Tulane/Xavier Center for Bioenvironmental Research Project
"Hazardous Materials in Aquatic Environments;
Subproject: Biomarkers and Risk Assessment in Bayou Trepagnier, LA"

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Introduction

Tulane and Xavier Universities have singled out the environment as a major strategic focus for research and training for now and beyond the year 2000. The Tulane/Xavier Center for Bioenvironmental Research (CBR) was established in 1989 as the umbrella organization to coordinate environmental research at both universities.

CBR projects funded by the DoE under the "Hazardous Materials in Aquatic Environments" grant are defining the following: 1) the complex interactions that occur during the transport of contaminants through wetlands environments, 2) the actual and potential impact of contaminants on ecological systems and health, 3) the mechanisms and new technologies through which these impacts might be remediated, and, 4) new programs aimed at educating and training environmental workers of the future. The subproject described in this report, "Biomarkers and Risk Assessment in Bayou Trepagnier, LA", is particularly relevant to the U.S. Department of Energy's Environmental Restoration and Waste Management programs aimed at solving problems related to hazard monitoring and clean-up prioritization at sites with aquatic pollution problems in the DOE complex.

Objectives

Bayou Trepagnier and its control site, Bayou Traverse, are situated in the wetlands that intervene between the Mississippi River and Lake Pontchartrain in the New Orleans area (Figure 1). Bayou Trepagnier is contaminated with effluent from petroleum and petrochemical plants. It contains heavy metals and organic contaminants dissolved in pore waters and adsorbed to sediments. Bottom sediments of the bayou are contaminated with lead, zinc, chromium, arsenic, and polyaromatic hydrocarbons that were dumped in the bayou in years prior to the passage of the Clean Water Act in 1972.

Similar hydrocarbon and metals contaminants are present at DOE National Laboratory sites (e.g., Savannah River Site, A/M Area, SC), DOD sites (e.g., McClellan Air Force Base, Operable Unit B/C, CA), and EPA Superfund sites (e.g., French Ltd. Superfund Site, TX).

Clean-up levels related to hydrocarbon contaminants at DOE sites have been based on conventional quantitative risk assessments that consider toxicity, dose, exposure duration and pathways, as well as induced health effects. As a result of the DOE Draft 1995 Risk Report to

Congress, the sites are being made aware of the need to determine risks to humans, the environment, and ecosystems and to identify and describe individual populations or segments of the environment that would be at risk from the hazards that are present at the sites. The Proposed Guidelines to revise EPA's 1986 Guidelines for Carcinogen Risk Assessment attempt to address the limitations of the 1986 Guidelines where identification of potential human hazard relied heavily on tumor findings, and in practice, seldom made full use of all biological information including eco-risk assessment.

The DOE, DOD and EPA are faced with a multitude of sites to cleanup, yet have neither the resources nor workforce to perform all cleanup tasks. Thus simple, easy, and reliable method of prioritizing sites for cleanup based on the ecological and/or human health risk is necessary. Our approach in the "Hazardous Materials in Aquatic Environments" project is to produce a prioritization scheme based upon eco-risk predicted by contaminant levels and validated by simple biomarker studies designed to assay bioavailability and reflect exposure. This process would occur in the following three steps:

Characterization of contaminants present at the sites.

I. Prediction of risks associated with specific sites.

II. Assessment of sensitive, molecular biomarkers in wildlife or animals placed on site or exposed to sediments from the site in controlled laboratory settings to determine if contaminants are bioavailable and hazardous.

III. Review data to design and prioritize clean-up at specific sites.

A first test of this approach, related to oil refining contaminants present in Bayou Trepagnier, LA. is presented in this report. Contaminant level profiles, risk assessments related to marine and estuarine contaminated sediments, and the measurement of changes in sensitive molecular biomarkers in frogs exposed to sediments, identify a site in Bayou Trepagnier as a source of exposure. These findings are being integrated into ongoing deliberations by the Louisiana Dept. of Environmental Quality, Shell Oil, stakeholders, and environmental groups involved in designing a remediation plan for the Bayou. The initial success of our approach indicates that this process will be of value to the DOE in prioritization of cleanup at aquatic sites.

Approach

Bayou Trepagnier was chosen by the Tulane-Xavier DOE/EM Project as a natural laboratory for studying the fate and transport of heavy metal and organic contaminants in wetlands, including characterization of contaminant loadings, water quality, hydrology, and biota. Bayou Trepagnier is located in St. Charles Parish at Norco, Louisiana. The drainage system is bounded on the south by US Highway 61, on the north by Lake Pontchartrain, on the west by the Lower Guide Levee of the Bonne Carre Floodway, and on the east by Bayou La Branche (DEQ, 1986). As stated in the DEQ impact assessment for Bayou Trepagnier, Bayou Trepagnier flows in a northeasterly direction through a bald cypress/tupelo gum swamp with mixed hardwoods on natural banks and spoil areas along the upper reaches of the stream. Approximately 4 miles from its headwaters, Bayou Trepagnier joins with Bayou La Branche. Bayou Trepagnier and Bayou La Branche are listed in the Louisiana Natural and Scenic Stream System.

Bayou Trepagnier has been heavily impacted by the discharge of heavy metals (lead, zinc, chromium, copper, and arsenic), crude oil, and refining intermediates from the Norco Manufacturing Complex over the past 80 years. Contaminants were mainly confined to the Bayou channel until extensive dredging in 1951 created spoil banks mainly on the spillway side of the Bayou. Over the past 45 years, the weathering of spoil bank material mobilized lead, zinc, and chromium, thus contaminating the marsh and swamp adjacent to the Bayou. Monitoring of the water column indicates that lead in sediments tend to be tightly bound as sulfides. The possibility of sporadic releases from the sediments exists due to the high content of dissolved organic carbon in the water column. Polyaromatic hydrocarbons, the dominant organic contaminants in the Bayou, are concentrated in the bottom sediments. The spoil banks are relatively free of these compounds. Several of these compounds that occur at high concentrations are known to have carcinogenic, embryotoxic, and endocrine disruptive effects on aquatic organisms.

Bayou Trepagnier is a tidally influenced stream approximately four miles in length. The headwaters of the stream originate at the Shell Oil Company Refinery north property boundary. The Shell Oil Company Refinery ceased dumping cooling water into the bayou in the fall of 1995. At this point, the only water movement that occurs in the Bayou originates from tidal flow from Lake Pontchartrain and from rain water and flooding.

The oil refinery at Norco was constructed at the headwaters of Bayou Trepagnier in 1916. Wastes from the plant were discharged into the Bayou through the 1950's, at which time the proprietors of the refinery dredged the Bayou to make it more navigable, dumping the dredged spoils onto the west bank of the Bayou. From that point, the Louisiana State Department of Environmental Quality (DEQ) stopped the release of refinery wastes into the Bayou. The CBR became involved in studying Bayou Trepagnier in 1992.

CBR research cores were established to streamline and coordinate sampling, contaminant characterization, and data analysis. The Field Work Core provides researchers with water, soil, or biotic samples, measures water quality parameters, and coordinates the overall plume characterization effort. The Analytical Core performs organic and inorganic analyses, insures uniformity of analysis protocols, and maintains Quality Assurance/Quality Control and instrument certification programs for each of the satellite labs involved in the project. The Data Management Core collects all data and enters it into the project database after review, develops tools for electronic access to the database, digitizes geographic features of the bayou, and uses Global Positioning System (GPS) to locate biotic and abiotic sampling locations. The Core also analyzes data and generates maps using a Geographic Information System (GIS).

To assess and validate risk relative to levels of PAH and metals contaminated sediments in Bayou Trepagnier, the following steps were taken:

First, Bayou Trepagnier was sampled at 20 sites along its route from the Norco/Shell oil refinery to its confluence with Lake Pontchartrain.

Second, a risk assessment methodology developed by (Long, MacDonald et al. 1995) related to contaminated marine and estuarine sediments was applied to contaminant level data from the Bayou.

Third, local amphibian species (frogs) were placed in cages at sites with contaminant levels associated with predicted high risk and at control sites.

Frogs were assayed for changes a molecular biomarkers of exposure, the cytokine IL-1B. Frogs were also exposed in the laboratory to sediments from contaminated and clean sites to confirm field biomarker data. Thus, this approach was designed to integrate site contaminant data via a quantitative model for predicting risk with molecular biomarker data.

Project Description

Measurement of metals and PAH's in Bayou Trepagnier

Bottom sediments from the Bayou were sampled to a depth of 15 cm at 20 sites equally spaced between the headwaters of the Bayou at the Norco refinery to the confluence of the Bayou with Lake Pontchartrain. The top 5 cm was retained for analysis. Heavy metals concentrations were analyzed using ICP spectroscopy and analysis of organic contaminants, for example, polycyclic aromatic hydrocarbons (PAHs), was carried out using GC-mass spectroscopy.

Risk assessment

Risk assessment was carried out using the method of (Long, MacDonald et al. 1995). Concentrations of metals and PAHs were examined, one at a time, to predict whether biota at the site would be rarely, occasionally, or frequently adversely affected by the presence of single contaminants. These predictions were extremely conservative in that they did not take into account the effects of mixtures of PAH's and/or metals. This method effectively identified specific sites along the Bayou most likely to be adversely impacted by the contaminants.

The applied method of risk assessment compares concentrations of contaminants at the sites to levels characterized in marine and estuarine contaminated sediments known to produce a variety of biological effects. These include toxicity endpoints, changes in community composition, and histopathological disorders. Predicted risk was grouped into 3 categories:

ERL: environmental risk low, rarely associated with adverse effects

IV. ERL-ERM: environmental risk low to environmental risk median, occasionally associated with adverse effects, and

V. >ERM: greater than environmental risk median, frequently associated with adverse effects.

Biomarker measurements in frogs

Risk assessment predictions employ a variety of toxicologic and community structure measurements to predict risk but offer no distinct, molecular biomarkers that can be rapidly measured and that correlate with the severity of exposure. Ideally, molecular biomarkers should offer insight into the molecular nature of adverse effects, such as the progression of environmentally induced disease or of the organisms response to the exposure. Cytokines, found in all vertebrates, represent excellent biomarkers for following the course of exposure and disease. (Silbergeld 1993) The cytokine interleukin-1ß (IL-1ß) was evaluated as a potential biomarker of exposure because 1) it initiates the cytokine cascade that controls the vertebrate immune response, 2) it activates immune system cells such as T-cells, B-cells, and phagocytic cells such as macrophages and brain microglia, and 3) it acts as a growth/survival factor during development of the nervous system. (Giulian and Lachman 1985; Watkins, Parsons et al. 1987; Cohen and Haynes 1991; Dinarello 1991; Sei, Vitkovic et al. 1995) Thus, changes in IL-1ß levels, as a consequence of exposure, are indicative of major changes in normal neuroimmune system functions. It is also indicative of serious changes in the course of neural development in young animals.

Measurements of IL-1ß levels relative to exposure are pertinent to studies related to mammals including humans, and, as described in this report, IL-1ß levels also indicate exposure in lower vertebrates in this case, amphibians. Since frogs are amphibians found throughout wetlands environments and are impacted by contaminants relevant to the DOE cleanup mission, they were employed as a sentinel species regarding changes in IL-1ß levels upon exposure to Bayou Trepagnier sediments. (Cairns and Mount 1990; Wake 1991)Thus, the following three experiments were carried out to establish IL-1β as a biomarker of exposure in frogs related to contaminated sediments:

Ranid frogs were placed in cages for up to 3 days at a site predicted by risk assessment to be the most hazardous, due to the presence of multiple PAH's and metals (Bayou Trepagnier) and at a nearby control site (Bayou Traverse) which showed virtually no metals or PAH's.

VI.To control for other unknown factors, possibly present at Bayou Trepagnier and not at Bayou Traverse, and that might influence frog IL-1β levels, sediments from both sites were brought into the laboratory. Frogs were exposed for 14 days to sediments under controlled laboratory conditions.

VII.To validate the relationship between exposure to contaminanted sediments and IL-1ß levels, frogs (in this case, developing *Xenopus laevis* laboratory frogs) were exposed to the environmental toxicant methylmercury chloride in a variety of doses.

Results

Distribution of Contaminated Sediments and Risk Assessment in Bayou Trepagnier

Heavy Metals

Lead, chromium, and zinc all occurred at levels exceeding ERM at sites along the bayou (Figure 2). Site 101, for example, as shown in Figure 5, exceeded ERM for all of these metals.

Polycyclic Aromatic hydrocarbons

Seven PAH compounds and total PAH occurred in concentrations above ERM along the Bayou. An additional six PAH's occurred at levels ERL-ERM (Figures 3 & 5). A large group of organic compounds listed in Figure 3 were either detected at levels below ERL or were looked for but not detected at the sites. Two areas consistently showed PAH levels associated with risk as predicted by the risk assessment method. For example, benzo(a)pyrene risk levels are plotted on a map of Bayou Trepagnier sample sites in Figure 4. Concentrations exceed ERM at 2 sites (#71 and #111).

Do Biomarkers of Exposure Confirm Risk Predictions?

IL-1ß is Reduced in Neural Tissues of Frogs Exposed to Bayou Trepagnier Sediments

Frogs Exposed in Field Studies

Frogs were placed in cages in Bayou Trepagnier at a location between site #105, shown to exceed ERM for lead, chromium, nickel, and zinc and site #111, shown to exceed ERM for these seven PAHs listed in Figure 5, as well as for total PAHs present at one site. Control cages were placed at a site in Bayou Traverse with similar ecological characteristics but with no measurable contaminants in the sediments. Frogs were removed from sites after one, two, or four weeks and analyzed for levels of IL-1ß protein in the brain using western blot and dot blot procedures (Schagger and von Jagow 1987; Danielpour 1993). Results indicated that frogs exposed to the contamination in Bayou Trepagnier for two weeks showed statistically significant decreases in brain IL-1ß protein levels (Figure 6; $p<.04$, one way ANOVA).

Frogs Exposed to Sediments in the Laboratory

To rule out the possibility that factors other than contaminated sediments at Bayou Trepagnier contributed to decreased levels of IL-1β protein, sediments from both sites were transported to the laboratory and used to expose frogs. Frogs placed upon Bayou Trepagnier sediments showed statistically significant decreases in IL-1ß protein after 10 days of exposure compared to frogs placed upon sediments from Bayou Traverse (Figure 7; p<.0001, one way ANOVA). Thus, the sediments themselves were sufficient to cause decreases in IL-1ß protein.

Exposure of Developing Frogs to Methyl Mercury Chloride also Reduces

IL-1ß Levels in Neural Tissues

Reduction of IL-1ß in the Vth Cranial Ganglion

In experiments done in collaboration with Anna Jelaso at Tulane University, developing *Xenopus laevis* embryos were exposed to different doses of methylmercury chloride (MMC) to determine if toxicant exposure caused decreases in neural IL-1ß levels. Western blot analysis confirmed reductions in the IL-1ß active protein in embryos exposed to 25 parts per billion (ppb) or greater (Figure 8). Computer assisted image analysis was used to measure IL-1ß protein levels in specific areas of the brain of developing frogs after exposure of up to 100 ppb MMC. IL-1β protein expression in the Vth cranial ganglion was significantly reduced in embryos exposed to 50 ppb (p<.006) and 100 ppb MMC (p<.0009).

Reduction of IL-1ß in the Neuromuscular System

Similar to results in the Vth cranial ganglion, exposure to concentrations of methylmercury of 50 ppb and greater produced statistically significant decreases in IL-1ß protein in the anterior myotomes, the site of ongoing development of neuromuscular connections (50 ppb, p<.0001; 100 ppb p<.0036).

Methylmercury toxicity related to changes in IL-1ß levels in frog embryos

Exposures of 50 ppb MMC not only produced decreases in IL-1ß protein but also produced significant tissue damage (in the spinal cord and musculature), and developmental defects such as curved and shortened body axis, and increased mortality (50 ppb, p<.0013; 100 ppb, p<.0001) compared to control embryos.

Suitability of neural IL-1ß as a biomarker of exposure to contaminated sediments

Risk assessment methods applied to Bayou Trepagnier sites predicted that sediments from several sites should produce adverse effects in animals exposed to them. Both field and laboratory sediment exposures on frogs produced dramatic decreases in neural IL-1ß. These frogs showed no overt signs of disease but in some cases, showed damage to organs such as the spleen. Laboratory based dose: response studies using the toxicant MMC confirmed that neural IL-1ß levels do indeed decrease upon exposure of environmental levels of 50 ppb or greater and that these decreases are concomitant with tissue damage and increased mortality. Field data, lab exposure data, and single toxicant exposure data, together indicate that IL-1ß is a sensitive, molecular biomarker of exposure to contaminated sediments and to single toxicants such as methylmercury.

Conclusions

Eco-risk predicted from contaminant concentration data implicated sediments from several Bayou Trepagnier sites as a source of contaminant exposure for wildlife.

VIII. Neural IL-1ß levels serves a biomarker of exposure in frogs placed at the site, or exposed to site derived sediments.

Application

Risk assessment methods are useful in predicting which environmental sites might act as sources of harmful exposure to wildlife and human populations. Validating risk assessment predictions with biomarker measurements from either field or laboratory based exposures of appropriate wildlife such as frogs may represent a fast and reliable method of prioritizing site cleanup and for determining remedial activity levels.

Future Activities

Additional studies are testing the risk biomarker method at different sites along the Bayou to determine if IL-1B responds more to exposure to metals or PAH contaminants. In addition, new studies have been initiated to determine if more sensitive biomarkers of exposure exist, especially biomarkers related to biological receptor based disruption in the environment. Examples of this include contaminants that disrupt hormone levels in wildlife and in humans, and the disruption of additional receptor based cytokines such as TNF and IL-6. The ultimate endpoint of contaminant: receptor binding studies is to produce new "molecular machines" composed of specific biological receptors linked to a reporter system that will sense cytokine or hormone disrupting contaminants in the environment. The magnitude of response should indicate both bioavailability and risk. These constructs would replace both the contaminant measurement phase of risk assessment, and the biomarker validation phase which, in current work is carried out by exposing animals to field sediments. The molecular machines will incorporate the use of functional bio-molecules, usually receptor molecules, coupled to a bio sensor that will detect the hazardous chemical receptor interaction by giving off or absorbing a photon or two of light. These biologically engineered constructs are fixed to support systems which may be solid or flexible but can be placed in the field and monitored by light storage devices or connected to electronic monitoring systems. Tulane and Xavier have the capacity to handle all stages, including formulation of the devices. The Department of Biomedical Engineering is applying for a major grant for a Laboratory for Artificial Cell Engineering which will create truely cutting edge technologies for the combination of biological systems with artificial materials. Application of new technologies such as these might represent a considerable savings for the Department of Energy in hazard assessment work related to risk based prioritization of sites to clean up. CBR researchers currently have a first step approach system where human estrogen receptors are expressed in yeast. These receptors are activated by contaminants and ultimately turn on a gene in the yeast that produces a color. The intensity of the color is indicative of the contaminant concentration. These methods have been used to show that DDTs and PCBs activate the estrogen receptor, disrupting its normal function. (Arnold, Klotz et al. 1996) A paper has been recently submitted that establishes an antagonistic relationship between Bayou Trepagnier PAHs and the estrogen receptor. CBR researchers are currently adapting this new technology for studying disrupting effects of contaminants on thyroxin receptors and on cytokine receptors. Ultimately, these "molecular machines" will be placed in aquatic environments and on dry land as hazard monitoring devices. Development of these technologies will produce a new generation of simple devices that not only will detect exposure, but will also be calibrated to predict risk and associated clean up levels. These constructs may also be adapted for use in binding contaminants to accomplish clean up.

Figure 1: Map depicting Bayou Trepagnier as it courses from the Norco/Shell Refinery (Industry) to its confluence with Lake Pontchartrain. GPS locations represent global positioning system located sites used in the study. The Mississippi River is to the lower left of the figure and Lake Pontchartrain is to the upper right.

Figure 2 The potential risk effects of inorganic compounds in Bayou Trepagnier. Elements detected or tested for appear in the first column. The ERM or "environmental risk median" limit appears in the third column. This value represents the concentration in parts per million that is associated with frequent adverse effects. The number of sampled stations that were found to be above ERM appears in the fourth column. The ERM value range, that is, those concentrations that occur above ERM appear in the fifth column. Concentrations for levels falling between ERM and ERL (environmental risk low), appear in the last five columns. Values for those elements (starting with lithium) that were detected at levels below ERL, occur in the bottom two thirds of the figure.

Figure 3: The potential risk effects of organic conataminants in Bayou Trepagnier. The compounds appear in the first column, their "environmental risk median" or ERM limit in parts per billion, appears in the second column, the number of stations that showed concentrations above ERM appears in the third column, and the value range above ERM appears in the fourth column. Similar data regarding the environmental risk low to environmental risk medium range (ERL-ERM) appear in the next three columns, and the final three columns show stations and values that were below environmental risk low (ERL).

Figure 4 Map of Bayou Trepagnier showing sites where one PAH contaminant (benzo(a)pyrene) either exceeds ERM, falls between ERM and ERL, or is below ERL. Two sites' risk predictions were >ERM for this compound. Data taken from other available environmental reports concerning Bayou Trepagnier also appear in this figure.

Figure 5 Risk values related to inorganic lead, chromium, arsenic, nickel, and zinc (in parts per million), and to PAH compounds (in parts per billion) found at two sites. The ERM or "environmental risk median" cut off value appears in the 2nd column and the value that was measured at the site occurs in the third and fourth columns. Both sites exceed ERM for total lead. Site 101 exceeds ERM for chromium, arsenic, nickel and zinc. Where both sites exceed ERM for dibenzene(a,h) anthracene. Site 111also exceeds ERM for all other PAH's listed in the table, as well as for the total PAHs..

Figure 6 Frogs were placed in cages for two weeks in Bayou Traverse, at a clean site, with low predicted risk, and Bayou Trepagnier, at a site characterized by high predicted risk . A reduction in brain IL-1B in frogs kept at the Bayou Trepagnier site was significant at $p<.04$, compared to frogs kept at Bayou Traverse.

Figure 7 Frogs were exposed in the laboratory to mud from Bayou Traverse, a site with no predicted risk, and from Bayou Trepagnier, a site with high predicted risk. Brain IL-1β, as measured by Western and dot blot analysis, decreased in frogs kept on Bayou Trepagnier mud ($p<.0001$).

Figure 8. Developing frog embryos were treated with methylmercury chloride to determine if exposure to a known toxicant would cause reductions in IL-1B. Western blot analysis as shown

revealed a reduction in both precursor and active product proteins starting at 25 parts per billion exposure. Immunocytochemistry experiments showed similar statistically significant reductions of IL-1 in specific brain areas, such as the trigeminal (Vth) cranial ganglion.

Contract Information

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