Toxicological Foundations of Ecological Risk Assessment: Biomarker Development and Interpretation Based on Laboratory and Wildlife Species

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Ecological risk assessments based on chemical residue analysis and species demographics tend to ignore the bioavailability and bioaccumulation of the chemicals of concern. This study describes the incorporation of mechanistically based biomarkers into an ecological risk assessment of a polycyclic aromatic hydrocarbon (PAH)-contaminated site. A combination of soil residue analysis, tissue residue analysis, biomarkers in on-site trapped animals and biomarkers in animals confined to enclosures was used. In particular, the use of captured deer mice (Peromyscus maniculatus) for these studies is compared to the use of laboratory-raised deer mice placed in enclosures. This study indicates that the higher degree of variability in the responses of wild deer mice make the use of enclosure studies advantageous. Positive control studies performed by dosing laboratory-raised deer mice with the same PAHs as found on the site were used to validate this approach. These studies indicate that immune suppression occurred at PAH concentrations an order of magnitude below those required for the induction of ethoxyresorufin-O-dealkylase activity. — Environ Health Perspect 102(Suppl 12):65-69 (1994)

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Currently, most ecological risk assessments are based on chemical residue analysis of soils, sediments, and surface waters along with determinations of wildlife species density and diversity. Chemical analyses have the advantage of being specific, quantitative, and very sensitive. The biological significance of chemical concentrations measured in soil, water, and air is not so specific or quantitative (1). These data are then used to predict species impact based upon previous studies with the species and chemicals in question and/or extrapolated from data obtained from other species. The biological end points most commonly selected are survival and reproduction. This method of performing an ecological risk assessment suffers from two major shortcomings. First, the bioavailability of the chemicals from the environmental matrix is assumed to be 100%. For most chemicals and environmental matrices, this tends to overestimate the availability of a chemical. Second, the chemical may be inducing behavioral, biochemical, physiological, or toxic responses in the impacted species that directly affect the stability of a population though they are not detected during field assessments that examine individual survival and reproductive success. The measurement of chemical residues in tissues gives a more accurate prediction of effects. However, this technique works best with metals and organic chemicals that bioaccumulate rather than with compounds that are readily metabolized or are present as complex mixtures (1). Moreover, residue analysis in tissues is also quite expensive. The use of mechanistically relevant biomarkers in an ecological risk assessment can provide information that overcomes the limitations of a residue-driven approach.

The National Academy of Sciences defines a biomarker as "a xenobiologically induced variation in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample" (2). Biomarkers can be indicators of exposure to a xenobiotic, of the effect elicited by a chemical or of susceptibility (3). Biomarkers selected for use in ecological risk assessments should, whenever possible, be indicative of the effect of a chemical in addition to exposure to that chemical. Furthermore, the biomarker should exhibit different responses to different classes of xenobiotics. They should be mechanistically based and each member of a class of chemicals should yield a quantitatively different dose-response relationship. The response elicited by a chemical may exhibit a high degree of correlation with carcass residue analysis, though with quickly metabolized compounds this correlation may be better applied to excretion products or may not apply at all. Ideally, a biomarker should hold the potential for nonlethal applications. Biomarkers may be used to detect genotoxic effects of xenobiotics as well as nongenotoxic effects. Moreover, careful biomarker selection can differentiate between direct-acting and indirect-acting genotoxic xenobiotics. Of particular interest to The Institute of Wildlife and Environmental Toxicology at Clemson University (TIWET) are those chemicals...
that alter the normal gene regulation processes.

The use of a biomarker-driven ecological risk assessment possesses other advantages. In general, biomarkers are selected to exhibit a greater sensitivity to a xenobiotic than traditional endpoints such as mortality or reproductive success. Moreover, the mechanistic specificity allows discrimination of the effects of different chemicals. Biomarker-driven ecological risk assessments also have the advantage of being much less expensive than chemical residue analysis. The development of a biomarker driven ecological risk assessment program requires the selection and verification of biomarkers in selected sentinel species.

There has been extensive research into the effects of xenobiotics on laboratory species for the purpose of establishing a human health-oriented data base. Few such determinations have been performed for wildlife species. It can not be assumed, however, that dose–response relationships determined for a xenobiotic in a laboratory species are directly applicable to wildlife. Therefore, there is a need for comparisons between laboratory- and wildlife-based biomarkers to determine relative sensitivity, disposition, mechanisms, and dose–response relationships. This can best be realized through side-by-side comparisons between laboratory rodents and laboratory reared wildlife such as the deer mouse (*Peromyscus maniculatus*).

We are currently investigating the effects of xenobiotics produced by day-to-day operations of a naval air station in northwestern United States (NAS Whidbey Island, Whidbey Island, Washington) on the indigenous and migratory wildlife. Operations at this site resulted in the release of polycyclic aromatic hydrocarbons (PAHs) into the soil at two fire fighting schools (old and new), at a pesticide rinsate station, and into a runway ditch system. Based on previous studies involving PAHs, induction of hepatic monoxygenase activity and immunotoxicity were selected as biomarkers for subsequent study. This report describes how a combination of chemical residue analysis and wildlife-based biomarker studies provides a better foundation for ecological risk assessment than does residue analysis alone. The results obtained from chemical residue analysis, trapping studies, rodent enclosure studies, and laboratory dosing studies will be described.

**Materials and Methods**

**Animal Care**

Deer mice used in the laboratory and field enclosure studies were bred in the TIWET animal unit from stock obtained from the University of South Carolina *Peromyscus* Stock Center. These deer mice are maintained in an in-house animal unit operated under NIH and USDA standards. All procedures are approved by the Clemson University Animal Research Committee. Animals receive food (Harlan Teklad Laboratory Mouse Breed Diet No. 8626 or Rodent Diet No. 8604) and water *ad libitum*. Feed is analyzed for contaminants on a lot-to-lot basis and the water is analyzed monthly. The animals are on a 12-hr light/dark cycle.

**Trapping Studies**

Mammal trapping was conducted by using a grid trapping as described by Brewer et al. (unpublished data). Sherman live traps were placed on the nodes of a 10 m × 10 m grid. The traps were set at dusk and inspected at dawn the next morning. The traps were not set during the day. Any small mammals collected were transported to the field laboratory, euthanized with CO₂, and the livers perfused and removed. The livers were then frozen in liquid nitrogen and transported to TIWET for microsomal preparation and EROD/PROD analysis. The remainder of the carcass was frozen, transported to TIWET and archived for later analyses.

**Enclosure Studies**

Laboratory-raised deer mice were placed five to eight in a rectangular enclosure constructed of 1-cm mesh hardware cloth attached to a 20-gauge steel base (0.93 m² in area). The enclosures were sunk 0.20 m into the soil and extended 0.25 m above the soil. The mice received residue-free food and water *ad libitum*. Enclosures were placed in multiple locations on both contaminated and reference soils. The mice were kept in the enclosures for 7 days. The mice were inspected daily for signs of morbidity. At the end of the 1-week period, the mice were euthanized by CO₂ asphyxiation and organs harvested for analysis.

**Positive Control Studies**

These studies used 7- to 8-week-old male deer mice that received PAHs dissolved in corn oil intraperitoneally (IP). The mice received the PAHs on days 1, 3, 5, 7 and 9. The mice received 4 × 10³ sheep red blood cells IP on day 6 and were euthanized on day 11. At this time, the spleens and livers were removed, weighed, and prepared for analysis. The remainder of the carcass was retained for subsequent residue analysis.

**Chemicals and Biochemicals**

Sheep red blood cells in Alsever's solution were obtained from Bio-Whittaker (Walkersville, MD). Lyophilized guinea pig complement was purchased from Gibco BRL (Gaithersburg, MD). The cytochrome P450 substrates, 7-ethoxy- and 7-pentoxyresorufin were obtained from Molecular Probes (Eugene, OR). All other chemicals and biochemicals were obtained from Sigma (St. Louis, MO).

**Hepatic Monoxygenase Activity**

EROD and PROD activities were measured by a modification of the method of Prough et al. (4). Protein concentration was determined by the bicinchoninic acid method, a modification of the technique of Smith et al. (5). Activity was measured by combining microsomes, substrate, and NADPH in a 96-well plate and measuring the emission at a wavelength of 585 nm with an excitation wavelength of 530 nm. Nine measurements were made in approximately 3 min and the activity transformed and reported as pmol resoruﬁn formed per minute per milligram protein.

**Splenic Plaque-forming Cell Assay**

The number of plaque-forming cells per spleen was determined by the Cunningham (6) modification of the Jerne Plaque Assay (7). The plaques were counted using a hand tally and an inverted microscope at low power. The cells in the suspension were counted using a hemocytometer and the results reported as plaques per million splenocytes. Immune function for each dose was subsequently calculated as a percent of control, nondosed plaque forming activity.

**Residue Analysis**

Tissues (up to 10 g) were homogenized using a Virtis homogenizer and extracted using 25 ml of a methanol/propanol/acetone mixture (50/25/25 vol %). The extraction was performed on a rotary shaker at 275 rpm for 1 hr. The samples were then centrifuged and the supernatant decanted into a 125-ml Erlenmeyer flask. The polarity of the extract was increased by the addition of 25 ml of 0.1 N NaCl. The extract was homogenized and then passed through a C18 solid phase extraction column. Retained analytes were eluted from the column with 3 × 1 ml portions of a
mixture of ether and hexane (50/50 vol %). The volume of the eluant was reduced to 2 ml with dry nitrogen. A 1-ml portion was evaporated to near dryness with dry nitrogen and reconstituted in methanol. The sample was then filtered through a 0.45-μm filter to remove particulates and stored in an amber archive vial. PAH components were analyzed on a Hewlett Packard 1090 high pressure liquid chromatograph with a C18 column (25 cm × 0.4 cm) using fluorescence and diode array UV detection. Calibration curves spanned 1 to 100 ppm for low fluorescence compounds (e.g., acenaphthylene) to 0.05 to 0.5 ppm for highly fluorescent compounds (e.g., chrysene).

Statistics
All statistical procedures used the SuperANOVA program (Abacus Concepts). Significance levels were set at p < 0.05.

Results
Surficial soil samples collected from the runway ditch system and from the old fire school were analyzed for polycyclic aromatic hydrocarbon content. The results of the analyses are listed in Table 1. Samples from the old fire school contained a wider variety of PAHs, and the amount present was generally greater than found in samples from the runway ditch system. In particular, PAHs known to exhibit high affinity for the Ah receptor were found in higher concentrations at the old fire school. This suggested the need for residue data from rodents collected on these sites.

A trapping study was performed on runway ditch system sites, the old fire school site, and a nearby reference site. The livers from wild deer mice trapped at these sites were removed and subsequently examined for PAH residues. The results of this study are listed in Table 2. There was a large degree of variation observed in samples collected from the same site. Analysis of the livers from mice trapped on the reference site showed moderate levels of acenaphthylene and benzo[a]anthracene. No other PAHs were detected in liver tissue from mice collected on the reference site. In contrast, liver specimens from the two contaminated sites exhibited moderate levels of a variety of PAHs. However, no traces of dibenzo[a,h]anthracene or indeno[1,2,3]perylene, potent PAHs with a marked affinity for the Ah receptor, were found in these samples.

The remaining livers and kidneys from the above trapping study were prepared for the determination of hepatic monooxynge-

| Compound                  | Location            | Old fire school |
|---------------------------|---------------------|-----------------|
| Naphthalene               | 0                   | 57.2 ± 104.5    |
| Acenaphthylene            | 0                   | 0.031 ± 0.073   |
| Acenaphthylene            | 0.0061 ± 0.0198     | 0.0041 ± 0.0134 |
| Fluorene                  | 0.112 ± 0.180       | 0.075 ± 0.138   |
| Phenanthrene              | 0.049 ± 0.087       | 0.224 ± 0.328   |
| Anthracene                | 0.103 ± 0.253       | 0.251 ± 0.569   |
| Fluoranthene              | 0.0182 ± 0.034      | 0.043 ± 0.096   |
| Pyrene                    | 0.009 ± 0.022       | 0.085 ± 0.156   |
| Benzo[a]anthracene        | 0.023 ± 0.050       | 0.041 ± 0.117   |
| Chrysene                  | 0                   | 0.015 ± 0.024   |
| Benzo[b]fluorene          | 0.143 ± 0.243       | 0.165 ± 0.302   |
| Benzo[k]fluorene          | 0.009 ± 0.022       | 0.042 ± 0.224   |
| Benzo[a]pyrene            | 0.0014 ± 0.0018     | 0.084 ± 0.221   |
| Dibenzo[a,h]anthracene    | 0.0018 ± 0.0028     | 0.017 ± 0.042   |
| Benzo[g,h,i]perylene      | 0                   | 0.062 ± 0.064   |
| Indeno[1,2,3]perylene     | 0.183 ± 0.394       | 0.229 ± 0.543   |

*Values in mg/kg.

| Compound                  | Runway ditch system | Old fire school |
|---------------------------|---------------------|-----------------|
| Acenaphthylene            | 3.2 ± 11.01         | 1.91 ± 3.63     |
| Fluorene                  | 0.14 ± 0.23         | 0.0973 ± 0.325  |
| Benzo[a]anthracene        | 0.24 ± 0.59         | 0.221 ± 0.583   |
| Chrysene                  | 0.01 ± 0.02         | 0.044 ± 0.243   |
| Benzo[b]fluorene          | 0.05 ± 0.12         | 0.24 ± 0.22     |
| Benzo[k]fluorene          | 0.07 ± 0.20         | 0.089 ± 0.131   |
| Dibenzo[a,h]anthracene    | 0                   | 0               |
| Indeno[1,2,3]perylene     | 0.183 ± 0.394       | 0.229 ± 0.543   |

*Values in mg/kg.

| Site         | Hepatic EROD | Hepatic PROD | Renal EROD | Renal PROD |
|--------------|--------------|--------------|------------|------------|
| Reference 1  | 195 ± 60     | 24.9 ± 12.1  | 41.3 ± 3.2 | 89 ± 3.8   |
| Reference 2  | 166 ± 52     | 27.2 ± 5.6   | 54 ± 2.3   | 2.3 ± 1.4  |
| Reference 3  | 193 ± 71     | 34.6 ± 17.6  | 74.8 ± 8.2 | 5.4 ± 3.7  |
| Old fire school | 234 ± 78   | 29.0 ± 11.7  | 10.4 ± 5.1 | 4.2 ± 3.9  |
| New fire school | 177 ± 65    | 31.1 ± 9.9   | 20.6 ± 4.9 | 5.2 ± 2.8  |

*Values in pmole/s/mg protein.

Samples collected from these mice. Notice that the hepatic EROD activities in both the laboratory control and the field reference mice are not different from each other and both are significantly lower than hepatic EROD activities from wild deer mice trapped on reference sites. In contrast, there were no differences observed between hepatic PROD activities in mice from laboratory controls, enclosures on reference sites, and trapped wild mice from reference sites. Significant differences were observed in hepatic EROD activity between mice in enclosures on reference sites and mice from enclosures located on pesticide rinsate sites and old fire school.
sites. No differences were observed in hepatic EROD activity between mice from control sites and sites located on the run- 
ditch system or the new fireschool.

No differences were observed in hepatic PROD activity from mice located in enclo- 

cures on control, runway ditch system, old fire school, or new fire school sites. There were 
a significant elevation in renal EROD activity in mice from enclosures located on sites at the old fire 
school. No elevation in renal PROD activity was observed in mice from any of the contaminated sites. The 
results of this study suggested that other, Ah receptor-mediated, manifestations of 
PAH-induced toxicity might be present in deer mice living on the old fire school or the pesticide 
runway station.

A series of positive control studies was therefore initiated, examining the induction of 
EROD and the suppression of antibody formation by PAHs. Table 5 shows the sup- 
pression of the splenic plaque-forming cell response to sheep red blood cells by three 
PAHs. All three PAHs elicited significant immunosuppression; however, 1,2,5,6-
dibenz[a,h]anthracene (dibenzo[a,h]anthracene) and 7,12-dimethylbenz[a]anthracene were 
more potent immunosuppressants than was 3-methylcholanthrene. The ED_{50} for 
immunosuppression, as judged by the plaque-forming cell assay, was less than 
0.14 mg/kg/day for all three compounds. Conversely, no significant induction of 
EROD activity was observed until a dose of 0.3 mg/kg/day of 1,2,5,6-dibenz[a]anthracene 
was administered. The same dose caused a 73% decrease in the splenic plaque-forming cell response. A dose of 3 
mg/kg/day of 3-methylcholanthrene was required to elicit significant EROD induc- 
tion, a dose that resulted in 80% suppression of the splenic plaque-forming cell response. Moreover, none of the doses of 
7,12-dimethylbenz[a]anthracene administered resulted in significant induction of 
EROD activity although doses as low as 0.03 mg/kg/day resulted in 81% suppression of the plaque-forming cell response to 
sheep red blood cells.

Chemical residue analysis of surface 

soils collected from two sites at the naval 

air station indicated the presence of a vari- 

ty of PAHs. However, there was a high 
degree of variability in the results obtained, 
indicating a nonhomogenous PAH pres- 
ence in the soil. This variability, plus a lack of 
definitive dose–response relationships for 
these PAHs in wildlife species, makes 
ecological risk assessments based solely on 
soil analysis of this type unreliable. 

Combining soil analysis with PAH analysis 
of tissues from animals collected on the same 
sites offers substantial advantages in 
the quality of information collected with 
little increase in the cost of the studies. For 
example, comparison of PAH levels in soil 
with that in tissues gives an indication of the 
bioavailability of the PAHs from the soil and the degree of bioaccumulation possible. 
In this study, the mean levels of PAHs found in the livers of trapped deer 
mice were approximately an order of 
magnitude higher than those found in soil samples from the same sites, indicating both 
bioavailability and a degree of bioaccumulation.

PAHs elicit the majority of their toxic and 
biological effects through interactions with the Ah receptor, resulting in the 
induction of cytochrome P450 isozymes 
causing the metabolic conversion of the 
PAHs to mutagenic, carcinogenic, or other- 
wise reactive forms (8,9). For certain 
PAHs, the higher the affinity between a 
PAH and the Ah receptor, the greater the 
resulting induction of cytochrome 
P450A1 as measured by aryl hydrocarbon 
hydroxylase activity (AH) or EROD activity (10,11). In this study, the amount of 
a PAH found in the tissues of the fetal 
deer mice was inversely proportional to its 
affinity for the Ah receptor. In particular, 1,2,5,6-dibenz[a]anthracene and indeno-
[1,2,3]pyrene had measurable soil levels but were not found during tissue analysis. 
This could be due to enhanced metabolism 
by the cytochrome P450A1-induced 
population (which could be examined using 
PAH metabolite analysis in bile or excreta). 
The higher levels of EROD activity in wild 

deer mice captured from control sites as 
compared to laboratory-reared deer mice 
tend to support this hypothesis as does the 
increase in EROD activity in wild deer 
mice captured from the PAH-contami-
nated sites in that the elevated hepatic 
monoxygenase activity in these animals 
may result in more PAH being excreted 
than stored in fat depots. However, the 
high degree of variability in EROD activity 
in the wild deer mice prevented the detec-
tion of significant differences between 
control and contaminated sites.

For this reason, a series of enclosure 

experiments was planned and enacted. 

These experiments were designed to reduce 
variability by using a more homogeneous, 
chemically clean line of laboratory-raised 
deer mice in enclosures on both reference
and contaminated sites. It was thought that the enclosures would reduce the variability in soil types to which an individual mouse would be exposed (12), thereby reducing the variation in dose levels. Furthermore, the controlled diet the mice were given prior to being placed on the site should eliminate dietary induction of EROD activity. The results obtained from these experiments indicate that this was the case. First, there was no difference between reference site mice and laboratory controls. Second, the enclosure animals had lower EROD activity than did the wild deer mice collected from the same reference sites. Third, the standard deviation in EROD activities measured among the animals in an enclosure were quite low compared to wild deer mice trapped on the same sites. These data indicate that enclosure studies may be an effective adjunct to capturing wild mammals on a contaminated site, providing a reasonable basis for ecological risk assessments.

The use of immune parameters as biomarkers for PAHs was suggested by the work of Malmgren et al. (13), Ward et al. (14, 15) and Stjernsward (16). Hepatic chlorodibenzo[al]furans were subsequently found to cause significant immunosuppression before EROD induction becomes apparent (17). The results obtained in our dosing studies with 3-methylcholanthrene and 7,12-dibenz[a]anthracene, which are synthetic PAHs, as well as 1,2:5,6-dibenz[a]anthracene, a PAH identified in soil samples from the old fire school and the runway ditch system, further indicate that immune suppression, as measured by inhibition of the plaque-forming cell response to sheep red blood cells, is a sensitive biomarker for PAH exposure and effects. It appears from the data obtained thus far that immune suppression is 10-fold more sensitive than is EROD activity induction. Furthermore, it appears that deer mice are approximately 10-fold more sensitive to the effects of PAHs than are C57Bl/6 mice (RL Dickerson, unpublished data).

**Conclusions**

Additional positive control studies are planned to further validate this biomarker approach to ecological risk assessment. These studies, designed to develop dose–response relationships between PAHs, EROD activity, and immune suppression, will provide a link between the field studies and the previously developed database using laboratory rodents. In addition, the susceptibility of PAH-immuno-suppressed deer mice to challenge with pathogenic bacteria, viruses and helminths will be determined.

These and other studies (18, 19) suggest that effective ecological risk assessments should incorporate a combination of soil and tissue residue analyses with a form of biomarker evaluation. It appears that the measurement of mechanistically relevant biomarkers in enclosed animals on contaminated sites is an effective means of measuring these biomarkers and is a useful adjunct to the use of field-captured animals. From these studies, it is evident that more work needs to be done in identifying biomarkers for use in ecological risk assessments of wildlife species and in the validation of their use in field investigations. In particular, there is a need for biomarkers that can differentiate between the genotoxic and nongenotoxic effects of a xenobiotic that has the potential for both types of effects.

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