2.3 bound to the antigen coated beads was inversely proportional to the concentration of 3- to 5- ring PAHs. A solution of 50% dimethylsulfoxide (DMSO) in deionized water was incorporated for line rinsing between each sample analysis to prevent cross-contamination of samples by residual PAHs. In total, quantitative assessments were obtained in approximately 3 minutes, with 7 minutes for preparation and introduction of the next sample. Up to seven samples can be loaded for analysis with all further steps performed automatically.

*Calibration curves for the biosensor*

Several PAH formulations were evaluated for biosensor detection (signal response) and eventual use in the generation of a calibration curve. The formulations were SRM 1647b Priority Pollutant PAHs (National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA), SRM 2260 Aromatic Hydrocarbons in Toluene (NIST), a solution containing equal concentrations (by mass) of 6 PAHs (anthracene, chrysene, fluoranthrene, fluorene, phenanthrene, pyrene), and a solution of only phenanthrene. Each solution was diluted in deionized water such that they contained less than 1% of the original solvent. The calibration curves represent the sum of the PAH concentrations for the respective formulations. For each formulation standard, a five-point calibration curve within its linear range was used.

*Matrix effects*

As the desired utility of this biosensor technology was for the detection of hydrophobic PAH molecules, different amounts of organic solvents were examined to determine how they would affect the performance of the biosensor. PBS buffer solutions containing 5 and 10% of the water miscible solvents, DMF, DMSO, methanol and acetone were
examined on the KinExA. The results were normalized to the antibody binding signal
generated in a PBS buffer solution containing no solvent.

Biosensor sampling of various water sources required the evaluation of matrix effects
(i.e., effects of varying solute components on biosensor performance). Various water
sources were anticipated to range from fresh to oceanic waters, therefore, salinities of 0 to
38 ppt were evaluated. Hawaiian Marine Mix (HMM) was diluted in deionized water
and salinities were assessed by a refractometer. These solutions of varying salinities
were assessed to determine the effect on biosensor function. Solutions of varying salinity
were spiked with two different concentrations of phenanthrene, 1 µg/l and 3.1 µg/l, and
the effect on the PAH detection assessed. Additionally, filtered (5 µm) York River water
and PBS were also spiked with 1 µg/l phenanthrene so that the impact of a natural water
source on biosensor function could be assessed. Similarly, dilutions of humic acid
(dissolved organic carbon; DOC) were spiked with 2.0 µg/l of phenanthrene to determine
if DOC interfered with PAH quantification.

**Biosensor environmental applications**

*Site description and sample collection*

The biosensor was employed in four different contexts; 1) LA Clarke & Son EPA
Superfund site in Spotsylvania, VA, USA (groundwater monitoring), 2) Money Point site
in the southern branch of the Elizabeth River near Norfolk, VA, USA (estuarine
monitoring), 3) Seawater Research Laboratory at the Virginia Institute of Marine Science
(VIMS), Gloucester Point, VA, USA (laboratory toxicological study), and 4) two road-
side drainage locations near VIMS (stormwater runoff monitoring).

**Groundwater monitoring**

The LA Clarke site was a creosote/coal tar wood treatment facility; which, as a result of
50 years of operation, had contaminated the groundwater with PAHs, benzene, and dense
non-aqueous phase liquids. Eight wells from this site were selected based on historical data, which indicated they would provide a wide range of PAH levels. The sampling order was from less to more contaminated starting with W7, W5, MW81, D8, PW01, D3, W6, and then MW80. The wells were purged prior to sampling, and then 30 ml of water were collected in amber vials for biosensor analysis and 4 L were collected in amber bottles for GC-MS analysis. The apparent outdoor temperatures were below 5°C, which caused some fittings to leak. So, the biosensor was placed in the back of a heated sport utility vehicle and powered with an extension cord connected to an outlet in an on-site work shed. Samples taken for GC-MS analysis were kept cool until laboratory analyses could be performed.

**Estuarine monitoring**

The Elizabeth River Project, an independent, non-profit organization, has targeted the Money Point area of the Elizabeth River (Norfolk, Virginia, USA) as a remediation site. This site has severely PAH-contaminated sediments due to historical creosote releases (ERP 2008). Remediation efforts include dredging of contaminated sediments, capping, and wetland restoration. On June 9, 2009, while the dredging operation was underway, the biosensor was employed to monitor PAHs released into the surrounding water column. All samples were collected outside of a sediment curtain that enclosed the dredging activity. Water samples were collected in 30 ml amber glass vials for on-site biosensor analysis and in 4 L amber glass bottles for laboratory-based GC-MS analysis. At this site, the biosensor was operated on board the 6-m vessel, R/V Oystercatcher. The biosensor was kept in the front cabin area and powered by a 12 V battery (Energizer E27RC Marine Battery; St. Louis, MO, USA) through a 110 V power inverter (Xantrex Xpower 1750 Plus; Vancouver, Canada). Ambient temperatures on board the vessel exceeded 35°C at times, which deteriorated sensor performance; therefore, temperature was stabilized by placing ice chips in the reagent block. Biosensor samples were filtered and analyzed on-site, while the samples taken for GC-MS analysis were kept on ice until laboratory analyses could be performed.
Toxicological study

The toxicological study was performed to monitor the phenanthrene dosing in an independent experiment. Zebrafish (Danio rerio) were held in 8 L freshwater aquaria with water temperature maintained at 28°C. Fish were exposed to phenanthrene at 2 nominal concentrations, one at 150 μg/l and a second at 400 μg/l, each with triplicate tanks. The tanks were fortified with phenanthrene every 12 hours and the water exchanged every 24 hours. Aliquots of water (~1 ml) were collected for biosensor and HPLC analyses from 4 tanks (2 low and 2 high exposure tanks) prior to and post phenanthrene renewal. Samples were collected for 5 consecutive days. The biosensor samples were assessed at room temperature, while the samples taken for HPLC analysis were kept cool until laboratory analyses could be performed.

Stormwater runoff study

Two sites located near the VIMS campus were selected for monitoring during a rain event (April 1-16, 2010). The first site was a retention pond located below the Coleman Bridge (CB; lat. 37° 14’ 52”, long. -76° 30’ 9”). The second site was along the Yorktown Creek (YTC) where it passes under the George Washington Memorial Highway/ US Route 17 (lat. 37° 13’ 55”, long. -76° 30’ 50”) on the west side. Because the sites were sampled simultaneously and were close to the laboratory (i.e., ~0.5 to 3 km away), the samples were brought back to the laboratory for analysis. Samples were collected at each site every other day until it began raining, whereupon the samples were collected hourly. For biosensor analysis, triplicate water samples were collected in 30 ml vials; larger samples (1 L) were collected for GC-MS analysis when biosensor results revealed that PAH concentrations were rapidly changing. Cumulative rainfall readings from rain gauges stationed at each sampling site and the observation times were recorded.

Analytical analysis of PAHs

GC-MS analysis was conducted using previously described methods (Unger et al. 2008) with the following modifications. The estuarine samples were filtered using a 0.45 μm Teflon® filter (Millipore Corporation, Billerica, MA, USA) prior to analysis because of
high levels of suspended solids, which could potentially clog the biosensor lines. For the groundwater, estuarine, and stormwater runoff studies, 1 L of water sample was transferred to a pre-cleaned separatory funnel, spiked with surrogate standards (1,4-dichlorobenzene-d₄, naphthalene-d₈, acenaphthene-d₁₀, phenanthrene-d₁₀, chrysene-d₁₂, perylene-d₁₂, PCB 30, PCB 65, PCB 204, 1,1'-binaphthyl, and perinaphthenone; Ultra Scientific, Kingston, RI, USA), and extracted three times with 40 ml of DCM. The volume was reduced under a gentle stream of nitrogen using a TurboVap® evaporator (Zymark Corp., Hopkinton, MA, USA) and the internal standard p-terphenyl (ChemService, West Chester, PA, USA) was added. The extracts were analyzed on a Varian 3800 GC using a Varian CP-8400 Autosampler coupled to a Saturn 2000 GC/MS/MS ion trap MS (Varian, Walnut Creek, CA, USA) operated in electron ionization mode (70 eV). It was equipped with a split/splitless injector maintained at 320°C. The carrier gas was He and injections were made in splitless mode on a DB5, 60 m x 0.32 mm x 0.25 µm film thickness capillary column from J&W Scientific (Folsom, CA, USA). The GC temperature program was 75°C to 320°C at 4°C/minute with an initial hold of 1 minute. The MS trap, manifold, and the transfer line temperatures were 220°C, 80°C, and 320°C, respectively. Scans were 100 to 500 m/z for 8 to 49 minute and then 100 to 650 m/z from 49 to 62.25 minute; selected ions were used to quantify the targeted analytes. Seven-point calibration curves and sample analyses of individual analytes were performed using the Varian MS Workstation software package, version 6.8 (Varian). The limit of detection was approximately 0.01 µg/l per analyte. Each set of extractions contained a laboratory blank and a replicate environmental sample.

HPLC analysis of the toxicological samples followed a modified method from Unger and colleagues (Unger et al. 2007). The HPLC method was performed using a Thermo Separation Product (TSP; Riviera, FL, USA) SpectraSYSTEM P4000 Binary Gradient Pump, a TSP SpectraSYSTEM AS3000 Variable-Loop Autosampler with column oven, and a Waters 474 Fluorescent Detector. The gradient was 100% water to 20/80% water/acetonitrile (Burdick & Jackson) through a Restek Allure C18 column (5 µm particle size, 250 x 4.6 mm; 60 Å pore size; Restek Corp., Bellefonte, PA, USA).
Biosensor analysis of PAHs

Most of the groundwater samples were run undiluted; the samples that exceeded the upper limit of quantitation (30 μg/l) were diluted with deionized water and analyzed. The estuarine water samples were filtered (0.45 μm Teflon®; Millipore) prior to analysis. The samples from the toxicological study were diluted in deionized water prior to analysis to bring them into the quantification range of the biosensor. The filtration and dilution steps were completed in no more than 5 minutes. Sample analyses were completed within 30 minutes of sample collection. The stormwater runoff samples were analyzed directly, without any manipulation. During the rain event, one to two of the triplicate samples were analyzed in near real-time (within 10 to 60 minutes of collection), to guide further sampling. The remaining samples were kept at 4°C and analyzed within 3 days. Standards and blanks were routinely analyzed to ensure the performance of the instrument during each study.

Statistical analyses

The calibration curves, detection limits and biosensor linear range were determined by log-linear regression analysis. Error estimates of the biosensor method were determined by triplicate analysis of laboratory standards. During laboratory development of the biosensor methodology, the detection limit was determined by performing the EPA method detection limit procedure 40 CFR Part 136 Appendix B. During field studies, the limit of detection was defined as a signal-to-noise ratio of 3:1. The PAH concentrations determined by biosensor and GC-MS or HPLC methods were compared using a Pearson correlation coefficient (r) analysis. Deming (orthogonal) regression analysis was performed to examine the relationship between the biosensor and the GC-MS or HPLC method. All statistics were performed using Minitab v16 software (Minitab, State College, PA, USA).
RESULTS

Validation of synthesized haptens

dibenzothiophene-4-acrylic acid – The synthetic pathway for the hapten DBTAA is shown in Figure 11A. TLC revealed the presence of two carbonyl compounds, the faster eluting compound was isolated as a colorless, crystalline solid (21% yield) and identified as dibenzothiophene-4-carbaldehyde on the basis of melting point, mass spectra and \(^1\)H NMR. M.p. 115-122°C (lit. m.p. 123-124°C, (Katritzky and Perumal 1990)); MW calculated for \(\text{C}_{13}\text{H}_8\text{SO}\) \(m/z\) 212.3; observed 212; \(^1\)H NMR: δ 10.31 (s, 1H), 8.45 (dd, J = 7.8 + 1.0 Hz, 1H), 8.24 (dd, J = 5.0 + 2.4 Hz, 1H), 8.01 (m, 2H), 7.69 (t, J = 7.5 Hz, 1H), 7.53 (m, 2H); The NMR spectrum is consistent with that reported by Katritzky and Perumal for this compound which they had prepared by a different route (1990).

The dibenzothiophene-4-carbaldehyde was used in the second step of the reaction sequence shown in Figure 11A. The final product was recrystallized from ethanol/water yielding colorless needles (109.6 mg, 49.4% yield) and identified as dibenzothiophene-4-acrylic acid (DBTAA) on the basis of the mass and \(^1\)H NMR spectra. M.p. 249-253°C; MW calculated for \(\text{C}_{15}\text{H}_{10}\text{SO}_2\) \(m/z\) 254.3, observed 254 (Figure 11B); \(^1\)H NMR results are listed in Table 3, the carboxyl H was not observed.

benzothiophene-5-acrylic acid – The synthetic pathway for BT5AA is shown in Figure 12A. The product was identified as benzothiophene-5-acrylic acid on the basis of mass spectra and \(^1\)H NMR. M.p. 226-231°C; MW calculated for \(\text{C}_{11}\text{H}_6\text{SO}_2\) \(m/z\) 204.2; observed 204 (Figure 12B); \(^1\)H NMR results are listed in Table 3, the carboxyl H was not observed.
Figure 11. A) Synthesis of dibenzothiophene-4-acrylic acid (DBTAA). B) Mass spectrum of DBTAA. The signal at 254 is the molecular ion.
Table 3. NMR assignments for synthesized compounds.

<table>
<thead>
<tr>
<th>Compound name (abbr.)</th>
<th>Compound structure</th>
<th>'H NMR:</th>
</tr>
</thead>
<tbody>
<tr>
<td>dibenzothiophene-4-acrylic acid (DBTAA)</td>
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<tr>
<td></td>
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<td>7.52 (m, 3H)</td>
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<td></td>
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<td>7.93 (m, 1H)</td>
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<td>8.08 (d, 1H, J=16.2 Hz)</td>
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<td>8.19 (m, 1H)</td>
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<td></td>
<td>8.22 (dd, 1H, J=7.8+1.0 Hz)</td>
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<td>6.53 (d, 1H, J=16Hz)</td>
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<td>7.25-7.59 (m, 9H)</td>
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Figure 12. A) Synthesis of benzo thiophene-5-acrylic acid (BT5AA). B) Mass spectrum of BT5AA. The signal at 204 is the molecular ion.
**thiophen-2-ylacrylic acid** – The synthetic pathway for 2TAA is shown in Figure 13A. The product was identified as thiophen-2-ylacrylic acid on the basis of melting point, mass spectra, and ¹H NMR. M.p. 148-149°C (lit. m.p. 144-146°C, (Zhu et al. 2007)); MW calculated for C₇H₆SO₂ m/z 154.2; observed 154 (Figure 13B); ¹H NMR results are listed in Table 3, the carboxyl H was not observed. The ¹H NMR spectrum is consistent with that reported by Zhu et al. (2007) for this compound which they had prepared by a different route.

**thiophen-3-ylacrylic acid** – The synthetic pathway for 3TAA is shown in Figure 14A. The product was identified as thiophen-3-ylacrylic acid on the basis of melting point, mass spectra, and ¹H NMR. M.p. 153-154°C (lit. m.p. 149-150°C, (Mamaev 1957)); MW calculated for C₇H₆SO₂ m/z 154.2; observed 154 (Figure 14B); ¹H NMR results are listed in Table 3, the carboxyl H was not observed.

**5-methyl-2-thiopheneacrylic acid** – The synthetic pathway for 5M2TAA is shown in Figure 15A. The product was identified as 5-methyl-2-thiopheneacrylic acid on the basis of mass spectra and ¹H NMR. M.p. 158-166°C; MW calculated for C₈H₈SO₂ m/z 168.2; observed 168 (Figure 15B); ¹H NMR results are listed in Table 3, the carboxyl H was not observed.

**2-methylfluorene-9-ylideneacetic acid** – The synthetic pathway for 2MF9AA is shown in Figure 16A. The product was identified as 2-methylfluorene-9-ylideneacetic acid on the basis of mass spectra and ¹H NMR. M.p. 199-201°C; MW calculated for C₁₆H₁₂O₂ m/z 236.3; observed 236 (Figure 16B); ¹H NMR results are listed in Table 3, the carboxyl H was not observed.

**3-methylfluorene-9-ylideneacetic acid** – The synthetic pathway for 3MF9AA is shown in Figure 17A. The intermediate product of 3-methylfluorenone was identified on the basis of mass spectra, giving a parent ion of m/z 194 (Figure 17B). The final product was identified as 3-methylfluorene-9-ylideneacetic acid on the basis of ¹H NMR. Melting point was not determined because of the very low yield obtained during the synthesis.
Figure 13. A) Synthesis of thiophen-2-ylacrylic acid (2TAA). B) Mass spectrum of 2TAA. The signal at 154 is the molecular ion.
Figure 14. A) Synthesis of thiophen-3-ylacrylic acid (3TAA). B) Mass spectrum of 3TAA. The signal at 154 is the molecular ion.
Figure 15. A) Synthesis of 5-methyl-2-thiopheneacrylic acid (5M2TAA). B) Mass spectrum of 5M2TAA. The signal at 168 is the molecular ion.
Figure 16. A) Synthesis of 2-methylfluorene-9-ylideneacetic acid (2MF9AA). B) Mass spectrum of 2MF9AA. The signal at 236 is the molecular ion.
Figure 17. A) Synthesis of 3-methylfluorene-9-ylideneacetic acid (3MF9AA). B) Mass spectrum of 3-methylfluorenone, the intermediate product. The signal at 194 is the molecular ion.
The mass spectra of the MW ion calculated for C\textsubscript{16}H\textsubscript{12}O\textsubscript{2} $m/z$ 236.3; could not be obtained because it would not dissolve in a solvent suitable for MS analysis; $^1$H NMR results are listed in Table 3, the carboxyl H was not observed.

As there was not enough product from the 3MF9AA preparation saved for adequate GC-MS results, a small portion of the activated derivatized hapten underwent an attempted hydrolysis (treatment with NaOH and extracted into chloroform and methanol) to once again yield the acid. However, after GC-MS analysis, a molecular ion of 263 appeared (Figure 18), while the expected product of 236 did not. It was determined that instead of the acid, the dimethylamine of the compound (C\textsubscript{18}H\textsubscript{17}NO, $m/z$ 263.3) was generated.

An effort to hydrolyze the amine product into the carboxylic acid was attempted. This involved refluxing the solution in excess NaOH for 24 hours and subsequently extracted into chloroform and methanol. After GS-MS analysis, a molecular ion of 263 again appeared, and an additional molecular ion of 250 (Figure 19). It was determined that the 250 ion was the methyl ester of the carboxylic acid (C\textsubscript{17}H\textsubscript{14}O\textsubscript{2}, $m/z$ 250.3), which was due to the presence of methanol in the solvent. Despite not identifying the carboxylic acid compound by GC-MS, sufficient evidence supports the validation of its synthesis (i.e., $^1$H NMR and GS-MS of derivatives of the desired product).

*biphenyl-2-ylacrylic acid* – 2BIPAA, was obtained as a gift from Dr. Harris. It was identified as biphenyl-2-ylacrylic acid on the basis of mass spectra. M.p. 200-204°C; MW calculated for C\textsubscript{15}H\textsubscript{12}O\textsubscript{2} $m/z$ 224.3; observed 224 (Figure 20). The melting point for the reduced form of 2BIPAA, 2-biphenylpropionic acid, was 112-114°C (lit. m.p. 122-124°C (Dice et al. 1950) and 110-113°C (Cook et al. 1950)), and its $^1$H NMR results are listed in Table 3 for the carboxyl H was not observed.

*biphenyl-4-ylacrylic acid* – 4BIPAA, was obtained as a gift from Dr. Harris. It was identified as biphenyl-4-ylacrylic acid on the basis of melting point and mass spectra. M.p. 231-235°C (lit. m.p. 225°C, (Braun and Nelles 1933); MW calculated for C\textsubscript{15}H\textsubscript{12}O\textsubscript{2} $m/z$ 224.3; observed 224 (Figure 21). The melting point for the reduced form of
Figure 18. Mass spectrum of the dimethylamine of 3MF9AA. The signal at 263 is the molecular ion.
Figure 19. Mass spectrum of the methyl ester of 3MF^9AA. The signal at 250 is the molecular ion.
Figure 20. Mass spectrum of 2BIPAA. The signal at 224 is the molecular ion.
Figure 21. Mass spectrum of 4BIPAA. The signal at 224 is the molecular ion.
4BIPAA, 4-biphenylpropionic acid, was 154-156°C (lit. m.p. 147-149°C (Kigawa et al. 1981)), and its $^1$H NMR results are listed in Table 3 and the carboxyl H was not observed.

**Hapten to BSA conjugation**

Addition of activated DBTAA to the BSA solutions resulted in visible precipitation in the 50% DMF/PBS reaction. No conjugate was retrievable from this preparation after filtration. The remaining DBTAA-BSA conjugates were quantified and ranged from 3.4 to 6.3 mg/ml. MALDI-MS analysis revealed DBTAA-BSA conjugation ratios (Table 4) were the highest (6.13:1 DBTAA:BSA) for reactions in PBS containing 25% DMF. Since the hapten had been added at a ratio of 10:1, but conjugation was 6.13:1, the efficiency for this reaction was approximately 60%. The borate buffered solutions resulted in ratios ranging from 4.17 to 4.67, while the PBS without added DMF yielded a ratio of only 2.96.

**Hapten to OVA conjugation**

Although the haptens (2TAA, 2TPA, 3TAA, 5M2TAA, and 4BIPAA) that were employed for conjugation to OVA were added in small aliquots, two different results were observed. In some instances, the protein would precipitate immediately upon addition of the activated hapten. Otherwise, a precipitate would form in 30 to 60 minutes. Either consequence resulted in little to no remaining soluble protein as determined by a BCA protein determination assay.

**Serum antibody titration**

All serum titrations to the various haptens were comparable to that depicted in Figure 22. The immunogen, in this case, 4BIPAA-KLH, was employed and an increase in titer was observed following each subsequent immunizations. Titer vales were defined as the units of activity in the volume of serum at 50% of the maximum rate. In almost all instances, titers were quantifiable after the 3rd and 4th bleed. Using the homologous hapten on
Table 4. Conjugation ratios of the DBTAA-BSA conjugates as concluded by MALDI-MS.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Buffer</th>
<th>% DMF</th>
<th>Observed Mass (Da)</th>
<th>Changed Mass (Da)</th>
<th>Molar ratio (DBTAA : BSA)</th>
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<tr>
<td>DBTAA-BSA</td>
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<td>67,479</td>
<td>1,048</td>
<td>4.44</td>
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<td>67,534</td>
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<td>na</td>
<td>66,431</td>
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</table>

a. Protein lost during sterile filtration.
Figure 22. A representative serum titration over the course of immunization. Serum was from mouse D (1 out of 4) immunized with 4BIPAA-KLH. ELISAs were conducted approximately one month after each immunization using plates coated with 4BIPAA-BSA. The antibody titer for the 4th sera was 500 units/ml (1 unit = 50% max rate = 2 μl).
dissimilar carriers enabled us to exclusively determine the anti-hapten titer (Figure 22, Table 5). All mouse titers are presented in Table 5. Antibody titers were approximately 10 to 100-fold greater for the carrier protein (e.g., KLH) than they were for the hapten of interest.

In one particular case, sera from 2MF9AA-KLH mice, one individual had an extremely high titer to the hapten compared to the others in this group. This discrepancy was investigated in a later cELISA study. It appeared that mice immunized with BT5AA-KLH had very little hapten response. In an effort to address if this was because of a failed conjugation, BT3AA-KLH sera, which demonstrated titers to the BT3AA hapten, were tested using BT5AA-BSA. If any of the cross-reactive specificities were present, a titer should be observed in the absence of any reactivity to BSA. Titers comparable to those observed against BT3AA-BSA were also observed against BT5AA-BSA (Table 5 and Figure 23), indicating that the conjugation was successful. The titer to the homologous hapten-carrier was 25-fold higher than for the heterologous hapten-carrier. The recognition of the free analyte by cross-reactive antibodies was then examined by cELISA (below).

**Sera inhibition screenings**

In order to determine the specificity of the serum antibodies, cELISA’s were conducted exploring various soluble inhibitors (e.g., heterocycles, PAHs).

Antibodies raised against 3TAA-KLH are shown to recognize only the 3TAA inhibitor, but not the underivatized thiophenes (Figure 24). Similarly, the other single-ring hapten antisera to 2TAA-KLH, 2TPA-KLH, and 5M2TAA-KLH sera could not be inhibited by any underivatized thiophenes, which included thiophene, methylated thiophenes, dibenzothiophene, and methylated dibenzothiophenes. As an additional positive control, all of these sera were inhibited by their respective hapten-KLH conjugate (data not shown).
Table 5. Hapten and carrier protein titers of experimental mice.

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<tr>
<th>Derivatized Hapten</th>
<th>Homologous Hapten</th>
<th>Heterologous Hapten</th>
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<td>(n=4)</td>
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<tr>
<td>2TPA</td>
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Carrier protein was KLH in all instances, except where noted in parentheses below the derivatized hapten.

*nd = not detectable

Blank boxes indicate assays that were not tested

a. Titration was not carried out far enough, 50% point exceeds dilution.
Figure 23. Titration ELISA of BT3AA-KLH sera against both BT3AA-BSA and BT5AA-BSA antigens coated onto microtiter plates. Serum was from mouse F (1 out of 5) after the 2nd bleed. The titer to the homologous hapten (BT3AA) was approximately 33,000 units/ml and the titer to the heterologous hapten (BT5AA) was approximately 1,400 units/ml.
Figure 24. Competitive ELISA of 3TAA-KLH sera. The coating antigen was 3TAA-BSA and the inhibitors were 3-methylthiophene, thiophene and 3TAA (structures shown).
Conversely, antibodies raised against DBTAA-KLH are shown to recognize all three inhibitors tested in this particular assay: dibenzothiophene, 4-methyl dibenzothiophene, and DBTAA (Figure 25).

The titers of the individual mice immunized with 2MF9AA varied greatly, in that one individual, mouse H (1 out of 5), had a hapten titer of 33,000, while the remaining 4 had titers lower than 1,000 despite having received the same immunogen conjugate at the same dosing regime. Mouse H serum was not able to recognize 2-methylphenanthrene, when employed as an inhibitor in the cELISA, while mouse B, a lower hapten titer serum, could, resulting in an IC₅₀ (inflection point; concentration of inhibitor at 50% of the response) of 4 µg/l (Figure 26).

Sera from both groups of mice immunized with the 4BIPAA-KLH and 2BIPAA-KLH were screened in a cELISA for activity towards biphenyl. Sera from mice immunized with the 4BIPAA-KLH exhibited high sensitivity to biphenyl with an IC₅₀ value of 0.3 µg/l, which is approximately 100 times more sensitive than mice immunized with 2BIPAA (Figure 27). Furthermore, when the other 4BIPAA-carrier immunized mice (4BIPAA-BSA and 4BIPAA-Thyro) produced detectable titers, when they were assessed for biphenyl specificity, biphenyl could not inhibit, while it could for the 4BIPAA-KLH antisera (Figure 28).

Then, 4BIPAA-KLH sera were also screened against a panel of PAHs (pyrene, naphthalene, phenanthrene, biphenyl and anthracene) to provide a profile of which PAHs were recognized. This was completed after both the 3rd and 4th bleeds. As shown in Figure 29, this sera was specific for the 3-ring PAHs anthracene (IC₅₀(4th) = 4 µg/l) and phenanthrene (IC₅₀(4th) = 400 µg/l), as well as, biphenyl (IC₅₀(4th) = 500 µg/l), but not for naphthalene or the larger PAH pyrene. However, one month earlier (i.e., 3rd bleed, Figure 30) naphthalene was recognized demonstrating an IC₅₀(3rd) of 400 µg/l. Also interesting is the minimum amount of change in sensitivity for anthracene (IC₅₀(3rd) = 6 µg/l) and phenanthrene (IC₅₀(3rd) = 300 µg/l), while sensitivity to biphenyl (IC₅₀(3rd) = 20 µg/l) decreased by 25-fold from 3rd to 4th bleed.
Figure 25. Competitive ELISA of DBTAA-KLH sera (mouse B, 1 out of 4). The coating antigen was DBTAA-BSA and the inhibitors were DBTAA, 4-methyldibenzothiophene, and dibenzothiophene (structures shown).
Figure 26. Competitive ELISA of 2MF9AA-KLH sera from mice B and H (2 out of 5). The coating antigen was 2MF9AA-BSA and the inhibitor was 2-methylphenanthrene (structure shown).
Figure 27. Competitive ELISA of 4BIPAA-KLH and 2BIPAA-KLH sera. Each plate was coated with its respective hapten-BSA and the inhibitor was biphenyl (structure shown).
Figure 28. Competitive ELISA of 4BIPAA sera generated to various protein carriers (KLH, BSA and Thyro). The coating antigen for the 4BIPAA-KLH and 4BIPAA-Thyro sera was 4BIPAA-BSA, while the coating antigen for 4BIPAA-BSA sera was 4BIPAA-Thyro. All of these assays employed biphenyl as the inhibitor (structure shown).
Figure 29. Competitive ELISA of 4BIPAA-KLH sera (mouse B, 1 out of 4) assessed after the 4th bleed. The coating antigen was 4BIPAA-BSA and the inhibitors were various PAHs: pyrene, naphthalene, phenanthrene, biphenyl and anthracene (structures shown)
Figure 30. Competitive ELISA of 4BIPAA-KLH sera (mouse B, 1 out of 4) assessed after the 3\textsuperscript{rd} bleed. The coating antigen was 4BIPAA-BSA and the inhibitors were various PAHs: pyrene, naphthalene, phenanthrene, biphenyl and anthracene (structures shown)
The 2MF9AA-KLH sera was screened against a panel of PAHs and alkylated PAHs to provide a profile of which compounds were recognized (Figure 31). The unsubstituted 3- and 4-ring PAHs exhibit the highest sensitivity with IC₅₀ values ranging from 10 to 60 µg/l. The mono-alkylated 3-ring PAH and heterocycle were recognized similarly with IC₅₀ values around 30 and 80 µg/l. The di-alkylated 3- and 4-ring PAHs exhibited good inhibition with IC₅₀ values ranging from 80 to 200 µg/l. The alkylated naphthalenes, both di- and tri-substituted, demonstrated less sensitivity (IC₅₀ values >1,000 µg/l) than all of the other PAHs used in this study. Overall, this sera was specific for 3- and 4-ring PAHs and alkylated PAHs, but not for alkylated 2-ring PAHs.

The titer screening of BT3AA-KLH sera against the heterologous hapten BT5AA produced a measurable response. Because the initial cELISA of BT3AA sera conducted on plates coated with its homologous hapten, BT3AA, did not demonstrate inhibition from benzothiophene, its surrogate target, a heterologous cELISA was performed. In this case, the BT3AA sera were first titered against BT5AA to determine its 50% point (refer to Figure 23). As a result of the cELISA, BT3AA-KLH sera were inhibited by benzothiophene when assayed on a plate coated with the heterologous BT5AA hapten (Figure 32).

**MAb production**

Numerous (15) fusions were performed on mice immunized with various hapten-carrier immunogens. As the goal was to isolate hapten-specific antibodies, fusions resulting in linking arm-specific or carrier specific antibodies, were discarded. A few of these were also tested for IgG secretion and were positive. The frequencies of these unwanted specificities were recorded – approximately 75% were secreting IgG and of those 30% were anti-KLH on a single plate.

Following the fusion of a DBTAA-KLH immunized mouse, 22 positive cultures expressed antibody activity towards DBTAA-BSA. From the cELISA screening of these fusion plates, 3 hybridomas demonstrated significant inhibition with dibenzothiophene
Figure 31. Competitive ELISA of 2MF9AA-KLH sera (mouse F, 1 out of 5) against various unsubstituted and alkylated PAHs. The coating antigen was 2MF9AA-BSA and the inhibitors were: phenanthrene, anthracene, benzo[a]anthracene, 9,10-dimethylbenzanthracene, 7,12-dimethylbenzanthracene, 9,10-dimethylanthracene, 3,6-dimethylphenanthrene, 2,3,5-trimethylnaphthalene, 1,4,5-trimethylnaphthalene, 1,3-dimethynaphthalene, 4-methyl dibenzothiophene and 2-methylphenanthrene (select structures shown).
Figure 32. Competitive ELISA of BT3AA-KLH sera (mouse E, 1 out of 5) against BT3AA-BSA and BT5AA-BSA coated plates and inhibited by benzothiophene (structure shown).
and were cloned. The mAb 7B2.3, an IgG2a isotype possessing kappa light chains, was selected for use in the biosensor.

After performing the magnetic bead isolation procedure during a 4BIPAA-KLH fusion, a total of approximately $6 \times 10^5$ cells were counted in the hapten-selected fraction. These were then plated into six wells of a 96-well tissue culture plate in order to maintain high cell density to aid in cell survival and growth. The remaining fraction totaled $1.1 \times 10^8$ cells and was plated into seven 96-well plates. Upon screening, one anti-4BIPAA-BSA positive hybridoma was revealed from the supernatant fraction of cells (i.e., not hapten-selected). Regardless, when screened against biphenyl, no reduction in activity was seen. Therefore, this hybridoma was positive for the linking arm and discarded. No hybridoma from the hapten-selected fraction revealed any anti-4BIPAA-BSA activity.

**MAb titration**

The titration profile of the purified 7B2.3 was obtained by screening against a coating concentration of 1 μg/ml DBTAA-BSA resulted in a 50% point corresponding to 200 μg/l of mAb (Figure 33). Thiomersal is a common preservative employed at a concentration of 0.01%. When used at this concentration in a titration ELISA for 7B3.2, there was no detectable response.

**MAb inhibition screening**

MAb 7B2.3 demonstrated differential sensitivities for a wide variety of PAHs (Figure 34). The 2-ring PAHs biphenyl and naphthalene exhibited virtually no inhibition, the 3-ring PAHs anthracene, phenanthrene, and fluorene exhibit good inhibition with IC$_{50}$ values ranging from 2 to 28 μg/l. The sulfur-containing heterocycle, dibenzothiophene, displayed an inhibition (IC$_{50}$ of 19 μg/l) similar to that of the 3-ring PAHs, whereas the oxygen-containing PAH dibenzofuran demonstrated a 5-fold less sensitivity (IC$_{50}$ of 105 μg/l). The 4-ring PAHs chrysene and benzo[a]anthracene were also sensitively detected with IC$_{50}$ values of 0.7 μg/l. The 5-ring PAH benzo[a]pyrene was recognized with the highest sensitivity with an IC$_{50}$ value of 0.5 μg/l. Furthermore, another cELISA was
Figure 33. Titer of 7B2.3 using antigen DBTAA-BSA coated at 1 μg/ml. Thiomersal (structure shown) was employed as a preservative in the buffer diluent at 0.01% and is shown here to inhibit the response.
Figure 34. Competitive ELISA of 7B2.3 against unsubstituted PAHs. The coating antigen was DBTAA-BSA and the inhibitors were anthracene, benzo[a]pyrene, benzo[a]anthracene, biphenyl, chrysene, dibenzofuran, dibenzothiophene, fluoranthene, fluorene, naphthalene, phenanthrene and pyrene (select structures shown).
conducted employing alkylated PAHs (Figure 35). Here, the alkylated naphthalenes (2-rings) exhibit more inhibition (IC$_{50}$ of 60 to 100 µg/l) than the unsubstituted 2-ring PAHs, yet still 10-fold higher than the alkylated 3- and 4-PAHs (IC$_{50}$ below 6 µg/l). While most of the 3- and 4-ring alkylated PAHs have comparable IC$_{50}$ values with the unsubstituted PAHs, the mAb is highly sensitive to 2-methylphenanthrene (IC$_{50}$ < 0.1 µg/l).

The inhibitors selected for a cELISA evaluation are often those compounds within the class of PAHs as they are most likely to cross-react with the antibody. GC-MS analysis of the stormwater runoff indicated that other unrelated pollutants were present in high concentrations. Therefore, to ensure that there was no cross-reactivity with these compounds, a cELISA was conducted employing the following analytes: bisphenol-A, triclosan, nonylphenol and caffeine (Figure 36). Phenanthrene was assessed as a positive control and for reference. Only at extremely high concentrations (> 1,000 µg/l) do these analytes exhibit any inhibition of the antibody response.

**Biosensor development**

*Antibody kinetic analysis*

Kinetic analysis of the 7B2.3 revealed an equilibrium association constant, $K_a$, of $2.5 \times 10^8$ M$^{-1}$ with an on-rate ($k_{on}$) of $1.52 \times 10^5$ M$^{-1}$ s$^{-1}$ and an off-rate ($k_{off}$) of $6.10 \times 10^{-4}$ s$^{-1}$. Equilibrium was also empirically determined to occur within approximately 10 seconds on the basis of a series of timed reactions. This demonstrated that the biosensor method did not require any additional time for antibody and sample incubation.

*Calibration curves for the biosensor*

The regression curves for the four calibration standards are shown in Figure 37. The concentrations of these solutions ranged from 0.3 to 30 µg/l total PAH. The calibration curves have comparable log-linear best fit lines with minimal variability in each line ($r^2 \geq 0.98$). Based on these results, and for simplicity, the phenanthrene standard curve was
Figure 35. Competitive ELISA of 7B2.3 against alkylated PAHs. The coating antigen was DBTAA-BSA and the inhibitors were 9,10-dimethylbenzanthracene, 7,12-dimethylbenzanthracene, 9,10-dimethylanthracene, 3,6-dimethylphenanthrene, 2,3,5-trimethylnaphthalene, 1,4,5-trimethylnaphthalene, 1,3-dimethylnaphthalene, 4-methyldibenzothiophene and 2-methylphenanthrene (select structures shown).
Figure 36. Competitive ELISA of 7B2.3 against other environmental contaminants – bisphenol-A, triclosan, nonylphenol, and caffeine – found in the stormwater runoff. The coating antigen was DBTAA-BSA and phenanthrene was assessed as a positive control and for reference.
Figure 37. Biosensor calibration curves for solutions containing either a mixture of PAHs; SRM 1647b, SRM 2260, 6 PAH (phenanthrene, anthracene, fluorene, chrysene, pyrene, and fluoranthrene) or phenanthrene. The log-linear lines of best fit are displayed for each solution tested. For the sake of clarity, standard deviation bars are shown only for phenanthrene, as determined by analysis of triplicate standard solutions at each concentration.
selected for use in field applications; therefore, biosensor assessments were reported as phenanthrene equivalents. The detection limit for phenanthrene equivalents, as determined by standard EPA method, was 0.3 μg/l PAHs with a linear range of 0.3 to 30 μg/l. For field analyses, the detection limit varied from 0.2 to 1 μg/l. The error in the method, from triplicate analysis of standard solutions, was usually no more than 10% of the signal response (Figure 37). The largest error is seen at the highest biosensor response as this is where the PAH concentration is the lowest and is approaching the detection limit.

**Solvent effects**

Various amounts of solvent buffer solutions were examined on the KinExA®3000 (Figure 38). All of the solutions containing DMF and DMSO reduced the signal substantially. The 5% solution of methanol and both the acetone-containing solutions increased the signal by about 20%, while the 10% solution of methanol increased it by almost 50%. A reduced signal was thought to lower the sensitivity of the instrument as it would require more antibody molecules to generate a larger signal, which in turn requires more analytes to reduce the signal, thereby not improving the detection limit. Therefore, methanol and acetone were the desirable solvents, and 5% methanol was the most often employed due to its lower evaporative properties.

**Salinity effects**

HMM was diluted to varying concentrations in order to simulate varying levels of salinity within the aqueous environment and its effect on the biosensor. Solutions containing 0 to 38 ppt of salinity did not adversely affect the biosensor signal (Figure 39). Similarly, analysis of samples spiked with known amounts of phenanthrene demonstrated no discrepancy in response due to the salinity of the sample. Likewise, when assessing phenanthrene spiked PBS and filtered YR water samples, no adverse affects were evident.
Figure 38. Solvent interactions on antibody binding to the antigen-coated beads. All values were normalized to the voltage change with no solvent, with the same amount of antibody present in each sample, in duplicate.
Figure 39. Salinity interactions on the biosensor. Varying salinity solutions of HMM were employed. Two phenanthrene spiked HMM solutions at varying salinities were also tested and shown above. Dashed lines represent ± 3SD around the mean of the standard solution containing no salts.
DOC effects

HA was employed to examine the effects of DOC on biosensor performance. When HA concentrations exceeded 800 μg/l, the biosensor signal was adversely affected (Figure 40). The antibody signal dropped approximately 10% from that of the standard solution containing no HA. Yet, when HA solutions were spiked with phenanthrene at 2.0 μg/l and assessed, the response did not deviate greater than 10% around that of the standard solution containing no HA.

Biosensor applications

Groundwater monitoring

Samples from wells W7, W5, MW81 and D8 did not require dilution for biosensor analysis. The samples from PW01, D3, W6 and MW80 were diluted before analysis. The estimated PAH concentrations from the biosensor and GC-MS analyses for each well are shown in Figure 41. The GC-MS results are presented as 1) total PAHs (including alkylated and unsubstituted PAHs, 2 rings or greater) and as 2) a sum of 6 PAHs (phenanthrene, anthracene, fluorene, chrysene, pyrene, and fluoranthrene). Since the antibody has greater specificity for the 3- to 5-ring PAHs, it should underestimate total PAH if the sample contains a large amount of the lower molecular weight PAHs (e.g., naphthalenes). To test this hypothesis, total PAH and 6 PAH were compared to the biosensor results.

Well samples W7, W5, and D8 were below the detection limit of the biosensor and the GC-MS analyses confirmed these results – total PAH concentrations were less than 0.02 μg/l. The remaining wells had detectable concentrations of PAHs. Biosensor results for wells MW80 and PW01 greatly underestimated total PAH concentrations compared to the GC-MS results, but corresponded well with the sum of the selected 6 PAHs. Based on GC-MS analysis, both samples contain substantial concentrations of the 2-ring PAHs (e.g., naphthalenes). In well samples W6, MW81, and D3, the biosensor overestimated
Figure 40. Humic acid interactions on the biosensor. Varying amounts of humic acid solutions were employed. Phenanthrene spiked solutions at varying humic acid concentrations were also tested. Dashed lines represent ± 3SD around the mean of the standard solution containing no humic acid.
Figure 41. PAH concentrations (expressed as phenanthrene equivalents, µg/l) measured by the biosensor and GC-MS for the wells sampled during the groundwater monitoring at the LA Clarke & Son site in Spotsylvania, VA. The white bars are the GC-MS results for total PAHs, the hatched bars are the GC-MS sum of the 6 PAHs (phenanthrene, anthracene, fluorene, chrysene, pyrene, and fluoranthrene), and the black bars are the biosensor results. There is a good correlation between the biosensor and GC-MS methods with respect to GC-MS total PAH \( (r = 0.58, n = 7) \) and the 6 PAH sum \( (r = 0.79, n = 7) \).
the total PAH concentration. Here, the GC-MS analysis detected quantifiable concentrations for polycyclic aromatic heterocycles (e.g., dibenzothiophene, methyl-dibenzothiophene, and benzonaphthofuran) which are not included in the total PAH concentration value and cross-react with the antibody. Overall, a better correlation was generated when comparing the biosensor results to the 6 PAH sum \( r = 0.79, n = 7 \) than to total PAH \( r = 0.58, n = 7 \). Similarly, using Deming regression analysis, the slope of the line of best fit for the biosensor results compared to the 6 PAH values was \( 0.65 \pm 0.21 \) (se), while to the total PAH values it was \( 0.11 \pm 0.06 \).

**Estuarine monitoring**

A spatial representation of PAH concentrations measured with the biosensor defines a plume ranging from 0.3 to 3.2 \( \mu g/l \) (phenanthrene equivalents) with higher PAH concentrations closer to the dredge site (Figure 42). Due to the biosensor’s rapid and on-site operation, an area of approximately 90,000 \( m^2 \) was surveyed around the operating dredge site, thus defining the extent of the plume relative to background PAH concentrations in the estuary. PAH concentrations at seven of the stations, where additional large volume samples were collected, demonstrated a very strong positive correlation between biosensor and GC-MS results \( r = 0.95, n = 7 \) (Figure 43). The GC-MS PAH value is the sum of all detected (LOD > 0.01 \( \mu g/l \) per analyte) alkylated and non-substituted 3- to 5-ring PAHs (11 to 39 compounds identified). As a comparison, the 16 EPA PAHs (excluding naphthalene) comprised 19 to 41% (with one sample containing 88%) of the reported total PAH concentration. The Deming regression slope of the line comparing the biosensor to GC-MS total PAH was \( 0.90 \pm 0.14 \) (se).

**Toxicological study**

The zebrafish toxicological study focused on measuring a single spiked PAH analyte, phenanthrene throughout a dosing experiment. Forty-eight samples were collected, 24 preceding renewal and 24 post renewal to monitor changes in phenanthrene concentrations over a span of 5 days. There was a strong correlation between HPLC determined PAH concentrations and those determined by biosensor \( r = 0.84, n = 48; \)
Figure 42. Near real-time PAH monitoring of the remedial dredging project completed at the Money Point site in the Elizabeth River in Norfolk, VA. The PAH concentrations determined by the biosensor are represented by the gray circles proportional to concentration. (Note, concentrations for the sites labeled beginning with a 'C' are reported in Figure 3 as these were validated by GC-MS.) The 'dredge' polygon represents the location of the dredging operation.
Figure 43. PAH concentrations (μg/l) measured by the biosensor and GC-MS (sum of 11 to 39 analytes) during the estuarine monitoring at the Money Point site along the Elizabeth River in Norfolk, VA. There is a strong correlation between the two methods ($r = 0.95, n = 7$). Based on Deming regression analysis, the slope of the line of best fit was $0.90 ± 0.14$ (se).
Figure 44. Based on Deming regression analysis, the slope of the line of best fit was $1.1 \pm 0.10$.

Although a strong correlation was observed, the biosensor, on average, overestimated the phenanthrene concentration. Since phenanthrene serves as the standard for biosensor calibration, overestimation of phenanthrene concentrations suggested that cross-reactive phenanthrene oxidation products could be entering the water. The reactivity of 1-hydroxyphenanthrene (a metabolic by-product detected in the zebrafish tissues) was tested on the biosensor and this oxidation product exhibited comparable reactivity to the parent compound, phenanthrene (Figure 45).

**Stormwater runoff study**

PAH concentrations measured with the biosensor and cumulative rainfall for the CB and YTC sites were documented before, during, and after the rain event (Figure 46, A and B). The rain event began at approximately 1 AM local time. At both sites, the initial increase of PAH pollutants was measured approximately 1 to 2 hours after the rain started. Although rainfall ceased at approximately 8 AM, sample collection continued until the PAH concentrations declined to background values. From the start of the rain event to the end of the intensive sampling session, 17 hours elapsed and 84 samples (14 time points, triplicate samples, 2 sites) were collected for biosensor analysis. Typically, two of the three replicate samples were analyzed within the hour. In total, the CB site received 1 cm of rain, while the YTC site received 0.8 cm. The peak PAH concentration (phenanthrene equivalents) measured was 4.4 µg/l for the CB site and 3.6 µg/l for the YTC site. Samples were collected periodically during the rain event for GC-MS analysis in an attempt to encompass a range of PAH concentrations. The biosensor analyses were used to guide this sampling effort due to its generation of near-real-time results. The GC-MS PAH value is the sum of all alkylated and non-substituted 3- to 5-ring PAHs (4 to 9 compounds identified). Good correlation ($r = 0.86$, $n = 7$) between biosensor and GC-MS results was observed (Figure 47). As a comparison, the sum of the 16 EPA PAHs (excluding naphthalene) comprised 50 to 78% of the reported total PAH concentration.
The Deming regression slope of the line comparing the biosensor to GC-MS results is $9.68 \pm 2.63$.

Figure 44. Phenanthrene concentrations (µg/l) determined by HPLC and biosensor for samples taken during the toxicology study. There is a strong correlation between methods in determining phenanthrene concentrations ($r = 0.84$, $n = 48$). Based on Deming regression analysis, the slope of the line of best fit (shown as a solid line) was $1.1 \pm 0.10$. The dashed line is the theoretical 1:1 relationship line.
Figure 45. Biosensor detection of 1-hydroxyphenanthrene at concentrations comparable to PAHs is shown by the black circles and solid line. For comparison, the results for phenanthrene are shown by the white circles and dashed line.
Figure 46. Near real-time monitoring of PAH concentrations in stormwater runoff during a rain event. Concentration profiles of PAHs assessed from samples collected from A) a retention pond located below the Coleman Bridge and B) a culvert into the Yorktown Creek located alongside U.S. Route 17. Error bars represent the standard deviation of three replicate samples. The bars indicate the PAH concentrations and the line shows the cumulative rainfall.
Figure 47. PAH concentrations (μg/l) from the stormwater runoff study as determined by the biosensor and GC-MS methods (sum of 4 to 9 analytes). There was a strong correlation between methods in determining PAH concentrations ($r = 0.86$, $n = 7$). Based on Deming regression analysis, the slope of the line of best fit was $9.68 \pm 2.63$ (se).
DISCUSSION

Haptenation efficiency with hydrophobic haptens

Changes in protein haptenation can influence the activity and specificity of the resulting antibody molecule. For example, Klaus and Cross (1974) report that an immunogen containing a high hapten:protein molar ratio generally stimulates an IgM response, while a low haptenation ratio (<30) results in high affinity IgG antibodies, perhaps governed by antigen-driven selection. Researchers have produced high affinity IgG mAbs using immunogens with as many as 26 PAH haptens attached to the BSA carrier molecule (Scharnweber et al. 2001, Matschulat et al. 2005, Glushkov et al. 2006); however, they were not always concerned with keeping the conjugate soluble. Altering the organic solvent concentration could improve conjugation efficiencies while still allowing the resulting conjugate to remain soluble despite the increased number of hydrophobic haptens. In turn, this would reduce the amount of hapten needed, which is beneficial for haptens that can only be obtained in small quantities. Although altering the molar ratio of hapten to protein had been previously explored (Malaitsev and Azhipa 1993, Vyjayanthi et al. 1995, Singh et al. 2004), to my knowledge altering the amount of organic solvent in the reaction mixture had not been investigated. The goal was to produce DBTAA-conjugates without causing conformational changes that would result in precipitation of the conjugate and/or compromising its ability to serve as a screening antigen or immunogen. I coupled approximately 6 haptens per molecule of BSA (Table 4) and feel that this served us well in terms of effectiveness and ease of use.

Researchers have historically relied on TNBS and other spectrophotometric methods to determine haptenation ratios (Scharnweber et al. 2001, Glushkov et al. 2006). Direct measurement by UV/vis spectroscopy is possible when haptens have strong chromophores that absorb at longer wavelengths than the protein. TNBS titrations take
advantage of the strong trinitrophenyl chromophores of the TNBS to estimate the amino groups of the protein remaining free after conjugation with the hapten (Habeeb 1966). Some studies comparing MALDI-MS with the spectrophotometric methods have concluded that they give comparable results (Singh et al. 2004, Matschulat et al. 2005). However, others have observed major discrepancies between these methods (Wengatz et al. 1992, Adamczyk et al. 1994). There is evidence that adducts of hydrophobic compounds can change the conformation of proteins altering the number of amino groups that can be titrated with TNBS. In addition, it may not be possible to completely remove unreacted hapten from BSA and other proteins that have hydrophobic pockets. MALDI-MS avoids these problems. However, there are several limitations with mass spectrometric methods: the level of adduction must be sufficiently high and the difference in mass of the adducted and unadducted must be sufficiently large that it can be measured accurately. Furthermore, the protein must be relatively homogeneous, the conjugates need to be soluble and there are mass limitations with the instrumentation. As a consequence, BSA adducts are well-suited for estimation by MALDI-MS, but KLH adducts are not as their molecular weights are highly variable and high (~1x10^6 Da).

In a comparison of this work with that of Singh et al. (2004), where the focus was on characterizing protein conjugates based on the amount of hapten added to a solution, this method provides a 20% increase in conjugation efficiency. This was achieved in the 25% DMF/PBS solution. Both studies added the hapten at a 10:1 molar ratio with BSA and made use of MALDI-MS for determining the haptenation ratios. Singh et al. succeeded in conjugating an average of 4 molecules, compared to 6 in this study. However, Singh et al. only used borate buffer and did not account for the volume of DMF that was being added when adding the activated derivatized hapten, although this only amounted to, at most, 3% DMF. The borate solutions used in the present study yielded an efficiency of 40-50%, which is comparable to those published by Singh (2004). This conjugation protocol is expected to apply to the other hydrophobic haptens and did apply to other common carriers, such as Thyro. This protocol did not prove effective with OVA, but it is unclear if this was because too many hydrophobic moieties were being attached to the
molecule causing it to precipitate or because the reagents in this method are somehow unsuitable for OVA.

**Generation of amines from DMF solvent**

Although it was not intentional to characterize the 3MF9AA hapten after activation, this generated a fortuitous result. The goal was to hydrolyze the ester activated product to regenerate the carboxylic acid. GC-MS analysis revealed an unexpected amine product. This was probably a product of using DMF as the solvent since this activated derivatized hapten was used after prolonged storage in the -80°C freezer. As a result, a better solvent choice might have been N-methyl-2-pyrrolidone or N,N-dimethylacetamide. Both of which are less susceptible hydrolysis in aqueous solutions. Furthermore, DMF instability may have had implications in the previous findings referring to haptenation ratios. By using DMF solvent, many of the activated haptens may have already been quenched and therefore unavailable to react with the free amine groups on the protein, perhaps producing a lower haptenation ratio.

**Thiomersal interference**

Thiomersal was completely incompatible with titration ELISA (Figure 33), such that it should never be employed as a preservative for prolonged storage with 7B2.3. An alternative preservative that has been employed without any adverse effects is sodium azide.

**Antisera specificities**

The analysis of serum screenings, both of titers (ELISA) and specificity analysis (cELISA), provided new insights into antibody recognition and hapten design. No inhibitions were observed with any of the single ring haptens (i.e., 2TAA, 2TPA, 5MTAA and 3TAA), but inhibition did occur with the 2-ring and larger haptens (i.e., BT3AA, 4BIPAA, DBTAA, and 2MF9AA). Although this observation suggests that a hapten the size of one benzene ring alone may be too small for an antibody to recognize,
trinitrophenyl is a very commonly employed hapten in immunological studies (Rittenberg and Pratt 1969). However a key feature of trinitrophenyl is that it possesses three nitro groups, which have polar characteristics not present in the hydrophobic PAH and arylthiophene haptens. Increased antibody recognition of polar characteristics is also evident in the fact that these same sera could be inhibited by the derivatized hapten (Figure 24), which possesses an oxygen molecule at the site of attachment to the carrier protein. Antibody recognition of the carboxyl group, which is on the distal end of the linking arm, suggests that the entire hapten structure, including the linking arm, is being recognized within the antibody binding pocket.

For the individual antiserum that demonstrated a high hapten-carrier titer (i.e., titer values >2,000; 4BIPAA-BSA, 4BIPAA-Thyro, BT3AA-KLH, 3MBT2AA-KLH, and 2MF9AA-KLH (one individual); Table 5), inhibition assays with the underivatized target analyte were not successful. Similar observations of high hapten titer with low sensitivity have been reported elsewhere employing other small molecules (Danilova 1994, Zhang et al. 2007). Furthermore, various protein carriers for the 4BIPAA adduct were employed in an attempt to generate more hapten specific antibodies. While this seemed to have worked – hapten titers were >2,000 – similar to the previous findings, neither of these sera recognized biphenyl. It is mentioned that the choice of carrier molecule is highly important (Fasciglione et al. 1996). However, an immunization scheme such as this, employing a variety of carriers with a single hapten, has not often been conducted. Varying the coating antigen’s carrier molecule has been explored (Marco et al. 1993a), as well as, varying the linking arm between the carrier and the hapten (Danilova 1994).

The demonstration of biphenyl inhibition with the 4BIPAA-KLH sera, and not with the 2BIPAA-KLH sera (Figure 27) is intriguing. The only change in hapten conjugation was the position of the linking arm for carrier attachment (Figure 10). For 2BIPAA, the attachment at the 2 position on the biphenyl molecule may inhibit free rotation around the central bond of the biphenyl moiety (Figure 48). Rotation about the central bond is not inhibited with the linker arm attached at C4. This emphasizes the importance of preserving identifiable structures of a hapten to allow for desired inhibition or affinity for
Figure 48. Spatial illustrations of biphenyl, 4BIPAA, and 2BIPAA. Although biphenyl alone appears planar, it can freely rotate around its central bond. This same free central bond rotation is preserved in the 4BIPAA where attachment of the linking arm is on the 4 position. However, as shown in the image of the 2BIPAA, linking arm attachment at the 2 position reduces the ability of the molecule to rotate as freely.
the underivatized target molecule (Marco et al. 1995). While it is common to preserve functional groups (e.g., carboxyls, ketones, amines), inherent structural characteristics must also be considered when designing a hapten-carrier immunogen.

Another consideration in hapten design is the length of the linking arm. A length of three to six atoms is frequently suggested to be ideal since it is distant enough from the carrier, to avoid steric hindrance with the recognition site of the B cell, yet not long enough to lead to folding (Marco et al. 1995, Song et al. 2010). Many researchers have been successful generating antibodies to PAHs by employing haptens with four carbon spacer arms (Meisenecker et al. 1993, Scharnweber et al. 2001, Suchanek et al. 2001). Many of the haptens synthesized in this study employed three carbon spacer arms (i.e., acrylic acid chains; Figure 10). Two-carbon linking arms were used as three immunizing haptens; BT5AA, 2MF9AA and 3MF9AA. Antibodies generated to BT5AA exhibited very low hapten titers (<50; Table 5), and the titers for 2MF9AA (excluding one individual) and 3MF9AA were only a little higher (<1,000, Table 5). As antibody screenings were conducted against antigens with the same hapten, but a different carrier, the BT5AA sera study suggests that a two-carbon linking arm attached to a two-ring molecule may not be sufficient to produce distinct recognition of the hapten. However, the additional ring on 2MF9AA and 3MF9AA may have led to a portion of the hapten to be distinct from the protein carrier.

A measurable titer could be observed when the BT5AA conjugate (containing a 2-carbon linking arm) was employed in a titration ELISA with BT3AA sera. Furthermore, PAH inhibition was demonstrated (Figure 32). Heterologous haptens have been employed to explore possible heteroclitic responses, wherein the binding of the antibody is greater for a hapten other than the one that induced it (Marco et al. 1993b, Abad et al. 1998, Lee et al. 1998, Zhang et al. 2007). Conversely, with the homologous hapten employing another 2-carbon linking arm-containing hapten, 2MF9AA, all but one individual were capable of recognizing the PAH (Figure 26 and Figure 31). In essence, both heterologous and homologous haptens can be employed in cELISAs with haptens containing 2-carbon linking arms, even though their success as immunogens was limited. Therefore, although
2-carbon linking arms with very small hydrophobic haptens (smaller than 2 benzene rings) should not be employed in designing an immunogen, they may still be valuable as screening antigens.

Not only is each individual’s antibody repertoire different, these antibody specificity profiles can change over time. With each subsequent antigen challenge, or simply by the process of affinity maturation, the specificity of the sera changes from recognizing one PAH with rather low affinity, to a related one with even higher affinity. This was demonstrated by the profiles of 4BIPAA sera over time (Figure 29 and Figure 30). While it is crucial to know the specificity of a serum response, it is also impossible to predict what profile will be obtained when ultimately generating a mAb, as the serum response is a composite of many different antibodies. In fact, not only does the specificity of the antibody change, so does the sensitivity. The detection limit was increased by approximately 10-fold in 7B2.3 as compared to the antiserum screened for dibenzothiophene (Figure 25 and Figure 34). This was expected, given that individual antibody-secreting cells are being isolated, fused, and selected based on their sensitivity and specificity. Therefore, less effort needs to be focused on screening sera with the entire suite of PAHs, and more effort devoted to mAb selection. While polyclonal sera could be employed for PAH detection, the changing affinity profile renders them an inconsistent option.

Employing cELISA during mAb development

The use of a cELISA with the underivatized PAH is essential as antibodies may recognize some portion of the hapten-linker-carrier and not strictly the hapten alone (Vanderlaan et al. 1988, Danilova 1994). This cELISA should be employed in the initial screening of the hybridoma supernatants to confirm adequate activity towards the underivatized PAH. A cELISA of the mAb ensures a better likelihood of selecting the most specific anti-PAH hybridoma, because fusions tend to yield a low percentage of positive cultures overall.
A cELISA was carried out by Quelven and colleagues, where they screened the mAbs after the hybridomas had been fully cloned and discovered that only one mAb had a high affinity towards anthracene, whereas the other mAb only recognized the modified anthracene (1999). Even though successful mAbs have been selected without performing a competitive screening (Quelven et al. 1999, Schamweber et al. 2001, Matschulat et al. 2005), the mAbs were eventually screened with the underivatized PAH inhibitor.

**Magnetic bead isolation comments**

Although 15 fusions were performed, only one yielded a suitable analyte-specific hybridoma. Of those that did not yield a suitable mAb, the fusion revealed the production of IgG antibodies specific for the carrier, indicating that the fusion was a success. In an effort to increase the likelihood of fusing a hapten-specific splenocytes instead of carrier-specific ones, magnetic beads were employed to concentrate these hapten-specific cells. However, because so few were isolated, plating them at a low density into only six wells may have reduced their chances of survival. One way to overcome the low density is to supplement the well with peritoneal macrophages (Bartal and Hirshaut 1987). Another alternative is to conduct subtractive panning, by removing the carrier-positive cells and leaving the hapten-specific cells and all of those without any surface IgG (Sheedy et al. 2007). Then, a microfluidic device can be employed that enables the pairing of these potentially hapten-specific cells to come into direct contact with their fusion partner cells while the fusion of membranes is occurring. In turn, this could yield a more efficient fusion overall (Skelley et al. 2009).

**Sensitivity and specificity of 7B2.3 compared to other anti PAH antibodies**

Antibody 7B2.3 in a cELISA format can detect dibenzothiophene as well as the other 3- to 5-ring PAHs in aqueous media at concentrations as low as 0.1 μg/l (0.1 ppb). Cross-reactivity analyses performed using select analytes demonstrated that this mAb recognized 3- to 5-ring unsubstituted PAHs, but not those with 2 rings, such as naphthalene or biphenyl (Figure 34). Previously, Quelven et al. (1999) developed an anti-anthracene mAb that recognized underivatized anthracene and it also cross-reacted
with the same 3- to 5-ring PAHs. An important difference between the Quelven antibody and 7B2.3 is that 7B2.3 has more than an order of magnitude higher sensitivity for PAHs. The Quelven antibody recognized underivatized anthracene at a concentration of 5 to 10 μg/l, whereas 7B2.3 did so at 0.1 to 10 μg/l. An anti-benzo[a]pyrene mAb developed by Scharnweber et al. could detect benzo[a]pyrene at a concentration as low as 0.3 μg/l (IC50 of 8.9 μg/l) and had specificity for mostly 4- to 6-ring compounds (2001). While the benzo[a]pyrene mAb seems as sensitive as 7B2.3, 7B2.3 recognizes the 3- to 5-ring PAHs, thus increasing its value as a total PAH detector for water samples as the larger 6-ring PAHs are highly insoluble and 3-ring PAHs are more soluble. Moreover, 7B2.3 has been tested with alkylated PAHs demonstrating its ability to quantify more than just the unsubstituted PAHs (Figure 35), perhaps providing a better determination of total PAHs in a sample.

**Immunoassay performance compared to commercially available technology**

The quantitative trace analysis of select individual PAHs by 7B2.3 was demonstrated in an ELISA-based immunoassay format. Thus it has potential as an alternative to HPLC and GC-MS techniques for PAH quantification. Overall, the assay takes 2 hours to complete and is highly sensitive (down to 0.1 μg/l) for a single analyte solution. For soil, there is a commercially available immunoassay test kit, EPA Method 4035 Ensys® PAH RIS soil test kit (SDIX), based on using an anti-phenanthrene mAb, which can detect PAHs at 1 mg/l (ppm) (McDonald et al. 1994). Moore and colleagues demonstrated that by using the mAb from the soil test kit in a biosensor format (screen-printed electrodes), the biosensor possesses a limit of detection of 1.6 ± 0.5 μg/l (ppb) for PAHs in water samples (2004). This assay, and that of Moore et al., are designed to detect water-soluble PAHs and are comparable in sensitivity to EPA Method 610 which detects PAHs in wastewaters down to 0.2 μg/l by means of HPLC. The method described here offers an increased likelihood of success, in terms of generating sensitive and selective mAbs, compared to previous reported polyclonal and monoclonal antibodies produced by other techniques.
Biosensor performance compared to the literature

The primary advantages of this biosensor are its speed, portability and sensitivity, as well as the small volumes required and limited sample manipulations. Overall, the biosensor provides rapid and precise quantification of PAHs, permitting delineation of small changes in concentration. Moreover, the instrument can be transported to the field and operated while on-site. This affords higher resolution than previous technology could easily allow, and has a myriad of uses in environmental monitoring and management.

On-site biosensor evaluation of water surrounding a contaminated sediment dredging operation documented a spatial distribution of PAH concentrations in near real-time (Figure 42). Information about the size and intensity of the plume was provided immediately to engineers monitoring the dredging operation from shore and was able to verify that the PAH plume never exceeded 10 µg/l. If the near real-time results had shown elevated concentrations were a concern, dredging could have been halted and remediative actions put in place. In addition, near real-time data provided information to scientists on board the vessel to guide the collection of large volume water samples for later analysis of specific PAH compounds. This saved time, effort, and money by eliminating the future analysis of samples that might contain PAHs below the detection limit of the GC-MS methods. Overall, the data provided by this near real-time assay allowed for rapid decision-making about sample collection and remediation effectiveness.

The biosensor was employed to document a temporal pulse of PAH concentrations at two locations during a rainfall event and to time the collection of validation samples. In the present study, sample collection for GC-MS validation began 2 hours after the rain had started, and continued 4 hours after the rain had stopped (Figure 46), which encompassed the period of all elevated PAH concentrations. Since field sites will differ in topography, runoff volume, and amount of contamination, the timing of the pulse of contaminants and the duration of the elevated fluxes will inevitably vary among sites (Hoffman et al. 1985). Moreover, input loads are a function of concentration of an analyte and flow volume over time. Since concentrations will vary widely over time, higher temporal resolution of measurements can reduce the inaccuracies in estimating input loads of contaminants to the system during a rainfall event (Hoffman et al. 1985). As shown in the present study,
measurements made after the rain has stopped (~4 hours later) revealed that elevated concentrations were still present (Figure 46).

By utilizing the biosensor in both of the above applications (estuarine and stormwater runoff monitoring), samples indicating a non-detectable PAH concentration (i.e., far from the dredge, before the pulse, and at the end of the flux) were not collected for additional, more costly laboratory-based analysis. These advantages can result in considerable savings of time and money for field PAH determinations.

The toxicological study permitted analysis of a single analyte (phenanthrene) rather than a complex mixture. By analyzing a single compound, superior accuracy was anticipated since the analyte was identical to the standard. However, overestimation occurred possibly due to the release of oxidized phenanthrene (Sun et al. 2006). These metabolites of phenanthrene, such as 1-hydroxyphenanthrene, cross-react with the antibody (Figure 45), which agrees with other authors’ suggestions (Li et al. 2000, Knopp 2006). These compounds were identified in fish tissues in the system (Prosser et al. 2011) and may have contributed to the biosensor overestimating the phenanthrene concentration in the water.

Validation of the immunoassay method for total PAH quantification in environmental samples is difficult as PAHs commonly occur as complex mixtures. Conventional validation methods (i.e., GC-MS) require the identification and quantification of the individual analytes before summation. However, immunoassays bind structurally similar groups of compounds resulting in one combined concentration estimate. Therefore, a defined number of these individual analytes detected in the conventional method must be pre-selected by the analyst to provide a single summed value to which the immunoassay method can be compared. To this end, most methods select the 16 EPA priority PAH pollutants for quantification. This has resulted in the publication of many studies in which immunoassays are reported as overestimating the PAH concentrations found in a sample (Barceló et al. 1998, Knopp et al. 2000, Li et al. 2000, Matschulat et al. 2005) with the explanation that this is due to the cross-reactivity of the antibody with similar
analytes. Much of the increased antibody reactivity could be cross-reactivity with alkylated PAHs, as it is well documented that alkylated species may comprise a large fraction of the PAHs in petrogenic samples (Neff et al. 2005, Pies et al. 2008). Therefore, the total PAH value determined by conventional methods (as defined as an arbitrary set of unsubstituted PAHs) may underestimate total PAH concentration in the environment. For this reason, the definition of total PAHs was broadened to include the sum of unsubstituted and alkylated homologs when using the GC-MS method. I found that the higher the number of analytes detected – 39 analytes in the estuarine study compared to 9 in the stormwater study – resulted in a better correlation when comparing the total PAH value in the GC-MS and biosensor method (Figure 43 and Figure 47; \( m = 0.90 \) and 9.68, respectively). Similarly, in the groundwater monitoring study, heterocycles were present, which were not included in the summation of analytes from the GC-MS validation results. This may have contributed to the lower slope for the total PAH metric \( m = 0.11 \); 6 PAH, \( m = 0.65 \). Nonetheless, immunoassay detection of these other compounds may actually be desirable, because it is detecting other structurally similar compounds, which exhibit toxicity at comparable or lower concentrations (Neff et al. 2005).

The second confounding issue with validation of an immunoassay method with a conventional method is the detection limit of the conventional method. When the target analyte is just below the quantitation limit of the conventional method, it will not be added to the total. However, the antibody, due to cross-reactivity, will generate an additive effect and the resultant value will be the sum of all low level analytes. For example, the nearly 10-fold difference seen between the estuarine and stormwater runoff studies \( (m = 0.90 \) and \( m = 9.68 \) respectively) could be a result of the limit of detection for individual analytes of 0.01 \( \mu g/l \) by GC-MS. If the sample were to contain, for example, a thousand possible alkylated and unsubstituted PAHs below this limit, a total of 10 \( \mu g/l \) could potentially be undetected. This is also suggested in the estuarine study (Figure 43) where the two highest concentration samples vary considerably in the biosensor response but are similar in total PAH concentrations as measured by GC-MS. The highest GC-MS value \( (3.1 \mu g/l) \) is a sum of 39 analytes with many analytes near the detection limit while
the next highest GC-MS value (2.9 μg/l) is a sum of only 29 analytes and it is likely that many additional compounds were just below the reporting limit. Cross-reactivity is considered to be a limitation of antibody-based analytical methods, but this feature may actually provide a better summation of low level PAHs in complex mixtures not possible with targeted analyte-based conventional analyses. A potential resolution, when directly comparable results are required, is to calibrate the immunoassay result to the source material based on a few confirmatory conventionally assessed samples (e.g., GC-MS) (Waters et al. 1997).

A concern of any conventional on-site field sampling is the need for large sample volumes and the preservation and transportation of samples. In these demonstrations, sample volumes for biosensor analysis never exceeded 30 ml, although as little as 1 ml could be used for biosensor analysis. Sample holding time is another important consideration to avoid sample degradation. This can be avoided when employing immunoassays as there is little need for sample pretreatment (i.e., extraction or concentration) and analysis is conducted on-site promptly after collection. In this study, these samples were, at most, filtered or diluted. This also minimizes the amount of harmful solvents needed to be taken into the field. The only solvent employed was the DMSO used in the biosensor to clean lines between samples; 100 ml of DMSO was more than adequate for 50+ measurements.

Sensitivity, speed, and portability are considered to be the key advantages of immunoassays over conventional analytical methods for field monitoring of PAHs. In an effort to compare this method with published literature, I found, to the best of my knowledge, that no immunoassay has been reported for on-site, quantitative measurements of PAHs in aqueous environmental samples. In terms of sensitivity, detection limits are often reported in the sub-ppb (μg/l) level without the need for any sample pretreatment (Barceló et al. 1998, Li et al. 2000, Fähnrich et al. 2003, Gobi and Miura 2004, Matschulat et al. 2005, Dostalek et al. 2007), which is consistent with these results. Of the many immunoassay methods being developed, only ELISA-based methods have been employed for the quantification of PAHs in naturally contaminated
water samples (Barceló et al. 1998, Knopp et al. 2000, Li et al. 2000). This antibody-based biosensor method required 3 minutes for a quantitative response (including sample and antibody mixing time) and an additional 7 minutes for regeneration. Two surface plasmon resonance immunoassays report a minimum response time of 15 minutes from sample input to data output; one requires an additional few minutes for sensor regeneration (Gobi and Miura 2004) and the other requires 30 minutes for antibody incubation (Dostalek et al. 2007). A piezoelectric immunosensor for benzo[a]pyrene requires 30 minutes for benzo[a]pyrene incubation and then approximately 5 minutes for a steady state response (Liu et al. 1999). Although many methods are being developed with the promise of quantitative PAH monitoring, none have been exploited for their rapid on-site assessment capabilities. (For a review of PAH biosensors, see Appendix A.)

Field immunoassays can be susceptible to extreme temperature; however, ideal operating temperature ranges have only been reported for immunoassays designed for the analysis of PAHs in soil. The RaPID PAH ELISA (now from SDIX) requires the reagents to be within 18 to 27°C (Product Manual). It is speculated by Waters and colleagues that this same ELISA could adequately operate in the range of 10 to 30°C (1997). The PAH RIS® (from Ensys) is reported to perform correctly between ambient temperatures of 4 and 32°C (McDonald et al. 1994). Similarly, with the biosensor, maintaining a temperature range of 10 to 35°C ensured proper equipment function and accurate analysis.

Imunoassays have their limitations and optimal operating conditions. Understanding what the antibody recognizes determines the value of the assay and how the results should be interpreted. I have shown that field-based biosensor analyses can be accomplished with good correlation to laboratory-based conventional analytical methods. The biosensor provides a rapid generation of results (10 to 30 minutes), which guided a sampling survey and was used to measure the kinetics of PAH concentrations. The biosensor quantified PAH concentrations rapidly in the field using a simple protocol with small sample volumes. This technology represents a new tool to allow researchers and
environmental managers to document PAH fate in aqueous systems with resolution that was previously cost prohibitive.

**Future Perspectives**

While the original goal of developing biosensors was for the rapid detection of environmental contaminants, it may be adapted for other applications. Antibody platforms could also be used as a preparatory method for isolating and concentrating a specific analyte from a sample (Fishman et al. 1998, Pérez and Barceló 2000). Multi-analyte detection has been briefly explored (Miura et al. 2003, Dostalek et al. 2007) and may yield extraordinary uses for biosensors. Namely, biosensors could be employed to survey an area with unknown contaminants. Traditional methods require prior knowledge about a pollutant in order to determine the proper extraction method. Information can be missed if a particular analyte is not retrieved or is lower than the detection limit of the method. Once detected on-site by a sensitive biosensor, a larger sample can be collected for compound-specific analysis by traditional methods saving time and money. Additionally, this current biosensor technology has already been used for TNT (Bromage et al. 2007b) and uranium (Melton et al. 2009) analysis. Therefore, it is conceivable that they could be combined for multi-analyte analysis. The biosensor described here accurately quantifies 3- to 5-ring PAHs, which does not include the smaller aromatic PAHs. While the analysis is satisfactory for weathered environmental samples, which lack the more volatile 2-ring PAHs (Sauer et al. 1998), it could be improved by employing antibodies with specificity for smaller aromatic compounds. This would allow accurate total PAH assessments of any aqueous sample. These new tools might also be useful to discern contaminate sources and identify their petrogenic or pyrogenic origin (Wang and Fingas 2003). Another possible use of biosensors is to monitor the receiving dose during a toxicological study. Especially important when volatilization, sorption, metabolism, etc. of the analyte is of concern and the dose may be changing on a time scale not conducive for traditional analysis.
APPENDICES

A. Summary of anti-PAH antibodies presently in the scientific literature
Antibodies are known to cross-react with molecules of similar structure (Nording and Haglund 2003), which presents a great challenge with PAH analysis. Therefore, mAbs are selected with the greatest specificity for the PAH of interest. This requires screening the mAbs against a broad panel of PAHs and other molecules (such as PCBs, NPs, etc.). Several anti-PAHs antibodies have been developed, varying in degrees of screening rigor, and incorporated into immunosensors (Table 6).

Review of PAH Biosensors (Table 7)
The most common assay format for the detection of small molecules is a competitive inhibition assay wherein a signal-generating antibody is blocked from binding to an analyte-coated surface by the presence of free analyte within a sample (Figure 3). The antibody and sample are mixed and introduced to the antigen-coated surface. The resulting signal is inversely proportional to the amount of analyte in the sample. Transducers of this binding event vary and are organized according to type (electrochemical, optical, and piezoelectric). For clarification, a table describing the various types of transducers and the principles behind the measured values is provided (Table 8).

Electrochemical Detection
Electrochemical devices measure electrical signals produced from a chemical reaction. (Electrochemical cells either produce electrical energy from a chemical reaction, or produce a chemical reaction by introducing electrical energy.) When biomolecules are involved, the biorecognition event is used to generate the chemical reaction. For
Table 6. Summary of antibodies to PAHs

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Type</th>
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<th>Reference</th>
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<td>BAP-13</td>
<td>mouse</td>
<td>monoclonal</td>
<td>benzo(a)pyrenyl-1-butyric acid</td>
<td>Suchanek et al. 2001, Scharmweb et al. 2001</td>
</tr>
<tr>
<td>--</td>
<td>sheep</td>
<td>polyclonal</td>
<td>np²</td>
<td>Gift from Abuknesha used in Goryacheva et al. 2007</td>
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<td>benzo(a)pyrene-6-isocyanate</td>
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</tr>
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<td>7B2.3</td>
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<td>monoclonal</td>
<td>dibenzothiophene-4-acrylic acid</td>
<td>Spier et al. 2009</td>
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<tr>
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<td>polyclonal</td>
<td>4-(1-pyrenyl)butyric acid</td>
<td>Meiseneker et al. 1993</td>
</tr>
<tr>
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<td>Quelven et al. 1999</td>
</tr>
<tr>
<td>4D5 &amp; 10C10</td>
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<td>monoclonal</td>
<td>benzo(a)pyrene-6-isocyanate</td>
<td>Gomes and Santella 1990</td>
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<td>mouse</td>
<td>monoclonal</td>
<td>γ-(7-benzo[a]pyrenyl)-butyric acid</td>
<td>Matschulat et al. 2005¹</td>
</tr>
<tr>
<td>--</td>
<td>rabbit</td>
<td>polyclonal</td>
<td>9,10-Dihydrobenzo[a]pyren-7(8H)-one-7-(O-carboxymethyl)-oxime</td>
<td>Roda et al. 1991</td>
</tr>
<tr>
<td>anti-BaP-BSA</td>
<td>mouse</td>
<td>monoclonal</td>
<td>4-oxo-4-(benzo[a]pyrene)butyrate</td>
<td>Miura et al. 2003</td>
</tr>
<tr>
<td>anti-fluorene</td>
<td>rabbit</td>
<td>polyclonal</td>
<td>fluorenyl-methylazide.</td>
<td>Professor F. Le Goffic of ENSCP, Paris France</td>
</tr>
<tr>
<td>anti-pyrene</td>
<td>rabbit</td>
<td>polyclonal</td>
<td>pyrene derivative</td>
<td>Used in Pérez and Barceló 2000</td>
</tr>
</tbody>
</table>

**Commercially available anti-PAH antibodies**

<table>
<thead>
<tr>
<th>Source</th>
<th>Antibody</th>
<th>Species</th>
<th>Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novus Biologics</td>
<td>BAP-13</td>
<td>mouse</td>
<td>monoclonal</td>
<td>see above</td>
</tr>
<tr>
<td>Abcam</td>
<td>--</td>
<td>rat</td>
<td>polyclonal</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>BAP-13</td>
<td>mouse</td>
<td>monoclonal</td>
<td>&quot;</td>
</tr>
<tr>
<td>ExBio</td>
<td>BAP-13</td>
<td>mouse</td>
<td>monoclonal</td>
<td>&quot;</td>
</tr>
<tr>
<td>Abraxis</td>
<td>--</td>
<td>mouse</td>
<td>np</td>
<td>&quot;</td>
</tr>
<tr>
<td>Santa Cruz</td>
<td>4D5 &amp;</td>
<td>mouse</td>
<td>monoclonal</td>
<td>&quot;</td>
</tr>
<tr>
<td>Biotechnology</td>
<td>10C10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDIX</td>
<td>α-Phen-33</td>
<td>mouse</td>
<td>monoclonal</td>
<td>Used in Moore et al. 2004, Fähnrich et al. 2003</td>
</tr>
<tr>
<td></td>
<td>--</td>
<td>rabbit</td>
<td>polyclonal</td>
<td></td>
</tr>
</tbody>
</table>

1. ‘--' no name designation
2. np = not provided
3. a total of 14 mAbs were examined in this paper
Table 7. Features and specifications of PAH immunosensors

<table>
<thead>
<tr>
<th>Transducer Detection</th>
<th>Target PAH analyte(s)</th>
<th>LOD - range (ng/l)</th>
<th>Speed (mins)</th>
<th>Vol (ml)</th>
<th>Matrix</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Capacitance</strong></td>
<td>Linear sweep voltammetry</td>
<td>BaP - BSA</td>
<td>na</td>
<td>15</td>
<td>10</td>
<td>buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BaP, acenaphthene, fluorene, fluoreanthene, pyrene, 1-pyrenebutyric acid</td>
<td>25-1,260 2-4</td>
<td>40</td>
<td>np</td>
<td>buffer</td>
</tr>
<tr>
<td><strong>Electrochemical</strong></td>
<td>Cyclic voltammetry</td>
<td>BaP</td>
<td>2-0, 25-2,520</td>
<td>180</td>
<td>5</td>
<td>buffer</td>
</tr>
<tr>
<td></td>
<td>Amperometric</td>
<td>phenanthrene</td>
<td>2-100</td>
<td>60</td>
<td>10</td>
<td>buffer</td>
</tr>
<tr>
<td></td>
<td>Screen printed electrodes</td>
<td>Phen and 16 PAHs</td>
<td>0.05-0.45</td>
<td>90</td>
<td>150</td>
<td>spiked river, tap, sea, and mineral</td>
</tr>
<tr>
<td><strong>Piezoelectric</strong></td>
<td>QCM</td>
<td>BaP, pyrene, naphthalene</td>
<td>1,260-2,520 756-2,520</td>
<td>30</td>
<td>100</td>
<td>buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BaP</td>
<td>0.05-0.18</td>
<td>45</td>
<td>300</td>
<td>buffer</td>
</tr>
<tr>
<td><strong>SPR</strong></td>
<td>macro-flow cell and micro-flow cell</td>
<td>BaP</td>
<td>0.05-0.300</td>
<td>20</td>
<td>300</td>
<td>buffer</td>
</tr>
<tr>
<td></td>
<td>Multichannel</td>
<td>BaP</td>
<td>0.05-0.18</td>
<td>45</td>
<td>300</td>
<td>buffer</td>
</tr>
<tr>
<td><strong>Optical</strong></td>
<td>Fiber optic</td>
<td>BaP</td>
<td>0.25</td>
<td>12</td>
<td>5</td>
<td>buffer</td>
</tr>
<tr>
<td></td>
<td>Fluorescence</td>
<td>Pyrene, BaP + 16 PAHs</td>
<td>~2-000 0.9</td>
<td>np</td>
<td>10</td>
<td>spiked river water</td>
</tr>
<tr>
<td></td>
<td>Polarization</td>
<td>3- to 5-nng PAHs</td>
<td>0.3-30</td>
<td>3</td>
<td>400</td>
<td>naturally contaminated river and run-off</td>
</tr>
<tr>
<td></td>
<td>Reflectometric</td>
<td>BaP</td>
<td>3-70</td>
<td>np</td>
<td>np</td>
<td>buffer</td>
</tr>
<tr>
<td></td>
<td>interference</td>
<td>White light</td>
<td>np</td>
<td>np</td>
<td>buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>spectroscopy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IR</td>
<td>BaP</td>
<td>1,260-2,520</td>
<td>60</td>
<td>150</td>
<td>buffer</td>
</tr>
</tbody>
</table>

Target analyte = The PAH molecules for which the immunosensor detected in the assay.

Speed = Defined as the amount of time (estimated to the best extent possible) it takes from sample introduction to electronic result. In most cases, protein immobilization is required, but is performed in advance. In the case where the sample requires incubation with the antibody reagent, external to the sensor, this time is included in the speed value.

Buffer = Solutions vary, such as some contain 10% ethanol. For those details, please refer to the reference provided.
Table 8. The types of transducers used in PAH biosensors, along with the principle of the resulting data.

<table>
<thead>
<tr>
<th>Transducer / Detection</th>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrochemical</td>
<td>Storage of electric energy (energy storage potential)</td>
</tr>
<tr>
<td>Capacitance</td>
<td>Change in current while applying a fixed potential</td>
</tr>
<tr>
<td>Amperometric</td>
<td></td>
</tr>
<tr>
<td>Piezoelectric</td>
<td>Change in shape producing a measurable frequency</td>
</tr>
<tr>
<td>Quartz crystal</td>
<td></td>
</tr>
<tr>
<td>microbalance</td>
<td></td>
</tr>
<tr>
<td>Optical</td>
<td>Change in light intensity</td>
</tr>
<tr>
<td>Fluorescence</td>
<td></td>
</tr>
<tr>
<td>Infrared</td>
<td></td>
</tr>
<tr>
<td>Surface plasmon</td>
<td></td>
</tr>
<tr>
<td>resonance</td>
<td></td>
</tr>
<tr>
<td>Reflectometric/</td>
<td></td>
</tr>
<tr>
<td>interference</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
immunosensors, typically the antigen is immobilized onto the surface of an electrode, which ensures that the biorecognition event is occurring near the sensing (or working) electrode surface (see review by Grieshaber et al. 2008). However, as the binding of antibody to the antigen-coated electrode does not produce a substantial current, the antibody is conjugated to an enzyme which is capable of producing the needed chemical reaction. These enzyme-labeled antibodies are then introduced to the solution and will bind to the antigen-coated electrodes. In a second step, unbound antibodies are removed and a substrate is added to the antibody-bound enzymes to generate an electroactive product (usually hydrogen peroxide or oxygen), altering the electrical properties of the buffer. These electrical properties generate a measurable change in the current (amperometric) or the energy storage potential of a solution (capacitance).

**Capacitance**

Electrolytic capacitance measures the ability of the cell to hold an electrical charge. The capacitance is dependent on the dielectric properties of the electrodes. For sensors employing a biological molecule, the biological molecule serves as an insulating layer, such that the distance between the electrodes is influenced by the thickness of the biological layer bound on the electrode surface. For biorecognition, either the antibody or the antigen is immobilized onto the surface of the electrode. As the corresponding antibody binds the immobilized antigen or vice versa, a decrease in capacitance is measured proportional to thickness of the dielectric layer, which is due to the amount of biological molecule bound.

A capacitance immunosensor for detecting PAH-conjugates has been described by Liu and colleagues (1998). In this assay, the PAH-BSA antigen and the anti-PAH mAb, are immobilized onto the surface of gold plated electrodes. For both formats, 10 μl of sample solution were transferred to the electrode surface and allowed to incubate for 15 minutes before linear sweep voltammetry was employed detecting the cell’s capacitance. With the antigen immobilized format, specificity for the anti-PAH mAb was demonstrated as compared with the nonspecific binding of a mouse IgG molecule. With the mAb
immobilized format, antigen samples containing pyrene-BSA and BaP-BSA both bound to the mAb while a solution of BSA did not. This was the extent of the investigation. Nonetheless, it demonstrated this immunosensor’s potential for PAH detection as it could employ competitive inhibition where the analyte conjugates could be blocked by PAHs.

**Amperometric transducers**

In amperometric devices, the current associated with the reduction or oxidation of the enzymatic product is monitored while applying a fixed potential between the cell’s electrodes. The current is proportional to the concentration of the electroactive product in solution, and therefore to the concentration of antibody binding to the electrodes surface. Consequently, the current is inversely proportional to the amount of PAH in solution, which inhibits antibody binding to the surface, dampening the current change.

Importantly, amperometric transducers are less affected by sample turbidity, quenching, or interference from absorbing and fluorescing compounds which can confound optical biosensors.

Liu and colleagues (2000) have described an amperometric immunosensor for the detection of BaP. A pyrene derivative was immobilized onto a gold plated electrode. BaP was detected through its competitive inhibition of anti-PAH antibodies binding to the pyrene-coated electrode. Presumably, the BaP and antibody solution were allowed to incubate with the analyte-modified electrode for 40 minutes, as this was noted as the amount of time provided for the antibody solution alone to incubate with the electrode. Then, the electrode was immersed in a solution containing the redox probe Fe(CN)$_6^{3-/4-}$, such that bound antibodies would increase the hydrophobic layer therefore decreasing the redox current. Cyclic voltammetry measurements were conducted and the authors report quantification of BaP from 25.2 to 1,260 μg/l.

Wei and colleagues (2009) described a similar electrochemical immunosensor for BaP employing ruthenium(II)polypyridine derivative as a redox label. Electrodes were coated with anti-PAH antibodies. Competitive inhibition was performed by depositing a 5 μl
solution containing a PAH analyte (BaP, acenaphthene, fluorene, fluoranthene, pyrene, 1-pyrenebutyric acid) and the redox-labeled-PAH onto the electrode and was incubated for 180 minutes. Cyclic voltammetry measurements were conducted and the LOD for BaP was 2.4 μg/l while the other PAHs were examined for cross-reactivity.

Recently, Wang and colleagues (2011) described another amperometric immunosensor for BaP. They increased the number of analyte (derivatized pyrene)-BSA antigens immobilized onto the electrode surface by employing dendritic-nanosilica to increase the surface area of the working electrode. This subsequently increased the number of antibodies taking part in the reaction. The electrode was incubated with a 5 μl mixture of anti-PAH mAb and BaP for 120 minutes for a competitive inhibition assay. An enzyme-labeled secondary antibody was then introduced to the electrode and incubated for 60 minutes, immersed in a hydroquinone solution, followed by exposure to a current. This sensor detected BaP with a linear range of 2.5 to 2,520 μg/l, and a LOD of 2.0 μg/l.

Guilbault’s laboratory (Fahnrich et al. 2003) has explored the use of screen-printed electrodes for the electrochemical detection of PAHs. They concluded that the most sensitive and fastest assay format was yielded by immersing the phenanthrene-BSA coated electrode in a solution of mAb and an enzyme-labeled secondary antibody, with or without the PAH sample, and controls for 90 minutes. The electrode was then rinsed and a +300mV potential was applied. After the current reached a flat baseline, a substrate was added, which produced a change in the current. This method detected phenanthrene from 0.5 to 45 μg/l with a LOD of 0.8 μg/l. Specificity analysis was conducted, resulting in varying degrees of cross-reactivities with the 16 EPA priority pollutant PAHs, most notably, the 3- and 4-ring PAHs. Lastly, spiked water samples (river and tap) were evaluated and the system showed slightly decreased sensitivity.

In a subsequent report, Guibault’s laboratory (Moore et al. 2004) refined this method for analysis of a single drop (10 μl) of a spiked environmental sample (sea, river, tap and mineral water). This method utilizes the displacement of pre-adsorbed mAbs onto the antigen-coated electrode surface by the introduction of phenanthrene in the sample. The
electrode was immersed with the sample solution for 30 minutes, then washed and incubated with an enzyme-labeled secondary antibody for 30 minutes. Following another wash step, the substrate was added and linear sweep voltammetry was employed to detect the current produced at the working electrode. The linear range of detection for phenanthrene was 2 to 100 μg/l.

**Piezoelectric transducers**

Quartz crystals have the unique property of resonating at a given frequency upon application of an electric field. A detectable shift in frequency is produced through a perturbation to the crystal, such as the adsorption of mass onto its surface, as is the case with most antibody-based piezoelectric biosensors. The greater the change in mass, the greater the change in frequency. As antibody molecules possess 600 times the mass of PAH molecules (i.e., 150 kDa compared to 250 Da), piezoelectric techniques employ an antigen-coated platform. PAH detection is conducted via competitive inhibition. Without PAHs present in a sample, antibodies can bind unimpeded to the surface, decreasing the frequency. However, with increasing PAH concentrations, there was a decrease in the frequency modulation by the antibody.

A piezoelectric immunosensor for PAHs was developed by Liu and colleagues (1999). This method employs the immobilization of a BaP-BSA antigen onto a quartz crystal. An anti-PAH mAb was then allowed to bind the sensor surface for 30 minutes before the introduction of a 100 μl BaP sample. At this step, the flow of the BaP sample was stopped for 30 minutes to allow for a competitive displacement immunoreaction to occur. The sample was then washed away and the frequency recorded. Because the sensor surface is pre-exposed to mAb molecules, an increase in PAH concentration reaching the sensor surface causes more antibodies to be displaced with an increase in resonance frequency. This technique provided a BaP quantification from 1,260 to 2,500 μg/l. Cross-reactivity was demonstrated with pyrene and naphthalene.
Boudjaj and colleagues (2010) describe another piezoelectric immunosensor for the detection of BaP. Instead of immobilizing the analyte-conjugate (a larger molecule), a layer of the derivatized analyte (a smaller molecule), pyrene butyric acid, was used to coat the surface of the crystals. Antibody and analyte solutions (500 µl) were incubated for 60 minutes prior to introduction to the sensor surface. The BaP and mAb solutions were pumped over the sensor surface at a flow rate of 25 µl/minute and the frequency shift of the antibody adsorption onto the crystal surface was recorded. This method resulted in quantification from 756 to 2,520 µg/l.

Optical

Optically-based transducers possess the broadest range of mechanisms for transduction. In the case of PAHs, light can be emitted from the analyte itself or can be generated from a fluorescent label affixed to the antibody, the antigen, or the hapten. Light can be detected as it is reflected or refracted from the sensor’s surface in response to antibody-PAH binding.

Surface Plasmon Resonance (SPR)

SPR-based biosensors operate by detecting a change in the refractive index caused by adsorption of a molecule onto the sensor surface. The larger the molecule, the larger the change in the refractive index. Since PAHs are much smaller than antibodies (~600 times smaller), analytes (derivatized PAHs) are covalently attached to the sensor surface to capture the larger antibody and effect a substantive change in the refractive index. SPR-based PAH immunosensors utilize a competitive inhibition format where the concentration of PAHs in the sample is inversely proportional to the change in refractive index (Figure 4). If there are no PAHs in the sample, all of the antibodies will bind to the sensor surface generating a greater shift response. Two SPR-based immunosensors have been described for the detection of BaP.

Gobi and Miura (Gobi et al. 2003, Miura et al. 2003, Gobi and Miura 2004) have described both a macro- and a micro-flow cell, SPR-based immunosensors for the
detection of BaP. Both methods used the same anti-BaP mAb developed against a BaP-BSA immunogen. This same immunogen, BaP-BSA, was also used as the antigen in all of their studies. These methods require an antibody and sample incubation step for 5 minutes and then incident angle reached a plateau in approximately 15 minutes resulting in a total time of 20 minutes for sample analysis. They reported a BaP quantification range of 0.01 to 300 µg/l and no-cross reactivity with a second analyte, HBP. This is the largest range of quantification (10^5) reported for any PAH immunosensor to date, however results were as much as +/- 2 times the actual BaP concentration. The microflow-cell method requires 300 µl volume of a sample.

A second SPR-based method was described by Dostalek and colleagues (2007). Their immunosensor used a 30 minute analyte and antibody incubation step. The sample and antibody mixture was permitted to flow over the sensing channel for 10 minutes, followed by a wash step for 5 to 7 minutes. In all, it took 45 minutes from the incubation of the sample with the antibody to obtaining a signal response. The LOD for BaP was 0.05 µg/l with quantification up to 0.18 µg/l. Selectivity of this method was examined in two ways; by testing the cross-reactivity of the antibodies (anti-ATR, anti-2,4-D, anti-4-NP, and anti-BaP) for recognition of the coated antigens (ATR-BSA, 2,4-D-BSA, BaP-BSA and 4-NP-OVA) and by incubating the anti-BaP antibody with a mixture of four analytes (BaP, ATR, 2,4-D, and 4-NP) at one specified concentration in buffer. Although the anti-BaP antibody significantly cross-reacted with the 4-NP-OVA antigen (64%), the anti-BaP antibody reportedly demonstrated no discrepancy when incubated with the BaP analyte alone or in the mixture.

**Fluorescence-based detection**

Fluorescence detection is considered a more sensitive technique than other spectrophotometric methods. Fluorescence is produced when sufficient energy is absorbed to excite a valence electron from its ground state to an excited state and a photon is emitted after its relaxation to the ground state. In the immunosensors that
follow, this fluorescence is produced from either the excitation of the PAH molecule itself or by a fluorescent tag conjugated to one of the reagents.

*Natural PAH fluorescence.* The first anti-PAH method employing fluorescence detection was conducted by Vo-Dihn (1987). The fluorescence was emitted by the BaP molecule upon exposure to a laser, an inherent property of BaP. Rabbit anti-BaP polyclonal antibodies were covalently attached to the tip of a fiber optic cable. This served as the probe, which was placed in a sample solution and incubated for 10 minutes. The probe was then rinsed with PBS and exposed to a helium cadmium laser in order to elicit fluorescence. In all, sample analysis was reportedly completed in approximately 12 minutes. The signal was proportional to the number of BaP molecules bound to the tip of the probe. The sample size needed was 5 μl, and in this volume 1 fmol could be detected, which is equivalent to 0.25 μg/l. However, this technology suffers from high non-specific binding of PAH to the cable itself.

*Polarized fluorescence.* Yadavalli and Pishko (2004) employed an anti-PAH mAb labeled with a fluorophore. A solution containing analyte and antibody was passed through a microfluidic channel where it was exposed to polarized excitation light. The antibody bound to the analyte resulted in a larger, slower rotating entity that was reflected by the increase in fluorescent anisotropy. It is worth noting that fluorescence polarization is the only PAH immunosensor method, described to date, that does not use any form of immobilized antigen nor antibody. The authors noted that typical assays were conducted on <10 μl samples, however it was unclear how much was used in their experiments. They noted a LOD of 10 to 40 nM, which, if this was detected in a 10 μl sample, would result in a LOD of approximately 2,000 μg/l. The authors analyzed other aromatics (benzene, toluene, and anthracene), but could not conclusively report their detection. No mention of antibody incubation time or speed of analysis was provided.

Goryacheva and colleagues (2007) employed fluorescence polarization, where contrary to the previous study, they fluorescently labeled an analyte tracer and not the antibody. Similar to the previous study, there was no need to separate the bound and unbound
antibodies, because as the fluorescently-labeled tracer is bound, there would be an increase in fluorescence polarization. In this investigation, the method was based on competitive inhibition with PAHs in the sample competing with the labeled tracers for antibody binding. Two anti-PAH antibodies, a mAb and sheep polyclonal, were employed. Different PAH tracers were explored (2, 4, and 5 rings), as well as characterization of cross-reactivity of the mAb/antisera with 16 PAHs. The polyclonal antibodies detected all analytes, while the mAb preferentially bound the 4- to 5-ring PAHs. No incubation time was required, as both mAb and polyclonal antibodies came to equilibrium immediately upon mixing the reagents, but total analysis time was not provided. No matrix effects were evident when environmental river samples were spiked with known amounts of BaP.

*Fluorescence intensity from a label.* Spier and colleagues (2011) report a completely automated PAH immunosensor based on detecting the intensity of a fluorescently-labeled mAb. Antibody characterization demonstrated that the mAb recognized 3- to 5-ring PAHs (Spier et al. 2009). Analyte-protein conjugates were immobilized on PMMA beads loaded into a flow cell positioned in front of a laser. As the mAb came to equilibrium quickly, no additional incubation with the sample was required. A 400 µl solution of sample and antibody were passed over the bead-packed flow-cell, where the fluorescently-labeled antibodies would either bind to the antigen-coated beads, or were inhibited from binding by PAHs in solution. For a sample containing no PAHs, the tagged mAbs provide the greatest fluorescence intensity. Thus, the fluorescent signal was inversely proportional to the concentration of PAH. The linear range of PAH quantification was 0.2 to 30 µg/l. Samples were analyzed in 3 minutes, with an additional 7 minutes for cleaning and preparing the sensor for the next sample. This study evaluated natural water samples contaminated with a complex mixture of PAHs. Validation analysis with GC-MS demonstrated good correlation and accurate quantification of 3- to 5-ring PAHs.
Reflectometric interference UV/VIS spectroscopy

One of the first automated immunosensors for BaP detection was described by Lange et al (2002). The aim of their study was characterization of anti-BaP polyclonal antibodies rather than to produce a rapid, portable instrument. They demonstrated that the polyclonal antibodies (Vo-Dinh et al. 1987) were specific for BaP and not for chrysene and pyrene. This method employed a flow-injection system, a analyte-coated transducer surface, and a UV/VIS spectrometer. The principle of this method was that as antibodies bound to the analyte-coated transducer surface, the reflectance pattern of the continuous white light aimed at the transducer surface changed and was measured with good resolution. The antibody reaction was based on competitive inhibition where the maximum signal was produced when no BaP present and all of the antibodies can bind the sensor coated surface. Increasing BaP concentrations caused less antibodies to bind to the surface and therefore less reflected white light was observed. This method had a working range of 3 to 70 µg/l of BaP. As speed was not a concern of this study, pre-incubation of antibody with sample was conducted, but no times were provided, however a signal response was generated in less than 250 seconds.

Infrared

Boudjay and colleagues employed IR absorption technology for the development of a BaP immunosensor (2009). An IR signal was produced when the antibody binds a BaP molecule – a new IR band formed, indicative of the presence and stretching of the aromatic C-H band of BaP upon interaction with the antibody. Therefore, the concentration of BaP in a sample is directly proportional to the integrated area of this band. This method relied on the direct immobilization of the mAb onto the sensor surface and required no additional labels. A 10 ml solution of BaP was exposed to the antibody-coated surface for 60 minutes, washed and then the IR spectrum recorded. This label-free, direct IR method demonstrated a LOD for BaP of 1,260 µg/l with a measuring range up to 2,520 µg/l. In a subsequent publication, Boudjay and colleagues (2010) reported the employment of this IR method in an indirect competitive format, where the
BaP-surrogate antigen was coated onto the immobilized surface, yielding the same BaP LOD of 1,260 μg/l.

**Current state of PAH biosensor technology**

The majority of PAH immunosensors possess detection limits in the μg/l range (Table 7) making them suitable for measuring environmentally relevant concentrations. Rapid analysis is a desirable trait for biosensors and most of these can complete PAH analysis in minutes. For sensors requiring more than 30 minutes, sample incubation was required to allow the antibody to come to equilibrium with any available analytes. While sensitivity and speed are desirable traits for biosensor methodologies, biosensors must transcend the controlled situation of the laboratory to make field assessments routine and reliable. Namely, matrix effects must be evaluated with complex environmental samples and PAH mixtures, as well as validation of results with traditional analytical methods. Portability, automation, and user-friendliness are issues of paramount importance.

**Comparisons of biosensor and classical analytical methods**

The main goal for developing PAH immunosensors is to perform rapid, on-site PAH analysis. This is to replace or assist traditional methods, which require samples to be analyzed by laboratory-bound techniques such as GC-MS or HPLC. To accomplish this, biosensor methods must employ samples that can be assessed in the field without extensive manipulations, such as extraction or concentration. The biosensor methods require small volumes, ranging from 5 to 500 μl per sample. In comparison, for low-level detection, classical analytical methods require up to 1 L per sample. Because the sample volume is large in classical methods, the analytes of interest must first be extracted into an organic solvent and then concentrated. For analysis of a single sample, the analytes are then separated and individually identified. Depending on the number of identifiable analytes, this process can take hours to days to complete. Although biosensor methods do not provide resolution of individual analytes, sample analysis can be completed in minutes. In return, a much greater number of samples can be assessed with less time, effort, and expense.
As reported in this review, most of these biosensors have only been tested with single analyte samples. However, PAHs in the environment commonly exist as complex mixtures. Moreover, these compounds possess similar molecular structures and electron density. As demonstrated in several anti-PAH antibody characterization studies, these antibodies are highly cross-reactive with other PAHs (Nording and Haglund 2003). Therefore, if the final goal is to employ the biosensor for precise analyte identification during environmental analysis, samples containing relevant complex mixtures of PAHs must be resolved and confirmed with traditional analytical methods (Rodriguez-Mozaz et al. 2006).

**Antibody incubation times**

The analysis time for the biosensors presented here is reported as the amount of time required from the moment the sample is introduced to the system. This includes incubating the sample with the antibody, typically conducted for 30 to 60 minutes. However, most of the methods described do not utilize antibodies for which kinetic analyses have been completed. If an antibody comes to equilibrium with a sample in a very short amount of time, this incubation step can be reduced or eliminated. This is the case for the antibodies employed in the Goryacheva et al. (2007) and Spier et al. (2011) studies. If kinetic analysis were to be conducted for the other antibodies employed, analysis times may be shortened in the future.

**Comparison of label-free and labeled reagents**

Both, label-free (Figure 49) and labeled methods have their advantages. With less reagents, less steps can be involved. When labels are employed, they require covalent attachment to the antibody or antigen. This conjugation process can place labels within the antibody binding site, thereby decreasing the antibody’s potential activity. The natural fluorescence of PAH molecules allow label-free detection; however, protein molecules and other natural organic substances may fluoresce at a similar wavelength. Fluorescent labels can provide a narrow targeted wavelength and optical filters can be incorporated to increase the specificity of the signal.
Figure 49. Label-free detection methods. A) infrared spectroscopy or natural fluorescence emitted from a PAH molecule, B) electrical storage (capacitance), surface plasmon resonance, or light reflectance/interference C) piezoelectric.
Reusability of biosensors

Reversible binding of antibody-antigen systems (i.e. sensor reusability) can be exploited to increase the useable life of some biosensors. To regenerate surfaces (removing previously reacted antibodies or antigens) a variety of chaotropic and proteolytic reagents have been used, including; urea (Liu et al. 1999), MgCl₂ (Liu et al. 2000), pepsin (Gobi et al. 2003, Miura et al. 2003), and NaOH with acetonitrile (Dostalek et al. 2007). An immunosensor has been reused successfully as many as 50 times, while others have experienced high retention of the previous reactants. This is very likely a function of the antibody’s affinity or non-specific binding with the sensor surface. Likewise, the choice of chaotropic reagent will vary depending on the types of materials incorporated into the lines and seals of the sensor instrument. While this is certainly an avenue that can be explored, it is unclear if it is critical for successful field deployment. Regeneration would involve more reagents, but may also eliminate the need for numerous sensing surfaces and could reduce the reagents required in preparatory stages (i.e., antigen or antibody coating).

Applications to other areas of health and disease

PAH detection is not only occurring in aquatic environmental samples, but also has been completed extensively in food sources (EU 2002) by GC-MS methods. Human exposure to PAHs is also conducted via examining urine samples for PAH metabolites (Jongeneelen et al. 1985), and antibody-based assays have already been developed for one PAH metabolite (Marco et al. 1993a). Similarly, human exposure to PAHs typically results in the formation of PAH-DNA adducts, which can serve as another biochemical molecule to detect via PAH biosensor (Vo-Dinh et al. 1987). Likewise, the methods presented in this review are only specific for PAHs due to the incorporated antibodies. By changing to other antibodies, these biosensors could be adapted to quantify any molecule for which an antibody can be developed.
## B. Inline biosensor sample handling protocols

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LITERATURE CITED


VITA

CANDACE RAESPIER