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Tulane/Xavier University
Center for Bioenvironmental Research

Hazardous Materials in Aquatic Environments of the Mississippi River Basin

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Annual Technical Report
(12/92-12/93)

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**Tulane/Xavier University
Hazardous Materials in Aquatic Environments
of the Mississippi River Basin
Annual Technical Report
Project # DE-FG01-93EW53023
(December 30, 1992 - December 29, 1993)**

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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. In 1989, the Tulane/Xavier Center for Bioenvironmental Research (CBR) was established as the umbrella organization which coordinates environmental research at both universities. The CBR is supported by major grants from the Department of Energy, Department of Defense, National Institute of Environmental Health and Safety, National Institute of Health, National Institute of Environmental Health Sciences, and other agencies. This joint venture is truly interdisciplinary, involving faculty and student participation from most schools and divisions at both universities and, thus, presents an integrated approach to environmental problems. Research ranging from creating new technologies for environmental clean-up to understanding the economics that drive environmental policy decisions are coordinated under the CBR auspices.

Founded in 1834, Tulane University is one of the major private research universities in the South. Over 11,000 students are enrolled in its 11 schools and colleges. Undergraduates are enrolled in Tulane's School of Engineering, School of Architecture, A.B. Freeman School of Business, Newcomb College, University College and Paul Tulane College. Over 4,500 graduate students are enrolled in liberal arts and sciences, engineering, public health and tropical medicine, social work, law, business, medicine and architecture. The recently dedicated J. Bennett Johnston Health and Environmental Research building provides state of the art laboratory space for core areas of bioenvironmental research including toxicology and environmental health sciences.

Xavier University, the only historically black Catholic institution in the United States, was founded in 1915 by a religious order dedicated to the education of American minorities. Enrollment is approximately 3,500 and offers preparation in thirty-six undergraduate majors. Xavier ranks first nationally in the number of black undergraduates receiving degrees in the physical sciences. Countering a national trend of declining African American enrollment in advanced degree programs, high numbers of Xavier graduates go on to professional and graduate schools. The University is moving ahead with plans to develop environmental curricula and research programs to help meet the nation's growing demand for environmental scientists. Recent renovations and a new addition to the College of Pharmacy building have expanded the laboratory facilities for Xavier researchers.

In December, 1992, the Tulane/Xavier CBR was awarded a five year grant to study pollution in the Mississippi River system. The "Hazardous Materials in Aquatic Environments of the Mississippi River Basin" project is a broad research and education program aimed at elucidating the nature and magnitude of toxic materials that contaminate aquatic environments of the Mississippi River Basin. Studies include defining the complex interactions that occur during the transport of contaminants, the actual and potential impact on ecological systems and health, and the mechanisms through which these impacts might be remediated. The Mississippi River Basin represents a model system for analyzing and solving contamination problems that are found in aquatic systems world-wide. These research and education projects are particularly relevant to the U.S. Department of Energy's programs aimed at solving aquatic pollution problems associated with DOE National Laboratories.

First year funding supported seven collaborative cluster projects and twelve initiation projects. Over 70 faculty from Xavier University (from the School of Arts and Sciences and College of Pharmacy) and Tulane University (from the Liberal Arts and Sciences, School of Engineering,

Medical School, and the School of Public Health and Tropical Medicine) participated during the first year. Additionally, more than 40 graduate and numerous undergraduate students worked on research problems associated with the project.

Study Sites

Sites in the Mississippi River basin were selected for studying how industrial contaminants enter aquatic ecosystems and how these compounds move through environmental phases and influence different species. The following areas were chosen as the major sampling sites:

- Devil's Swamp, a cypress swamp that lies northwest of Baton Rouge, is adjacent to the Mississippi River and includes a man-made lake. The swamp is polluted by a variety of surrounding industrial operations, including an abandoned hazardous waste disposal facility.
- Bayou Trepagnier (designated a "natural and scenic stream" within the Natural and Scenic River Act of 1970) serves as the receiving stream for large volumes of water used in many oil processing activities. The 3 1/2-mile bayou flows in a northeasterly direction through a cypress-tupelo swamp and was selected for study based upon its known contamination by metals, oil and grease.
- Tunica Swamp is a relatively pristine water body located approximately 20 miles up river from Devil's Swamp near St. Francisville. It is the control site.

Other sampling sites include: Lake Pontchartrain, Atchafalaya River, Bayou St. John and Barataria Bay.

Methods of Communication

Since this project involves numerous investigators at the two institutions, it is imperative to have well-organized modes of communication among researchers and project administrators. This is facilitated through monthly meetings that rotate among the three participating campuses (Xavier, Tulane uptown and Tulane downtown). All investigators are encouraged to attend. At each meeting, investigators from one or two selected projects present their current research findings. These presentations foster interactions among participants across projects and have resulted in the development of new, interdisciplinary research teams.

Additionally, a poster session is being planned for February, 1994. Investigators from each project will be able to present their research and answer questions from reviewers, DOE administrators, faculty, students and representatives from state and federal regulatory agencies. This venue will provide investigators with important feedback related to the next year's proposed work.

Oak Ridge National Laboratory (ORNL) is working closely with this consortia by providing research support and expertise in a variety of areas. Interactions with ORNL include visits to Oak Ridge by Tulane/Xavier researchers and administrators and student internship programs. In addition, there is close contact among project investigators and ORNL scientists. A joint seminar series has been planned in which ORNL staff will visit and give presentations on studies related to aquatic pollution that are relevant to the Tulane/Xavier project. For more details on these ORNL interactions, please see Administrative Activities (pages 6-7).

Technical Highlights

The Biological Fate, Transport and Ecotoxicity cluster has completed a detailed report concerning the history and present status of heavy metals concentrations in soil collections from Devil's Swamp and has prepared an encompassing QA/QC document. Ecotoxicity studies charted the incidence of tumors in the fish population from Devil's Swamp, while other studies measured biomarkers for neuro, immuno and developmental toxicity in frogs from Devil's Swamp Lake. Laboratory studies were instigated to define dose: response relationships among contaminant levels and biomarkers. Studies to evaluate the physiological and biochemical effects of cadmium in

the red swamp crayfish provided the first evidence in a crustacean that cadmium exposure results in hyperglycemia. Preliminary analysis of cypress cores from Bayou Trepagnier for heavy metals suggests cypress is a good indicator of long-term contamination patterns.

The Bioremediation cluster has performed anaerobic serum bottle studies to determine the toxicity of carbon tetrachloride in samples taken from Devil's Swamp, Bayou St. John and Lake Pontchartrain. This group has also used site-directed mutagenesis to introduce amino acid substitutions into the first eight amino acid residues at the amino terminal end of cytochrome P450 protein in an attempt to alter the degradative abilities of this enzyme.

The Natural and Active Chemical Remediation cluster has successfully synthesized a new polymer material designed to remove heavy metal ions from waste water. Researchers also investigated the role of sediment acid volatile sulfides (AVS) in limiting the concentration of heavy metals in the water column. Preliminary data suggest that the Barataria estuary has a limited capacity to absorb heavy metals via exchange reactions with sediment AVS.

The Expert Geographical Information System cluster has developed a set of guidelines regarding the required capabilities and features of the GIS facility that is being designed for use by fate and transport modelers with little or no experience in GIS, database management and computer graphics.

The Assessment of Mechanisms of Metal-Induced Reproductive Toxicity cluster has focused field studies on 5 sites along Bayou Trepagnier. The group found that sediments contained high amounts of iron and aluminum at all sites and found significant amounts of lead, chromium, manganese and zinc. Additionally, all sites revealed the presence of saturated and unsaturated hydrocarbons over the 4 to 20 carbon chain length. Lab studies in crayfish indicate that metal treatments at the studied concentrations and lengths of exposure do not appear to interfere with oocyte maturation.

The Pore-Level Flow of Microorganisms cluster integrated experimental and computational models of pore-level behavior of microorganisms. Studies included the detailed analysis of convection and diffusion within the pores, and the convection and chemotactic responses of swimming microorganisms to the local contaminant concentration.

The Enhancement of Environmental Education cluster is developing a comprehensive education program aimed at producing graduates who can successfully carry out DOE's mission of environmental restoration and waste management. Xavier introduced an Environmental Studies minor and an Environmental Science track within the science disciplines. A B.S. degree in Environmental Engineering has been implemented at Tulane. Also, six new environment-related courses were offered at the two universities.

In addition to research cluster activities, initiation project investigators carried out research involving the following examples: using asymmetric polyvinylidene fluoride membranes for organic/water separations; assessing the toxicological effects of heavy metal mixtures on medaka fish; creating a novel hybrid chemical-enzymatic technique to remove aromatics from aqueous streams; and developing an interactive hypermedia model of hazardous materials risk communication. It is anticipated that many of the initiation projects will be expanded in the second year to become cluster projects.

Administrative Activities

Highlights of the past year's administrative activities are summarized below:

January

- Tulane/Xavier notified of grant award
- RFP distributed

February

- External review panel formed

March

- 35 concept papers were received
- Concept papers were peer reviewed and panel made recommendations to: combine education PIs to form one interdisciplinary, inter-university proposal; encourage PIs working in similar areas to collaborate to enhance the research and avoid duplication of efforts
- 35 initiation project proposals and 11 collaborative cluster project proposals were received

April

- Proposals were peer reviewed
- 13 initiation projects were funded and 8 collaborative clusters were funded
- Award notification sent to PIs
- Equipment Committee formed to determine equipment needs and purchases for cluster groups
- Additional committees were formed to coordinate sampling and analyses associated with the clusters

May

- The Coordinated Instrumentation Facility (CIF) sponsored three seminars concerning environmental sample preparation techniques to education investigators on the use of microwave digestion systems for sample preparation and the use of inductively coupled plasma and atomic absorption spectroscopy for analyses
- Relationship with Oak Ridge National Laboratories (ORNL) was established
- Student internship program developed with Oak Ridge

June

- Four students (2 from TU and 2 from XU) went to Oak Ridge for 10-week internship program

July

- Approval by DOE for equipment purchases
- Sampling started in Devil's Swamp Lake, Devil's Swamp, Bayou Trepagnier and Bayou St. John
- One professor from Xavier and one from Tulane spent 5 weeks at ORNL working with researchers and discussing future collaborations

August

- Plans were made with ORNL for an expanded summer internship program in Summer 1994
- PI meetings are planned monthly along with presentations from investigators
- Co-director toured Hanford site

September

- Drs. Carl Gehrs, Lee Shugart and Marshall Adams from ORNL visited Tulane and Xavier and presented two seminars for the project investigators
- Project administrators participated in the Office of Technology Development's (OTD) "New Technologies and Program Exhibition" at the Rayburn House Building and the Hart Senate Building in Washington, D.C.

October

- Project co-director participated in the Natural Resource Recovery Technology Forum sponsored by the National Environmental Waste Technology, Testing, and Evaluation Center that was held in Montana

November

- Project administrators (along with three undergraduate students that participated in the summer internship program) participated in two OTD exhibitions, one at the DOE Forestall Building and the other at DOE Germantown
- RFP for second year funding was issued

December

- Planning began for the academic poster session to be held in February, 1994
- Review panel for second year funding was identified

Collaborative Cluster Projects

Biological Fate, Transport, and Ecotoxicity of Toxic and Hazardous Waste in the Mississippi River Basin

A. Abdelghani, W. Hartley, H. Bart, C. Ide, E. Ellgaard, T. Sherry, M. Devall, L. Thien, E. Horner, M. Mizell, R. Thompkins, A. Apblett, M. Fink, M. Fingerman, J. Barber, H. Ensley, A. Anderson, T. Akers, A.J. Englande, R. Reimers, C. Thomas, A. Thiagarajah, T. Huang, C. Hill, B. Howard, S. Phadtare, T. Mandal, Y. Pramer, P. Martinat, M. Polito, P. Obih

Performance Sites:

Tulane University (Departments of Environmental Health Sciences, Cell and Molecular Biology, Ecology, Evolution and Organismal Biology, Anatomy, Medicine); Xavier University (College of Pharmacy)

Abstract

The objective of the cluster investigators is to develop a dynamic model for the evaluation of the biological fate, transport, and ecotoxicity from multiple chemical contamination of the Mississippi River Basin. To develop this environmental model, FY 93-94 most of cluster investigators focused on Devil's Swamp Site (DSS), a cypress swamp which lies just Northwest of Baton Rouge, Louisiana, adjacent to the Mississippi River. The DSS which includes a man-made lake has contaminated sediment, water and biota. The DSS receives flood water from the Mississippi River during high flow periods and the Baton Rouge Bayou drains through the DSS. The DSS receives toxic substances and hazardous waste from a wide variety of surrounding industrial operations including an abandoned hazardous waste disposal facility. In addition, some investigators studied Bayou Trepannier. This research cluster will continue studying Devil Swamp. The large number of investigators in this cluster resulted from incorporating related research proposals based on reviewer recommendations. The specific aims of the cluster for the first year were to conduct a physical, chemical, ecological survey and baseline toxicological characterization of the DSS from existing databases maintained by State and federal agencies, field studies (assessment) of sediment, air, water and biota, and laboratory screening studies. This assessment will provide critical information and focus for the next two years in-depth studies of critical transport and fate processes, ecotoxicity, biomarkers of effect, and uptake, metabolism and distribution of toxicants.

The primary significant outcome of the cluster researchers will be the development of an ecological risk assessment model combining biotic and physical/chemical variables for DSS with a projection of model reliability and accuracy for use at other typical Mississippi River Basin sites. A reasonable assessment of accuracy will be possible based on the confirmatory and investigatory laboratory studies completed in association with the field studies at DSS.

Introduction

The overall theme of the cluster is to develop an ecological risk assessment model for wetland environments typical of the Mississippi River Basin. This project is relevant to DOE restoration of wetlands with multiple chemical contaminants. This will would incorporate biotic and abiotic variables that impact on aquatic ecosystems and environmental fate. This cluster brings together scientists from Xavier and Tulane that have expertise in field ecology, aquatic toxicology, pathology, physiology/biochemistry of aquatic organisms, plant physiology, biological chemical

SUBCLUSTERS OF BIOLOGICAL FATE AND TRANSPORT PROJECT

* T = Tulane
** X = Xavier

and fate, environmental chemistry and engineering, neurotoxicology, immunotoxicology and other disciplines for a comprehensive and interactive study of DSS. The cluster will consist of three interactive subcluster research groups as follows with investigators primarily assigned to each group.

The Ecology Subcluster will continue to evaluate the impact of DSS contaminants on biological diversity (species diversity), trophic structure and other population studies on fish, amphibians, plants and birds in addition to collecting aquatic organisms and media (soil, air, sediment, and water) for the other two sub-cluster research groups. The Biomarkers Subcluster will continue to evaluate specific biomarkers of toxic effect in fish and frogs with focus on cancer and disease, developmental effects, neurotoxicity and immunotoxicity. Biomarker evaluation of these endpoints will be conducted in the field and validated in laboratory "in vivo" aquatic animal models. The Exposure Subcluster will continue to study the uptake, metabolism and bioaccumulation, and acute and chronic toxicity of site contaminants in fish, macroinvertebrates, aquatic plants and terrestrial plants and birds. Chemical specific field studies will be followed by confirmatory and detailed laboratory studies. Studies in the field will be conducted to characterize the distribution of site contaminants and conduct analysis using Environmental Health Sciences laboratories (ENHS), School of Public Health and Tropical Medicine of all animal and plant tissues for DSS contaminants. This continues to include characterization of the fate, distribution (transport) of contaminants in the sediments, water, air and biota, and evaluation of the bioavailability of metals and organics.

Summary Of Achievements In Fy '93-'94

Based on the program plan of year 1 FY 93-94, sampling and laboratory activities and staff have been organized and a database review has been completed. The report of database literature review and DSS field and laboratory screening studies are in the process of being published.

A detailed quality assurance/quality control (QA/QC) document was prepared and distributed to all DOE Investigators. If followed, all QA/QC procedures will be the same for all researchers.

A report about the history and chemical concentrations of metals in soil collection from Devil's Swamp was also prepared and distributed.

Ecology Subcluster

- Samples of fish, frogs, crayfish, bottom sediments, vegetation and water have been taken from the Devil's Swamp, the southernmost lateral floodplain habitat on the Mississippi River situated on the eastbank just west of Baton Rouge. These samples were provided to the ecology and other subcluster investigators. The scope of the sampling has been limited by the absence (until just recently) of electrofishing equipment. However, the addition of the electrofishing boat will permit sampling of the four remaining riverine sites and give us access to the control area (Tunica Swamp).
- Cores have been taken from cypress trees in Tunica Swamp, Devil's Swamp and Bayou Trepagnier. Preliminary analysis of cypress cores from Bayou Trepagnier for heavy metals suggests cypress is a good indicator species and can be used to record past and present effects of natural and manmade events in the environment. For example, the lead levels from 1960 to 1993 are twice as high as that found in rings prior to 1960; this correlates with the time course of known lead contamination in the area.
- Most of the gastrointestinal tracts of fish from the first two collections and black crappie from the third collection have been examined. The greatest abundance of aquatic organisms was found in the stomachs of black crappies. Most organisms were entirely intact, allowing

preliminary identification to family. Identification to genus or species should be possible. The most abundant were larvae and pupae of the Chironomidae and Chaoboridae.

Biomarkers Subcluster

- Comprehensive histopathological examination and documentation of approximately sixty fish from Devil's Swamp representing nine different species is in progress. Initial histopathology results indicate that pathology biomarker studies should focus on the liver, kidney, spleen, thyroid, pancreas and gills.
- Lesions identified of particular interest with regard to exposure to chemical contaminants and disease in Devil's Swamp fish include the following: Channel Catfish (proliferation of alarm cells in the skin and kidney mesangiosclerosis); Yellow Bullhead Catfish (high incidence of spongiosis hepatis, telangiectasis of the liver, probable hemangioma/tumor, white pulp lesion in spleen, dilated Bowman's space in the kidney, and hyperplastic thyroid); Garfish (inflammation of muscle, melanin macrophage centers in the liver and inflammation of the pancreas; and Carp Fish (ectopic thyroid tissue in the kidney, telangiectasis of the gill, and periductal inflammation of the liver and vascular elements).
- Laboratory studies on the effects of methyl mercury on laboratory frogs show that extremely low concentrations alter gross morphological and behavioral development. Neural circuitry related to escape swimming appears altered in early swimming embryos. In adult animals the mitotic capacity of immune system cells is highly sensitive to low concentrations of methyl mercury. Laboratory studies indicate that gross morphology and behavior are dependable developmental biomarkers of exposure. Further studies will determine if neuroimmune system biochemicals such as glucocorticoids will serve as sensitive indicators of compromised immune system and nervous system function. Wild frogs caught at Devil's swamp are under comprehensive neurological and immunological evaluation with no adverse effects noted to date.

Exposure Subcluster

- The toxicity, uptake and accumulation of heavy metals by Lemna gibba (duckweed) is in progress. The acute toxicities of arsenic, cadmium, chromium, lead, thorium, and uranium have been determined with regard to vegetative reproduction of axenic cultures, grown under defined laboratory conditions. With regard to organic pollutants, statistically accurate toxicity curves for phenol, p-chlorophenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol, pentachlorophenol and ethylene glycol have been determined. The structures of the metabolites of the chlorophenols (chlorophenylglucosides) have also been determined.
- Studies were initiated to evaluate the physiological and biochemical effects of the heavy metal cadmium in the red swamp crayfish, Procambarus clarkii and the fiddler crab, Uca pugilator. This model approach provided the first evidence in a crustacean that cadmium exposure results in hyperglycemia in the intact crayfish. This hyperglycemia was shown to be mediated, at least partially, by the crustacean hyperglycemic hormone from the sinus glands, neurohemal organs, in the eyestalks. Exposure of crabs to cadmium resulted in decreased lactate dehydrogenase activity in the hepatopancreas but, in contrast, this enzyme activity in the abdominal muscles increased.
- Laboratory studies on the toxicity of arsenic, cadmium and mercury to crawfish and bluegill sunfish showed an order of toxicity as follows:

A.	Fish	Hg > As and Cd
B.	Crawfish	Hg > As > Cd

Mercury showed the highest toxicity ($LC_{50} = 0.5$ and 6.5 mg/l to fish and crawfish respectively). Arsenic and cadmium had the same toxicities to fish ($LC_{50} = 13$ mg/l) and arsenic was twice as toxic as cadmium to crawfish ($LC_{50} = 45$ and 85 mg/l).

Bioassays on other species including microorganisms are planned to continue in FY '94-'95. Uptake, distribution, storage and depuration studies following the determination of LC_{50} 's for study chemicals will be conducted. Aquatic organisms will be exposed to concentrations based on the results of bioassays. Studies will include subchronic exposure of crawfish, bluegill sunfish to different concentrations for at least 3 months of uptake followed by 3 months of loss.

- Environmental samples including fish, frogs, crawfish, vegetation, water, soil, birds blood, feathers, and tree cores were collected from Devil's Swamp and brought to the Environmental Health Sciences Laboratories for analysis. Most of the samples have been prepared for metal analysis (lead, mercury, arsenic, chromium, and cadmium) and organics such as hexachlorobutadiene. Results will be distributed to all DOE investigators upon completion of chemical analysis. Preliminary results of these analysis indicate that cadmium concentrations in different fish species might pose a human health risk if these are consumed.

Cluster Project Descriptions

Ecology Subcluster

Comparison of Contaminant Levels in Aquatic Organisms from Different Flood Regimes in the Lower Mississippi River Basin

Abstract

We proposed to compare levels of heavy metals and organic contaminants in selected fish and invertebrate species from sites representative of the different lateral flooding regimes that exist in the lower Mississippi River Basin as part of a three-year study of the biological fate, transport and ecotoxicity of toxic and hazardous wastes in the system. The ultimate goal of the research is to assess the role of lateral floodplain habitats in the natural processing of hazardous materials. This paper focuses on samples taken in Devil's Swamp, one of five sites selected for sampling. In it we attempt to show how the ecology of the different fish species associated with the Swamp relates to exposure to-, uptake of-, and pathology from contaminants in the system. A total of 120 fish representing 24 species were collected. Preliminary result of stomach analysis, histopathology screening and analysis of heavy metals are presented and discussed in relation to the ecology of the species involved. Fish species encountered in Devil's Swamp exhibit a range of ecologies and conditions that make them valuable indicators of environmental contamination. Knowledge of dietary habits, habitat preferences and movements of the species is important for understanding the variance in reported levels of important contaminant in the system. Preliminary analytical results suggest that species with prolonged associations with the swamp and/or those with diets or trophic positions that predispose them to exposure through the food chain, exhibit high levels of contaminants and higher incidence of disease and tissue abnormality.

Introduction

As part of a three-year study of the biological fate, transport and ecotoxicity of toxic and hazardous wastes in the lower Mississippi River Basin, we proposed to compare levels of heavy metals and organic contaminants in selected fish and invertebrate species, serving as models of key components of the aquatic food chain, from sites representative of the different lateral flooding regimes that exist in the basin. We proposed to periodically sample five sites differing in opportunities for lateral flooding and extent of forested lateral flood-plain habitat in the Mississippi and Atchafalaya river systems. The five sites are Mississippi River and Tunica Swamp near St. Francisville, Mississippi River and Devil's Swamp near Scotlandville, Mississippi River below

New Orleans, Atchafalaya River and swamp at Melville (upper Atchafalaya), and Atchafalaya River and swamp below Interstate 10 (lower Atchafalaya). Proposed trophic levels include bottom-feeding fish (either a common sucker species, carp or catfish), planktivorous fish (e.g., bluegill sunfish), top level predator (e.g., largemouth bass), benthic detritivores (crayfish, midge larva), and aquatic sediments over which the organisms are collected.

The primary objective of the study is to determine the effect of lateral flooding on levels of contaminants in water, and on the cycling of contaminants through aquatic organisms. The ultimate goal of the research is to assess the role of lateral floodplain habitats in the natural processing of hazardous materials. In the lowermost section of the Mississippi River system (especially below Baton Rouge), lateral flooding opportunities are limited by high levees that lie close to the river on both banks restricting it to a relatively narrow channel (Baker et al., 1991). Forested lateral floodplain habitat is virtually nonexistent in this reach. In the Atchafalaya River system, by contrast, the flow is spread over a wide forested flood-plain system. Lateral flooding in the Atchafalaya River system, and that which occurs on a smaller scale along the east side of the Mississippi River above Baton Rouge should increase the biological cycling and filtering of contaminants from the flood waters, resulting in higher concentrations at different trophic levels within aquatic animal communities - relative to levels in the water - in these areas compared to the lowermost parts of the Mississippi River.

Our initial thrust, upon which this report is largely based, has been to survey of the literature on the ecology of the lower Mississippi River system, and to sample one of the primary study areas, Devil's Swamp, for the purpose of characterizing the aquatic biota and obtaining samples for analysis by ourselves and a number of the other investigators in the Biological Fate and Transport Cluster Group. A number of locations in Devil's Swamp have been sampled, and the samples have been subjected to a variety of analyses. However, most of the samples still await analysis of their contents of specific environmental contaminants. In this paper we attempt to show how the ecology of the different fish species associated with Devil's Swamp relates to exposure to, uptake of, and pathology from contaminants in the system. We identify the key components of the fish community in Devil's Swamp and, using information from the literature, characterize the different species in terms of their natural habits and preferred habitats. We analyze stomach contents of the fishes to characterize the important invertebrate links in the food chain. Results of health screenings and chemical analyses are reported where available, and an effort is made to relate these results to the ecology of the species concerned.

The Study Area

Devil's Swamp, situated on the eastbank of the Mississippi River just north and east of Baton Rouge, is the southern most lateral floodplain/swamp system on the Mississippi River proper. Devil's Swamp is drained by a small stream, Bayou Baton Rouge, which originates on the natural high bluff on the east side of the river, enters the northern most part of Devil's Swamps swamp, flows through the swamp and a portion of the river's expanded floodplain, and finally exits the swamp through a deep channel in route to the Mississippi River (Fig. 1). At low river stages much of the swamp sits above the channel of the river. During these times, the natural pattern of water flow is southward through the swamp and Bayou Baton Rouge to the Mississippi River. As the river rises it drowns the lower channel of Bayou Baton Rouge and backs up into Devil's Swamp. At very high river stages, the river completely inundates the swamp flooding all of the land between the river and its eastern bluff.

Devil's Swamp Lake is a man-made "borrow pit" lake situated at the head of a man-made harbor (Baton Rouge Harbor) which borders the east side of the swamp. The harbor, formerly part of the swamp, was dredged to form a side channel and terminal for unloading barges. The material excavated to form Devil's Swamp Lake now forms the levee that isolates the harbor from Devil's Swamp. The lake retains water year-around and may serve as a settling basin for water and materials traveling through the swamp (including contaminants). Most of the water entering the

swamp from Bayou Baton Rouge flows through Devil's Swamp lake en route to the Mississippi River. Water from the Mississippi River backs up into the lake at high river stages, and riverine fishes gain access to the lake. As river water recedes and water drains from the swamp, fish and other aquatic life from the swamp retreat to the lake. At low river stages, and during times of low flow from Bayou Baton Rouge, fish and other aquatic organisms become isolated in lake. A number of the species - those characteristic of oxbows and other seasonally isolated water bodies associated with the Mississippi River floodplain - are permanent residents in the lake. The lake is only accessible by boat through the swamp when the river stage is 30 ft or higher; at lower stages the lake may be accessed by portaging across the levee from the Baton Rouge Harbor.

A hazardous waste site, the Brooklawn site of Petroleum Processors, Inc., situated along Bayou Baton Rouge near the head of the swamp, introduced contaminants into the swamp during the 1960's and 1970's. Upper portions of the swamp are contaminated with volatile aromatic hydrocarbons, chlorinated hydrocarbons, polyaromatic hydrocarbons and heavy metals. An eco-risk assessment conducted by a company involved in the cleanup of the hazardous waste site revealed levels of contaminants, specifically hexachlorobenzene (HCB) and hexachlorobutadiene (HCBd), in fish and other aquatic animals from the upper parts of Devil's Swamp in excess of EPA action levels (NPC Services, Inc. personal communication). Levels of HCB and HCBd are reported for pelagic and benthic fish (whole and fillet) from 18 and 19 stations, respectively, in Devil's Swamp. Variance in the reported levels in fish (whole and fillet) is great (3.04 - 3600 ug/kg and 3.35 - 1900 ug/kg, respectively for HCB and HCBd) and undoubtedly relates to factors such as the age and ecology of the fish species analyzed. Lower parts of the swamp are undoubtedly affected because water from Bayou Baton Rouge and upper parts of the swamp drains through the lower swamp en route to the Mississippi River. The extent to which the biota of the lower swamp is affected is yet to be determined.

Methods

Between September and December of 1993, samples of fish, frogs, invertebrates, bottom sediments, water, and shoreline vegetation and soil were collected from four locations in Devil's Swamp, three sites in Devil's Swamp Lake and one site in the lower portion of the swamp near the lower channel of Bayou Baton Rouge (Fig. 1). Fish samples were taken with a combination of gears including trammel nets, baited set lines, and boat electrofishing. Frogs were taken by gigning at night. Crayfish were taken in baited traps. An Ekman grab sampler was used to sample benthic invertebrates and bottom sediments. A wash bucket was used to rinse benthic invertebrates taken with the grab sampler. The invertebrates were preserved in 70% ethanol in the field. Samples of water and bottom sediments were placed in glass containers with teflon-lined lids. All fish, frog, crayfish, water and sediment samples were transported on ice. Samples for heavy metals analysis were acidified immediately on return.

Frogs were delivered whole to Drs. Horner, Ide and Thomkins of the Biomarkers group who are investigating immunological and neurological effects of exposure to amphibians. Fish specimens were identified, enumerated and then delivered to the laboratory of Dr. Hartley of the Biomarkers Group. The fish were necropsied within 14 hours of capture. Gonads, gills, skin samples, spleen and pieces of liver were removed from the fish and fixed in 10% neutral buffered formalin for histopathological analysis by Drs. Hartley, Mizell and Thiyagarajah. Remaining livers were frozen and later delivered to Drs. Hill, Howard, Huang and Phadtare of the Biomarkers Group investigating the influence of contaminants on xenobiotic metabolizing enzymes and mutagenic effects of the enzymes' products. Brains were removed and delivered to Dr. Obih of the Exposure group.

Stomachs and intestines from the fish were removed and fixed in 10% neutral buffered formalin. The GI tracts were later dissected and all contents placed in 70% ethanol. Invertebrates from the stomachs were identified to the family level using a variety of invertebrate identification keys

(Bryce and Hobart 1972, Merritt and Cummins 1984, Pennak 1969, Peterson 1962, Stehr 1987 1991, Thorp and Covich 1991). All food items were retained for future reference.

The remaining fish carcasses were frozen for subsequent analysis of heavy metal and organic contaminants by Dr. Abdelghani.

Results And Discussion

Table 1 lists numbers of individuals of all fish species collected in Devil's Swamp by sample location and date. A total of 120 fish representing 24 species were collected. The varying number of species and individuals taken on the different sampling trips is a reflection of the complexities involved in sampling a swamp associated with a river as large as the Mississippi. Companies involved in the cleanup of the hazardous wastes sites denied us the direct road access to northern portions of Devil's Swamp (above Devil's Swamp Lake) through their right-of-ways. Thus, the northern most area in the swamp we could sample was Devil's Swamp Lake, and it was accessible through only two routes: through the swamp by boat from the river, which requires a river stage of 30 feet or higher; and portaging across the levee that separates the lake from Baton Rouge Harbor (Fig. 1). During a reconnaissance trip in August 1993, the river stage was 31 feet and we were able to reach the Lake by boat. By September the river had fallen to 24 feet. The lowermost site in the swamp, which was sampled in September, was the farthest point we could reach by boat through the swamp. The sample (SLT 93-1), which contained 43 individuals and 18 species, was taken with a boat electrofisher. To obtain the second sample (SLT 93-2) we entered Devil's Swamp Lake across the levee from Baton Rouge Harbor. The electrofishing boat was too large to carry across the divide. We obtained the sample by suspending a backpack electrofishing off the front of a small flatboat which we paddled along the shore of the lake. The effort yielded only four species and nine fish total. The river remained low through our October sampling trip and we again forced to use the over-land crossing to access the lake. Trammel nets and baited set lines were used to obtain sample SLT 93-3. The effort yielded 63 fish representing 13 species. The river had risen to 31 feet by the December sampling trip, permitting us, once again, to travel through the swamp by boat. An electrofishing equipment malfunction was responsible for the low catch in the sample from that date (SLT 93-6).

The fish species encountered in Devil's Swamp are typical of tributaries and backwaters of the lower Mississippi River (Lambou 1959, Guillory 1979, Conner and Bryan 1974). A number of the species are known to use floodplain habitats only during high river, with the majority of individuals returning to the river as the water recedes (Guillory 1979). Other species from the list are primarily adapted to backwaters and likely spend most of their lives in lakes associated with the swamp (Lambou 1959). The following accounts of aspects of the natural history of species collected in Devils Swamp are summarized from accounts in Lambou (1959), Conner and Bryan (1974), Guillory (1979) and Baker et al. (1991). Data on feeding and longevity is primarily from Carlander (1969, 1977). Histopathology results were obtained by Hartley et al. (this report) from specimens from Devil's Swamp Lake. Heavy metals analysis results were obtained by Abdelghani et al., (this report).

The spotted gar, *Lepisosteus oculatus*, though found in the river, favors backwaters and likely maintains a population in Devil's Swamp throughout the year. The species may reach 30 years in age. It is primarily piscivorous as an adult, but consumes crayfish and insects as young and occasionally as an adult. The stomach of one of the spotted gar from the lake contained a crayfish pereopods. Like other gar, the spotted spends most of its time near the surface of the water, and surface oriented prey likely compose a large proportion of the diet. This portion of the diet would not be expected to contain significant amounts of HCB (which is virtually insoluble in water) and other contaminants that associate more readily with sediment than with water, unless, the prey organisms themselves consumed prey that were associated with bottom sediments. Nevertheless, the spotted gar's long life span, its year-around residence in the swamp, and its position near the top of the food chain, make it a good candidate for study. Gar from Devil's Swamp Lake were

found to have inflamed muscle and pancreas tissue (Hartley et al., this report). Specimens from the lake were found to contain lead and cadmium in excess of human risk levels (Abdelghani et al., this report).

The bowfin, *Amia calva*, is similar to the spotted gar, in that it is long lived (survives 30 years in captivity, Carlander 1969), is primarily a piscivore, favors backwaters, and undoubtedly is a permanent resident in Devil's Swamp. As a candidate for exhibiting effects of exposure to a wide range of contaminants, it has the added benefit of being more structure and bottom oriented than gar and having a larger portion of its diet composed of bottom-oriented organisms. Stomach contents analysis, histopathology, and analytical chemistry have yet to be performed on any of the four specimens taken.

The gizzard shad, *Dorosoma cepedianum*, and threadfin shad *D. petenense* are planktivorous fishes which feed primarily on phytoplankton near the surface of the water column. Gizzard shad have been known to reach nine years in age, but the vast majority of individuals survive less than four years. Threadfin shad live only two years. As primary consumers, the two shad species would not be expected to bioaccumulate contaminants from other trophic levels. The diet of threadfin shad does include dipterans such as *Chaoborus* spp. and chironomids which live or feed in bottom sediments, however. Both species are long distant migrants which tend to move up and down the river and back and forth between the river and lateral flood plain habitats. Thus, they would not be expected to have a prolonged association with Devil's Swamp. Preliminary analyses have revealed high levels of lead (136 ppb) in gizzard shad from Devil's Swamp Lake (Abdelghani et al., this report).

The common carp, *Cyprinus carpio*, has been known to survive 47 years in captivity. It feeds primarily on invertebrates (chironomids, fingernail clams) strained from bottom sediments. It thrives in virtually every kind of freshwater habitat and is extremely tolerant of environmental stress. Preliminary histopathology tests have revealed ectopic thyroid tissue in the kidney, telangiectasis of the gill and periductal inflammation of the liver and vascular elements (Hartley et al., this report).

The golden shiner, *Notemigonus crysoleucas*, is a species that favors floodplain backwater habitats such as swamps. They live a maximum of nine years, and feed primarily on small crustaceans, other zooplankton and algae. All four specimens taken were from the extreme lower part of Devil's Swamp. The specimens have yet to be analyzed.

The river carpsucker, *Carpiodes carpio*, smallmouth buffalo, *Ictiobus bubalus*, bigmouth buffalo, *I. cyprinellus*, and black buffalo, *I. niger*, are all sucker species with similar ecologies. All move back and forth between the river and its main flood plain habitats on a regular basis. The river carpsucker survives 8-9 years and is the shortest lived; the black buffalo is the longest lived reaching a maximum of 23 years. River carpsuckers, smallmouth buffalo and black buffalo feed on chironomids, algae and small crustaceans such as amphipods strained from bottom sediments. The bigmouth buffalo differs in that a significant portion of its diet is made up of pelagic zooplankton.

Blue catfish, *Ictalurus furcatus*, also migrate between the river and its flooded lateral habitats, and thus would not be expected to have a prolonged association with Devil's Swamp. Blue catfish live more than ten years. They consume zooplankton and insects as young, and fish and crayfish as adults. Preliminary histopathological examination of specimens taken in Devil's Swamp Lake has revealed evidence of kidney irregularity (mesangiosclerosis) and proliferation of alarm cells in the skin (Hartley et al. this report). Two of three blue catfish analyzed from the lake had high levels of lead (287 and 411 ppb), and one of two specimens of catfish analyzed had a cadmium level of 4453 ppb (Abdelghani et al., this report).

Yellow bullhead, *Ameiurus natalis*, in contrast to blue catfish, are more typical of backwater habitats than riverine habitats in the lower Mississippi River system, and would be expected to have a prolonged association with a lentic environment such as Devil's Swamp Lake. The life span of the bullhead is only half that of blue catfish (5 years), and the diet consists mainly of fish, crayfish and benthic insects. A stomach from one of the specimens from Devils Swamp Lake contained a whole crayfish, while another contained aquatic insect larvae (chironomids). Histopathology results have revealed a number of conditions in specimens from the lake including: hyperplastic thyroid, cystic structures in the liver believed to be a precancerous lesion related to environmental pollution; dilated sinusoids in liver with flocculent material and pooling of blood in perisinusoid spaces; possible hemangioma tumor (in two of four specimens examined); and dilated Bowman's space in kidney (Hartley et al. this report).

The assortment of sunfishes obtained from Devil's Swamp are all typical of backwater habitats in the lower Mississippi River but all of the species exhibit seasonal movements through tributaries and occasionally foray into the river proper (Guillory 1980). The flier, *Centrarchus macropterus*, lives a maximum of seven years and feeds on cladocerans, chironomids and hemipterans most likely picked from vegetation. Green sunfish, *Lepomis cyanellus*, lives a maximum of ten years. Young strain zooplankton from the water column. As they mature, insects, crayfish and fish are consumed with increasing regularity. Warmouth, *Lepomis gulosus*, live a maximum of ten years. Like the green sunfish, it has a relatively large mouth and switches its diet from zooplankton to fish, crayfish and insects with maturity. Largemouth bass live the longest (15 years) and is the most piscivorous of the sunfish species, switching to a diet predominated by fish at an earlier age. Stomach contents analysis and histopathological examinations have yet to be performed on specimens of these species.

Black crappie, *Pomoxis nigromaculatus*, is another centrarchid typical of backwater habitats such as Devil's Swamp, and large numbers of adult-sized specimens were taken in Devil's Swamp Lake. The species is described as a planktivore with zooplankton comprising 50% of the diet of even adult-sized specimens. Fish and a variety of insects are also taken by adults. A detailed analysis of stomach contents was performed on 17 of the 19 specimens taken in Devil's Swamp Lake 12-13 October 1993. The results (Table 2) indicate that the diet is made up primarily of aquatic diptera (Chironomid, Culicid and unidentified dipterans). A large portion of the dipterans in the diet were in the pupae stage and were likely encountered near the water surface. However, larvae from these groups pass the larval stage burrowed in bottom sediments (Pennak 1989). Chironomid larvae were also found in large numbers and may have been in the process of emerging from the sediments in preparation for pupation. Chaoborid larvae, also found in large numbers, are active swimmers which migrate daily from the bottom to upper layers of the water column in search of their zooplankton prey. The chaoborid larvae found in crappie stomachs may have been picked from the water column. Thus, the fraction of benthic organisms in the diets of crappie can be quite high at least during some seasons (i.e., emergence) and it is possible that fish such as crappie, though not top-level consumers, still concentrate significant quantities of insoluble organic contaminants such as HCB. As yet there are no histopathology or analytical chemistry results for the crappie specimens from Devil's Swamp Lake.

Crayfish are an important prey item in the diets of many of the predatory fish encountered in this study. Preliminary analysis of muscle and fat from a sample of crayfish from the lowermost site in Devil's Swamp revealed levels of cadmium of 2301 and 4123 ppb, respectively, and levels of lead of 87 and 31 ppb, respectively (Abdelghani et al., this report). At this level of potential contamination it is easy to understand how fish species near the top of the food chain (e.g., gar) and those that consume crayfish in high numbers (e.g., blue catfish) would concentrate these contaminants in their tissues.

Conclusions

Fish species encountered in Devil's Swamp exhibit a range of ecologies and conditions that make them valuable indicators of environmental contamination. Knowledge of dietary habits, habitat preferences and movements of the species is important for understanding the variance in reported levels of important contaminant in the system. Preliminary analytical results suggest that species with prolonged associations with the swamp and/or those with diets or trophic positions that predispose them to exposure through the food chain, exhibit high levels of contaminants and higher incidence of disease and tissue abnormality.

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Tree Cores as Biomarkers of Pollution

Tree growth rings provide a permanent record of the effects of climate on growth and can indicate population perturbations in the environment including the effects of pollution (Devall, Grender and Koretz, 1991). Changes in ring width not only provide information on individual trees, but can be averaged (standardized) to form indices for a population (Fritts, 1976). Thus growth rings can provide environmental data over time even though a forest may not have been studied in the past (Van Deusen, 1987, 1989a; Cook, Johnson and Blasing, 1987). The common signal among sampled trees in a stand thus can provide information about the history of the site on a year to year basis (Cook, Johnson and Blasing, 1987).

Chronologies of yearly tree rings have been used to study various ecological problems including fires (Dieterich, 1980), floods (Sigafos, 1964), volcanic eruption (Yamaguchi, 1985), carbon dioxide levels (LaMarche, Graybill, Fritts and Rose, 1984), the effects of harmful insects (Swetman, Thompson, and Sutherland, 1985), etc. In addition, tree growth rings of some species have been used to provide a record of heavy metal uptake (Bondietti, et al., 1989; Baes, and Ragsdale, 1981; Cutter and Guyette, 1993; Baes and McLaughlin, 1986).

The change in environmental concentration of heavy metals actually occurs by means of translocation. Heavy metals can be found throughout the environment; lead, cadmium, and zinc have been measured in major vegetation types in leaf litter and soils around smelters (airborne; Hutton, 1984). Metal deposition rates have been shown to increase with height in woodland systems while leaf surface characteristics have a strong influence on metal retention (Little, 1974). Within vegetation lead for example tends to accumulate in bark, although wood contains less lead, total amounts in the ecosystem depend upon standing crop (Freedman, 1989; Friedland and Johnson, 1985). The elevated metal status of litter results partly from contamination of foliage by aerial deposition and partly from throughfall and washoff. Most lead, cadmium and zinc accumulate in the upper few centimeters of soil; the soil is a major long-term sink for metal inputs (Hutton, 1984). Root uptake by ground flora is a major factor in recycling heavy metals (Hutton, 1984).

This paper reports our progress in using tree cores of cypress (*Taxodium distichum*) as a biomarker of pollution in populations on the floodplain of the Mississippi River. Two polluted populations, Devil's Swamp and Bayou Trepagnier, were selected to determine the feasibility of utilizing cypress growth rings for cross dating to record past environmental history and to monitor heavy metal contamination. One additional site was selected as a control.

Cypress is a long-lived, wide-spread, deciduous gymnosperm capable of growing in water or dry ground. The species ranges along the Gulf Coast through Florida to New Jersey on the Atlantic coast; it occurs in the Mississippi River embayment to southern Illinois and forms numerous populations in the interior of the southeastern United States (Fernald, 1950). Individual trees can become quite large and are considered to be one of the largest trees in height and girth in the southeastern United States (Brown, 1965). The wood decays very slowly and it is also prized for its beauty and ease of processing.

Cypress is a suitable tree species to use for tree ring analysis and heavy metal uptake in that it is a common species, occurring in stands along the immediate floodplain and drainage of the Mississippi River. The United States Forest Service has previously cored trees of cypress and successfully established that crossdating is feasible for the species (M. Devall, 1991).

Sites

Devil's Swamp is located just northwest of Baton Rouge, Louisiana. The site is known to be contaminated by a wide array of chemicals, which would be expected to affect tree development

and growth. Pollution from a large landfill contaminates the swamp which empties into the Mississippi River. We sampled cypress trees along the bayou that empties into the Mississippi River. The swamp in this region is a cypress-tupelo dominated community with an understory that is usually flooded but containing large numbers of button-bush (*Cephalanthus occidentalis*). Many of the cypress trees are very large with diameters at DBH (diameter at breast height) exceeding 2 meters; however, the tops of most of these trees have been lost (hurricanes, etc.) and the trunks are hollow.

Bayou Trepagnier is located between the Mississippi River and Lake Pontchartrain (Fig. 1), just northwest of New Orleans, Louisiana. The river is approximately 2 meters higher than Lake Pontchartrain and water in the ecosystem drains into the lake. Several petroleum cracking plants are located at the head of the bayou and waste from the cracking and other operations was dumped or drained into the bayou for approximately 15-20 years until 1972 (enactment of the Clean Water Act). The plants now operate within the regulations of the act but continue to deposit wastes. Near the point source of pollution, lead in the soil exceeds 400 ppm and high levels of other heavy metals can be recorded.

The entire bayou contains a forest with many large cypress trees plus, maples, tupelo, oaks, etc. In periods of heavy rainfall the water in the stream (bayou) floods the forest. Eventually all the water in the winding bayou drains into Lake Pontchartrain (ca 5 miles from the source of pollution). The forest gives way to a brackish marsh near the lake. A gradient of high to low polluted soils should occur along the bayou.

A high levee abuts Bayou Trepagnier to the north. This levee (Fig. 1) forms one side of the Bonne Carre Spillway which runs from the Mississippi River to Lake Pontchartrain. Water from the Mississippi River can be diverted (flood gates on the river) through the spillway into Lake Pontchartrain and eventually into the Gulf of Mexico.

The control population, Sarah Bayou or a portion of Tunica Swamp, is located approximately 45 miles upstream of Devil's Swamp on the east bank of the Mississippi River. The swamp is not leveed and borders the Mississippi River.

Methods

The cypress trees were cored using a 5 mm in diameter tree core drill. Each tree was cored twice and the cores placed in plastic straws and stored and dried at the U. S. Forest Service, New Orleans, Louisiana. Eleven trees in Devil's Swamp and 20 trees in Bayou Trepagnier were cored (Fig. 1); 60 trees were cored in Sarah Bayou. Dried cores were mounted on strips of wood and then polished using three different grades of sandpaper. The widths of the cores were measured by projecting the rings onto a TV screen and then measured via computer.

The components that comprise ring widths of a single tree at time t are as follows (Graybill, 1982): $R(t) = C_t + B_t + D1_t + D2_t + e_t$, etc. C_t is the climate component at time t , common to all trees; B_t is the biological growth trend; a disturbance signal unique to the individual tree; $D2_t$ is a disturbance signal that could be caused by pollution, etc. which is common to most all individuals and e_t is random disturbance. It is necessary to remove as many as possible of the signals of B and $D1$, in order to maximize the C and $D2$ (Devall, Grender and Koretz, 1991; Van Deusen, 1988).

The biological growth trend is the slowly changing capacity to respond to climate changes unique to each tree (Fritts and Swetman, 1986). To remove this trend, cores are standardized via inverse hypersine transformation and then taking the first difference of the ring widths; $D1$ and e are treated as noise (Van Deusen, 1987). Thus the standardized ring widths are expressed as a linear function of climate variables, a common disturbance variable and a random component (Devall, Grender and Koretz, 1991).

Fig. 4. Average Concentrations of Copper in Cypress Tree-Cores Over Time

Fig. 5. Average Concentrations of Iron in Cypress Tree-Cores Over Time.

Fig 6. Average Concentrations of Manganese in Cypress Tree-Cores Over Time.

Fig 7. Average Concentrations of Zinc in Cypress Tree-Cores Over Time.

Fig. 8. Bayou Tresegnier, Louisiana.

After standardization, the Kalman filter is used to rid the data set of "noisy data" and to reduce the ring widths to a single chronology (Devall, Grender and Koretz, 1991). Varying parameters are used to determine if the conditions of the past are the same as the present. The Kalman filter provides a system for predicting, updating and smoothing (Van Deusen, 1987; Anderson and Moore, 1979).

The equations needed to implement the Kalman filter are: $Y_t = F_t x_t + v_t$, where Y_t is a vector of transformed ring widths at time t , F_t is a fixed matrix of order $n_t \times p$, n_t is the sample size at time t , x_t is a $p \times 1$ vector of state parameters, and v_t is an $n_t \times 1$ vector of residuals with zero expectation and variance matrix V_t (Van Deusen, 1987, 1988). The state parameters are variables that evolve over time according to a first order Markov process defined by the system equation, where x_{t-1} represents parameters like the common chronology that vary over time, G is a fixed $p \times p$ matrix, and u_t is a $p \times 1$ vector of residuals with zero expectation and variance matrix W_t (Devall, Grender and Koretz, 1991). The Kalman filter provides a recursive procedure for inference about the state vector, x_t . See Devall, Grender and Koretz, (1991) for an application of the Kalman filter.

Tree cores were cut to prescribed year increments dependent upon a minimum weight of wood set at 0.100 grams. Thus 0.100 grams equaled approximately 7-10 years of growth. A Wiley mill was used to grind these samples which were then pressed into wafer form with a 10 mm die normally used for infrared spectrometry. A Siemens SRS200 wavelength dispersive X-ray fluorescent spectrometry was used to analyze for heavy metals (Central Instrument Facility, Tulane University). The tree cores of cypress were analyzed for the following elements: Mn, Ni, Cu, Zn, As, Sr, and Pb.

Results

The present investigation was initiated in September, 1993 and the results described are preliminary, but cautious conclusions can nevertheless be offered.

To date, the cores from all three sites (total, 92 cores) have been sanded and the widths of the rings measured via computer. Trees in the three populations have not been cross-dated. Only tree cores from Bayou Trepagnier have been analyzed for the heavy metals lead, zinc, arsenic, copper, chromium, iron, arsenic, and manganese. In Bayou Tregagnier tree cores of 7 of the 21 trees cored have been analyzed: numbers 6, 8, 10, 11, 15, and 16 (Fig. 8). The tree core data for heavy metals were arranged in three groups: group 1-numbers 16, 17, 18, 19, and 21; group 2- numbers 1-10, 20; group 3-numbers, 11-15 (see Fig. 8). Heavy metal concentrations are presented in Figs. 1 to 7; only one tree from group 1 has been analyzed.

The concentration of heavy metals in tree rings varies depending upon the elements, location of the trees, and distribution of elements in the growth rings.

Copper (Fig. 4): For 70 years copper varied between 2-4 ppm. Then in 1920-1925 the levels began to rise reaching 28 ppm in trees group-2. Trees in group 3 (farthest from pollution source) showed the least variation and lowest levels of copper in the wood. However, the overall trend has been toward increasing levels.

Manganese (Fig. 6): This element shows only a slight increase since 1895, although groups 2 and 3 show spikes in some years. Interestingly, group 1 shows distinctly low level of manganese.

Zinc (Fig. 7): No trend can be detected in this element except for a high spike from 1965 to 1985 in group 3.

Arsenic (Fig. 2): High levels of arsenic are found in the single tree in group 1.

Chromium (Fig. 3): Varying high levels beginning in 1915 with the highest peak in 1985 to 1988.

Iron (Fig. 5): Similar to copper in showing an upward trend (increase) in concentration from 1915.

Lead (Fig. 1): In the period 1915 to 1930 a large peak of lead appears in group 3. Group 1 shows very high lead levels.

Discussion

Cores from cypress trees growing along Bayou Trepagnier were collected for cross-dating and heavy metal analysis. The soils bordering this bayou are designated as being contaminated by the Department of Environmental Quality, State of Louisiana and the bayou runs from a known pollution point source, a petrochemical cracking plant to Lake Pontchartrain.

Analysis of the distribution of heavy metals within the tree cores presents a historical account of the year by year accumulation of the heavy metals and thus would appear to monitor the species of heavy metal contamination and its chronology. Assuming that this is the case, our preliminary results indicate that the major contaminants in the area since the beginning of the 20th century are copper, iron, and possibly lead and chromium. Both copper and iron increase in the growth rings beginning in the period from 1920 through 1935. The overall average concentration of iron for all trees increases from about 400 ppm (for 5 years growth rings) during the period 1895 to 1920 to levels between 700 and 1200 in the 1980's and 1990's. During this same period copper increases from about 3 ppm to levels between 7 and 17 ppm.

In contrast to copper and iron, the year by year accumulations of manganese, arsenic, and zinc are relatively constant throughout the period from 1895 to 1993.

Of the two remaining heavy metals, lead is distributed in the rings in a pattern similar to that of manganese, arsenic, and zinc, i. e., constant throughout, with one major exception. Notably during the period from 1910 to 1929 there is a significant increase in the accumulation of lead. Following the increase in 1910 through 1929 the levels of lead decrease again to finally remain fairly constant through 1993. Chromium also shows an increase during the period from 1910 to 1929, going from 2 to 8 ppm, and its concentration during the years from 1930 to 1993 fluctuate dramatically reaching levels as high as 13 ppm in 1985.

As discussed by Cutter and Guyette (1993) the critical problem with tree rings as environmental monitors is the mobility of elements across ring boundaries. Mobility in xylem is based on ion solubility, sapwood-heart-wood concentrations, charge/ionic radius ratio, essential nature, sap pH, and bonding in the xylem matrix. Cutter and Guyette (1993) assign a value of NR to cypress, which means recommended with limitations. However, the species may be useful for relatively immobile elements. For example, preliminary data indicates cypress may be useful to record levels of copper, iron, chromium and lead in environments. Obviously further analysis is needed particularly of very old trees from contaminated as well as control sites.

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Effects of Environmental Contaminants on the Top Consumers in Aquatic Ecosystems

Evaluating the effects of persistent environmental contaminants on the higher level consumers of an aquatic ecosystem, namely the wading birds, involves assessing the contaminant concentrations in both the consumer and the available prey. Fish-eating birds are vulnerable to contaminant effects due to the probability of biomagnification in the aquatic food chain (Heinz, et al, 1985). Fish readily take up metals suspended in the water column (Atchison, et al, 1977) and persistent, lipid-soluble, organohalogen compounds are absorbed by all species, slowly metabolized, rapidly accumulated (Pentreath and Windom, 1974), and stored in fatty tissues and eggs (Magee, 1974). Elevated levels of trace metals in the environment have led to high concentrations in feathers (Hoffman and Curnow, 1979) and hexachlorobenzene (HCB) has been detected in eggs of Little Blue Herons (*Florida caerulea*), Great Egrets (*Casmerodius albus*), Cattle Egrets (*Bubulcus ibis*), and Glossy Ibis (*Plegadis falcinellus*) in Louisiana (Ohlendorf, et al, 1979).

The edible portions of fish taken from Devil's Swamp in 1986 and 1987 by the Louisiana Department of Environmental Quality (LDEQ) contained 0 - 0.122 ppm HCB and 0 - 0.27 ppm hexachlorobutadiene (HCBd). LDEQ guidelines for both of these organohalogens is 0.06 ppm. NPC Services conducted an ecorisk assessment in 1992-1993 which found elevated levels of heavy metals in Devil's Swamp (Table 1).

There is a breeding colony of 3000 wading birds less than eight (8) miles northwest of Devil's Swamp in Bueche, La. The colony, censused by Martin and Lester in 1990, contains 600 Little Blue Herons, 450 Cattle Egrets, 750 Snowy Egrets (*Egretta thula*), 300 Great Egrets, and 900 White Ibis (*Eudocimus albus*). Observations made this summer by Spahn show that Tricolor (Louisiana) Herons (*Egretta tricolor*), Great Blue Herons (*Ardea herodias*), and Anhingas (*Anhinga anhinga*) are also present. Data collected on flight directions taken to and from the colony indicate that almost 19% of the birds observed are probably foraging in Devil's Swamp (Table 2).

Samples of prey items were collected from Grand Bay, Chenal Bayou, and Devil's Swamp during the summer and are currently undergoing analyses for heavy metal and organohalogen contamination. The spring breeding season will allow the collection of eggs, feathers and blood from chicks, and prey brought to nestlings. These data will assess food chain contamination as

Table 1: Levels of Heavy Metals in Soils of Devil's Swamp

(NPC Services Ecorisk Assessment 1992-3)

	Arsenic	Cadmium	Chromium	Lead	Zinc
Background (mg/kg)	0.7	3.0	5.0	30.0	1.0
Devil's Swamp (mg/kg)	1.3-5.6	0.7-8.9	3.4-13.7	28.8-73.9	27.2-43.8

Table 2: Flight directions taken by the two most commonly observed wading birds in the Bueche, La. colony.

Dir.*	Great Egret number observed (%)		Little Blue Heron number observed (%)		Total (%)
NE	62	(36.9)	68	(50.0)	130 (42.8)
NW	27	(16.1)	15	(11.0)	42 (13.8)
SW	47	(28.0)	28	(20.6)	75 (24.7)
SE	32	(19.0)	25	(18.4)	57 (18.8)
TOTALS	168		136		304

* The most probable foraging location associated with the direction taken is: NE--Profit Island, NW--Grand Bay/Chenal Bayou, SW--farmlands/ditches, SE--Devil's Swamp.

wading birds feed their young the same size and composition of prey items as they themselves consume (Kushlan, 1978).

Comparison of these data with similar data collected from non-contaminated colonies will allow the evaluation of the effects of environmental contaminants on these top consumers of aquatic ecosystems.

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Dietary Analysis Of The Fish Of Devil's Swamp And Devil's Swamp Lake

Abstract

As a first step in a three-year DOE project, we analyzed the contents of gastrointestinal tracts of fish collected in Devil's Swamp Lake, near Baton Rouge, LA. We wished to determine dietary preferences of fish in order to design an aquatic macroinvertebrate sampling plan. In the next step of this project, the macroinvertebrates will be sampled directly, and analyzed for heavy metal and organic pollutants. To date, the GI tracts of 18 catfish, 2 gar, and 17 black crappie have been examined. The most abundant aquatic macroinvertebrates were immature Diptera: Chironomidae, Chaoboridae, Culicidae, and Ceratopogonidae.

Introduction

An integral part of any environmental toxicology study is to determine the extent to which toxic substances or pollutants enter the food chain of the organisms in the study site. To this end, we

planned to sample aquatic macroinvertebrates in Devil's Swamp, Baton Rouge, LA. However, it is difficult to develop an unbiased sample of all macroinvertebrates present in a habitat. Therefore, we decided to sample only those aquatic macroinvertebrates that actually enter the food chain of the most abundant fish present at the site. As the first step in a three-year project, we characterized the stomach contents of fish from Devil's Swamp in terms of macroinvertebrate taxonomic diversity. The next step of the project will be to sample directly the taxonomic groups found in the gastrointestinal (GI) tracts of the fish. We will identify the invertebrates in these samples, then subject them to analysis to determine their uptake of heavy metals or other hazardous materials.

Methods and Materials

Fish were collected by Dr. Hank Bart of the Tulane University Museum of Natural History. Three collecting trips have been conducted so far; the third was the most successful (Table 1). Immediately upon capture, fish were placed on ice in coolers and brought back to the laboratory for preliminary dissection. GI tracts from the first collection were removed and frozen with no fixation. GI tracts from the second and third collections were fixed in 10% neutral buffered formalin. Xavier University research students funded by the NIH (Minority Access to Research Careers) dissected the GI tracts, made notes and sketches, and placed all invertebrates in 70% ethanol for later identification. We conducted preliminary identifications to family (Bryce and Hobart, 1972; Merritt and Cummins, 1984; Pennak, 1969; Peterson, 1962; Stehr, 1987, 1991; Thorp and Covich, 1991). All specimens were saved for later species identification.

Results and Discussion

Most GI tracts of fish from the first two collections and black crappie from the third collection have been examined (Table 1). No identifiable contents were found from the catfish from the first collection. This was possibly due to the fact that the GI tracts were kept frozen unfixed until dissection. Stomach contents may have been digested beyond recognition. A few GI tracts from the second collection revealed identifiable contents. A complete crawfish was found in the stomach of one bullhead, crawfish pereopods were found in the stomach of one of the gars, and a few chironomid larvae found in the stomachs of the other bullheads.

A great abundance of aquatic organisms was found in the stomachs of the black crappies from the third collection (Table 2). Most organisms were entirely intact, allowing preliminary identification to family. Identification to genus or even species should be possible with many specimens. The most abundant were larvae and pupae of Chironomidae and Chaoboridae, all 5 mm or less, in size. If an organism numbered greater than 50, we did not obtain an absolute count. Almost no identifiable material was recovered from the intestines.

Most chironomid larvae are benthic and microphagous, feeding on finely divided detritus, algal cells, and other microorganisms. The Chironomidae are a large and diverse group. Many genera exhibit a large degree of aquatic habitat specialization. Larvae of Chaoboridae are predaceous, feeding unselectively on a diversity of aquatic organisms.

We will continue analyzing the diets of collected fish of all species, in order to obtain between-species and seasonal estimates of dietary preferences. We will also attempt to further identify specimens to genus and species, contacting specialists if necessary. Species identification of the Chironomidae may allow us to determine to some extent the movement of the fish.

The immature aquatic Diptera guild is easily sampled with conventional aquatic sampling equipment, such as an Ekman bottom grab sampler (Merritt and Cummins, 1984). As originally proposed, in the second year of the project, We will accompany the collecting trips and sample this guild directly with the grab sampler. These samples will then be analyzed for heavy metal and organic pollutants.

Table 1. Status of project as of 14 December 1993: fish species to date whose gastrointestinal tracts have been examined for determination of dietary preference.

COLLECTION DATE	LOCATION	SPECIES	GI TRACT EXAMINATION COMPLETED AS OF 14 DEC 1993
5 Aug.	Devil's Swamp	14 catfish	yes
9 Sept.	Devil's Swamp	4 yellow bullhead, <i>Ictalurus natalis</i>	yes
		2 gar, <i>Leiostomus sp.</i>	yes
	Lake	1 carp, <i>Carpiodes sp.</i>	no
13 Oct.	Devil's Swamp	17 black crappie, <i>Pomoxis nigromaculatus</i>	yes
		12 smallmouth buffalofish, <i>ictiobus bubalus</i>	no
	Lake	3 bigmouth buffalofish, <i>I. cyprinellus</i>	no
		2 highfin carpeucker, <i>Carpiodes velifer</i>	no

Table 2. Identification of gastrointestinal tract contents of 17 black crappie collected from Devil's Swamp Lake, 13 October 1993.

CRAPPIE NUMBER	DEPT PUPA a	CHIRO PUPA b	CHIRO LARVA c	CHAO LARVA d	CULIC PUPA e	CERAT LARVA f	CORIX g	OTHERS
1	21		30	20				
2	>50		45					
3	29				6		3	1 Carabidae larva 1 Formicidae
4						2	7	
5	>50	>50	>50					
6	3	>50		14				2 Amphipoda, 7 unknown Dipera
7		17	18					1 Amphipoda
8	43		>50	>50		1		1 Serranomyiidae larva
9								
10	20		20		9			
11		>50	>50					
12	>50	10		>50			1	
13	18		2					
14	4		4					
15								
16	>50		8					
17	35		20	>50				

a. Unknown Diptera pupae, probably Chironomidae

b, c. Diptera: Chironomidae

d. Diptera: Chaoboridae

e. Diptera: Culicidae

f. Diptera: Ceratopogonidae

g. Hemiptera: Corixidae

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Biomarkers Subcluster

Histopathology Of Fishes From Devil's Swamp

Introduction

Naturally occurring tumors of aquatic animals have served as useful model systems for toxicity (Mizell, 1985). Harshbarger and Clark (1990) have extensively reviewed the epizootiology of tumors in North American fishes. They report that epizootics fall into two broad categories. The first category, hemic, neural, pigment cell, connective tissue, and gonial neoplasms are probably not related to environmental pollution. The second category includes epithelial neoplasms, including the liver, pancreas, gastrointestinal and epidermis, in which tumor incidence is strongly related to environmental pollution. The freshwater and estuarine fish species at Devil's Swamp, including many large species-specific populations, represent an opportunity to evaluate background tumor incidences particularly in bottom feeding fish which are believed to be at risk for epizootic liver tumors from polluted waters.

The specific three year study aims were as follows: to identify lesions and diseases in fish as biomarkers of exposure to environmental contaminants; to characterize the progression and prevalence of lesions in feral fish; to identify sensitive fish species for use as sentinels of ecotoxicity in wetland environments; and to verify field observations in a laboratory animal model, the Japanese medaka (*Oryzias latipes*). Approximately one hundred fish representing twenty different species are under histopathologic evaluation. Initial developmental studies with the Japanese medaka fish (*Oryzias latipes*) exposed to Devil's Swamp water are in progress.

The slides/tissues with lesions will be submitted for peer review/outside confirmation. A sample of suspect tumors will be sent for confirmation to the Registry of Tumors in Lower Animals, National Museum of Natural History, The Smithsonian Institution, Washington, D.C.. Laboratory studies for developmental and cancer effects will be conducted using the existing Tulane University medaka (*Oryzias latipes*) fish culture. In the future, microinjection of Devil's Swamp contaminants will be used for developmental and cancer studies.

Materials and Methods

Fishes were caught, from 2 sites, Devil's Swamp Lake, and Bayou Baton Rouge, in the Devil's Swamp Basin, using electroshocking (boat mounted apparatus or backpack shocker), crab trap or rod and line on September 9, October 11-12, and December 16, 1993. Smaller fish species were placed in 10% neutral buffered formalin on site for fixation. Fishes were placed on ice and transported to the laboratory on the same day. Fishes were necropsied either on the same day or on the following day. The time between the fish catch and the necropsy varied from 4 hours to 14 hours. No post-mortum changes were noted based in examination of the integrity of nucleated red blood cells. A complete necropsy was performed on all fishes. Various organs such as stomach, gonads, gills, skin, spleen, and kidney were removed and fixed in 10% neutral buffered formalin for histopathological analyses. Multiple pieces (2-3 mm thick) of liver were taken and fixed in 10% neutral buffered formalin. The remaining livers and brain were frozen separately in ziplock plastic bags. The carcasses were frozen separately for the chemical analyses. Formalin-fixed tissues were processed for histopathological analyses. Tissues were dehydrated in an automatic tissue processor and embedded in paraffin. Paraffin blocks were cut at 6 μ m thick, and sections were stained with hematoxylin and eosin, and evaluated from morphological changes using a bright field light microscope. Selected blocks were recut and stained with Periodic Schiff's stain or Congo Red stain. Slides were coded and evaluated.

Results

Fish species collected from Devil's Swamp, currently under histopathological examination, are as follows: Black Crappie (Pomoxis nigromaculatus); Small Mouth Buffalo (Ictiobus bubalus); Big Mouth Buffalo (Ictiobus cyprinellus); River Carpsucker (Carpionodes carpio); Gizzard Shad (Dorosoma cepedianum); Striped Bass (Morone saxatilis); Striped Mullet (Mugil cephalus); Yellow Bullhead Catfish (Ameiurus natalis); Channel Catfish (Ictalurus punctatus); Golden Shiner (Notemigonus crysoleucas); Tidewater Silverside (Menidia beryllina); Black Buffalo (Ictiobus niger); Yellow Bass (Morone mississippiensis); Gizzard Shad (Dorosoma cepedianum); Redfin Shad (Dorosoma petense); Green Sunfish (Lepomis cyanellus); Dollar Sunfish (Lepomis marginatus); Mosquito Fish (Gambusia affinis); Common Carp (Cyprinus carpio); Black Crappie (Pomoxis nigromaculatus); and Spotted Gar (Lepisosteus oculatus).

The total lengths of Channel Catfish (Ictalurus punctatus) ranged from 19.5-28.5 cm, and the Yellow Bullheads (Ictalurus natalis) ranged from 27-39 cm. Histological preparations of various tissues from 10 Channel Catfish and 4 Yellow Bullheads were examined. The prevalence of lesions in Channel Catfish (Table 1) and Yellow Bullheads (Table 2) are tabulated below. The most striking lesions were found in Yellow Bullheads. All the lesions are classified into the following categories: infectious conditions (parasitic, bacterial) and non-infectious conditions (cytotoxic, proliferative nonneoplastic, proliferative neoplastic neoplasm).

Table 1
The Prevalence of Lesions in Channel Catfish from Devil Swamp

Lesions	Number affected
Frayed fins	8/10
Eroded upper lips	2/10
Parasitic infections:	
Gills	7/10
Viscera	1/10
Bacterial thrombi in kidney	1/10
Telangiectasis:	
Gills	1/10
Liver	0/10
Gastritis	1/10
Enteritis	1/10
Inflammation	3/10
Neoplasms	0/10
Proliferative lesions:	
alarm substance cell	1/10

Table 2
The Prevalence of Lesions in Yellow Bullheads from Devil Swamp

Lesions	Number affected
Frayed fins	0/4
Eroded upper lips	0/4
Parasitic infections:	
Gills	0/4
Viscera	1/4
Bacterial thrombi in kidney	0/4
Telangiectasis:	
Gills	0/4
Liver	2/4
Gastritis	0/4
Enteritis	0/4
Inflammation	0/4
Neoplasms (suspected)	2/4
Proliferative lesions:	
alarm substance cell	1/4
gill epithelium	2/4
Amyloidosis	3/4
Glomerulo nephrosis	1/4

The protozoan parasites (epitheliocystis, trichodina, ambiphrya, henneguya, myxosporidians) were frequently found in gills. Monogenean flukes were also seen in the gills of Channel Catfish.

In Channel Catfish, proliferation of alarm cells (fright-substance cells or shreckstoffzellen) in the skin was noted (Figure 1). Normal Catfish skin has only several layers of alarm cells. This may be viewed as a local immune response where cutaneous antibody levels can rise independent of systemic humoral responses. In the liver of Yellow Bullheads, spongiosis hepatitis was noted (Figure 2). This lesion consists of cystic structures around hepatic arteries. There are also dilated sinusoids (endothelial lining) with flocculent material in the perisinusoid space. The lesion is considered to be precancerous. In the liver of the Yellow Bullhead, telangiectasis of the liver (abnormal dilation of the sinusoids with pooling of blood in the spaces) was observed (Figure 3). This lesion is a possible hemangioma (tumor). In the Yellow Bullhead, white pulp lesions in the spleen were noted. The lesions consists of deposits of pale eosinophilic material in the follicle arteries.

The kidneys of Yellow Bullheads and Channel Catfish had lesions. The Yellow Bullheads had dilated Bowman's space with eosinophilic material and inflammatory cells accumulating in the Bowman's space. (Figure 4). The Channel Catfish had mesangiosclerosis of the kidney demonstrated by pinkish material in the central portion of the glomerular capsule. Hyperplastic thyroid tissue was also present in the Yellow Bullheads.

In Garfish, inflammation of muscle tissue was noted in several fish (Figure 5) and there were high density melanin macrophage centers in the liver (Figure 6). The environmental significance of the high density melanin macrophage centers is unknown. However, the environmental variability of this lesion will be further studied.

In Carp, ectopic thyroid tissue was noted in the kidney (Figure 7). Although this was noted in a single fish, the unusual pathology will be studied further. Other lesions observed in Carp include teleangiectasis of the gill possibly related to environmental conditions (Figure 8) and periductal inflammation of the liver and vascular elements.

Discussion and Conclusions

Two of the 4 Yellow Bullheads had liver telangiectasis where the abnormally dilated sinusoids contained large amounts of blood. The same Bullheads also had a suspected benign neoplasm "Cavernous hemangioma." We will send these slides to Registry of Lower Animals at Smithsonian Institution for further diagnosis and archiving. In addition, all 4 Yellow Bullheads had a lesion which could be an early lesion of spongiosis hepatitis. These lesions were characterized by dilated sinusoids devoid of blood and a dilated perisinusoidal space containing pale eosinophilic flocculent material. There was no damage to the endothelial lining. The multilocular cystic structures were seen around the larger blood vessels and also along the sinusoids. These lesions were somewhat similar to those described in rats (Bannach et al. 1981) and in other fish species (Couch and Courtney, 1987; Grizzle and Thiagarajah, 1988; Wester and Canton, 1986) after exposing to chemical carcinogens, and also in fish collected from polluted rivers (Bruno and Ellis, 1986; Myers et al. 1987). An extensive study conducted on Bullheads collected from Silver Spring, New York failed to demonstrate any lesions in Yellow Bullheads (Bowser et al. 1991).

An analysis of initial results indicate we have identified several possible biomarkers for disease and chemical exposure from Devil's Swamp. In the future, pathology biomarkers studies will focus on liver, spleen, thyroid, pancreas, and gill.

Figure 1: Proliferation of alarm substance cells in channel catfish skin collected from Bayou Baton Rouge. Alarm substance cells (A), and dermis (D). H&E x 1900.

Figure 2: Spongiosis lesion in the liver of a yellow bullhead collected from Devil's Swamp Lake. Hepatic artery (HA), spongiotic lesion and accumulation of flocculent material in the perisinusoidal space (arrows), hepatocytes (H), and dilated sinusoids (S). H&E x 2600.

Figure 3: Probable cavernous hemangioma of the liver in a yellow bullhead collected from Devil's Swamp Lake. Cavernous space (C), hepatocytes (H), and endothelial cells (arrows). H&E x 3300.

Figure 4: Dilated Bowman's space in the kidney of a yellow bullhead collected from Devil's Swamp Lake. Bowman's space (B), Bowman's capsule (arrows), and inflammatory cells (arrowheads). H&E x 8000.

Figure 5: Inflammation of muscle in a gar fish collected from Devil's Swamp Lake. Muscle bundles (MB), inflammatory cells (arrows), and hemorrhage (H). H&E x 3300.

Figure 6: Melanomacrophage centers in the liver of a gar fish collected from Devil's Swamp Lake. Melanomacrophage centers (M). H&E x 3300.

Figure 7: Ectopic thyroid tissue in the kidney of a carp collected from Devil's Swamp lake. Thyroid follicles (T), and kidney tissue (K). H&E x 1900.

Figure 8: Telangiectasis of the gill of a carp collected from Devil's Swamp Lake. Telangiectasia of gill lamella (T). H&E x 1300.

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Developmental, Immunological, and Neurological Biomarkers of Exposure in Frogs: Ongoing Studies and Progress to Date

Immunotoxicology and Neurotoxicology of Frogs from Devil's Swamp

Wild caught *Rana catesbiana* (N=5) from the banks of Devil's Swamp Lake had normal immune function as measured by PHA and CON A mitogen stimulation of T-cells. The animals were of robust size and appeared healthy. Immunocytochemistry for neural IL-1 β and the glial fibrillary acidic protein (GFAP) appeared normal in the brain.. Both of these proteins have been shown to be upregulated during exposure to heavy metals such as lead (O'Callaghan and Miller, 1988; Dinarello, 1991).

Developmental Laboratory Studies with Water and Sediments from Devil's Swamp

In studies related to field studies, spawnings from the laboratory frog, *Xenopus laevis*, were obtained from mating pairs stimulated with hormone. Early cleavage stage embryos were placed in 6 bowls (50 embryos/bowl). Two bowls contained water from Devil's Swamp Lake, 2 contained water plus sediments from the lake, and 2 bowls contained laboratory water. All groups showed normal development and good swimming behavior through feeding tadpole stages when the experiment was terminated.

Methyl Mercury Studies

Since mercury is one of the major contaminants of the Clinch River system at Oak Ridge, and since mercury is also a contaminant found in Devil's Swamp, we chose to focus on how low levels of mercury equivalent to those found in areas of Devil's Swamp might influence frog physiology and development. Expected endpoints of these studies are to isolate specific biomarkers of exposure to levels of mercury found in the environment.

Adult Frogs: Immunotoxicity Studies

Cultured splenocytes from adult *Xenopus laevis* laboratory frogs were treated with methyl mercury. Mitogen assays were inhibited by concentrations of methyl mercury from 0.25 to 50 ppb. Concentrations above 50 ppb were lethal to the cultured cells.

Developmental Studies:

Mortality Studies

Pilot studies using approximately 250 embryos showed that methyl mercury in low concentrations caused mortality and severe developmental defects in *Xenopus laevis* embryos. To provide quantitative data, additional embryos were placed 25 to a bowl at morula stage. Bowls contained 0 µg/L, 1 µg/L, 5 µg/L, 50 µg/L, or 500 µg/L methyl mercury respectively. As shown in figure 1, mortality over the first 9 days of development, well into tadpole feeding stages was normal for 1 µg/L (1 ppb) compared to controls. Major changes in mortality occurred at 50 and 500 ppb, with most animals dying by day 5 which represents the end of embryonic development. Animals treated with 5 ppb showed some changes in mortality after day 5.

Gross Morphology as a Biomarker of Exposure to Methyl Mercury

Some survivors at 50 to 500 ppb showed morphological changes such as bent anterior-posterior body axis, shorter tail, disorganized posterior somites, and in some extreme cases, proximal-distal duplication of the digestive tract, and ventralization of the eyes. In these animals, the eyes were found facing downward, latero-ventral in the head, in the normal gill position. The eyes are normally face laterally and are found dorsally in the head. Animals treated at 5 ppb showed no overt changes in morphology through the first 4 days of development. Some animals began to appear shorter by 5 days of treatment. We must point out that not all animals showed gross changes in morphology by day 7. Some animals appeared normal throughout early development, even at 50 ppb. However, animals generally showed changes in morphology a day before death.

Escape Behavior as a Biomarker of Exposure to Methyl Mercury

In another experiment, embryos were placed 25 to a bowl at morula stage. By day 3 of treatment, control animals were swimming vigorously in response to changes in ambient illumination (an escape response) or to tactile stimulation with a fine hair. Embryos treated at 25 ppb showed a sluggish response, even though no overt changes in morphology had yet occurred. Embryos treated at 50 ppb barely responded, showing an occasional tail flip after strong tactile stimulation. These animals showed no overt changes in morphology at this early day of treatment. They showed strong heartbeats and otherwise appeared healthy.

Neurophysiological Correlates to Altered Escape Behavior in Treated Embryos

To determine if lack of escape behavior is due to neurological or muscular deficiencies as a consequence of treatment with methyl mercury, a spawning of *Unresponsive* mutant *Xenopus* embryos was obtained and treated with either 0 µg/L, 1 µg/L, 5 µg/L, 25 µg/L, or 50 µg/L, methyl mercury respectively. *Unresponsive* is a recessive mutation which renders embryos incapable of movement up through the first 5 days of development (Reinschmidt and Tompkins, 1984; Dudek, Ide and Tompkins, 1987). Paralysis is due to lack of excitation:contraction coupling within the

muscle fiber. Hindbrain swimming pattern generator circuits as well as neuromuscular synapses function normally (Dudek, Ide and Tompkins, 198). Since the embryo is naturally paralyzed, placement of an intracellular electrode within a single muscle fiber can be accomplished with only cold anesthesia.

During a bout of escape swimming induced by changes in ambient illumination or by tactile stimulation, the electrode acts as a monitor of escape circuit function. First, the pattern of excitatory junctional potentials (ejp's) impinging on the muscle follows swimming commands generated by hindbrain pattern generator neurons. The pattern generator usually runs for a set period (from 5 to 45 seconds). The size and duration of the junctional potentials reveals the number and synchrony of motor neurons in the spinal cord that are following commands from the pattern generator cells. Finally, at peak junctional potential activity, the muscle fibers sometimes generate their own action potentials, although muscle fiber spikes are not required for fiber contraction. The fibers appear to contract with increasing depolarization brought about by excitatory junctional potentials. Thus, a single intracellular electrode can reveal the health of many of the elements of swimming circuitry.

Recordings done in a number of *ur/ur* control embryos followed previously published work as described above. Recordings done in embryos treated with 50 ppb methyl mercury showed healthy muscle cells as gauged by robust resting potentials of up to 90 mv negative. However, neither changes in ambient light nor direct tactile stimulation produced ejp's as in control embryos. In embryos treated with 25 ppb methyl mercury, some ejp's were evident, but were of smaller size and duration. The pattern was also of shorter duration. Thus, our initial hypothesis is that the primary focus of methyl mercury effects on escape circuitry appears to reside in the brain and spinal cord, and not in the muscle fiber itself.

Conclusions

1. To date, wild caught frogs from Devil's Swamp Lake appear to be in good health and show apparently normal neurological and immunological function. We are awaiting analyses of levels of metal and hydrocarbon contaminants from our collaborators. We predict that these frogs will show low levels of contamination. We also predict that frogs from known polluted areas of Bayou Trepanger will show adverse health effects compared to frogs from known unpolluted areas of Bayou Trepagnier.
2. To calibrate our controlled laboratory approach to problems experienced by frogs in the wild, we are treating both adult and developing laboratory frogs (*Xenopus laevis*) with water and sediments taken from both Devil's Swamp and Bayou Trepanger. We predict that results from these studies will mirror data obtained from wild caught frogs.
3. Our laboratory studies on the effects of methyl mercury on laboratory frogs show that extremely low concentrations alter gross morphological and behavioral development. Neural circuitry related to escape swimming appears to be altered in early swimming embryos. In adult animals, the mitotic capacity of immune system cells is highly sensitive to low concentrations of methyl mercury.
4. Laboratory studies indicate that gross morphology and behavior are dependable developmental biomarkers of exposure. Further studies will determine if neuroimmune system biochemicals such as glucocorticoids or IL-1 β will also serve as sensitive indicators of compromised immune system and nervous system function.

(Figure 1)
The Effects of Mercury Concentrations on Tadpole percent Mortality
during the first 9 days of development.

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Exposure Subcluster

A Multifaceted Study of Heavy Metal and Anthropogenic Organic Pollution Ecosystems.

1) Toxicity, uptake and accumulation of heavy metals by *Lemna gibba*

Considerable heavy metal pollution has resulted from the high concentration of energy-related industries in the industrial corridor between Baton Rouge and New Orleans. Much, if not all, of this pollution enters aquatic ecosystems. One plant (probably more so than any other) can be expected to be a part of those aquatic environments and therefore to be exposed to those toxicants. Duckweed, an ubiquitous aquatic macrophyte that can be found in almost any permanent surface fresh to brackish waters. As the name implies, duckweed forms a prominent part of the diet of ducks but also occupies a basal position in a great many other food chains; certain of these involve economically-important species and several culminate with man. It is important therefore, to understand these trophic interactions in order to evaluate the potential of heavy metal pollution for ecological as well as economic damage. This becomes even more critical in geographic locations such as S. Louisiana where so much of the area consists of wetlands and where the population depends heavily upon the productivity of those wetlands.

Plants in general are known to take up certain ions and will often accumulate them to greater than external concentrations. Duckweed is no exception and, in fact, this very property of duckweed has been commercialized and is marketed as "duckweed-based technology" for wastewater treatment by industry and even municipalities. The ability of duckweed to bioaccumulate inorganic ions has at least two important potential ramifications. First, there are the implications for biomagnification with regard to duckweed's position in so many food webs. Second, there is the possibility of utilizing duckweed as a bioharvester of heavy metal ions, present at relatively low sub-lethal but potentially dangerous concentrations and combining this with methods of chemical remediation. It was for this later reason that the actinides, thorium and uranium, were investigated.

These elements are characteristically found in the gypsum piles that are the by-products of phosphate fertilizer production. An inevitable consequence of these piles is the leaching from them of relatively small amounts of actinides which then end up in surface waters. The low levels of radioactivity that these actinides emit present little direct danger but the potential for biouptake and biomagnification makes them considerably more threatening. However, if duckweed is able to "scavenge" the actinides that are present in the water at low concentrations, then the possibility exists of subsequently harvesting the plants and disposing of the actinides in a more environmentally-acceptable manner.

There are, of course, many plants that share most of the features (e.g. widespread distribution, ecological value, ability to bioaccumulate, etc.) of duckweed that are described above, however, none are as experimentally-approachable as is duckweed. Because of its diminutive size and rapid rate of vegetative reproduction, duckweed can be treated almost as though it was a bacterium. It can be grown, in large numbers, in a relatively small space, in sterile culture, in defined media and under closely-controlled environmental conditions. Results obtained in the laboratory can be tested under field conditions and, where appropriate, extrapolated to other aquatic angiosperms if not angiosperms in general.

Lemna gibba, the largest member of the duckweed family (Lemnaceae), was routinely maintained, in sterile culture, in 125 ml Erlenmeyer flasks, in 50 ml of a minimal media (pH 4.6) with added sucrose and tryptone to detect contamination. The plants were grown under a 24 h photoperiod and a constant 82°F thermoperiod. Experimental cultures were started from stocks using six 3- to 4-frond colonies (i.e. approx. 20 fronds) and were grown for 7 days. The number of fronds at Day 0 were counted and then again at Day 7. Vegetative reproduction was calculated in terms of per cent frond number increase over the 7-day growth period. Toxicities of heavy metals were determined by adding, under sterile conditions, known concentrations of ions and calculating per cent frond increases. To date, the following heavy metals, added in the forms shown in parentheses and over the concentration ranges indicated, have been investigated: arsenic (as sodium arsenate; 0 - 2.5 mM), cadmium (as cadmium nitrate; 0 - 100 μ M), lead (as lead nitrate; 0 - 2.5 mM), thorium (as thorium nitrate; 0 - 1 mM) and uranium (as uranyl nitrate; 0 - 500 μ M). For each heavy metal, five different concentrations, with at least 10 replicates at each concentration, were employed. Growth curves depicting the effects of increasing concentrations of the different heavy metals upon the vegetative growth were constructed and are shown in Figure 1 a - e.

Quantitative studies of the uptake and accumulation of heavy metals by *L. gibba* have only recently been initiated so that the results that are currently available are still somewhat preliminary. Cadmium uptake has been studied indirectly by measuring the disappearance of cadmium from the growth medium of *L. gibba* using an ion selective cadmium electrode. Twenty fronds, grown for 7 days in 50 ml of medium plus 5.0×10^{-5} M cadmium, reduced the concentration of cadmium in the growth medium by half (see Table 1). Starting with larger plant inocula, cadmium in the growth medium was reduced to less than detectable amounts in the 7 day growth period (see Table 2). The most obvious interpretation of these results is that cadmium disappearance from the growth medium was due to plant uptake. Certainly, control flasks that contained medium with no added cadmium showed no similar depletion. However, the definitive evidence showing cadmium accumulation in the duckweed concomitant with its disappearance from the growth medium has not yet been sought.

Similar results have been obtained for uranium, supplied to the plants as uranyl nitrate and assayed by fluorescence spectroscopy. The growth medium of *L. gibba* containing 100 μ M UO^{+3} showed a strong emission peak at approx. 500 nm (see Fig. 2b); the emission spectrum of control media containing no uranium was without any discernible peaks (see Fig. 2a). After 2 days of growth (20 frond inoculum) the emission peak at 500 nm was dramatically reduced (see Fig 2c) and by 4 days had disappeared entirely to be replaced by a broad band of non-specific emission at the

shorter wavelengths (see Fig. 2d). The appearance of the non-specific emission presumably relates to the release and build up in the medium of organic metabolites by the plants. Again, the most obvious explanation of these results is that the disappearance of uranyl from the duckweed growth was due to plant uptake but the experiments to confirm this have not yet been performed.

The results cited above, though preliminary, provide strong evidence that *L. gibba* grown in sub-lethal concentrations of cadmium and uranium is able to take them up and concentrate them. We anticipate that Lemna will do the same for the other ions under investigation. This being the case, the possibility now exists of utilizing the duckweed to "scavenge" heavy metals, including the actinides, from aqueous environments and of linking the physiological abilities of the plant to methods of chemical remediation through the development of stable waste forms in which the harvested ions can be encapsulated and immobilized. These aspects are discussed in section 5, below.

Table 1. Uptake of cadmium from growth medium of *L. gibba*. 20 fronds of *L. gibba* were grown in 50 ml of medium + 5.0×10^{-5} M Cd^{+2} . Cadmium concentrations were determined by ion selective electrode.

Days of growth	Cd^{+2} concentration
0	4.9×10^{-5} M
2	3.8×10^{-5} M
4	3.2×10^{-5} M
7	2.7×10^{-5} M

Table 2. Uptake of cadmium from growth medium of *L. gibba*. Differing numbers of fronds were grown in 50 ml of medium containing 5×10^{-5} M Cd^{+2} . Cadmium concentrations were determined after 7 days of growth by ion selective electrode

Number of fronds	Cd^{+2} conc. at Day 7
10	1.8×10^{-5} M
25	8.8×10^{-6} M
50	1.5×10^{-6} M
100	N D

Figure 1. Effect of heavy metals on the vegetative growth of *L. gibba*. a) thorium; b) uranium; c) arsenic; d) cadmium and e) lead.

Figure 2. Determination, by fluorescence spectroscopy, of uranium in growth medium of *L. gibba*. The various emission spectra, obtained by excitation at 320 nm, are, a) control medium containing no uranium; b) medium containing 100 μM UO^{+3} , supplied as uranyl nitrate at Day 0; c) medium, originally containing 100 μM UO^{+3} , in which plants had been growing for 2 days; d) medium, originally containing 100 μM UO^{+3} , in which plants had been growing for 4 days.

2). Toxicity, uptake and metabolism of organics by *Lemna gibba*.

The study of plant metabolism of organics is a study of the methodology that nature uses to cleanse itself. Unlike the heavy metals discussed in the section immediately above, plants have the capability to metabolize organic pollutants which enter their aquatic habitat. This capability can be either advantageous or disadvantageous. One important aspect to remember is that the plant metabolites can be benign products or products that are just as toxic as the parent compound as far as other members of the duckweed's ecosystem are concerned.

We have previously studied the toxicity and metabolism of 2,4-dichlorophenol (2,4-DCP) by *Lemna* (Ensley et al, 1994). It was demonstrated that duckweed can metabolize the 2,4-DCP to the corresponding 2,4-dichlorophenoxy-B-glucoside which was then released back into the plant's growth medium. The metabolite is even more water soluble than the parent phenol from which it was derived and should be equally toxic to mammalian species since the glucoside would hydrolyze to glucose and 2,4-DCP on exposure to the low pH of gastric secretions. In this case, the metabolism by duckweed masks the presence of 2,4-DCP contamination to standard analytical techniques thus exacerbating the detection of organic contamination.

The toxicities of aniline, n-butanol (BuOH) and tetrachloroethylene (TCE) toward *L. gibba* have been studied by the incubation of various concentrations of each chemical with axenic cultures of the duckweed (see section 1, above, for culture conditions). Aniline, BuOH and TCE are all relatively toxic with EC50's of 12, 12 and 8 μ M, respectively (see Fig. 3).

The fate of the organic contaminants has been studied by incubation of *Lemna* in sub-lethal concentrations (2 μ M) of either aniline, BuOH or TCE, spiked with the radiolabelled substrate under study (under the incubation conditions described earlier). The media were sampled at Day 0 and every 48h thereafter and analyzed by reverse phase HPLC (conditions - Waters uBondapak C18 column, 3.9 x 300 mm, linear gradient from H₂O to acetonitrile - 40 minutes) with an IN/US radionuclide detector in the eluate line. The radiochromatograms so obtained depicted the removal of the labeled compound from the growth medium and the concurrent appearance of metabolite(s), if any. When the labeled substrates had disappeared from the incubation media, the plants were harvested, extracted with ethanol and the ethanol extracts were analyzed by reverse phase HPLC to show the production, if any, of metabolite(s) that were retained within the plants. Representative reverse phase HPLC radiochromatograms for BuOH and aniline are shown in Figures 4 and 5, respectively. In the case of BuOH it can be seen that the labeled compound disappeared from the growth medium over a 10-day period and was not replaced, in the plant's growth medium, by a metabolite (Fig. 4). Essentially all of the label was recovered in the plant extract as a distinct metabolite which was slightly less polar than the BuOH. This data indicates that *Lemna* converts BuOH to a metabolite which is stored within the plant and is not returned to the aqueous medium, an ideal form of natural remediation with potential for exploitation.

The HPLC data for aniline (Fig. 5) shows a drastic reduction in labeled substrate over a 14-day incubation period and provides evidence for the formation of two metabolites, both of which were more polar than aniline. The minor, most polar, metabolite was found predominantly in the growth medium but was also present in plant extracts. The major metabolite was primarily retained in the plants.

The metabolism data for TCE is not complete at this time but preliminary results indicate the formation of at least one very polar metabolite which is produced by *L. gibba* and the returned to the medium. The isolation and identification of this, and the other organic metabolites, is currently under investigation.

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3) X-ray Fluorescence Spectroscopy as a Method for Constructing Dendrochemical Histories for Tree Species from the Southeastern United States and Abroad

The idea that trees could be used as environmental records date to as early as 1737 (Amato, 1988). Because trees partition wood in rings seasonally over long periods of time, tree rings have long been a source of data pertaining to growth trends and past environmental events such as hurricanes or droughts (Fritts, 1976). *Dendrochronology* is the scientific discipline developed to apply this idea.

Dendrochemistry, however, is a relatively new field in which workers can examine the record of chemical deposition in annual tree-rings. The earliest uses of dendrochemistry tended to involve examination of tree-rings for contamination that arose from industrial point sources or heavy metal deposition (Stone and Skelly 1974; Nash et al., 1975). One recent study (Vroblecky et al., 1992) examined the effect of increased levels of pollutants in groundwater on the concentration of potassium in heartwood of tulip poplar (*Liriodendron tulipifera* L.). In addition there is a significant literature on the determination of concentration of a variety of chemical wood treatments (Reistad and Pettersson 1973).

The use of tree rings as records of levels of the environmental constituency of chemical elements is increasing in use. Current methods of dendrochemical analysis include atomic absorption spectrophotometry (AA) (Berish and Ragsdale, 1985), proton-induced X-ray emission spectroscopy (PIXE) (Hall, 1986, Legge et al., 1984), proton-induced gamma ray emission spectroscopy (Hall, 1986), neutron activation analysis (NAA), inductively coupled plasma optical emission spectrometry with optional mass spectrophotometer (ICP/MS) (Baes and McLaughlin, 1984), and electron probe microanalysis (EPMA) (Wardell and Hart, 1973). These methods focus on micro-level concentrations and determine several to many elements at one time, however they are generally extremely expensive. Obviously some method of sub-sampling and compositing of samples is necessary if estimates of trends and background levels are to be obtained for a reasonable cost. Currently, X-ray fluorescence (XRF) methods are increasing in use in that they require minimal sample preparation (a host of problems may arise in preparation processes) and tend to be far less expensive (MacLauchlan et al., 1987). Furthermore, X-ray fluorescence techniques are non-destructive and samples may be used for duplicate runs or further confirmation. There are two types of XRF instruments available: energy dispersive and wavelength dispersive. Previous studies have utilized energy dispersive methods (MacLauchlan et al., 1987; El Kassaby and McLean, 1985; Bondietti et al., 1989; Kocman, 1991). Wavelength dispersive systems, however, generally show superior resolution for lighter elements.

Objectives

Our principle objective for the first phase of this project has been to develop reliable wavelength dispersive X-ray emission techniques for a suite of both heavy metal and nutritive elements. These techniques will provide information on past and present nutritive uptake and any pollution of sites over time. This first objective has resulted in sets of machine parameters and novel sample preparation protocols for the analysis of age incremented tree cores. Our second objective was to test our protocols on several sets of cores. We investigate trends in chemical constituents with an emphasis on recent histories that might include both nutrient state and possible pollution events.

Instrumentation

Our lab utilizes a Siemens SRS200 wavelength dispersive X-ray fluorescent spectrometer. Our only modification to the standard system is the use of a Moxtek prototype ultra-thin window which

Figure 3. Effect of aniline, n-butanol and tetrachloroethylene on the vegetative reproduction of *Lemna gibba*. The error bars represent standard errors

Figure 4. HPLC-radiochromatography of *Lemna gibba* growth medium containing 2 μ M butanol at Day 0, 4 and 8 (top three traces). The fourth trace shows HPLC-RC of an ethanol extract of plants that were harvested at Day 10. The bottom trace shows coinjection of the 10-day plant extract with authentic, radiolabeled butanol.

Figure 5. HPLC-radiochromatography of *Lemna gibba* growth medium containing 2 μ M aniline at Day 0, 4, 8 and 14 (top four traces). The bottom trace shows the HPLC-RC of an ethanol extract of plants that were harvested at Day 14

we have found to enhance the X-ray signal of low molecular weight elements, e.g. Sodium (Latimer and Mills, in prep). Instrument quality control is checked on a regular basis and secondarily by means of a variety of monitor-samples of known elemental concentration present in all runs.

Sample Collection and Preparation

Selection of tree species for analysis is important to the types of questions that may be addressed (Cutter and Guyette 1993). Considerations include tree habitat (e.g. aquatic or terrestrial), the potential for elemental radial translocation, and other xylem based and elemental factors. Bald cypress (*Taxodium dystichum* (L.) L.C. Rich) has characteristics which make it attractive for chemical analysis, including its longevity, and slow transition to heartwood in the inner portions of the stem. Selectivity by cypress of research target elements is not yet full known, but may influence analytical approaches to the apparent temporal distribution of the unknown chemicals. Our principle site is located near the Shell NORCO refining plant which had existing biological sample locations and offered high probability of revealing heavy metal influence on cypress. Our secondary sample sets consist of pine cores collected from the Southeastern U.S. and a small set of samples from a site in Eastern Europe near Russian nickel smelting operations.

Tree cores are extracted with a 4.3mm, Teflon coated increment borer, dried, and stored in plastic tubes. Dendrochronological analysis is performed by the United States Forest Service, Southern Experiment Station, Institute for Quantitative Studies. Cores are dated and increments indicated by notches made with a sharp knife blade. Cores are then cut to prescribed year increments with a minimum weight-of-use set at 0.100 grams. Through experimentation, sample thickness was found to become a factor in elemental concentration calibrations when sample weight is below approximately 0.8 grams. We employ a Wiley mill for grinding samples. Samples are then pressed into wafers (10 tons for 1 minute) with a 10mm die normally used for infrared spectrometry. We found that production of a sample pellet for a standard 25mm XRF sample holder unfeasible, due to the large amount of material needed. Use of a samples supported by thin film was also found to be unsatisfactory due to attenuation of light element signal. We therefore developed inserts to accept samples 10mm in diameter, thus allowing the use of a much smaller sample. The inserted are milled from spectroscopically pure graphite and fit securely in the standard sample holders. The prototype set of 10mm inserts was used for some time, but determined through standard monitoring and subsequent testing to be sufficiently irregular to warrant replacement. The second, more refined set of inserts, now in use, has shown no irregularities.

For samples approaching the 0.0100 gram threshold, it often becomes necessary to stabilize the sample pellet within the insert. We utilize a 10mm wafer made of spec-pure crystalline cellulose as a backing to prevent movement of the sample wafer in the sample cup. Additionally, a polymer sponge is placed snugly in the sample holder to prevent unwanted sample movement.

Elemental Measurement Parameters

We measured the following elements based on the needs of two separate projects: Na, Mg, Al, Si, P, S, Cl, K, Ca, Ti, Mn, and Fe, utilizing a chromium X-ray source and Mn, Fe, Ni, Cu, Zn, As, Sr, and Pb utilizing a molybdenum source (see Table 3 for measurement parameters). We utilized 10 international certified plant reference materials for calibrations (see Appendix A for a list of reference materials). Due to the complexity of some reference materials matrices, calibrations were not always strait forward. Because of the discrepancy of matrices of wood and leaves, natural--wood standards would be ideal and would most likely simplify calibrations. We are currently planning to construct such materials.

After determination of target elements, i.e. those elements we want to measure, we begin by using recommended elemental measurement parameters from a number of sources, e.g. instrument and accessory manufacturer(s) or previous studies. More precise parameters are determined by plotting scans of the areas encompassing the K-alpha peak of the target elements. This procedure provides

information concerning the optimum detector positions (corresponding to a wavelength) used to record peak intensities and whether background measurement and correction will be needed. Background measurement becomes necessary when peaks are located on the curved background caused by tube scatter or when signal to background ratios are low. The latter case is referred to as noise and is associated with low relative target element concentration. We test measurement parameters statistically to ensure acceptable output. Also, through the use of alternative crystal arrays, we have avoided peak overlap.

Calibration

Calibration of the instrument follows determination of measurement parameters for target elements. Calibrations are made for each element by determining the ratio of x-ray counts (x-axis) to the known concentrations of the target element (y-axis) for each reference material used (see Appendix A for a list of known elemental concentrations for each reference material). A calibration curve is constructed by fitting a curve (all linear for our calibrations) through a set of points covering the range of concentrations expected or known to occur in experimental samples. Counts may then be collected from samples of unknown concentration, the resulting count number corresponding to a specific elemental concentration on the calibration curve. Our primary test of calibration quality is accomplished by running the reference material samples from which the calibration was constructed, as unknowns, compare the two sets of values, and determine if the retrieved values are both sufficiently accurate and precise. If necessary, the calibration may then be modified to improve quality and the testing process repeated. This entire process may require several rounds to achieve acceptable results. The original list of target elements was considerably smaller than the lists presented above. Through a number of calibrations in which a subset of a variety of reference and core samples were used, it was apparent that while our sample matrices were simple (mostly cellulose; carbon, oxygen, and hydrogen), relatively small concentrations of heavier elements or higher concentrations of lighter elements could greatly bias results. Therefore, the measurement of several elements was added so as to allow adjustment for their effect, either absorbance or enhancement, in the calibration.

Quality Control Formal quality control measures are under development following methods used in other labs. Currently, a reference material monitor is included in every run and periodic retrieval of all reference material values is conducted. Quality control data from other projects is also incorporated for overall instrument reliability.

Results

We present, as major product for this year, two products. First a tabular presentation of standard calibration parameters and conditions for determining quantitative amounts of elements (Table 3). Second we present an example plot for chemicals from cypress cores collected in Bayou Trepagnier (Figure 6 and 7). In addition, we give a sample graph of aluminum, one of a several elements we have quantified in Southeastern U.S. pine cores (Figure 8). Table 3 presents information on the source tube, Siemens configuration parameters and counting time required for samples in the ranges given for our reference materials in our collection. Corrections for the tube scatter are given as degrees above and below the K-alpha peak for the element. Elements that interfere with the analyte are presented for the source tube associated with the particular element. These elements indicate possible additional analyses which may be necessary to apply correction formulae.

Analysis of cores obtained from cypress growing in Bayou Trepagnier has been undertaken and a more complete analysis of results from preliminary samples are being presented by a cooperating program science cluster (Thien et al., 1994). In this paper we present graphs based on two of the sampled trees to illustrate the possible trend in lead (Pb) and copper (Cu) concentration (figures 6 and 7). Examination of the plots in figures 6 and 7 show that there is an increase in the amount of lead in the cypress rings beginning in the 1920's. This corresponds with the initial construction and start up of the New Orleans Refining Company (now Shell, NORCO) near Bayou Trepagnier

Table 3. Measurement Parameters**X-ray Source: Cr - Chromium Source; Mo - Molybdenum Source**

Element	X-ray Source	XRF Config	Count. Time (sec)	Low Bkgd (degrees)	Peak (degrees)	High Bkgd (degrees)	Infr. Elem. (Cr Source)	Infr. Elem. (Mo Source)
Na	Cr	71010	200	23.5	25.23	26.8		
Mg	Cr	71010	200	19	20.83	22.5	Si, Cl, K, Ca, Mn, Fe	
Al	Cr	51010	100	143.7	145.12	148	Si, Ca, Ti, Mn, Fe	
Si	Cr	51010	20		109.189			
P	Cr	41010	100		140.879	143.5	K, Ca, Mn, Fe	
S	Cr	51010	80		75.8	78.5	Ca, Mn, Fe	
Cl	Cr	41010	20	91.75	92.662		K, Ca, Fe	
K	Cr	51010	40	50.6			Ca, Mn, Fe	
Ca	Cr	51010	40		45.11		Cl, K, Ti, Mn, Fe	
Ti	Cr	21010	20		86.183		Fe	
V	Cr, Mo	05010	100	121.6	123.265		?	
Cr	Mo	51010	100	105.5	107.275	109	----	Fe, Ni
Mn	Cr, Mo	21010	20	61.6	62.99	64.5	Ti, Fe	Fe, Cu, Zn
Fe	Cr, Mo	21010	40		57.513		K, Ca, Mn	Mn, Cu, Zn, As
Ni	Cr, Mo	25010	100	48.63	50		?	Mn, Fe
Cu	Cr, Mo	25010	40	44.2	45	45.92	?	Fe, Zn
Zn	Cr, Mo	25010	80	40.9	41.73		?	Fe, Cu
As	Mo	25010	100	33.28	33.99	35		
Sr	Mo	25010	100	24.4	25	25.92		
Pb	Mo	25010	200	27.6	28.27	29.1		Mn, Fe, Cu, Zn

in the 1920's. While this is preliminary data, it does seem to validate the initial hypothesis that heavy metal record of the industrial development in the area would be found in the rings of cypress. Much additional work is now needed to characterize its distribution and persistence in time. Finally, in order to address the issues, including detectability and signal to noise ratio in the tree-core samples, we present Figure 8, showing data from another project. They confirm that aluminum concentrations are well above the baseline and that the standard errors are an indication of signal to noise ratio.

Discussion

The X-ray analysis component of the scientific cluster is well under way. We have achieved notable success in developing sample preparation methods, analyte interactions, amelioration protocols and machine procedures for the quantitative determination of chemicals in cypress cores. There are questions that remain, but at this point many of these are related to sample sizes and locations. We expect to continue the analysis of data obtained from the X-ray emission spectra and to provide continuing support to other projects which will use the results of these methods (Thien, et al., 1994)

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Fig 6. Average Concentrations of Lead in Cypress Tree-Cores Over Time.

Fig 7. Average Concentrations of Copper in Cypress Tree-Cores Over Time.

Fig. 8 Inorganic chemicals in cores

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4) Effect of cadmium on physiological activities in crustaceans

The research has centered on analyzing the effects of a heavy metal, cadmium, on fine physiological processes.

A. Effect of cadmium on lactate dehydrogenase activity.

Lactate dehydrogenase (LDH) activity in the hepatopancreas and abdominal muscle of fiddler crabs, *Uca pugilator*, was determined after 24 and 48 h of exposure to 2 ppm cadmium chloride. For the cadmium exposed crabs, LDH activity in the hepatopancreas decreased, whereas that in the abdominal muscle increased. The increased LDH activity in the abdominal muscle may reflect increased dependence on anaerobic carbohydrate metabolism in fiddler crabs exposed to cadmium in their environment.

B. Effect of cadmium on the blood glucose concentration.

Exposure to 5 ppm cadmium chloride for up to 72 h produces hyperglycemia in intact *Procambarus clarkii*, but not in eyestalkless individuals. Extracts of eyestalks, regardless of whether the eyestalks are from cadmium exposed crayfish or crayfish kept in clean water, produce a greater hyperglycemia in eyestalkless crayfish kept in clean water than in cadmium exposed eyestalkless crayfish. This difference in effectiveness of the extracts may represent a decreased responsiveness of cadmium exposed crayfish to the crustacean hyperglycemic hormone. These results support the hypothesis that cadmium induced hyperglycemia is mediated at least in part by the crustacean hyperglycemic hormone that is produced in the eyestalk.

C. Effect of cadmium on the digestive juice and amylase activity.

Exposure to cadmium chloride (10 ppm) results in an increase of the pH of the digestive juice and a decrease in the amylase activity in the digestive juice of intact *Procambarus clarkii*. The pH optimum for amylase activity is 5.8 in both the digestive juice and hepatopancreas. The results are discussed in terms of the toxicity of cadmium to the secretory cells in the hepatopancreas.

D. Effect of cadmium on pigmentation.

Color changes of crustaceans are hormonally regulated endocrine physiological processes. In our previous studies we reported the heavy metal, cadmium chloride, interfere with normal physiological functions of crab and crayfish. To further examine how cadmium acts on other endocrine processes, we started working on the cadmium effect on color changes of fiddler crabs.

We noticed a significant change in color, i.e. chromatophore stage, of crabs exposed to cadmium. Irrespective of the background (either dark or light) the animals become more pale when exposed to 10 ppm cadmium. When we changed the background from light to dark after 4 days of exposure, to find out if there is any effect on the functional responses, the exposed animals exhibited less pigment dispersion than the controls.

The sinus gland complex in the eyestalk is the source of a pigment dispersing hormone in crustaceans. To learn whether the pigment dispersing hormone is not being released or the amount of the hormone is lowered by cadmium exposure, we injected eyestalk extract of 10 days cadmium exposed crabs as well as control animals into eyestalk ablated crabs. We observed that the eyestalks of the cadmium exposed animals were less potent in producing pigment dispersion than were the control eyestalks. Since the pigment concentrating hormone is also being synthesized and released from the eyestalks, to avoid the influence of this, currently we are separating these two hormones by using column chromatography and we will bioassay the fractions. We are also processing eyestalk and brain tissues for histological studies to observe if there is any effect of cadmium exposure at the cellular level.

E. PCR amplification of putative metallothionein gene homologs.

Crayfish tail muscle was used for molecular cloning of metallothionein cDNA. Metallothioneins are proteins that bind heavy metals, thereby aiding in detoxification. DNA of the crayfish was isolated. The Polymerase Chain Reaction (PCR) was carried out with putative crustacean metallothionein DNA primers. The PCR was performed under Ampli Wax Gems (Perkin-Elmer/Cetus) by changing several factors to get putative metallothionein specific bands. The PCR amplified cDNA was digested with EcoRI and ligated with T4 DNA ligase. *Escherichia coli* (Strain MC1061) cells were transferred with the ligation mix. Colonies were screened. Plasmid DNA from positive clones was isolated and digested with EcoRI. Both strands of clones containing appropriate inserts were subcloned into the single strand sequencing vectors M13mp18 or M13mp19 and sequenced. The use of degenerate primers for the PCR may lead to less than perfect matches between the template (DNA of the crayfish) and the primer, resulting in amplified products with sequences in the primer region that may not accurately represent the sequences of the original template. Four crayfish metallothionein sequences have been found in this study Whether all four are indeed coded for in the crayfish is under investigation.

Publications Resulting from Grant Support

Devi, M., P. S. Reddy, and M. Fingerman (1993) Effect of cadmium exposure on lactate dehydrogenase activity in the hepatopancreas and abdominal muscle of the fiddler crab, *Uca pugilator*. Comp. Biochem. Physiol. 106C: 739-742.

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- 5) Xenobiotic Metabolizing Esterases In The Liver And Brain Of Two Catfish Species, *Ictalurus Punctatus* and *Ictalurus Natalis*

Abstract

The B-type esterases which include acetylcholinesterases and the carboxylesterases were measured in the liver microsomes and cytosol and brains of two catfish species, *Ictalurus punctatus* and

Ictalurus natalis. A series of five esters of *p*-nitrophenol and acetylthiocholine were used as colorimetric substrates to assay the carboxylesterases and acetylcholinesterase activities, respectively. In vitro exposure of brain acetylcholinesterase from *Ictalurus punctatus* to cadmium, lead, and malathion at 5 ppm concentrations lead to significant inhibition of acetylcholinesterase activity. The enzyme inhibition was in the order: cadmium > lead = malathion. The substrate specificity of liver microsomal and cytosolic carboxylesterases were remarkably different from each other. The valerate ester of *p*-nitrophenol was most rapidly hydrolyzed by the microsomal carboxylesterases whereas the propionate ester was the best substrate for cytosolic carboxylesterases. The results are discussed in relation to the responses of xenobiotic metabolizing esterases to environmental pollutants and their possible use as biomarkers.

Introduction

The responses of various xenobiotic metabolizing enzymes in fish models are rapidly evolving as important biomarkers of exposure to environmental contaminants. Ethoxyresorufin-O-deethylase, a specific cytochrome P450-dependent monooxygenase, is often used as an indicator of polyaromatic hydrocarbon pollution (Jimenez et al 1990). Acetylcholinesterase, a well-known esterase, is another useful biomarker of exposure to organophosphorous and carbamate insecticides (Trundle and Marcial 1988). In order to effectively interpret the biomarker response and predict toxic risks, it is crucial to develop an understanding of the interaction of xenobiotics and toxicants on the biochemistry and regulation of these and other important xenobiotic metabolizing enzyme systems.

The xenobiotic metabolizing esterases reported in this study are the B-type esterases (Aldridge 1953) which are inhibited by organophosphates and include cholinesterases and carboxylesterases. Within this class, acetylcholinesterases (EC 3.1.1.7) are associated with nerve synapses and responsible for the hydrolysis and termination of the function of the endogenous chemical mediator acetylcholine. Inhibition of this enzyme leads to an accumulation of acetylcholine which may lead to tetany, paralysis and finally death. Therefore, the use of this enzyme for monitoring purposes is of considerable importance. The carboxylesterases (EC 3.1.1.1) represent a heterogenous group of isozymes that can catalyze the hydrolysis of a wide range of xenobiotic esters. For most carboxylesterases, their natural substrates are not known, therefore, their physiological functions remains to be elucidated. Although there is a plethora of literature on mammalian carboxylesterases and to a lesser extent on avian carboxylesterases, little is known about fish carboxylesterases. Although these enzymes occur very widely in animal tissues, they are found in high levels in liver microsomes. There is evidence that release of these enzymes into the serum, as a result of exposure to certain toxicants, is an indication of tissue damage (Huang et al 1993a, Hammock et al 1984).

This report describes the preliminary characterization of fish esterases in the brain and subcellular fractions of liver. The substrate specificity of these enzymes and the effect of cadmium, lead nitrate, and malathion on brain acetylcholinesterase are reported.

Materials And Methods

Chemicals

p-Nitrophenyl acetate, *p*-nitrophenyl propionate, *p*-nitrophenyl butyrate, *p*-nitrophenyl valerate, *p*-nitrophenyl caproate, acetylthiocholine, and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO). BCA protein determining reagent was purchased from Pierce Chemical Co. (Rockford, IL). All microtiter plates were purchased from Dynatech (Chantilly, VA).

Sources of catfish species

Ictalurus punctatus catfish weighing approximately 15 gms were obtained from the Department of Veterinary Medicine, Louisiana State University in Baton Rouge. *Ictalurus natalis* catfish were collected from devil swamp site (DSS), a cypress swamp which lies just northwest of Baton Rouge, Louisiana, adjacent to the Mississippi river. The DSS receives toxic substances and

hazardous waste from a wide variety of surrounding industrial operations. Fish were transported alive to the laboratory for dissection and analysis.

Enzyme preparation

Fish hepatic microsomes and cytosol were prepared by differential centrifugation. The livers were weighed and minced in ice-cold 0.1 M sodium phosphate buffer and 0.25 M sucrose at pH 7.4. The minced tissues were homogenized in 10 volumes of buffer and centrifuged at 10,000g for 15 min with a Beckman L7-65 ultracentrifuge. The resulting supernatants were centrifuged at 105,000g for 65 min. The resulting supernatants were collected as the cytosol fractions. The microsomal pellets were washed with 0.1 M sodium phosphate buffer and 0.25 M sucrose at pH 7.4 and resuspended in the same buffer. Fish brain tissues were minced and homogenized (approximately 20 mg of tissue per ml of 0.1 M sodium phosphate buffer at pH 8.0) in a Brinkman polytron homogenizer. The homogenates were then centrifuged at 20,000g for 10 min. The resulting supernatants were collected as the source of brain acetylcholinesterases. The liver microsomes and cytosols, and brain extracts were stored at -86 °C until used for enzyme assay.

Enzyme Assays

The esterase activity was measured in a continuous assay with 96-well microtiter plates with a Vmax plate reader (Molecular Devices, Palo Alto, CA) as described (Huang et al 1993b). The associated Softmax software provided with the instrument was used to calculate the standard curves of *p*-nitrophenol, rate of product formation, and protein concentrations used in the assay. A Perkin Elmer Lambda 2S spectrophotometer was also used to record the reaction rates. Independent studies demonstrated that the plate reader gave rates very similar to those observed with the Perkin Elmer spectrophotometer.

The hydrolysis of the *p*-nitrophenyl esters was determined as described previously (Huang et al 1993b). The incubation mixture contained 20 µl of enzyme solution in 278 µl of 0.1 M sodium phosphate buffer, pH 7.4. The reaction was started by the injection of 2 µl of the substrates (in acetone) to give a final concentration of 5×10^{-4} M. The liberation of *p*-nitrophenol was monitored for 2-5 min at 405 nm.

The esterase activity on acetylthiocholine was assayed by a modification of the method of Ellman et al (1961). In a typical assay using the plate reader, 278 µl of 0.015% DTNB in 0.1 M sodium phosphate buffer at pH 8.0 and 20 µl enzyme solution were added to individual wells. The reaction was initiated by the addition of 2 µl acetylthiocholine (in water) to give a final concentration of 5×10^{-4} M and the rates were recorded at 412 nm for 2-5 min. Reagent blanks containing no enzyme were used as controls. For the Perkin Elmer instrument, the assay mixture in the cuvette contained 0.2 ml enzyme solution, 1.3 ml of 0.1 M sodium phosphate buffer at pH 8.0, 0.05 ml of DTNB reagent (0.01 M), and 0.01 ml of acetylthiocholine (0.075 M). The absorbances were recorded at 412 nm.

The enzyme assays were carried out under conditions where the initial hydrolytic rates were linear with time for the protein and substrate concentrations used. The assay was carried out in triplicate. The measured rates were corrected for the spontaneous hydrolysis of the substrate where this was significant. The final concentration of organic solvents (acetone) used in the assay mixture was less than 1% and caused little or no effect on enzyme activity. The esterase activities are expressed as product formed per minute per milligram of protein. Protein concentrations were determined using the standard protocol version of the Pierce BCA assay. Bovine serum albumin was used as a protein standard.

Table 1 : Acetylcholinesterase activity in the liver and brain of two catfish species, *Ictalurus punctatus* and *Ictalurus natalis* with acetylthiocholine as substrate.*

Species (organ)	Specific activity (nmol/min/ mg protein)
<i>Ictalurus punctatus</i> (n=3)	
Liver microsomes	24.6 ± 1.24
brain	26.4 ± 0.29
brain (exposed to 5 ppm of cadmium)	18.2 ± 0.96
brain (exposed to 5 ppm of lead nitrate)	21.2 ± 2.17
brain (exposed to 5 ppm of malathion)	21.4 ± 1.50
<i>Ictalurus natalis</i>	
fish #1 liver microsomes	10.1 ± 0.48
brain	25.7 ± 2.74
fish # 2 liver microsomes	3.63 ± 0.36
fish # 3 liver microsomes	9.35 ± 1.12
fish # 4 liver microsome	1.72 ± 0.73

* Assay conditions are described under materials and methods. each value represents the means ± SD of three determinations.

Table 2 : Substrate specificity of liver microsomal and cytosolic carboxylesterases in two catfish species, *Ictalurus punctatus* and *Ictalurus natalis* with p-nitrophenyl esters as spectrophotometric substrates *

species	specific activity (nmol/min/mg protein)				
	acetate	propionate	butyrate	valerate	caproate
<i>Ictalurus punctatus</i> (n=3)					
microsome	14.6 ± 1.72	63.0 ± 3.69	99.0 ± 9.26	147 ± 18.3	92 ± 9.17
cytosol	6.85 ± 0.55	78.6 ± 3.36	31.2 ± 4.14	46.8 ± 1.08	37.3 ± 3.36
<i>Ictalurus natalis</i>					
Fish # 1 (microsome)	91.0 ± 9.73	300 ± 17.1	276 ± 7.82	425 ± 27.9	315 ± 22.9
cytosol	17.0 ± 0.29	254 ± 3.76	147 ± 6.31	211 ± 14.0	147 ± 5.64
Fish # 2 (microsome)	1.57 ± 0.004	3.63 ± 0.49	5.76 ± 1.01	56 ± 7.23	10.7 ± 0.96
cytosol	9.28 ± 0.241	123 ± 6.81	53.9 ± 3.74	95.2 ± 6.33	58 ± 5.11
Fish # 3 (microsome)	76.3 ± 5.47	230 ± 3.87	168 ± 11.2	186 ± 3.53	12.6 ± 0.39
cytosol	13.9 ± 0.82	194 ± 7.54	117 ± 9.98	192 ± 1.41	124 ± 4.31
Fish # 4 (microsome)	37.1 ± 0.51	9.47 ± 1.37	20.6 ± 4.27	301 ± 18.7	N.D.
cytosol	3.63 ± 0.33	25.9 ± 1.87	N.A.	14.4 ± 0.63	10.7 ± 0.33

* Assay conditions are described under materials and methods. Each value represents the means ± SD of three determinations. not determined (N.D). no detectable activity (N.A).

In vitro exposure studies

Brain tissues from *Ictalurus punctatus* were incubated for 30 min at 20 °C with either cadmium, lead nitrate, or malathion at a concentration of 5 ppm. Following the exposure period, the acetylcholinesterase activities were analyzed as described above.

Results And Discussion

Table 1 shows the acetylcholinesterase activity in the liver and brain of *Ictalurus punctatus* and *Ictalurus natalis*, and the effect of cadmium, lead, and malathion at concentrations of 5 ppm on brain acetylcholinesterase in *Ictalurus punctatus*. The acetylcholinesterase activity in the brain of both species appear to be very similar, although it should be noted that the brain sample from *Ictalurus natalis* was analyzed in only one fish. A larger sample needs to be analyzed in order to determine if there is a difference in acetylcholinesterases between the two species. Significant acetylcholinesterase activity was observed in the liver microsomes of both species. The acetylcholinesterase activities in the liver microsomes and brain of *Ictalurus punctatus* were comparable. Whereas, the liver microsomal acetylcholinesterase activities in *Ictalurus natalis* were lower (0.07- to 0.39-fold) than the acetylcholinesterase activities in the brain of both species. It is not known whether the catfish (*Ictalurus natalis*) from the devil swamp site has been exposed to any organophosphorous or carbamate insecticides. Control fishes of the same species from uncontaminated sites are required for further studies. The in vitro exposure studies show that acetylcholinesterase was most inhibited by cadmium, followed by lead and malathion.

In Table 2, the substrate specificity of liver microsomal and cytosolic carboxylesterases in the two catfish species were investigated with a series of five *p*-nitrophenyl esters. Unlike mammals, significant carboxylesterase activities in the cytosol of both species were observed in comparison to microsomal carboxylesterases. The microsomal and cytosolic carboxylesterases from both catfish species were strongly influenced by the length of the acyl moiety of *p*-nitrophenol. The specific esterase activity of liver microsomes for both species were observed to increase up to four carbon atoms (valerate) in the acyl moiety and then dropped with further elongation of the acyl chain (caproate). This data held true for all the fish tested except fish #3 where the liver microsome exhibited highest activity with the propionate ester. There was a marked difference in the substrate specificity of cytosolic carboxylesterases when compared to the microsomal enzymes. The highest activity of cytosolic carboxylesterases were observed with the propionate ester with two carbon atoms in the acyl chain.

In conclusion, this study shows that cadmium, lead nitrate and malathion caused inhibitory effects in the brain acetylcholinesterase activity of the channel catfish *Ictalurus punctatus* at a concentration of 5 ppm in in vitro studies. Measurement of acetylcholinesterase activity is a very important parameter for detecting and monitoring the effects of organophosphorous and carbamates pollution in the environment. Although the carboxylesterases have a wide range in their substrate specificity, their role in the detoxification of xenobiotics and in the metabolism of endogenous esters in aquatic organisms awaits further clarification.

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6) Development Of Ion-Exchange Materials For Immobilization Of Toxic Metals

The problem with the application of constructed wetlands (and the like) to heavy metal remediation is that the generated biomass necessarily contains high concentrations of heavy metals. These will be released back to the environment upon decomposition of the plants. Therefore, methodology must be devised for the removal and/or immobilization of the toxic metals. Some of those metals (e.g. chromium and cobalt) may be of significant commercial value and "critical" in the sense that the U.S. is dependant on foreign supplies (Clark, 1985). It would be economically sound to recover those metals and, in doing so, prevent further deterioration of the environment. The plants provide the first step in this process by taking up and concentrating the metals. What is then required is a means by which the toxic metal ions may be separated from the environmentally--benign ones. Preferably, the metals should be concentrated to the point where they may be economically disposed of or used as a feedstock in conventional smelting operations.

Ion-exchange materials provide an ideal method for removing and concentrating hazardous metals from aqueous solution. This is particularly true when the toxic ions bind irreversibly to the ion-exchanger so that it may be "loaded" to its theoretical limit. This may be accomplished by using either sulfur-containing exchange sites that will bind the heavy metals (which are extremely thiophilic) or phosphate moieties which could remove multivalent oxyphilic metals from solution. Since, our intent is to ultimately incorporate the toxic metals into a ceramic wasteform, we plan to design ion exchange materials that, once loaded, may be converted directly at low temperature to a ceramic material.

One such ion-exchanger that we have already developed in this project is based on polyacrylic acid that has been cross-linked with the metal ions required for Synroc (Apblett, in press). Synroc is a ceramic based on the minerals hollandite ($\text{BaAl}_2\text{Ti}_6\text{O}_{16}$), perovskite (CaTiO_3), and zirconolite ($\text{CaZrTi}_2\text{O}_7$) that has received considerable interest for the encapsulation and immobilization of radioactive waste due to its excellent leach-resistance (Ringwood, 1988). However, the standard methodology used for incorporation of the radionuclides into the ceramic does not provide for their homogeneous mixing with the ceramic matrix precursors. As well, the addition of titanium is required to prevent production of highly-oxidized water-soluble species, leading to a more complex wasteform. The tremendous success of co-precipitation methods for the preparation of mixed metal oxide phases suggests that they might provide an alternative method of preparation of Synroc

(Apblett, 1993). The introduction of appropriate elements required for Synroc mineral phases to a simulated radioactive waste stream (PUREX waste-4B) followed by the sodium salt of polyacrylic acid leads to separation of a Synroc precursor in which the components have been intimately mixed at the molecular level. This results in low sintering temperatures (950°C) and removes the necessity of adding buffering reagents to control the oxygen fugacity since the thermal decomposition of polyacrylic provides a reducing atmosphere. We have found that similar results may be obtained with more dilute wastestreams if we first react sodium polyacrylate with the elements required for the ceramic matrix (Ba, Al, Ti, Zr, and Ca) in a ratio that leaves 20% of the carboxylates as the sodium salt. The product is obtained as a white precipitate that has excellent ion-exchange properties that allows removal of all metal ions (except alkali metal ions) from solution. The success of the treatment of a radioactive wastestream has two important ramifications to the development of a duckweed-based bioremediation process. Firstly, radwaste contains representatives of all groups of metals in the periodic table and all of these, with the exception of the alkali metals, were successfully incorporated into the ceramic wasteform. Secondly, the ideal method of isolating the inorganics from duckweed is dissolution of the plants or their ash in nitric acid. This takes advantage of the high solubility of metal nitrates but leads to the possibility of oxidation reactions involving the anion when the conversion to a ceramic is performed. Since many highly-oxidized metals are very water soluble this could lead to unacceptable leaching of these species. The above process replaces nitrate with polyacrylate avoiding this complication. The demonstrated ability of duckweed to absorb actinides (see sect. 1, above) in tandem with this Synroc precursor may eventually provide a useful process for remediating low-level radioactive waste.

A second approach toward useful ion-exchange materials that we have adopted is the derivitization of minerals to enhance their ability to adsorb and immobilize heavy metals. This has the significant economic advantage of using readily available very-inexpensive starting materials. Furthermore, the clay minerals are natural ion-exchangers that readily calcine to ceramic aluminosilicates providing an ideal route to a rock-like wasteform. One such mineral is kaolin, $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$, which is composed of stacks of lamellae, each composed of a pair of alumina and silica sheets. Three of the four hydroxyl groups lie adjacent to the interlamellar space and provide the ion-exchange properties. Our intention was to improve on this capability by replacing the hydroxyls with phosphate groups which have superior affinity for multivalent or heavy metals. As well, such derivitization would also increase the interlamellar space providing greater access to the toxic metal species. We have intercalated ammonium phosphate into the clay mineral kaolinite by stirring the reagents together in 50:50 DMSO:H₂O. Heating the intercalate to 250°C leads to a condensation reaction that irreversibly binds the phosphate to the aluminosilicate layers. It was determined that this material uptakes Cd^{2+} readily from water (0.1g of ion-exchanger reduces the concentration of Cd^{2+} in 30 ml of water from 65 ppm to 11 ppm, very rapidly). The derivitized kaolin performs three-times better than the parent mineral in this respect (it reduced the concentration to 33 ppm). Unfortunately, the equilibrium concentration of Cd^{2+} was unacceptably high but this might be improved by buffering the solution to promote removal of the protons from phosphate (such studies are in progress). We also synthesized a second ion-exchange material, $\text{Na}_2[\text{LiAl}_2(\text{OH})_6(\text{PO}_4)]$, based on a layered aluminum oxide mineral. This was prepared by reaction of $\text{LiAl}_2(\text{OH})_7$ with disodium phosphate. Under the same conditions as the kaolin ion-exchangers, this material reduced Cd^{2+} concentrations below the detection limit of the ICP.

All of the above ion-exchangers are not expected to be specific for heavy metals but may be used to remove all multivalent metal ions from solution (their specificity is currently being determined by competitive binding Cd^{2+} of versus Ca^{2+}). The separation of heavy metals from other metal ions so that they may be recycled or immobilized individually would be highly beneficial. Fortunately, their thiophilicity (affinity for sulfur) make the heavy metals chemically-distinct. We have used this thiophilicity to develop an ion-exchanger that is specific for heavy metals. We found that TiS_2 ,

Appendix A

International Plant Reference Materials

Total Elemental Constituents for USFS Standards

Certified values are listed in
boldface type
Reference values listed in italics are
not certified

Standard	Na	Mg (ppm)	Al (ppm)	Si (%)	P (%)	S (ppm)	Cl (ppm)	K (%)
1 Aq. Pint 060	6700	6030	4180	28.5	0.516	5200	10000	1.14
2 Aq. Pint 061	3000	3900	10740	75.3	0.922	2300	2300	1.25
3 Olive lvs 062	70	1200	448	0.13	0.105	1600	700	0.31
4 Chlorella		3300			1.7			1.24
5 Cotton	88	53	44			54	600	
6 Hay	500		47		0.23			2.1
7 Peach lvs	24	4320	240		0.137		300	2.43
8 Pine ndls			546		0.12			0.37
9 Sargaseo	17000	6600	215		0.26	12000	51000	6.1
10 Tomatoe lvs		7000	1200		0.34			4.46
11 Citrus Lvs	180	5800	92		0.13	4070	414	1.82
12 Cellulose	15.7		0.77					

Standard	Ti (ppm)	V (ppm)	Cr (ppm)	Mn (ppm)	Fe (ppm)	Ni (ppm)	Cu (ppm)	Zn (ppm)
1 Aq. Pint 060	240	6	26	1780	2400	40	51.2	313
2 Aq. Pint 061	780	6	532	3771	9300	420	720	586
3 Olive lvs 062	240	1	2	57	280	8	46.6	16
4 Chlorella				89	1850		3.5	20.5
5 Cotton		0.09	0.11	0.15	11	0.09	0.59	
6 Hay			6.5	47	185	4	9.4	24
7 Peach lvs		0.37		98	218	0.69	3.7	17.9
8 Pine ndls			2.6		200	3.5	3	
9 Sargaseo	9	1	0.2		187			15.6
10 Tomatoe lvs								
11 Citrus Lvs			0.8	23	90	0.8	16.5	29
12 Cellulose								11

another layered material, can remove up to an equimolar amount of cadmium from solution (10 mmol of TiS_2 reduced the concentration of a 2250 ppm Cd^{2+} in 500 ml of water to a level below CP detection limits). Presumably, this occurs by solubilization of the oxyphilic TiO^{2+} ions. If so, adjustment of the pH of a heavy-metal depleted wastestream would precipitate the oxyphilic metals in a form ideal for preparation of a titanate ceramic wasteform. In such a manner, the heavy metals and the lighter metals may be immobilized in separate more-suitable wasteforms that closely resemble their natural repository in the earth's crust. We are also developing a similar system based on high-surface area iron sulfides. Other layered or porous three-dimensional sulfur-containing species which can bind heavy metals as highly-insoluble sulfide phases (e.g. KCu_4S_3 and $(\text{NH}_4)\text{Cu}_7\text{S}_4$) are being investigated as well.

Another class of natural ion-exchangers is zeolites which are complex aluminosilicates with well defined void spaces and channels (Dyer, 1988). They have remarkable size selectivity for metal ions and as such, have been utilized for the selective extraction of ^{137}Cs from "pond" water wastes which contain large excesses of Na^+ , Ca^{2+} , and Mg^{2+} ions. In this investigation, we found that Cd^{2+} efficiently exchanges for three out of four Na^+ sites in sodio-zeolite X-even with a thousand-fold excess of Na^+ in solution. Studies involving competitive exchange in the presence of dications (e.g. Ca^{2+}) are currently in progress. Since, ion-exchange for most metal cations into zeolites is reversible, we are studying the application of volatile organosilanes to chemically seal zeolite pores. It was determined that the reaction of phenylsilane with protio-zeolite X leads to formation of a silicate "plug" at very low temperatures. The mechanism of this reaction is currently being probed by MS, FTIR, and ^{29}Si CPMAS solid-state NMR.

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Toxicity and Uptake/Aquatic Organisms

Acute Lethal Toxicity Testing Of Heavy Metals On Crayfish And Bluegills

Abstract

Acute toxicity bioassays of heavy metals have been conducted on crayfish (*Procambarus* spp.) and bluegills. Preliminary data on crayfish shows the metal toxicity in this order: $\text{Hg} > \text{Cd} > \text{As}^{+3}$. The approximate LC50 values of crayfish for mercury, cadmium, and arsenic(III) are 9, 34, and 43 ppm respectively. The LC50 values of bluegills for mercury and cadmium are 0.4 and 6.1 ppm

respectively. Acute toxicity tests are in progress for arsenic(III), arsenic(V), cadmium, chromium(VI), mercury, and lead.

Introduction

There is great concern over the fate of heavy metals that have been released into the environment. Toxic effects on wildlife and movement up the food chain present possible threats to health and quality of life. Aquatic organisms provide a means of income and food for many Louisiana residents. Therefore, how pollutants such as metals affect fish and crayfish and whether they can bioaccumulate and be consumed by humans is of interest.

Short term toxicity tests (Bioassays) are used to evaluate acute toxicities of chemicals to aquatic organisms (catfish, bluegill fish, crawfish, etc.) and microorganisms. Mortality is used as the end point to determine the response to a certain toxicant. Test results are expressed as 96 hour lethal concentration (LC_{50}). Both refer to the concentration or dose which kills fifty percent of test animals at the end of 96 hour period. (American Public Health Association - 1992)

Acute tests can also be used to determine toxicant concentrations for intermediate and long term tests.

An acute bioassay is conducted in two phases:

A. Phase I: This includes the range finding to explore the approximate concentrations to be used in actual test (Phase II). Usually organisms are exposed to different concentrations (logarithmic ratio: 0.1, 1.0, 10, 100, 1000 ppm, etc.). This range must include the concentration which kills all test organisms and the other concentration which kills none.

B. Phase II: Animals are exposed to different concentrations ranging from the one which kills all test animals, and the other which kills none and 3-5 others in between.

This study will focus on measuring the acute lethal toxicity of As^{+3} , As^{+5} , Cd, Cr^{+6} , Hg, and Pb to crayfish and bluegills. Later studies will assess the toxicity of combinations of metals, the bioaccumulation in aquatic organisms, and the histopathology caused by these heavy metals. At present, preliminary results exist for the toxicities to crayfish of all the above metals except for lead. For the bluegills, toxicity to mercury and arsenic (III) have been assessed.

Literature Review

The acute lethal toxicity testing is an important step in assessing the bioaccumulation and ultimately understanding the movement of the compound in the environment (Anderson et al. 1983). Some acute toxicity studies have been done on crayfish (Del Ramo 1987; Mirenda 1986). Others have studied the bioaccumulation of metals in crayfish and bluegills (Abdelghani, A.A., 1976, 1980, 1981, Khowley and Abdelghani 1993) (Naqvi 1990; R.V. Anderson & Bower 1978), and some have measured the levels of metals in crayfish caught in the wild (Finerty 1990; Alikhan 1990; Madigosky 1991). This study desires to be a more complete look at heavy metal poisoning by examining six metals for acute toxicity, histopathology, and the bioaccumulation.

Materials and Methods

Crayfish (*Procambarus spp.*) were obtained from Belle River in the Atchafalaya River Basin in Louisiana. On the same day as their capture, they were declawed to prevent predation and cannibalism. Two weeks were allowed for the crayfish to acclimate to laboratory conditions. Fifty-gallon glass aquariums were used as holding tanks where the crayfish were allowed to disperse on crab-trap wire. They were fed oats biweekly, but were not fed 48-hrs prior to a test. Only intermolt crayfish were used for the tests.

Bluegills were obtained from the Louisiana Wildlife and Fisheries Department. They are commonly used as stock for Louisiana's ponds and rivers. They were fed twice daily with goldfish flake food. The bluegills used for testing measured between 3-4 cm in length.

A 96-hour static renewal bioassay was employed to study the acute effects of heavy metals as discussed in the 1992 APHA Standard Methods. Ten organisms were allocated to each test tank containing 16 liters of dilution water plus the particular concentration of metal to be tested. The dilution water used was New Orleans tap water which had been dechlorinated, filtered, and aerated for at least 24 hours. The test water was renewed daily in order to maintain the appropriate metal concentration and replenish the dissolved oxygen. The water in the test tanks were not aerated during the experiment.

Range-finding tests were done to narrow the concentrations tested. The following are the test concentrations, in parts per million of the metal, used for crayfish: As⁺³ (40,80,120,160,200) from As₂O₃; Hg (5,10,20,40,80) from HgCl₂; Cd (10,25,50, 75,100) from CdCl₂; Cr⁺⁶ (200,400,600,800,1000) from CrCl₃·6H₂O. For the bluegills, the test concentrations used are: Hg (0.1,0.2,0.4,0.6, 0.8); Cd (5,7.5,10,15,20).

Temperature throughout the experiment was 18.5 ± 1°C. The dissolved oxygen of the dilution water was 90-100% of saturation at the start of each renewal period.

The test organisms were examined and water parameters were measured daily. Dead crayfish or bluegills were removed promptly. Death was defined as cessation of movement, especially of the antennae and pleopods for the crayfish.

Results and Discussion

Preliminary results of the crayfish bioassays show the heavy metal toxicity in this order: Hg > Cd > As⁺³. The approximate LC₅₀ values for *Procambarus spp.* crayfish for mercury is 9 ppm, cadmium is 34 ppm, arsenic(III) is 43 ppm. The range-finding tests indicate that the LC₅₀ for chromium(VI) and arsenic(V) are approximately to 310 ppm and 400 ppm respectively. The final results of the acute toxicity of these five metals plus lead will be completed in the first few months of 1994.

For bluegills, the acute toxicity of two metals has been completed. The LC₅₀ of cadmium and mercury were measured to be 6.1 ppm and 0.4 ppm respectively. This shows the same trend of mercury being more toxic than cadmium as seen with the crayfish. The LC₅₀ values are much lower for bluegills than for crayfish as would be expected.

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Levels of Chemical Contaminants in Devil's Swamp Environment

Summary

Initial data generated on metal analysis from Devil's Swamp in Baton Rouge, Louisiana show that the metals cadmium and lead are present in levels exceeding EPA and DEQ regulatory limits, especially found in aquatic organisms (USEPA, 1991) These high concentrations are believed to have migrated from an adjacent Superfund site established to the norht of Devil's Swamp known as Brooklawn.

In contrast, a previous ecorisk assessment by NPC, Inc., showed no heavy metal buildup in soils surrounding Devil's Swamp but did find organic buildup of hexachlorobutadiene (HCBd) and hexachlorobutane (HCB). An in-depth investigation consists of exposure studies involving, Toxicity Characteristic Leaching Procedure, Elutriation Tests, Bulk Density Analysis, with analysis by Atomic Absorption, Inductively Coupled Plasma Emission, and Gas Chromatography/Mass Spectroscopy. Along with this analysis, a Mass Transfer Sorption Column Study will be performed.

Introduction

In September 1993, an investigation began to assess the presence of pollutants in the Devil's Swamp Area northeast of Baton Rouge, Louisiana. Devil's Swamp is a fifty square mile river basin swamp which is bordered by the Mississippi River and Bayou Baton Rouge. The area being assessed is adjacent to a Superfund site established to the north known as Brooklawn which was used for both recreational and commercial purposes. The biological fate and transport is being studied.

The investigation is being conducted to determine levels of metals: arsenic, cadmium, chromium, lead, mercury, and the organics: hexachlorobutadiene and hexachlorobutane, and inorganic compounds, in environmental samples including: water, sediment, soil, vegetation, tree leaves and cores, terrestrial vegetation, fish and benthic organisms, birds' blood and birds' feathers. Testing is being performed at the Department of Environmental Health Sciences, School of Public Health and Tropical Medicine, Tulane Medical Center by personnel from both Tulane and Xavier Universities.

Toxicity, uptake, storage, and elimination studies are in progress on aquatic organisms collected from the swamp. In addition abiotic studies including chemical adsorption capacities of Devil's Swamp soil are in progress.

Background

Devil's Swamp is located in East Baton Rouge parish and is bordered by the Mississippi River and Bayou Baton Rouge. It is bordered on the north by the Brooklawn site of Petro Processors, Inc. (PPI). In the past, Devil's Swamp was used for recreational and commercial purposes such as fishing and swimming. Presently it is partially contaminated with volatile aromatic hydrocarbons, volatile chlorinated hydrocarbons, polyaromatic hydrocarbons, and metals. Of particular concern is the high concentration of hexachlorobenzene (HCB) and hexachlorobutadiene (HCBd). To better understand the source of contamination the following is a history of activities noted in the area around the swamp, particularly the history of Petro Processors, Inc.

- 1959, The McVea Family, owners of the property, leased a 20 ft "borrow" pit to a local company to use as an industrial waste dump. Various waste products, including polyethylene, rubber, and carbon black, were placed in the pit.
- June 1964, PPI purchased the property from the McVea family.
- October 1964, PPI began operations as an industrial waste dumping facility. Non-hazardous and hazardous waste, including HCB, HCBd, scrap rubber, plastic, concrete, chlorinated solvents and organics were dumped in the pit.
- December 1964, levees were installed to prevent overflow of the waste pit.
- April 1965, the East Baton Rouge Parish Health Unit asks the Louisiana State Board of Health to investigate the PPI chemical waste discharge site.
- November 1966, a state inspection revealed black tar material being unloaded in the facility. Wash water used to clean trucks flowing away from the site. Also a fire burning at the site was producing offensive and irritating fumes.
- November 1968, a routine inspection by the Louisiana Wildlife and Fisheries Division of Water Pollution Control revealed: a) the pit overflowing, b) Trash trucks dumping to raise the levee, and c) the pit considered a primary source of constant pollution.

- November 1969, a state inspection noted leaking pits and waste was sited in Bayou Baton Rouge.
- December 1969, a levee broke at the site dumping thousands of gallons of waste in Bayou Baton Rouge. Over 100 head of cattle died in the area within a few days. A "cease and desist order" was issued. PPI resumed business within a few weeks.
- 1971, a report entitled "Waste Discharge from Petro Processors, Inc." is released.
- June 1974, PPI received permission to continue to handle solid industrial wastes.
- 1975, Civil damages awarded to the Ewell family for suit filed in 1970 after cattle died. The Ewells were awarded \$30,000.
- 1979, PPI's permit is revoked due to violations.
- 1980, the United States of America, The State of Louisiana, The Parish of East Baton Rouge, and the City of Baton Rouge Filed suit against PPI, U.S. Steel, Copolymer, UniRoyal, Dow, Ethyl, Shell, American Hoechst, Exxon, Exxon Chemical, Allied, and Rubicon for violation of EPA and local pollution regulations.
- 1984, The site is declared a Superfund site and Remedial Planning Investigation begins.
- 1986, Devil's Swamp Hazard Ranking System package was submitted to the EPA. Due to scoring the site does not make the National Priority List. The EPA approves the Remedial Planning Activities Report.
- 1988, A Supplemental Remedial Plan is submitted due to leakage problems.
- 1989, The consent decree is amended to provide for the implementation of a new cap/pump/treat remedy.
- 1990 NPC submitted a work plan covering site preparation and earthwork at the facility.
- 1993, An ecorisk assessment conducted by NPC show organisms in and around the site exceed EPA and DEQ action levels for HCB and HCB.

An assessment is currently being conducted to determine the environmental fate of metals and organic compounds in the area of Devil's Swamp surrounding the Superfund fund site.

Table 1 Shows levels of metals in Devil's Swamp soil by NPC Services.

Table 1

Summary of Metals in the Soil of Devil's Swamp
(Preliminary Results from NPC Services 1992-3 Ecorisk Assessment)

	Arsenic (mg/kg)	Cadmium (mg/kg)	Chromium(m g/kg)	Lead (mg/kg)	Mercury (mg/kg)	Zinc (mg/kg)
Action ¹ Levels	20	40	40 0	5 00	20	7 0
Background ² Levels	0.7	3	5	3 0	1	1
Range	<0. 45 to 11.7	< 0.35 to 23.4	2. 6 to 14.6	8. 7 to 38	< 0.02 to 16	5. 3 to 68
Mean	2.1 9	2. 39	10 .0	4 2.32	0. 07	3 5.1

¹ Federal Register, 1992

² Federal Register, 1992

Materials and Methods

Environmental samples including water, sediment, fish, crawfish, etc. were collected by the ecology subcluster headed by Dr. Bart according to the following quality assurance/quality control procedures prepared by Dr. Abdelghani's group.

(1) Sampling Sites Selection:

Before samples were collected, sampling points were selected following an initial visit to Devil's Swamp. These points were chosen to determine concentration of the metals: arsenic, cadmium, chromium, lead, and mercury in the relation to the surrounding river outlets. Two samples each of: water, composite sediment, soil (core), fish, terrestrial vegetation and leaves surrounding the swamp were collected.

(2) Equipment:

- a) Boat with electric shocking device
- b) Ekman Grab Sediment Sampler (acid washed)
- c) Precision Scientific Water Sampler (acid washed)
- d) Precision Scientific Core Sampler (acid washed)
- e) Type II Deionized Water
- f) Stainless Steel Bowls (acid washed)
- g) Miscellaneous equipment (rope, markers, labels, ice, nitric acid, sterile bags).

** Acid wash = 10% nitric acid qs with Type II deionized water.

(3) Containers:

- a) Containers used for collection were the Texberry 32 oz., wide mouth STRT side glass jar with phenolic caps P/AF.
- b) Prior to sampling, each jar was rinsed with Type II deionized water, 10% nitric acid solution and rinsed again with Type II deionized water.

c) To ensure sample integrity from collection to data reporting each sample was issued a chain of custody. On each container label was affixed prior to collection and after collection, each container was sealed and placed in an ice chest.

(4) Sample Collection and Transport:

Quality control of samples was ensured by the use of a field blank consisting of Type II deionized water placed in the sample jars. The field blank was opened in the boat and shows any potential contamination which could affect the sample. In between each collection, all equipment was acid rinsed and rubber gloves were used to protect from any cross contamination of samples. All samples were placed on ice during transportation to the ENHS Laboratories in New Orleans.

(5) Sample Preparation and Analysis:

All samples were acidified using nitric acid to a pH < 2. They were digested as follows:

(a) Aqueous Samples

This method provides for the acid digestion of water following a modified version of the SW 846 Method 3015 in a closed vessel using pressure controlled microwave heating for the determination of metals by spectroscopic methods.

Procedure:

1. Measure 40 ml of sample and 5 ml of HNO₃ into each vessel.
2. Seal all vessels.
3. Place the vessels into the turntable. Connect the vent tubes from the vessels to the collection vessel.
4. Place the turntable into the system. Connect the pressure sensing lines to the control vessel.
5. Program as follows:

STAGE	(1)	(2)
% POWER	60	60
PSI	35	70
TIME	20:00	30:00
TAP	5:00	30:00
FAN SPEED	100	100

6. Run the heating program to completion.
7. Cool the samples for a minimum of 5 minutes then manually vent.
8. Transfer the solution to a storage container then analyze.

(b) Sediment and Soil (Core)

This method provides for the acid digestion of sediment in a closed vessel using pressure controlled microwave heating for the determination of metals by spectroscopic methods.

Procedure:

1. Weigh 0.5 gm of sample into each vessel. Add 10 ml of deionized water, 5 ml of HNO₃, 4 ml of HF and 1 ml of HCl to each vessel.
2. Seal all vessels.
3. Place the vessels into the turntable. Connect the vent tubes from the vessels to the collection vessel.
4. Place the turntable into the microwave. Connect the pressure sensing line to the control vessel.
5. Program the microwave as follows:

STAGE	(1)
% POWER	100
PSI	120
TIME	30:00
TAP	20:00

FAN SPEED 100

6. Run the program to completion.
7. Cool the samples for a minimum of 5 minutes then manually vent the vessels.
8. Add approximately 2 gm of Boric acid crystals to each vessel. mix the samples well.
9. Transfer the solution to a flask, with a filtration step if needed, bring up to volume.

(c) Fish Tissue

This method provides for the acid digestion of fish tissue and fish fat in a closed vessel using pressure controlled microwave heating for the determination of metals by spectroscopic methods.

Procedure:

1. Weigh 0.5 gm of sample into each vessel. Add 10 ml HNO_3 each vessel.
2. Seal all vessels.
3. Place the vessels into the turntable. Connect the vent tubes from the vessels to the collection vessel.
4. Place the turntable into the microwave. Connect the pressure sensing line to the control vessel.
5. Program the microwave as follows:

STAGE	(1)	(2)	(3)	(4)	(5)
% POWER	40	40	40	40	40
PSI	20	40	85	135	175
TIME	10:00	10:00	10:00	10:00	10:00
TAP	5:00	5:00	5:00	5:00	5:00
FAN SPEED	100	100	100	100	100
6. Run the program to completion.
7. Cool the samples for a minimum of 5 minutes then manually vent the vessels.
8. Transfer the samples to a storage container then analyze.

(d) Leaves and Vegetation

This method provides for the acid digestion of leaves and vegetation in a closed vessel using pressure controlled microwave heating for the determination of metals by spectroscopic methods.

Procedure:

1. Weigh 0.5 gm of sample into each vessel. Add 2ml of deionized water, 5 ml HNO_3 and 1 ml of HF to each vessel.
2. Seal all vessels.
3. Place the vessels into the turntable. Connect the vent tubes from the vessels to the collection vessel.
4. Place the turntable into the microwave. Connect the pressure sensing line to the control vessel.
5. Program the microwave as follows:

STAGE	(1)	(2)	(3)
% POWER	100	100	100
PSI	40	85	150
TIME	6:00	6:00	10:00
TAP	3:00	3:00	5:00
FAN SPEED	100	100	100
6. Run the program to completion.
7. Cool the samples for a minimum of 5 minutes then manually vent the vessels.
8. Transfer the samples to a storage container then analyze.

(6) Sample Analysis:

Each sample of water, composite sediment, soil (core), terrestrial vegetation, leaves and fish were analyzed for arsenic, cadmium, chromium, lead, and mercury by graphite furnace atomic absorption spectroscopy according to Standard Methods, 1993.

<u>Metals</u>	<u>Method</u>
Arsenic (As)	3113
Cadmium (Cd)	3113
Chromium (Cr)	3113
Lead (Pb)	3113
Mercury (Hg)	3500

(7) Analysis QA/QC

Along with the analysis of samples, blanks were also run through the same method of analysis. A standard curve was made of each metal analyzed by atomic absorption with several solution concentrations encompassing the concentrations found in the sample. To test for interference from the matrix, samples were randomly spiked with standard concentrations of the analyte to be tested and analyzed along with the sample.

Results and Discussion:

Devil's Swamp and it's surrounding area have been shown to contain levels exceeding EPA and DEQ criteria for the metals, cadmium and lead. Preliminary data elucidates that aquatic organisms (small fish, catfish, gar, shad, and crawfish) contain concentrations of cadmium and lead in excess of regulatory limits (Figures, Appendix A) (USEPA, 1991). Also, the data shows concentrations of cadmium and lead to exceed regulatory criteria in water, composite sediment and soil (core). Uncertain of the exact origin of these high levels, our group believes the adjacent Superfund site is the major contributor to such increased concentrations of cadmium and lead.

Interesting, in 1992-93 NPC, INC. conducted an ecorisk assessment of Devil's Swamp and found no high levels of any metals in or around the region (Table 2-3), although they did find increased concentrations of the organics, hexachlorobutadiene and hexachlorobutane.

Over the next three years, Tulane/Xavier Universities will thoroughly investigate the origin, biological fate and transport of metals and organics in Devil's Swamp and its surrounding area.

Table 2 Concentration of Cadmium in Devil's Swamp Oct 93 (µg/L)		
Number	Sample	Concentration
1	Blank	0.00
2	Standard 1	20.00
3	Standard 2	40.00
4	Standard 3	60.00
5	Standard 4	80.00
6	Standard 5	100.00
7	Sample Blank	0.00
8	Gar 1	2381.61
9	Gar 2	29.24
10	Gar 3	87.82
11	40 ppb STD	45.21
12	Smallfish 1A	68.64
13	Smallfish 1B	60.51
14	Smallfish 2B	57.48
15	Smallfish 2A	36.67
16	Catfish 1A	61.76
17	Crawfish Muscle A	3587
18	Duckweed A	75.20
19	Duckweed B	66.01
20	20 ppb Std	27.62
21	Core	1256.32
22	Catfish 3A	3874.71
23	Sed 9	1943
24	Sed 4	1152.87
25	Terr Veg A	5257
26	Crawfish Fat A	2002.30
27	Terr Veg B	1001.15
28	Shad A	1697.70
29	Leaves	1126.44
30	Water - 4 TOTAL	33.09
31	Water - 4 DISS	30.36
32	WATER - 10 TOTAL	184.18
33	WATER _ 10 DISS	22.73

Table 3 Concentration of Lead in Devil's Swamp Oct 93
($\mu\text{g/L}$)

Number	Sample	Concentration
1	Blank	0.00
2	Standard 1	40.00
3	Standard 2	80.00
4	Standard 3	120.00
5	Standard 4	160.00
6	Standard 5	200.00
7	Blank 2	0.00
8	Catfish 1A	20.52
9	Catfish 3A	410.92
10	Catfish 5A	286.55
11	Duckweed	3.56
12	Terr Veg	24.23
13	10 ppb	10.04
14	Leaves	12.28
15	Core	2285.17
16	Sediment 4	635.53
17	Sediment 9	1416.55
18	Crawfish Fat	31.0
19	80 ppb	65.39
20	Crawfish mus	87.3
21	Sm. fish 1A	87.3
22	Sm. fish 2A	17.74
23	Shad	136.14
24	120 ppb	98.65
25	Gar	93.87
26	Frog Mus. 1	3.57
27	Frog Mus. 2	3.94
28	Frog Mus. 3	5.98
29	Frog Mus. 4	5.12
30	Frog Mus. 5	5.47
31	Frog Mus. 6	11.29
32	Frog Mus. 7	4.05
33	Frog Fat 8	4.05
34	200 ppb	192.11
35	Frog Fat 9	2.79
36	Frog Fat 10	3.27
37	Frog Fat 11	1.77
38	Frog Fat 12	2.72
39	Frog Fat 13	0.00
40	80 ppb	63.7
41	Frog Fat 14	0.00
42	Frog Liver 15	1.16
43	Frog Liver 16	0.69
44	Frog Liver 17	0.03
45	120 ppb	117.92
46	Frog Liver 18	0.59
47	Frog Liver 19	3.62

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APPENDIX A

DOE Devil's Swamp - Cadmium Standard

A-1

DOE Devil's Swamp - Cadmium (Water - Oct 1993)

A-2

DOE Devil's Swamp - Cadmium (Soil - Oct 1993)

A-3

DOE Devil's Swamp - Cadmium (Vegetation - Oct 1993)

A-4

Cadmium found in Devil's Swamp (Smallfish - Oct 1993)

A-5

DOE Devil's Swamp - Cadmium (Crawfish - Oct 1993)

A-6

DOE Devil's Swamp - Cadmium (Catfish - Oct 1993)

A-7

DOE Devil's Swamp - Cadmium (Gar - Oct 1993)

A-8

DOE Devil's Swamp - Lead Standards

A-9

DOE Devil's Swamp - Lead Catfish - Oct 93

A-10

DOE Devil's Swamp - Lead Crawfish - Oct 93

A-11

DOE Devil's Swamp - Lead Fish - Oct

A-12

A-13

A-14

A-15

A-16

Cadmium in Soil of Devil's Swamp (NPC Services 1992-3 Ecorisk Assessment)

A-17

Chromium in Soil of Devil's Swamp (NPC Services 1992-3 Ecorisk Assessment)

A-18

Lead in Soil of Devil's Swamp (NPC Services 1992-3 Ecorisk Assessment)

A-19

Zinc in Soil of Devil's Swamp (NPC Services 1992-3 Ecorisk Assessment)

A-20

Assessment of Mechanisms of Metal-Induced Reproductive Toxicity in Aquatic Species as a Biomarker of Exposure

M. Anderson, W. George, S. Sikka, B. Kamath,
J. Preslan, K. Agrawal, A. Rege

This project is designed to identify heavy metals and organic contaminants of concern which could impact on the biota in the Louisiana wetlands by assessment of uptake and bioaccumulation of contaminants and their effects on reproductive processes as biomarkers of exposure. Heavy metals (lead, cadmium, cobalt, and mercury) have been demonstrated to have toxic effects on reproduction in mammals and several aquatic species. Hexachlorobenzene (HCB) is a persistent environmental contaminant which has been measured in human serum, fat, semen, and follicular fluid. HCB has been shown to be a reproductive toxin in rats and primates. Polychlorinated biphenyls (PCBs) are prevalent chlorinated hydrocarbons currently contaminating our environment. PCBs resist degradation and are insoluble in water; however, they bioaccumulate in aquatic species. Disturbances of the reproductive systems are not only sensitive indicators of toxicity but threatens the propagation of a species.

Bayou Trepagnier

Bayou Trepagnier, designated a "natural and scenic stream" within the Natural and Scenic River System Act of 1970", is located in St. Charles Parish, Louisiana (Figure 1). The headwaters of the bayou originate at the Shell Oil Manufacturing Complex. Bayou Trepagnier serves as the receiving stream for the large volumes of water used in the many different processes of the Shell Complex. The outflow from the Shell Complex has been the primary source of flow in Bayou Trepagnier since 1966. Bayou Trepagnier flows in a northeasterly direction through a cypress-tupelo swamp. The stream, which has a very small drainage area, is about 3 1/2 miles in length and is bounded on the west by Engineer's Canal which borders the Bonnet Carre spillway, on the south by the Mississippi River flood control levee, and on the north by Lake Ponchartrain. Bayou Trepagnier joins Bayou La Branche which then flows about 1 mile through an intermediate marsh before entering Lake Pontchartrain. Bayou Trepagnier is identified as a portion of the Lake Pontchartrain Basin by the Louisiana Water Quality Management Plan. The Trepagnier-La Branche system is subject to tidal influences with fresh water at its upper end and brackish water near its drainage into Lake Pontchartrain. Tidal actions in Lake Pontchartrain affect the water levels and flow in the Trepagnier-La Branche system. Thus, extreme tidal changes can result in an increase in problems associated with contaminants and disruption of biological ecosystems.

Bayou Trepagnier was selected for study based upon its known contamination by metals, oil and grease. The Water Pollution Control Division of the Louisiana Department of Environmental Quality conducted an investigative survey of Bayou Trepagnier in July 1985. Their analyses of soil sediments revealed a high concentration of oil and grease, chromium, and lead. Sulfide odors were also noted. As a result of this initial investigation, a full study of Bayou Trepagnier was conducted by the Water Pollution Control Division of the Louisiana DEQ from May 1986 to March 1987 (1). They selected monitoring stations located at or near the headwaters of the stream and near its termination where it drains into Bayou La Branche. They evaluated water quality, characterized sediments, and evaluated the aquatic life present. Water analyses detected zinc and chromium but they were not considered high. Sediments were analyzed for metals, phenols, and oil and grease. Elevated levels of zinc, chromium, and lead were found in the sediments. Highest concentrations were observed at the headwaters of the stream at monitoring stations T3 and T4 (Cr, 69 to 8,164 ppm; Pb, 278 to 8,114 ppm; Zn, 69 to 7,785 ppm). These data indicate that zinc, chromium and lead have accumulated in the bottom sediments as a result of industrial discharge. Evaluation of the aquatic life in the Bayou revealed a disturbance in both the macroinvertebrate life

forms and the fish communities. The most widely distributed and abundant fishes observed to be present were mosquitofish (*Gambusia affinis*), sailfin molly (*Poecilia latipinna*), and sheepshead minnows (*Cyprinodon variegatus*). The absence of reptilian life was also reported.

In June of 1993, we explored the full length of Bayou Trepagnier by boat and by foot from its confluence with Bayou LaBranche to its headwaters just below the construction site for the new Shell Refinery hurricane protection levee. Eight sites along its length were originally sampled (Figure 1).

- Site 1: Headwaters
- Site 2: Outflow to engineers canal
- Site 3: Upstream inflow
- Site 4: Downstream inflow
- Site 5: Hard woods
- Site 6: Soft woods
- Site 7: Grassy marsh
- Site 8: Confluence with Bayou LaBranche

Site 1 is located close to the DEQ monitoring station T4.

Site 2 is located close to the DEQ monitoring station T5.

Site 7 is located close to the DEQ monitoring station T6.

Sites 3, 4, 5, or 6 are not located near any DEQ monitoring stations. Sites 3 and 4, near the midpoint of the stream where inflow streams are located, drain wetlands adjacent to the refineries and chemical plants of the town of Norco, Louisiana.

Five of these sites (1, 3, 4, 7, and 8) were selected for monitoring during the course of this study. Water, sediment and biological samples were collected in June according to EPA protocols.

Table I
Water Conditions in Bayou Trepagnier in June 1993

	<u>Headwaters</u>	<u>Upstream</u>	<u>Downstream</u>	<u>Grassy</u>	<u>At Bayou</u>
		<u>Flow</u>	<u>Flow</u>	<u>Marsh</u>	<u>La Branch</u>
Temp (°C)	30.0	33.1	32.6	31.4	30.2
Dissolved solids (g/L)	1.22	1.43	1.41	1.37	1.17
Conductivity	2.45	2.82	2.82	2.74	2.33

There are no significant differences among the 5 sites for the parameters measured.

Sediment digests from the 5 sites were prepared according to EPA Method 3050 and were analyzed by inductively coupled plasma emission spectroscopy (ICP).

Table II
Concentration of Metals
in Sediments from Bayou Trepagnier
(mg metal/kg sediment)
June 1993

<u>Metal</u>	<u>Headwaters</u>	<u>Upstream</u> <u>Flow</u>	<u>Downstream</u> <u>Flow</u>	<u>Grassy</u> <u>Marsh</u>	<u>Bayou</u> <u>La Branche</u>
Iron	>6000	3312	5586	>6000	5920
Aluminum	7044	1265	2778	7473	3284
Lead	605	256	499	41	34
Chromium	48	34	315	37	29
Manganese	229	41	85	263	175
Zinc	160	88	163	111	97
Vanadium	17	5	10	15	9
Cobalt	15	4	7	13	8
Nickel	13	3	6	11	7
Copper	12	5	11	13	6
Selenium	Present at low levels				
Arsenic	Present at low levels				
Cadmium	All values less than practical quantitation for ICP				
Beryllium	All values less than practical quantitation for ICP				

Control values for each metal were within 13% of nominal value. Recovery of pre-digestion aqueous spike (Cr, Pb, and Zn) was greater than 97%.

Iron and aluminum were found to be in high concentrations at all sites. These metals were not reported by the DEQ in 1989. Lead, chromium, manganese and zinc were also present in significant amounts. While the current values for lead, zinc and chromium fall within the range of values previously reported by the DEQ, we did not observe the higher concentrations which they reported. The lowest metal concentrations were observed at site 3 (upstream inflow). There is a substantial outflow of water from Bayou Trepagnier into Engineer's Canal at site 2, which may be a contributing factor to the lower concentrations of metals observed at site 3. However, at site 4 (downstream inflow) the concentrations of the metals in the sediments increase again. At sites 3 and 4 there is a strong, volatile odor recognized as sulfur and possibly organics. In addition, these two sites are essentially void of aquatic life. The findings at sites 3 and 4 are new in that they were not reported in the 1989 DEQ report on Bayou Trepagnier.

Water samples were taken from the 5 sites and were screened for the same metals as the sediments. Iron and manganese were the only metals found to be in high enough concentrations to be within detectable range for measurement. Maximum observed concentrations for iron and manganese were 2110 ppb and 622 ppb, respectively, at the site 3 (upstream inflow). Zinc and chromium which were measured by the DEQ in 1989 were not detected in this study.

Methylene chloride extracts of the soils from the 5 sites were analyzed by gas chromatography/mass spectroscopy (GC/MS) and purge and trap flame ionization detector gas chromatography (Figure 2). Saturated and unsaturated hydrocarbons over the 4 to 20 carbon chain length and sulfur were the most abundant constituents (Figure 3). While some hydrocarbons and a large quantity of sulfur were present in all sediments collected, the highest concentrations were found at sites 1 and 4 (Figures 2 and 3). Absent were the light weight substituted benzenes that are characteristic of gasoline. Oil and grease, and non-volatile petroleum constituents may also be significant contaminants in these soils.

The same species of fish were observed as reported in the 1989 DEQ report and the absence of reptilian life was still quite evident. Mosquitofish (killifish) were collected throughout the Bayou for whole body analyses of hydrocarbons. The fish were found to contain 8 different chemical compounds with chemical library matches to substituted forms of octane, hexadecane, cycloundecane, hexene, undecene, hexacosane, heptadecane, and pentatriacontane (Figure 4).

Laboratory Studies

Exposure of Crayfish to Lead or Chromium

Materials and Methods:

Red swamp crayfish (*Procambarus clarkii*) were purchased from a local vendor. They were separated according to sex and size. Only those with a carapace measuring 20 to 48mm from the tip of the rostrum to the posterior margin of the carapace were included in the study. Twenty to twenty-four crayfish of one sex were kept in a plastic aquarium containing 4 liters of tap water, which resulted in a water level of approximately 1 1/2 inches. Each aquarium was aerated using an air pump with a air dispersal bar. All crayfish were maintained under controlled conditions of temperature (24°C) and light with a light/dark cycle of 12 hours each. After two weeks of being kept in the above conditons, the crayfish were used for metal-exposure studies.

Based upon the results of previous metal analyses of Bayou Trepagnier, two metals (lead and chromium) were selected for these initial studies. There were two experimental groups and one control group for both males and females.

Group I (Pb): Crayfish were maintained in water containing 150 ppb lead which was prepared using lead nitrate, $[Pb(NO_3)_2]$, dissolved in tap water.

Group II (Cr): Crayfish were maintained in water containing 150 ppb chromium which was prepared using potassium dichromate, $[K_2Cr_2O_7]$, dissolved in tap water.

Group III (Cont): Crayfish were maintained in tap water to which no metal salt had been added.

Water solutions in which the crayfish were maintained, were prepared fresh and changed on a daily basis over a 7 week period. Twice a week water samples were collected at the time of preparation of the water solutions and at the end of a 24 hour exposure period to monitor the metal concentrations in the water. Crayfish were evaluated at the end of 4 weeks and 7 weeks. At the end of the exposure-periods, crayfish were weighed and hemolymph was collected for determination of metal concentrations. Hemolymph was collected using a 3cc heparinized syringe and 22 gauge needle which is inserted into the space under the carapace. The crayfish were then sacrificed by decapitation. Gonads, hepatopancreases, gills, and abdominal muscle were collected either for determination of tissue metal concentrations or for histological study. Those tissues for metal determinations were placed in plastic tubes and stored in a freezer at -20°C. They were subsequently microwave digested and analyzed by atomic absorption spectrometry. Tissues for histology were immediately placed in 10% buffered formalin. After at least 48 hours of fixation, tissues were washed in tap water, dehydrated in increasing concentrations of ethanol, cleared in xylene, and infiltrated and embedded in paraffin. Tissues were sectioned on a rotating paraffin microtome, affixed to clean glass slides and stained with either hematoxylin and eosin (H and E) or periodic acid Schiffs (PAS) reagent.

Results

Organ weights are expressed as percent of body weight and are designated as the organ index:

$$\text{Index No.} = \frac{\text{Organ weight (g)}}{\text{Body weight of crayfish}} \times 100$$

Table III
Body Weights and Organ Indices of Metal-Exposed Crayfish
After 4 Weeks of Exposure

	<u>Body Wts (g)</u>	<u>Gonadal Index</u>	<u>Hepatopancreas Index</u>
<u>FEMALES</u>			
Control (N=10)	19.96 ± 0.84	2.71 ± 0.28	4.17 ± 0.27
Lead (N=8)	18.81 ± 1.43	3.95 ± 0.29*	5.20 ± 0.61
Chromium (N=10)	16.52 ± 1.29	2.89 ± 0.39	4.43 ± 0.31
<u>MALES</u>			
Control (N=7)	19.96 ± 0.64	0.043 ± 0.005	3.92 ± 0.35
Lead (N=9)	18.81 ± 1.41	0.062 ± 0.011	3.62 ± 0.24
Chromium (N=9)	17.77 ± 1.40	0.072 ± 0.022	4.12 ± 0.43

Values are expressed as mean ± S.E.M.

*There is a significant increase in the ovarian index of the lead treated group when compared to the control group, P<.01.

Table IV
Body Weights and Organ Indices of Metal-Exposed Crayfish
After 7 Weeks of Exposure

	<u>Body Wts (g)</u>	<u>Gonadal Index</u>	<u>Hepatopancrease Index</u>
<u>FEMALES</u>			
Control (N=5)	20.53 ± 3.04	3.24 ± 0.37	3.43 ± 0.24
Lead (N=8)	18.47 ± 0.97	3.55 ± 0.50	4.02 ± 0.18
Chromium (N=10)	18.64 ± 1.18	3.25 ± 0.37	3.68 ± 0.28
<u>MALES</u>			
Control (N=5)	25.60 ± 3.62	0.017 ± .002	3.60 ± 0.44
Lead (N=3)	17.13 ± 0.82	0.073 ± .058	2.96 ± 0.56
Chromium (N=3)	16.37 ± 1.15	0.054 ± .002	2.83 ± 0.56

Values are expressed as Mean ± S.E.M.

There are no significant differences in body weights, gonadal indices, or hepatopancreas indices in the 7 week study.

The ovarian index for both the 4 and 7 week studies indicate that oocyte development for the control group, 150 ppb lead group and the 150 ppb chromium group are at the midvitellogenic or late vitellogenic stages of development (2). In fact, one female in each of the three groups had released her eggs and the eggs were attached to their abdomens. The eggs were black indicating that they had been fertilized (3). Current histological study of the ovaries should confirm these findings. It appears exposure to low concentrations of lead (150 ppb) or chromium (150 ppb) for 7 weeks does not interfere with oocyte development.

Table V
Lead Concentrations in Gills of Crayfish
Exposed to 150 ppb Lead
($\mu\text{g Pb/gm}$ gill tissue)

	<u>4 Week Exposure</u>	<u>7 Week Exposure</u>
<u>FEMALES</u>		
Lead Group	188.8 $\pm$ 48.3 (N=4)*	521.8 $\pm$ 156.7 (N=5)*
Control Group	1.5 $\pm$ 0.7 (N=4)	0.5 $\pm$ 0.4 (N=6)
<u>MALES</u>		
Lead Group	296.0 $\pm$ 80.0 (N=4)*	190.1 $\pm$ 23.8 (N=3)*
Control Group	3.7 $\pm$ 3.1 (N=3)	1.2 $\pm$ 0.7 (N=5)

Values are expressed as mean $\pm$ S.E.M.

*Lead groups are significantly different from the corresponding control groups, $P < .01$.

Tap water contains >5 ppb lead.

There are no significant differences between males and females in lead accumulation in the gills after 4 or 7 weeks of exposure.

Metal analyses for lead and chromium concentrations in other tissues are currently in progress.

The hepatopaneas is the major digestive gland of the crayfish. Most of the digested food passes into the hepatopaneas and is absorbed there (3). Histological study of this tri-lobed structure demonstrated a central lumen into which the tubular structures of this organ open. These tubular structures appear to be lined by a stratified or pseudostratified epithelium composed of several different cell types. Some cells appear to be secretory. They contain large amounts of a basophilic material in H and E stained sections. PAS positive material, indicative of carbohydrate moieties, was also observed in these cells. Some areas contain cyst-like structures which contain few cells and a flocculent type of material. The appearance of these structures suggests that they are fluid filled cavities containing a proteinaceous material. These areas may represent the areas involved in absorption. There is very little connective tissue present. Endothelial lined vessels are seen running through the hepatopaneas. There appears to be no overt pathology in the hepatopaneases among the different treatment groups after 4 weeks of metal-exposure. Results of the histological study of the hepatopaneas from the 7 week study are forthcoming.

Statistical Analyses

Data were analyzed statistically using a non-paired t-test for single group analyses. Analysis of variance (ANOVA) test is utilized for multigroup data followed by Scheffe's test for post-hoc comparisons.

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FIGURE 3
ANALYSIS OF SEDIMENT EXTRACT FROM BAYOU
TREPAGNIER HEADWATERS WITH CHARACTERISTIC
PETROLEUM SPECTRUM
SITE 1

FIGURE 4

EXTRACT OF KILLIFISH FROM BAYOU TREPAGNIER

Peaks with high percentage chemical library matches to substituted forms of octane, hexadecane, cycloundecane, hexene, undecene, hexacosane, heptadecane, and pentatriacontane.

Bioremediation of Selected Contaminants in Aquatic Environments of the Mississippi River Basin

**S. Bhattacharya, J. Bennett, A.J. Englands, V. Law, D. Mullin, H. Mielke,
J. Eckert, R. Fulginiti, B. Kamath, J. Ross**

Executive Summary

This bioremediation cluster includes several researchers from Tulane and Xavier representing departments of Biology, Cell and Molecular Biology, Chemical Engineering, Civil and Environmental Engineering, Environmental Health Sciences, and Pharmacy. Bioremediation is generally accepted as a long-term and economic treatment option. However, quantitative information on bioremediation and biosorption is required before this option can be adopted successfully. The primary goal of this on-going project is to determine the extent of natural biodegradation of hazardous organics and biosorption of hazardous organics and heavy metals by the consortia of bacteria (aerobic, anaerobic, sulfate reducing, methanotrophic, etc.), fungi (mycorrhizal, white rot, etc.), and plants. Methods to enhance the biodegradation process will be studied during the second and third years of this 3-year proposed project. The Devil's Swamp area near Baton Rouge and Bayou St. John in New Orleans have been selected as the first set of test sites. Some samples from Lake Pontchartrain, bordering New Orleans on the north, have also been analyzed.

It is expected that many of the contaminants found at the test site(s) are present at other sites of DOE's interest. Further, technology resulting from the proposed research involving enhanced natural biodegradation processes should be transferable to other DOE sites.

During the first few months (Year 1), field samples have been collected from selected sampling points. Appendix 1 shows maps of Devil's Swamp, Bayou St. John, and Lake Pontchartrain with the sampling points marked.

Anaerobic serum bottle studies were performed to determine the toxicity of selected organic compounds found in the samples. Appendix 2 contains a report sent to Dr. Tommy Phelps at Oak Ridge National Laboratory relative to some of this work. Figures 1 to 41, Appendix 3, are related to the anaerobic studies, with Figures 1 to 38 relating more specifically to the report in Appendix 2. As part of the anaerobic study, the focus was on isolating the organisms which could tolerate carbon tetrachloride. *Pseudomonas cepacia* appears to be the predominant organism in these samples.

Twenty species of white rot fungi and three molds have been analyzed for the ability to decolorize the polymeric dye, R-481. Decolorization of this dye is dependent on lignin-degrading enzymes useful in the biodegradation of various xenobiotics.

The schematic for the strategy for altering substrate specificity of cytochrome is shown in Figure 42, Appendix 3. Site-directed mutagenesis was used to introduce amino acid substitutions into the first eight amino acid residues at the amino terminal end of the protein, and thus far 169 mutants have been constructed, some of which have multiple amino acid substitutions. The wild type BM-3 and two mutant proteins have been purified, and a mutation of M5R was shown to alter the ability of laurate to bind to BM-3.

Samples of sediments from Orleans and Harrison Avenue bridges over Bayou St. John in New Orleans were taken on two separate dates and analyzed for heavy metals as a function of depth. The results are shown in Figures 43-44, Appendix 3. Heavy metals analysis results of Lake Pontchartrain sediments are shown in Figure 45, Appendix 3.

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Relative to a goal of developing a rapid, sensitive, and specific method to quantitatively detect polycyclic aromatic hydrocarbons in solution, the polymerase chain reactions (PCR) for amplification of a 600 bp region of the cloned human *c-myc* proto-oncogene have been optimized. A linear response in the amount of PCR when starting with less than 50 ng to over 500 ng of plasmid DNA has been demonstrated, Figure 46, Appendix 3.

Modeling work included sensitivity analysis of our model for substrate consumption and methane production. A model was also developed to quantify the interaction of methane-producing bacteria (MPB) and sulfate-reducing bacteria (SRB). Appendix 4 contains a summary of the work done on modeling their interactions.

During the first few months, a thorough literature search was performed. The articles studied have been included in the bibliography.

Summary Of Experimental Results

Tables 1 and 2 summarize the experimental results of each investigator for the first year of the project, and Appendix 4 contains a summary of the modeling results.

Table 1. Summary of Results Through December 30, 1993.

Investigator	Results
Bhattacharya (Tulane)	<p>a) Sediment and water samples have been collected from Devil's Swamp and Bayou St. John. The focus of this part of the study was to determine degradability of carbon tetrachloride (CT) and other selected organic compounds in samples from these sites.</p> <p>b) Serum bottles (150 ml) were used for Anaerobic Toxicity Assays (ATA) to determine any toxic effects of the selected compounds on anaerobes. Stock cultures for both glucose-enrichment, non-sulfate anaerobes, and lactate-enrichment sulfate reducing bacteria (SRB) have been developed and maintained in our lab for several years. Forty serum bottles were anaerobically seeded with non-sulfate anaerobes and another forty serum bottles with SRB. All the test bottles were spiked with selected concentrations of CT, methylene chloride (MC), and chloroform (CF). MC and CF were selected because these compounds were reported to be intermediates or end products in anaerobic CT degradation.</p> <ul style="list-style-type: none">• Glucose enrichment culture (summary of results):<ul style="list-style-type: none">CT: Acclimation to 9.6, 25.1, and 50 ppm in 21, 35, and 35 days respectively.<ul style="list-style-type: none">No acclimation to 100.5 ppm in 52 days.CF: No acclimation to any concentration over period of study.MC: Slight inhibition over first ten days with a 10.7 ppm spike.<ul style="list-style-type: none">Acclimation to 26.3 ppm in 28 days.No acclimation to 51.6 and 99.4 ppm in 52 days.• Lactate-SRB culture (summary of results):<ul style="list-style-type: none">CT: Concentrations up to 99.9 ppm inhibitory but not toxic.<ul style="list-style-type: none">Response of culture identical to different concentrations.CF: Concentrations up to 100.2 ppm inhibitory but not toxic.<ul style="list-style-type: none">Response of culture identical to different concentrations.MC: Concentrations up to 99.8 ppm inhibitory but not toxic.<ul style="list-style-type: none">Acclimation to 10.5 ppm in 48 days.Increasing concentrations only slightly more inhibitory.<p>c) Our graduate student, Mr. Richard Madura, went to ORNL in August, 1993, and worked under the supervision of Dr. Tommy Phelps.</p><ul style="list-style-type: none">• Sulfate reducing acetate culture used for dechlorination experiments on CT, CF, and TCE.<ul style="list-style-type: none">TCE: No dechlorination observed after 7 day incubation.CT: Dechlorination of 51 ppm greater than 90% when high substrate concentrations and sulfate present.<ul style="list-style-type: none">Dechlorination of 51 ppm up to 65% when sulfate absent.CF: Dechlorination of 4.3 ppm greater than 50% when sulfates present.<ul style="list-style-type: none">Dechlorination of 4.3 ppm greater than 75% when sulfates absent.<p>d) Serum bottles filled with Bayou St. John sediments showed little methanogenic activity when spiked with acetic acid.</p>

- e) Developed GC methods. Pentane extraction method presented by Henderson (1976) utilized for analysis of CT, CF, and MC. Detection limit for CT and CF reported to be approximately 0.1 ppb.
- Mielke
(Xavier)
- a) Prepared protocols, collected sediments and water samples, prepared and extracted sediment samples and conducted trace metal analysis, and updated ICP with ultrasonic nebulizer and state-of-the-art software.
- b) Figures 43-44, Appendix 3, summarize results from the top, middle, and bottom 5 cm of the sediment cores for Pb, Zn, and Cd for the samples from Orleans and Harrison bridges in New Orleans during June and October, 1993.
- c) Figure 45, Appendix 3, summarizes the results of bottom sediment samples from Lake Pontchartrain Pb, Zn, Cd, and Cu.
- Bennett
(Tulane)
- a) Isolated and cultured over 70 strains of white rot and other fungi from contaminated and control sites in Louisiana.
- b) Twenty species of white rot fungi and three molds have been analyzed for the ability to decolorize the polymeric dye, R-481. Decolorization of this dye is dependent on lignin-degrading enzymes useful in the biodegradation of various xenobiotics. Decolorization rates for effective white rots were: *Marasmius* sp. 55%, *Pluteus cevinus* 50%, *Pleurotus sapidus* 41%, and *Phanerochaete chrysosporium* 69%. The non-lignolytic molds, *Aspergillus niger*, *A. parasiticus*, and *Cunninghamella elegans* gave 100% decolorization.
- Eckert
(Xavier)
- Developed protocols, collected samples from Bayou St. John, and obtained preliminary data on polycyclic aromatic hydrocarbons. Relative to a goal of developing a rapid, sensitive, and specific method to quantitatively detect polycyclic aromatic hydrocarbons in solution, the polymerase chain reactions (PCR) for amplification of a 600 bp region of the cloned human *c-myc* proto-oncogene have been optimized. A linear response in the amount of PCR when starting with less than 50 ng to over 500 ng of plasmid DNA has been demonstrated, Figure 46, Appendix 3.
- Englande
(Tulane)
- a) The focus of this part of research was to study the effect of CT on aerobes and microaerophiles. Microbes were isolated from water and sediment samples collected from Devil's Swamp and Bayou St. John. Cultures were identified by standard techniques. *Pseudomonas cepacia* appears to be the predominant organism in these samples.
- b) From literature it appears that *E. coli* K-12 is the key organism which can grow in the presence of CT (Criddle, et al, 1990).
- c) Methods were developed for analyzing CT by EPA Method 601. Appropriate QA/QC procedures have been developed.
- Fulginiti
(Xavier)
- Developed protocols, collected sediments and water samples, considered alternative biota involved in removal and sequestering of pollutants. Clonal stock populations of the moss *Physcomitrium pyriforme* were used to inoculate replicas of three soil samples from Devil's Swamp. Initially, growth of the bryophyte was supported by all three soil samples, but the plants cultured on the soils died between 14 and 31 days.
- Jones

(Tulane post-
doctoral
associate)

a) Set up eighteen serum bottles (100 ml) using an acetate enrichment culture for testing the toxicity of hexachloro-1,3-butadiene (HCBD) and hexachlorobenzene (HCBZ). Established approximate toxicity levels using 10 to 100 ppm HCBD. Began testing HCBD in the 1-10 ppm range, with controls, Figures 39-41, Appendix 3.

b) Began determination of biodegradation kinetic rate constants from chemostat experiments using the Runge-Kutta method for solution of the coupled differential equations.

Kamath
(Xavier)
Law
(Tulane)

Examined alternative kinetic programs and their use in environmental models.

a) Using recently developed statistical approach (Law, et al., 1992; Todd, et al., 1992), determined biokinetic constants from the experimental data obtained in Bhattacharya's lab.

b) Ms. Michelle Todd, Law's graduate student, went to ORNL in August, 1993, and worked under the supervision of Dr. T. Phelps.

Mullin
(Tulane)

a) Focused on the use of protein engineering methods to reshape the substrate binding site of the cytochrome P450BM-3 from *Bacillus megaterium* (Narhi and Fulco, 1986 and 1987) to produce isozymes that are capable of oxidizing selected toxic organic compounds that contaminate Devil's Swamp.

b) Obtained from Dr. Tom Poulos (UC, Irvine) a plasmid called pT7BM3 that encodes the BM-3 protein (Darwish, et al., 1991), and have successfully overproduced soluble BM-3 in *E. coli* at levels up to about 20% of total cell protein and have shown that the overproduced protein is enzymatically active.

c) Site directed mutagenesis was used to introduce amino acid substitutions into the first eight amino acid residues at the amino terminal end of the protein and thus far 169 mutants have been constructed, some of which have multiple amino acid substitutions. The wild type BM-3 and two mutant proteins have been purified, and a mutation of M5R was shown to alter the ability of laurate to bind to BM-3.

Ross
(Xavier)

Developed protocols, collected sediments and water samples, isolated organisms and inoculated organic media. Bacterial growth was obtained when material from Bayou St. John and Lake Pontchartrain samples were inoculated into a mineral salts medium containing inorganic nitrogen, phosphorus, calcium, magnesium, trace minerals, and 1% sterilized crude oil.

Table 2. Summary of Research for the Remainder of Year 1.

Investigator	Tasks
Bhattacharya (Tulane)	a) Determine the biokinetics using serum bottles. b) Study the degradation mechanism and rates of CT.
Mielke (Xavier)	Expand analysis of Devil's Swamp samples and increase the collection of Bayou St. John samples for analysis in order to compare and obtain a diverse group of sediment types, pollutants, and organisms.
Bennett (Tulane)	a) Continue isolation of microbial isolates, both fungi and bacteria, from contaminated sites. b) Bacteria of the genus <i>Pseudomonas</i> will be characterized and used for studies on mixed bacterial-fungal consortia.
Eckert (Xavier)	Continue developing immuno-polymerase chain reaction techniques for identification and monitoring of specific polycyclic aromatic hydrocarbons.
Englande (Tulane)	Investigate biokinetics and abiotic factors ability to enhance assimilative capacity.
Fulginiti (Xavier)	Conduct field work to obtain various plant and alternative biota which survive both mild and harsh contamination by pollutants.
Jones (Tulane post-doctoral associate)	a) Coordinate and conduct sampling trips to Devil's Swamp and Bayou St. John approximately monthly. b) Continue development of toxicity data for HCBd and HCBZ, including a literature search relative to their toxicity. c) Develop kinetic constants for biodegradation of HCBd and HCBZ. d) Kinetic rate constants from chemostats via Runge-Kutta methods.
Kamath (Xavier)	Evaluate preliminary data and identify promising trends to test kinetic modeling.
Law (Tulane)	a) Coordinate with the GIS group (PI: Regens) for database development. b) Determine the necessity of additional data to refine the model.
Mullin (Tulane)	a) Use a molecular graphics program called Quanta in conjunction with another program called Charmm in order to simulate the effects of amino acid substitutions on the structure of BM-3. b) Attempt to measure the accessibility of smaller substrates to the heme iron of BM using a set of aliphatic amines of different chain length.
Ross (Xavier)	Identify organisms that actively biodegrade pollutants using standard microscopic techniques, membrane lipid analysis, and appropriate biochemical tests.

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APPENDIX 1
MAPS

APPENDIX 2

REPORT TO OAK RIDGE NATIONAL LABORATORY

TULANE UNIVERSITY DOE PROJECT

Richard Madura

Dr. Sanjoy Bhattacharya

January 1994

For Dr. Tommy Phelps

OVERVIEW OF WORK PERFORMED

ANAEROBIC TOXICITY ASSAY (ATA)

Anaerobic toxicity assays were performed with carbon tetrachloride, methylene chloride, and chloroform using either anaerobic glucose-acetate or sulfate-reducing lactate culture. Several concentrations were selected and are shown in Table 1. The concentrations are adjusted using Henry's constant for 35°C, 50 mL of liquid volume, and 100 mL of gas volume.

Table 1

Compound	Glucose-Acetate Culture		Lactate SRB Culture	
	Concentration Added mg/L	Concentration in Solution mg/L	Concentration Added mg/L	Concentration in Solution mg/L
Carbon Tetrachloride	9.6	2.1	9.4	2.0
	25.1	5.4	24.6	5.3
	50	10.8	49.1	10.6
	100.5	21.6	99.9	21.5
Methylene Chloride	10.7	7.4	10.5	7.3
	26.3	18.2	25.8	17.8
	51.6	35.7	50.6	35.0
	99.4	68.7	99.8	69.0
Chloroform	9.1	6.3	8.9	6.2
	24	16.6	23.5	16.3
	50	343.6	49	33.9
	99.5	68.8	100.2	69.3

Results: Gas production data for the glucose acetate culture indicated that carbon tetrachloride was inhibitory at all concentrations for a period of at least 23 days. At this point, the bottles spiked with 9.6 mg/L recovered and produced a cumulative volume of gas equal to the control at 29 days after spiking. The bottles spiked with 25.1 mg/L recovered after 37 days and resumed full gas production. Similarly, the bottles spiked with 50 mg/L renewed near full gas production at the same time. The bottle spiked with 100.5 ppm did not recover in 60 days. Gas production suggested that the conversion of glucose to fatty acids occurred (small amounts of CO₂ are produced by the conversion). This was further substantiated by no methane production following an acetic acid feed.

Chloroform completely inhibited gas production in the glucose acetate culture at all concentrations. Small volumes of gas, probably attributable to glucose conversion, were produced. Only the bottle spiked with 9.1 mg/L showed signs of gas production after 43 days.

Methylene chloride was the least toxic of the compounds. The 10.7 mg/L spike only slightly inhibited the culture. A spike of 26.3 mg/L produced inhibition of methanogenesis until day 29, at which point the bottles quickly produced a cumulative volume of gas comparable to the control. The bottles spiked with 51.6 and 99.4 mg/L only produced small amounts of gas over 60 days, suggesting that methanogenesis was inhibited.

The response of the lactate SRB culture was interesting. Gas production for all three compounds at all concentrations were very nearly identical. Inhibition over a 50 day period was approximately 33% of the total gas production. Only methylene chloride showed some slight differences in gas production, with lower concentrations producing increasing volumes of gas. Only the bottles spiked with 10.5 mg/L showed signs of recovery after 41 days.

SULFIDE TOXICITY STUDIES WITH LACTATE SRB CULTURE

Chemostats were set up to study the interaction of microorganisms in our lactate SRB culture under different COD/SO₄²⁻ ratios. Two sets of triplicates were fed 500 mg COD/L-day of lactate as lactic acid at ratios of 0.82 (the stoichiometric ratio) and 3.33 and a SRT of 10 days. These ratios corresponded to influent sulfate concentrations of 4000 and 1600 mg/L, respectively, and a lactic acid concentration of 5000 mg/L. I found after one week that my soluble sulfide concentrations were above the reported toxic thresholds for most bacteria (~800 mg/L). However, titration data indicated that my effluent volatile acids concentrations in the chemostats were still very low. Since I was only allowing the chemostats to reach steady state at this point, I was only performing titrations and sulfate analysis to monitor the health of the systems and was not collecting samples for GC analysis of individual volatile acids. At this point, I began collecting samples for analysis and monitored by effluent sulfide and sulfate concentrations to determine if I was indeed seeing complete utilization of the acids under high sulfide conditions.

Results: The chemostats at the COD/SO₄²⁻ of 3.33 seemed to reach steady state quickly, showing an effluent sulfide concentration of approximately 220 mg/L with a maximum of approximately 400 mg/L at the onset of monitoring. The effluent acetic acid and propionic acid concentrations did not exceed 200 mg/L throughout the period of the study. The effluent sulfate concentrations were always between 6 and 12 mg/L, indicating that some sulfides were lost as hydrogen sulfide gas (one would expect the sulfide concentration to be approximately one-third the influent sulfate concentration if none is lost as gas). This is expected since the pH of the chemostats was approximately 7 (the pK_a for HS⁻-H₂S). Throughout the experiment, lactic acid was completely converted to intermediates and could be found only in trace quantities in the effluent. An interesting observation from the GC analysis was the presence of several forms of butyric acid. This is unusual since lactic acid is a C₃ compound while butyric acid is a C₄ compound. Isobutyric acid was measured at approximately 3 to 5 mg/L, effluent n-butyric acid concentrations were typically 25 mg/L, and effluent methylbutyric acid concentrations ranged from 5 to 18 mg/L (although a few samples contained none of this acid periodically).

The chemostats at the COD/SO₄²⁻ ratio of 0.81 provided interesting results. At the onset of monitoring, the effluent acids concentrations (acetic, propionic) were comparable to the other set of chemostats. The effluent acetic acid concentrations were approximately 100 mg/L and very little propionic acid was detected when the sulfide concentrations were approximately 600 mg/L, supporting the titration data. In the first five days of monitoring, the sulfide concentration held steady and little propionic acid was detected. However, acetic acid accumulated rapidly, with an average concentration of approximately 700 mg/L in the effluent. Within 20 days from this time, sulfide concentrations dropped dramatically to nearly 100 mg/L while acetic acid concentrations were between 800 and 1500 mg/L (the chemostats behaved very differently although sulfide

concentrations were comparable). Likewise, propionic acid concentrations began to slowly increase, going from small amounts to 500 mg/L in 5 days, 1500 mg/L in 12 days, and ultimately to 2000 mg/L in 25 days (although a 750 mg/L decrease occurred over approximately 10 days after the 1500 mg/L peak). Effluent sulfate concentrations steadily increased until finally the influent concentration was reached. Interestingly, not all of the organisms were washed out. Lactate was still degraded by an unknown organism which may or may not be responsible for the degradation while sulfate reduction was occurring. A recent check on the chemostats shows that sulfate reduction has resumed, although complete utilization of it is occurring.

Like the chemostats with the COD/SO_4^{2-} of 3.33, the butyric acids were produced. However, under high sulfide concentrations, none were detected in the influent. Only after the sulfide concentration dropped below 500 mg/L were the acids formed. Very small amounts of methylbutyric acid were in the effluent (<2 mg/L). Isobutyric acid appeared sporadically for 25 days after which in two of the chemostats began to accumulate, although the concentrations were still very small (<4 mg/L). n-Butyric acid began to accumulate after 5 days and eventually reached approximately 150 mg/L in 35 days in two of the chemostats (the third only had 25 mg/L, reiterating the different behavior of the chemostats).

I have found only two papers discussing reductive methylation of propionic acid to butyric acid (one is a research project report which I probably will not be able to obtain). It seems that sulfide is an inhibitor of methylation of propionic acid since the high sulfide concentration (>500 mg/L) effluent did not contain the acids. The high COD/SO_4^{2-} chemostats contained the acids from the onset when the sulfide concentration was approximately 400 mg/L. The accumulation of acetic acid was due to inhibition (or absence) of acetate utilizing methanogens or SRB's. The accumulation of propionic acid could be due to one of two factors. First, propionate utilizers could themselves be inhibited by sulfides. Second, hydrogen utilizing methanogens and/or SRB's could be inhibited. The conversion of propionate to acetate is dependent upon low partial pressure of hydrogen. I measured the hydrogen concentration during toxicity and found that it was extremely high (it saturated the column). This might explain the accumulation of the acid.

METHOD DEVELOPMENT FOR GAS CHROMATOGRAPH

I have spent a great deal of time researching and testing methods for analyzing these volatile compounds with the new gas chromatograph. I am using a Supelco Vocol column and have tested both headspace gas and extracted solutions using the temperature program presented by Supelco. One method which I have had some success with is a pentane extraction followed by direct injection of the pentane. The extraction can be performed in the serum bottle (if no headspace is present) and is simple and reproducible. The paper I took the method from reported detection limits in the range of 0.5 ppb when an ECD is used. Since my concentrations are higher in the extraction solvent, I have switched to using an FID with very promising results. I have the potential to perform purge and trap (EPA Method 624), but I have the difficulty of filtering the samples prior to injection, thus losing some of the volatiles. Unfortunately, the GC was not operational at the time I was performing the ATA's. If it were, I could have analyzed the bottles for degradation or loss by gas bleeding over the course of the experiment.

APPENDIX 3
GRAPHS & FIGURES.

Figure 7. Methylbutyric Acid

Figure 8. Lactic Acid

Figure 9. 9.6 ppm CCl₄
Glucose-Acetate Culture

Figure 10. 25.1 ppm CCl₄
Glucose-Acetate Culture

Figure 11. 50 ppm CCl₄
Glucose-Acetate Culture

Figure 12. 100.5 ppm CCl₄
Glucose-Acetate Culture

Figure 13. ATA - Carbon Tetrachloride
Glucose-Acetate Culture

Figure 14. 9.1 ppm CHCl_3
Glucose-Acetate Culture

Figure 15. 24 ppm CHCl_3
Glucose-Acetate Culture

Figure 16. 50 ppm CHCl_3
Glucose-Acetate Culture

Figure 17. 99.5 ppm CHCl_3
Glucose-Acetate Culture

Figure 18. ATA- Chloroform
Glucose-Acetate Culture

Figure 19. 10.7 ppm CH₂Cl₂
Glucose-Acetate Culture

Control MC1 MC2 MC3

Figure 20. 26.3 ppm CH₂Cl₂
Glucose-Acetate Culture

Control MC4 MC5 MC6

Figure 21. 51.6 ppm CH₂Cl₂
Glucose-Acetate Culture

Control MC7 MC8 MC9

Figure 22. 99.4 ppm CH₂Cl₂
Glucose-Acetate Culture

Control MC10 MC11 MC12

Figure 23. ATA - Methylene Chloride
Glucose-Acetate Culture

Control 10.7 ppm 26.3 ppm 51.6 ppm 99.4 ppm

Figure 24. 9.4 ppm CCl₄
Lactate SRB Culture

Control CT1 CT2 CT3

Figure 25. 24.6 ppm CCl₄
Lactate SRB Culture

Figure 26. 49.1 ppm CCl₄
Lactate SRB Culture

Figure 27. 99.9 ppm CCl₄
Lactate SRB Culture

Figure 28. ATA - Carbon Tetrachloride
Lactate SRB Culture

Figure 29. 8.9 ppm CHCl₃
Lactate SRB Culture

Figure 30. 23.5 ppm CHCl₃
Lactate SRB Culture

Figure 31. 49 ppm CHCl_3
Lactate SRB Culture

Figure 32. 100.2 ppm CHCl_3
Lactate SRB Culture

Figure 33. ATA - Chloroform
Lactate SRB Culture

Figure 34. 10.5 ppm CH_2Cl_2
Lactate SRB Culture

Figure 35. 25.8 ppm CH_2Cl_2
Lactate SRB Culture

Figure 36. 50.6 ppm CH_2Cl_2
Lactate SRB Culture

Figure 37. 99.8 ppm CH₂Cl₂
Lactate SRB Culture

Control MC10 MC11 MC12

Figure 38. ATA - Methylene Chloride
Lactate SRB Culture

Control 10.5 ppm 25.8 ppm 50.6 ppm 99.8 ppm

FIGURE 41. 10 ppm HEXACHLORO-1,3-BUTADIENE with METHANOL CONTROL

Figure 42. Strategy for altering substrate specificity of cytochrome P450BM-3

We have a large collection of cytochrome P450BM-3 mutants, and cells expressing these mutant enzymes will be tested for ability to oxidize aromatic compounds and chlorinated aliphatic compounds.

Dawson, et al (1991), Protein Engineering 5:701-708.

Figure 43

Figure 44

Figure 45

Figure 46. Quantitative PCR experiment showing the effect on PCR product concentration due to the concentration of starting target DNA. λ = lambda DNA digested with Hind III (size standard), lane 1 = 30 ng of ϕ -copy DNA, lane 2 = 125 ng, lane 3 = 250 ng, lane 4 = 375 ng, and lane 5 = 500 ng.

k_1	=	Rate constant for MPB'S (day^{-1}).
k_{11}	=	Inhibition coefficient for MPB'S (mg/L).
k_{s1}	=	Monod half velocity coefficient for MPB'S (mg/L).
X_1	=	MPB concentration (mg/L).
b_1	=	Decay rate for MPB'S (day^{-1}).
Y_1	=	Yield coefficient for MPB'S ($\text{mg of MPB/mg of substrate}$).
$[\text{SO}_4]$	=	Sulfate concentration (mg/L).
$[\text{SU}]$	=	Sulfide concentration (mg/L).
k_2	=	Rate constant for SRB'S (day^{-1}).
k_{12}	=	Inhibition coefficient for SRB'S (mg/L).
k_{s2}	=	Monod half velocity coefficient for SRB'S (mg/L).
X_2	=	SRB concentration (mg/L).
b_2	=	Decay rate for SRB'S (day^{-1}).
Y_2	=	Yield coefficient for SRB'S ($\text{mg of SRB/mg of substrate}$).

The parameters for the model were assumed as follows:

1. k_1 = 2.5 day^{-1}
2. k_{s1} = 10 mg/L
3. k_{11} = 8 mg/L
4. k_2 = 2.5 day^{-1}
5. k_{s2} = 1 mg/L
6. k_{12} = 8 mg/L
7. Y_1 = 0.05 $\text{mg of MPB/mg of substrate}$
8. b_1 = 0.01 day^{-1}
9. Y_2 = 0.01 $\text{mg of SRB/mg of substrate}$
10. b_2 = 0.002 day^{-1}

The Runge-Kutta fourth order correct method was used to solve Equations (3) to (8) which are coupled first order differential equations.

SENSITIVITY ANALYSIS

1. The effect of varying the initial substrate concentration was studied and the results are as follows:

When the initial substrate concentration is increased the amount of MPB, SRB and sulfide should increase more rapidly with time and the concentration of sulfate should decrease more rapidly with time. The model predicts these and the results are shown in Figures 1-4.

2. The effect of varying initial MPB concentration was studied and the results are as follows:

When the initial MPB concentration is increased, the concentration of substrate should decrease more rapidly with time and there should be no effect on the variation in SRB, sulfate and sulfide concentrations with time. The prediction by the model agrees with this and the results are shown in Figure 5-8.

3. The effect of varying initial SRB concentration was studied and the results are as follows:

From Figures 9-12 it can be seen that as the initial concentration of SRB is increased, the concentration of substrate and sulfate decrease more rapidly with time and the concentration of sulfide increases more rapidly with time which are as expected. There is a no appreciable change in the concentration of MPB with time.

4. The effect of varying initial sulfate concentration with time was studied and the results are as follows:

APPENDIX 4

MODELING

INTERACTION OF MPB'S AND SRB'S IN ANAEROBIC ENVIRONMENTS

INTRODUCTION

A model has been developed for the interaction between Sulfate Reducing Bacteria (SRB) and Methane Producing Bacteria (MPB) under anaerobic conditions. This type of situations occur typically in anaerobic soils (E.g. wetlands and rice fields).

BACKGROUND

Acetate is a substrate which is consumed by both MPB'S and SRB'S. SRB'S can sustain and consume acetate only in the presence of sulfates. Sulfate cause inhibition to MPB'S in a non-competitive manner. SRB'S on consumption of substrate (acetate) produce sulfides which inhibit their growth itself in a competitive manner. The stoichiometry of MPB and SRB reaction with acetate is given by:

SRB

MPB

MODEL DEVELOPMENT

The reactions (1) and (2) are assumed to take place in a batch reactor. Consumption of substrate by MPB'S and SRB'S are assumed to follow Monod kinetics.

Writing a mass balance of each species yields:

Substrate

$$\frac{dS}{dt} = - \left[\frac{k_1 X_1}{k_1 X_1 + S} \left(1 + \frac{[SO_4]}{k_n} \right) + \frac{k_2 X_2}{k_2 X_2 + S} \left(1 + \frac{[SU]}{k_{12}} \right) \right] S \quad (3)$$

MPB

$$\frac{dX_1}{dt} = \frac{Y_1 k_1 S X_1}{k_1 X_1 + S} \left(1 + \frac{[SO_4]}{k_n} \right) - b_1 X_1 \quad (4)$$

SRB

$$\frac{dX_2}{dt} = \frac{Y_2 k_2 S X_2}{k_2 X_2 + S} \left(1 + \frac{[SU]}{k_{12}} \right) - b_2 X_2 \quad (5)$$

SULFATE

$$\frac{d[SO_4]}{dt} = \frac{0.984 k_2 S X_2}{k_2 X_2 + S} \left(1 + \frac{[SU]}{k_{12}} \right) \quad (6)$$

SULFIDE

$$\frac{d[SU]}{dt} = \frac{0.984 k_2 S X_2}{k_2 X_2 + S} \left(1 + \frac{[SU]}{k_{12}} \right) \quad (7)$$

where,

S = Substrate concentration (mg/L),
t = Time (days).

From Figure 13 it can be seen that as sulfate concentration is increased the sulfide concentration increase as expected. From Figure 14 it can be seen that as the initial concentration of sulfate is increased, the concentration of substrate increases more rapidly with time. The reason is, since sulfate inhibits MPB in a non competitive manner less amount of substrate is consumed by MPB. This can also be seen from Figure 15 where the concentration of MPB decreases more rapidly with time as the initial concentration of sulfate is increased. Figure 16 shows the variation of MPB concentration as the initial sulfate concentration is increased. As it can be seen there is no variation in SRB concentration with time as the the initial sulfate concentration is increased. This is due to the fact that the substrate utilized by SRB in a given time remains a constant.

CONCLUSIONS

A model has been developed to describe the kinetics of interaction between MPB'S and SRB'S in anaerobic environments and the sensitivity of the model has been analyzed.

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

Figure 12

Figure 13

Figure 14

Figure 15

Figure 16

APPENDIX 5

ACRONYMS

ATA	anaerobic toxicity assay
CF	chloroform
COD	chemical oxygen demand
CT	carbon tetrachloride
ECD	electron-capture detector
FID	fire-ionization detector
GC	gas chromatography
GIS	geographical information system
HCBD	hexachloro-1,3-butadiene
HCBZ	hexachlorobenzene
ICP	inductively-coupled plasma
MC	methylene chloride
MPB	methane-producing bacteria
ORNL	Oak Ridge National Laboratory
PCR	polymerase chain reaction
ppb	parts-per-billion
ppm	parts-per-million
QA	quality assurance
QC	quality control
SRB	sulfate-reducing bacteria
SRT	solids retention time
TCE	trichloroethylene
UC	University of California

Pore-Level Flow, Transport, Agglomeration, and Reaction Kinetics of Microorganisms

L. Fauci, D. Gaver, P. Moore, K. Papadopoulos, and B. Sharma

Introduction

The development of effective strategies for *in situ* bioremediation depends upon understanding the detailed pore-level behavior of contaminants and microorganisms within porous media. This is due to the fact that bioavailability of microorganisms to the toxin site depends upon the local physicochemical conditions (*e.g.* pH, temperature, concentrations of dissolved gasses). First, these conditions are primary determinants of bioavailability because they influence flocculation, the propensity of microbes to aggregate and adhere to each other and the local pore structure. Increased flocculation hinders microbial migration by lessening forced convection and diffusive transport of the colloidal mixture through small pores. The local physicochemical conditions influence bioavailability because microbes swim preferentially by chemotaxis, the directional motion induced by variations of chemical concentrations. Thus, if concentration gradients are appropriate, microbes may more readily swim towards contaminated regions and aid in the uniform elimination of toxic waste. Once the microbes are at the contamination site, restoration will be governed by metabolic kinetics, which in turn are functions of the local physicochemical state. All of the aforementioned processes occur in a moving viscous fluid, and therefore the fluid dynamical events must be included in any realistic model. As is evident by the above description, factors controlling the local environments of microbial communities in the subsurface interstices are critical for any *in situ* remediation technology. Unfortunately, knowledge of this small-scale system has yet to be fully investigated, and is extremely complex due to the many components that govern the physicochemical and flow conditions.

Current analysis of bacteria transport through porous media (Hornberger *et al.*, 1992) relies solely on continuum modelling. This study used a one-dimensional convection-dispersion model that includes entrainment and deposition in governing equations that describe the concentration of bacteria. Using results from their own experiments, the authors determined values of the transport coefficients used in their model. This investigation was not predictive, since these coefficients were determined in order to best fit the experimental data. Clearly, a better understanding of deposition and entrainment mechanisms will lead to a superior understanding of transport phenomena.

Efficient penetration of microbes into a porous media requires motility and chemotaxis (Soby & Bergman, 1983). Motile bacteria have been identified that degrade a variety of toxins. For example, *Pseudomonas putida*, found widely distributed in soil and freshwater environments, exhibits positive chemotaxis toward aromatic acids and chlorinated benzoates. Moreover, these compounds are also metabolized by the organisms (Harwood *et al.*, 1984, 1990). Strains of motile *Pseudomonas* have been identified that are capable of using benzene, chlorobenzene, or toluene as the sole source of carbon and energy (Alexander *et al.*, 1991). Bacterial chemotaxis has been studied experimentally and theoretically (for example see Berg, 1975; Ford & Lauffenburger, 1991; Keller & Segel, 1971; Othmer *et al.*, 1988 and Rivero *et al.*, 1989). A common feature of the theoretical models is that explicit dependence upon fluid dynamics is ignored. Recent studies of bacterial movement in microchannels suggest that surface interaction and hydrodynamic forces must be included in models at the micropore scale (Berg and Turner, 1990; Harkes *et al.*, 1992).

Controlled experiments (Alexander *et al.*, 1991) investigated the transport of bacteria through homogeneous pore sizes (40-60 Åm). They demonstrated that bacterial movement and adsorption is influenced by the fluid ionic strength. The deposition and agglomeration of bacteria determine the breakthrough of bacteria to the contaminant sites. It is clear from this study that aggregates

alter the geometry of the pores and thus the convection field. Since pores range from at least 1-100 bacteria diameters in size, it is appropriate to model this system in a manner such that the fluid is modelled as a continuum while the bacteria are defined as discrete objects of finite size. The results from such models will provide detailed information concerning transport that is not available from purely continuum representations.

Further evidence suggests that physicochemical surface interactions play an enormous role in the transport of colloidal pollutants or microorganisms through subsurface porous media and soils. These forces determine whether suspended particles are transported through or collected by the walls of a porous medium. Preliminary experiments in Dr. Papadopoulos' laboratory have shown that it is possible to visually observe the movement or adhesion behavior of particles and bacteria in straight as well as random pores. Solution parameters such as pH, ionic strength, the presence and nature of surfactants, and the morphology of the medium influence the interfacial forces, but must be investigated. There have been significant discrepancies between predicted and measured rates of particle transport through porous beds (Elimelech and O Melia, 1990). Commonly, this discrepancy is attributed to inaccurate interaction models (Mills et al, 1991). Traditional interaction models, however, do not account for the effects of the geometrical configuration of the particle-porous medium interaction. For this reason, an objective of our project will be the development and evaluation of improved interaction models. Building upon the current state of the theory of colloidal interactions, these models will specifically address the role of the geometry and morphology of the surfaces involved in the interaction. The geometrical configuration studies will look at deformable, shape-changing particles, interacting with the walls of tortuous pores. To date the only studies that address particle-pore surface interactions are those of Smith and Deen (1980, 1983) and Papadopoulos and Kuo (1990).

Research Accomplishments

The overall goal of this research project is to improve the characterization and assessment techniques currently being used to evaluate bioremediation alternatives. This will be accomplished by investigating the phenomena and processes that affect the fate and transport of pollutants and microorganisms at the microscopic level. The specific research objectives are:

1. quantify the physical constants relevant to the interfacial adsorption of bacteria. These constants describe the capture rate of adhering microbes (a function of flow rate, particle and pore diameter and the surface charge), the bacterial swimming speed in a quiescent fluid, chemotactic response in a spatially and temporally varying concentration field, and the adhesion energy;
2. examine the bacteriological properties associated with the bioremediation of a toxin using a microscopic viewpoint;
3. determine the detailed pore-level behavior of contaminants and microorganisms in a system with adsorbed contamination.

Computational Investigations

In the first year of the computational work, the following two-dimensional model has been developed. We make use of state-of-the-art methods in computational fluid dynamics which enable us to study flows in simple pore geometries and the interactions of the microbes with the fluid and surrounding pore structure.

The dynamic evolution of a single contaminant that is initially deposited within a pore filled with a viscous fluid depends upon the fluid motion induced by motile bacteria, background flow, diffusion and microbial uptake. Moreover, microbes move in direct response to the surrounding contaminant field (chemotaxis). This nonlinear coupled system of equations is described below.

The governing equations describing the fluid dynamics are:

$$\rho(u_t + (u \cdot \nabla)u) = -\nabla P + \mu \nabla^2 u + F_{\text{external}} \quad (1)$$

and

$$\nabla \cdot u = 0 \quad (2)$$

which are the Navier-Stokes and continuity equations for the incompressible fluid. These equations represent the balance of momentum and conservation of mass, and hold within the fluid domain. Here, ρ is the fluid density, u is the fluid velocity vector, p is pressure, μ is the fluid viscosity and F_{external} are forces on the fluid due to suspended microorganisms and the pore walls (described below). The no-slip/no-penetration boundary conditions are applied to the suspended microorganisms and pore walls.

The equation describing the convection and diffusion of the contaminant species within the fluid-filled pore is

$$c_t + (u \cdot \nabla) c = D^L \nabla^2 c - R(c) c, \quad (3)$$

where c is the concentration, and D^L is the molecular diffusivity in the liquid phase and $R(c)$ is a concentration-dependent consumption rate for the microbe.

The presence of microorganisms influence both the flow dynamics and the contaminant field. In turn, the microorganisms respond to the fluid and contaminant fields. For this reason, we incorporate discrete representations of microorganisms that are mechanically coupled to the fluid-contaminant system described above. These organisms have finite volume and exert stress on the fluid and thus alter convection and contaminant transport. Moreover, the swimming orientation of these organisms is determined by chemical concentration gradients. Contaminant concentrations are modified locally due to consumption by these organisms. Note that $R(c)$ in equation (3) is nonzero only at the site of a microbe. The microorganisms influence the fluid through F_{external} in equation 1. This F_{external} represents the force created by the N microorganisms and the walls on the fluid and has the following components:

$$F_{\text{external}} = \sum [F_{\text{microbe}(i)} + F_{\text{swim}(i)}] + F_{\text{walls}} \quad (4)$$

A single microorganism is modelled as a neutrally buoyant elastic ring, whose configuration is defined by $X_i(s, t)$, where s is a Lagrangian label, t is time and i denotes the i^{th} microbe. The boundary force per unit length $f_i(s, t)$ at each point in the ring is determined by the boundary's configuration at time t . This elastic force is transmitted directly to the fluid through

$$F_{\text{microbe}(i)}(x, t) = \int f_i(s, t) \delta(x - X_i(s, t)) ds \quad (5)$$

Here, the integration is over the ring structure and δ is the two-dimensional Dirac delta function. This force gives the microbe its material integrity. Note that each organism contributes such forces to the flow field and therefore their interactions, mediated through the fluid, are included in this model.

The walls are modelled in the same manner as the microbe rings, that is as neutrally buoyant elastic filaments immersed within the fluid domain. However, these walls are not free to move throughout the fluid, since they are elastically tethered to fixed points in space. F_{walls} is thus expressed in a manner analogous to equation (5). We choose this representation of the walls so

that the geometry of the pore can be easily changed. In our models to date, the pore is assumed to be straight.

Since inertial forces are negligible for bacterial swimming (Reynolds numbers are small), the forces induced by the microbe's locomotory movements on the fluid sum to zero. These forces do, however, result in a swimming velocity relative to the fluid (Pedley & Kessler, 1992), and are incorporated into our model in the following manner:

$$\mathbf{F}_{\text{swim}}(\mathbf{i})(\mathbf{x}, t) = \int \mathbf{f}_{\text{swim}}(\mathbf{i})(s, t) \delta(\mathbf{x} - \mathbf{X}_i(s, t)) ds \quad (6)$$

where the direction of the swimming force is given by $\nabla c(\mathbf{X}_{\text{centroid}}(\mathbf{i}))$ rotated by a random angle θ_i . Here $\nabla c(\mathbf{X}_{\text{centroid}}(\mathbf{i}))$ denotes the principal direction of chemotaxis as determined by conditions at the microbe centroid, and θ_i represents a random variable that reflects a bacteria's inability to exactly align its swimming direction towards the concentration gradient. Note that in order to conserve momentum, the sum of $\mathbf{f}_{\text{swim}}(\mathbf{i})$ around each organism equals zero. These opposing forces are distributed near the microbe ring so as to simulate the flagellum, which does indeed contribute such opposing forces (Fauci & Peskin, 1988).

Finally, the system is closed by requiring microbes to move at the local fluid velocity using

$$d\mathbf{X}_i(s, t)/dt = \mathbf{u}(\mathbf{X}_i(s, t), t) \quad (7)$$

The salient feature of this representation is that suspended organisms are replaced by suitable contributions to a force density term in the fluid dynamics equations. A single set of fluid equations holds in the entire domain and there are no internal boundary conditions. Consequently, the fluid dynamics equations may be solved efficiently using finite-difference methods on a uniform computational grid. We are able to model the interaction of more than one organism in the same domain of fluid. It is not assumed that the motion is steady-state, and therefore, transient effects can be modelled.

The numerical method that we use couples microbial motion with fluid dynamics, and is known as the *immersed boundary method*. This method was introduced by Peskin (Peskin, 1977) to model blood flow in the heart. Subsequently Fauci and Peskin (Fauci & Peskin, 1988) have used this approach to simulate the swimming of microorganisms, and Fogelson (Fogelson, 1984) to model platelet aggregation in the blood's clotting response. The full incompressible Navier-Stokes equations are solved in a domain of fluid within which neutrally buoyant elastic objects (*i.e.* microorganisms) undergoing time-dependent movements are immersed. Fluid quantities are represented on a grid (Eulerian description), and the swimming organism is modelled by a discrete collection of moving points (Lagrangian description) connected by elastic links. The external force of an organism on the fluid is represented as a delta-function layer of force supported only by the region of fluid which coincides with material points of the organism as described in equations 4-6, away from these points the external force is zero. The strength of this delta-function force is determined at each instant by the local configuration of the organism and the local contaminant field. The solution of the velocity field from this stage of the computation is then used to update the contaminant field.

The algorithm for the numerical solution of Equations 1-7 may be summarized as follows: at the beginning of each timestep n , we have the fluid velocity field $\mathbf{u}^n$, the configuration of the elastic boundaries, and the chemical concentration field c^n . In order to update these values to those occurring at the next time step we: (1) calculate the force density $\mathbf{f}_i^n$ from the boundary

configuration defined by the elastic boundaries; (2) calculate the swimming forces $f_{swim(i)}$ imposed on the fluid by each organism; (3) spread the force densities to the grid to determine the $F_{external}$ on the fluid; (4) solve the Navier-Stokes equations (equations 1-2) for u^{n+1} ; (5) solve the convection-reaction equation for c^{n+1} (equation 3) and (6) interpolate the fluid velocity field at each immersed boundary point and convect that point at the local fluid velocity (equation 7).

Figure 1 shows four 'snapshots' of a simulation intended to demonstrate the behavior of the coupled fluid/contaminant/microbe system. In this computational experiment, 18 microorganisms were initially placed randomly within a pore with a small bolus of contaminant. The contaminant diffuses and convects due to fluid motion induced by the microbes, which swim preferentially towards the region of higher contaminant concentration. The microbes simultaneously ingest the contaminant, which in turn modifies the concentration field. In these figures the vectors represent the fluid velocity field, and shading represents contour levels of contaminant. Note the net migration of cells is towards the contaminant site. However, at any instant any individual cell may be moving away from the contaminant due both to random motion and convection induced by other cells. Also, the consumption and the microbe swimming clearly affect the contaminant distribution.

We have now set up a controlled environment in the form of a computational model where microbial motility, contaminant evolution and fluid dynamics can be measured and visualized. This "experimental apparatus" will be used to systematically evaluate the influence of various physical parameters on contaminant depletion and microbial behavior. This computational work couples with the laboratory experiments in a number of ways. The comparison of simulations and laboratory experiments provides the means for parameter estimation. These estimates will be developed using simple models; and can then be used in computational experiments that are not easily replicated in the laboratory. In addition, once these parameters are known, our computational simulations will provide predictions of system variables (*i.e.* local concentrations) that are nearly impossible to measure *in situ*. Furthermore, simulation results can be used as a guide to design fruitful laboratory experiments, as described below.

Experimental Investigations

In the first year of the experimental work our primary emphasis was on the development of experimental methods to investigate bacterial chemotaxis at the pore-level.

Chemotaxis of *E. coli*

E. coli were chosen as the model bacteria to be chemotaxed in the direction of increasing concentration of two different nutrients, glucose and fucose. The strain *E. coli* K12 was obtained from American Type Culture Collection (ATTC Cat No. 10798). These *E. coli* were recovered in Tryptone with NaCl (TNa) medium at 30°C overnight, then transferred to culture plates that contain TNa agar and cultured overnight. Single colonies were selected from the plates, transferred to swarm plates (TNa with 0.3% agar) and grown at 30°C. Motile cells were selected from the edge of the colonies of the swarm plates, and incubated on TN agar slant overnight. The slants were stored at 4°C. For chemotaxis experiments, bacteria were scraped from the slant with an incubating loop, and were incubated in 25ml TNa medium inside a 250 ml PYREX flask. The culture was grown to stationary phase in a rotary shaker at 30°C and 180 rpm. Bacteria were harvested by centrifugation at 3500 rpm for 10 minutes and the supernatant was disposed. The *E. coli* were then resuspended in a 1 lt pH buffer containing 0.029g EDTA, 11.2g K₂HPO₄, and 4.8g KH₂PO₄. The bacterial suspension was then centrifuged again at 3500 rpm for 10 minutes and the process was repeated three times. Care was taken to ensure that the bacterial culture remain uncontaminated. Also, conditions were set so that cell multiplication was negligible during the course of each experiment.

The novel chemotaxis cell consists of two reservoirs (chambers) on a watchglass, communicating through a narrow capillary. The chambers are made of rubber O-rings of inside diameter ~ 1.3 cm. The connecting capillary has the macroscopic appearance of a thin hair, with a length of 0.8 cm and an inside diameter of 60 μ m. Since the chemotactic movement of *E. coli* is observed inside the capillary, it is advantageous to have as linear a concentration profile (constant gradient) for the nutrient (glucose or fucose) as possible. Nutrient solution was inserted in one chamber and the chemotaxis pH buffer (with no bacteria nor nutrient) in the other, and diffusion was allowed to take place for several hours before experiments commenced. The bacteria were then introduced into the other chamber, termed as the *E. coli* reservoir, and the migration was observed.

The video-microscopy/image analysis system, shown in a schematic in Figure 2, was used to capture the bacterial movement in real time, to make video movies of numerous experiments, and to extract qualitative understanding and quantitative measurements from the experiments. This initial experiment focussed on visualizing the results of bacterial chemotaxis in a capillary. As described above, the comparison between these laboratory experiments and the computational simulations are useful in determining the validity of our physical assumptions. One quantitative measurement that we will use for this purpose is the chemotactic velocity. Some typical results on cell motility are shown in Figure 3. A few observations can be made by studying the behavior of the four curves shown. In all cases chemotactic velocity increases, not only with increasing concentration gradient, but also with increasing concentration, as the distance from the entrance increases. This strongly suggests that in the computational model described above, the magnitude of f_{swim} should depend upon the local concentration, c . Presently, more experiments are being conducted to generalize and quantify these observations in ways that can provide laws for the mathematical models. A video still, showing a micrograph of the capillary with *E. coli* in motion, is shown in Figure 4a. This experiment was also simulated using the computational approach described above, and a "snapshot" is provided as Figure 4b. Note that the computations demonstrate the concentration field as well as the fluid velocity field. This information is not readily available from the laboratory experiments.

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Figure 1: Simulations of bacteria in a pore with contaminant. Velocity field results from swimming organisms.

Figure 2: Schematic of the experimental apparatus.

Figure 3: Results of bacteria motility experimental studies.

Figure 4: a) micrograph of *E. coli* in capillary tube. b) simulation of bacteria in concentration gradient. Velocity field results from bacteria motion.

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Natural and Active Chemical Remediation of Toxic Metals and Radionuclides in the Aquatic Environment

G. McPherson, P. Pintauro, S. O'Connor, J. Zhang, R. Gonzales, and G. Flowers

Abstract

The focus of this research is the non-biological, chemical remediation of toxic heavy metals and radionuclides in aquatic environments. This Tulane/Xavier group includes researchers from Chemistry, Chemical Engineering, and Geology. Active methods using novel zeolites and ion exchange membranes are currently being evaluated for use in removing heavy metals from natural waters. In addition, field and laboratory studies of metal ion exchange reactions and competitive, heavy metal adsorption on clay substrates are underway to determine sediment metal sequestering capacity. A summary of progress to date and future work is presented.

Introduction

The Mississippi River basin contains numerous DOE weapons production facilities, nuclear power plants, and industrial operations. In fact, one of the world's greatest concentration of energy-related industries is located on the lower Mississippi River between Baton Rouge and the Gulf of Mexico. This concentration of industry has led to many environmental problems, particularly in aquatic environments of the Mississippi delta region. This problem brings together a group of Tulane-Xavier researchers from the Departments of Chemical Engineering, Chemistry, and Geology with a common interest in active remediation and natural processes that reduce the bioavailability of inorganic pollutants, specifically heavy metals and radionuclides.

In general, contamination in an aquatic environment is caused by either the discharge of relatively concentrated waste streams from point sources, or surface runoff that contains low levels of pollutants derived from a wide geographic area. The elimination of point source pollution requires a reduction in waste volume and active chemical remediation. Non-point source pollution is a more difficult problem because economic constraints prohibit the treatment of low-level contamination in estuaries, swamps, and other aquatic environments.

Over the past six months, this research cluster has initiated a wide range of projects focused on active and natural remediation of toxic materials in the environment. In order to determine the metal sequestering capacity of sediments, an understanding of chemical reactions that partition metals into the bottom sediments is required. The extent to which sediments absorb and partition heavy metals and radionuclides is, among others, a function of the: 1) cation-exchange capacity of clays and other minerals present in the bottom sediments; 2) physicochemical conditions, such as temperature, salinity, Eh, pH, dissolved oxygen, etc., at the sediment-water interface; 3) sediment texture; 4) nature and concentration of organic compounds both in the sediments and water column; 5) adsorption capacity of clays and other large surface area solids; and 6) abundance of acid-volatile sulfides (AVS) that preferentially partition heavy metals in their lattices via exchange reactions with the aqueous solution. Specifically, we will study the effects of salinity, pH, and dissolved organic compounds on metal adsorption on suspended sediments at the fresh water-salt water interface and AVS cation exchange reactions in bottom sediments. Active remediation processes being investigated include the use of synthetic ion exchange materials (phosphazene polymers and synthetic phosphate-based microporous solids). Specifically, the sequestering capacity of these materials as a function of the concentration of various dissolved salts is being determined. Any active remediation technique must consider the effects of salinity on performance given the extreme variation of salinity in aquatic environments of the Mississippi delta. The purpose of this report is to summarize the progress made thus far in understanding natural sequestering processes and developing active chemical remediation techniques for use in aquatic environments.

Metal-Sediment Interactions At The Fresh Water-Salt-Water Interface

The Delta Region of the Mississippi River is characterized as having an extensive fresh-saltwater interface. At this interface, the pH changes from approximately 5 (fresh water) to about 8 (salt water). Because of this fluctuation in acidity, several changes that impact water quality and sedimentation occur. Specifically, we are currently addressing the adsorption of metal ions on river sediments at this interface. When changes occur in the direction of increasing pH, the speciation of metal ions may change substantially. In the limit, ionic species which exist as anions under acid conditions may become cations at higher pH. When these changes are coupled to changes in the surface charge of sediments, increased deposition of metals may occur in the interface. For example, alumina has an isoelectric point (point of zero charge) at a pH between 6 and 8. Below 6 it is positively charged, but above 8 it becomes negatively charged. Similarly, the negative charge on the surface of alumina is observed to increase sharply when a pH of 6 is exceeded.

A preliminary study of the adsorption of cadmium and lead, two metals which have a deleterious effect on the environment, on montmorillonite is presently underway. Montmorillonite is a well defined, naturally occurring clay. The approximate unit-cell formula of an Upton, Wyoming montmorillonite is $M^{+3}_{0.21}(Al_{3.06}Fe_{0.32}Mg_{0.66})(Al_{0.1}Si_{7.9})(OH)_4$ (Ross and Mortland, 1966). The M^{+3} cations can be readily exchanged by treating clay suspensions with saturated NaCl solutions. Following removal of excess salt, the pH of the resulting suspension is approximately 9.1. Sodium ions can easily be back exchanged with divalent or trivalent ions. When trivalent rare earth cations are used (e.g., Yb^{+3} , Eu^{+3} , Ho^{+3}), the pH drops to 5.7 (Miller, et al., 1982). This observation immediately suggests that these heavy metals will be released into the water column at the fresh-salt water interface. The extent to which adsorption of metal ions occurs on this particular clay is currently being investigated. Preliminary studies during the current funding period have been focused on the adsorption of Pb on the clay. At a pH below 7 the speciation of Pb is primarily in the form of Pb^{+2} , and the cations are stable in solution. When the pH exceeds 9, a precipitate of lead hydroxide is formed. For this reason, studies are currently confined to solutions with a pH below 9. Under these conditions, Pb is readily adsorbed and does not precipitate as the hydroxide.

Lead Adsorption Results

The adsorption of Pb as a function of pH was measured using atomic absorption to analyze supernatant liquids in the experiments. In these studies, approximately 50 mg samples were suspended in a solution which contained 3.66 ppm of Pb. The pH was adjusted through the addition of HCl. The supernatant liquid was analyzed, and the quantity of Pb adsorbed was determined by difference. In the present study, a sampling time of 40 min was used following the adjustment of the initial pH. The pH was accurately recorded prior to performing the AA measurements. In these measurements (see Table 1), the pH was varied within the range of pH expected at the salt-fresh water interface, i.e., between 5 and 9. The pH of the solution changed over a 24 hr period to give a final equilibrium pH of approximately 9.2. This final equilibrium pH did not depend on the initial pH and is consistent with previous studies on a fully Na-exchanged montmorillonite clay (Miller, et al., 1982). These results are in accord with studies in the literature (e.g., Fushimi and Uchmra, 1983) which show that the adsorption of Pb increases with pH. It is not yet clear whether this increase is due to an increase in adsorption or perhaps the formation of a Pb-hydroxide precipitate. This possibility and adsorption reversibility when the pH is cycled back to its initial value are currently under study.

pH	Pb Adsorbed ($\mu\text{g/g}$ of clay)
5.37	0.53
7.94	1.21
8.23	1.48
8.71	1.68

Table 1. Adsorption of Pb as a function of solution pH.

In order to obtain a better understanding of the adsorption of Pb the following studies are currently in progress:

- (1) The Pb content of the montmorillonite is being determined using ICP spectrometry. A major problem encountered with these measurements is incomplete digestion of the mineral.
- (2) The change in the surface charge (zeta potential) as a function of pH is currently under study. It is important to make accurate measurements of this surface charge in the pH region of interest (i.e., pH 5-9).
- (3) Continuous cycling experiments are in progress where the pH is changed between 5 and 9 in order to assess the importance of dynamic changes in pH on the adsorption behavior of Pb.

Future Work

During the upcoming funding period, we plan to continue current studies on the adsorption of lead with the help of the newly installed ICP spectrometer, and will initiate studies on the adsorption of cadmium. Finally, we will study the competitive adsorption of both lead and cadmium as a function of pH. Because we suspect that adsorbed organic materials may interfere with the adsorption of cations, an adsorption study of both lead and cadmium in the presence of adsorbed organic materials (paraffinic hydrocarbons) will be performed.

The speciation of both Co and Fe as a function of pH are well known (Baes and Mesmer, 1976). When the pH of the solution is below 8.5, the primary cationic species is Co^{2+} . At a higher pH the speciation changes to give $\text{Co}(\text{OH})^+$ at a pH of 9 and finally $\text{Co}(\text{OH})_2^0$ at a pH of 11. Small amounts of $\text{Co}(\text{OH})_3^-$ are observed at even higher pH values. For Fe the speciation behavior is even more complex. When the pH is below 3.0, the dominant Fe species in solution is Fe^{3+} . As the pH is increased, the speciation is observed to change to yield $\text{Fe}(\text{OH})^{2+}$ first, followed by $\text{Fe}(\text{OH})_2^+$. When the pH reaches 6, the concentrations of $\text{Fe}(\text{OH})_3^0$ and $\text{Fe}(\text{OH})_4^-$ begin to increase. Because the speciation of both cobalt and iron changes sharply at a pH which corresponds to the isoelectric point of montmorillonite, a competitive adsorption process sharply dependent on the pH is expected to occur. We think that an understanding of competitive adsorption at or near this isoelectric point may be important in predicting changes in the concentrations of heavy metals in sediments within this fresh-salt water interface.

If time permits, Mississippi river sediments will be extracted and studied in order to make comparisons with results obtained using well defined clays. The sample site is located on the Azezu canal that connects the Mississippi river to Quarantine Bay. The site is easily accessible from Empire, LA and is characterized by large variations in salinity as the water level of the river changes. The pH of the sampling site will be closely monitored as a function of the level of the Mississippi River.

The Role Of Acid Volatile Sulfides In Sediment Metal-Ion Exchange Reactions

As mentioned above, heavy metals are discharged into aquatic environments from a variety of point and non-point sources in a given drainage basin. The fate of heavy metal loadings depends on many factors, such as salinity, elemental chemistry, water hardness, dissolved oxygen, redox potential, sediment texture and mineralogy, etc. (Forstner and Wittman, 1981). In fine-grained sediments of the Mississippi delta, metals are readily absorbed where they tend to accumulate over time (Horowitz, 1985). According to Gambrell et al. (1980), the bioavailability of metals in sediments depends, in part, on how metals are distributed among various sediments phases (e.g., organic phase, exchangeable phase, sulfide phase, crystalline phase, etc.). Metals bound in the clay mineral lattice, for example, are unavailable, whereas metal dissolved in the pore water phase may be problematic. The bioavailability of metals dissolved in pore waters, in turn, is a complex function of water chemistry (see Sunda, et al. , 1978; Forstner and Wittman, 1981).

The Environmental Protection Agency, due to the passage of the Clean Water Act, has imposed water quality criteria for a variety of effluent contaminants, including heavy metals. Quality criteria or action levels have not, as yet, been extended to sediments because it is often difficult to assess the toxicity of contaminated sediments. Di Toro, et al., (1990), however, recently proposed that acid volatile sulfide (AVS) content be used to measure the heavy metal absorption capacity of bottom sediments. Their experiments indicate that iron monosulfides (i.e., greigite and mackinawite) in sediments readily react with dissolved heavy metals to form heavy metal sulfides. Exchange reactions of the form

where M refers to a divalent heavy metal are believed by Di Toro, et al. (1990) to limit the concentration of heavy metals in surface waters. However, once the supply of FeS in the sediment is exhausted, spillover into the water column and the biota is expected to occur. Although this phenomenon has been documented in laboratory experiments, only a few, limited field studies have been published. The general applicability of the AVS sediment criterion has not been established.

Environmental Sedimentology of Barataria Bay

During the first year of this project, we initiated a field study of AVS in the bottom sediments of Barataria Bay located approximately 50 miles south-southeast of New Orleans (Figure 1). Bottom sediment samples were taken from 97 locations in Barataria estuary, which is located near Grand Isle, Louisiana. Because the estuary is a wide, open body of water, a Magellan GPS receiver was used for navigation and to locate each sample site to within 100 meters. Sediment samples were taken using a grab sampler, transported back to the laboratory on ice, and frozen as soon as possible.

A split of selected samples was used to determine the acid-volatile sulfide (AVS) content of the bottom sediments. AVS content is measured by reacting 10-15 g of sediment with cold 6M HCl in an oxygen-free atmosphere. The evolved H₂S reacts with a deaerated silver nitrate solution to form Ag₂S, which is removed from the solution and weighed. Each sediment analysis is expressed as μ moles AVS per gram of dry sediment. Another sample split was reacted with aqua regia in Teflon PFA vessels using a CEM, Inc. MDS-2000 microwave digestion system. The recoverable metal content of bottom sediment samples (Pb, Cd, Cu, Fe, Ba, V, Ni, Zn, and Cr) was determined using the Perkin-Elmer Optima 3000 ICP spectrometer. Sediment size analyses were performed using standard ASTM procedures.

Sedimentology

It can be seen in Table 2 that bottom sediments of Barataria Bay are enriched in sand relative to the other Louisiana estuaries (Lakes Pontchartrain and Maurepas). The sand content of bottom sediments is comparable to that of Perdido Bay and other estuaries along the Mississippi-Alabama-Florida coast, but this has not always been the case. Comparison of

	Barataria Bay 1969 *	Barataria Bay 1993 **	Lake Pontchartrain	Lake Maurepas	Perdido Bay
Sand	23	44	19	7	50
Silt	52	23	38	50	27
Clay	23	33	43	3	23
Ave. Sed. Type	Sand-Silt-Clay	Sand-Silt-Clay	Silty Clay	Clayey Silt	Sand-Silt-Clay

* data from Barrett (1971)

** N = 97

Table 2. Average percentages of sand-, silt-, and clay-sized sediments in northern Gulf Coast estuaries (modified from Flowers and Isphording, 1990).

the sand-silt-clay diagrams (Figure 2) for 1969 (data from Barrett, 1971) and 1993 indicates that a significant change in bottom sediment texture has occurred over the past 24 years. In 1969, the bottom of Barataria Bay consisted predominantly of sandy and clayey silts; presently, sand-silt-clay is the most abundant sediment type. Presumably, the passage of eight hurricanes near Barataria Bay, including Hurricane Andrew in 1992 caused the relative increase in sand content of bottom sediments. Isphording, et al. (1989) documented a similar coarsening of sediment texture in Mobile Bay after the passage of Hurricane Frederick in 1979.

Heavy Metal Chemistry of Bottom Sediments

As can be seen in Table 3, bottom sediments contain an average of approximately 5 μ moles/g AVS, which is considerably lower than averages determined by Di Toro, et al. (1990) for the

	AVS μ moles/g*	Fe wt% **	Cu ppm	Zn ppm	Ni ppm	Pb ppm	V ppm	Cr ppm	Ba ppm
Ave.	4.8	1.35	8.3	49.2	20.9	9.8	17.6	26.1	168
SD	3.6	0.43	4.1	12.5	78.3	2.8	8.96	119.8	83
Ave. Shale	-	4.72	45	95	68	20	130	90	580

* N = 66

** N = 93

Table 3. Average heavy metal and acid volatile sulfide (AVS) content of Barataria Bay sediments.

Hudson River (12.6 μ moles/g) and Long Island Sound (15.9 μ moles/g). AVS content, unlike recoverable metal concentrations, does not correlate positively with the clay content of the sample (Figure 3). Assuming the sediment AVS is due to the presence of monosulfides, the average scavenging capacity of bottom sediments is approximately 5 μ moles divalent heavy metals/g of sediment. Di Toro, et al. (1990) indicate that in sediments containing 1 μ mole/g or less other sorption phases may control the toxicity of heavy metals. Preliminary data suggests that the Barataria estuary has a limited capacity to absorb heavy metals via exchange reactions with sediment AVS. Fortunately, recoverable metal data for Barataria Bay bottom sediments indicate that heavy metal contamination is not a problem in this estuary. But one sample was found with Cr

(1168 ppm) and Ni (768 ppm) values in excess of the average shale value, which is used as a baseline for determining contaminated sediments (Forstner and Wittman, 1981). Because bulk metal content is controlled largely by sediment texture, low metal concentrations are expected in a bay containing relatively coarse-grained bottom sediments (Horowitz, 1985). This is because fine-grained sediments have a greater capacity to scavenge metals from the water column. In the case of a major spill, water quality in the Barataria estuary would be impacted more than an estuary containing finer-grained sediments (e.g., Lake Pontchartrain).

Although no data are available for comparison, the average heavy metal content of the bay probably was higher in 1969 than it is today. With the passage of a major hurricane, significant volumes of fine-grained sediment can be resuspended and flushed out of an estuary with the retreat of the storm surge (Isphording, et al., 1989). In addition, significant changes in bathymetry can occur depending on the magnitude of currents generated by the storm (Isphording, et al., 1987). The net effect is to decrease the average concentration of heavy metals in the bottom sediments of the estuary.

Future Work

Next year we plan to investigate AVS and heavy metals in the bottom sediments of a fresh water bayou, Bayou Trepagnier, located near the Bonnet Carre' spillway. The upper reaches of this water body are known to be heavily contaminated with Pb, Zn, and Cr (LaDEQ, 1989). A series of cores will be taken along the bayou to document the contamination history of the site. Samples taken at different depths will be used to determine the relationship, if any, between AVS and the heavy metal content of sediment pore waters. A similar sampling program will be carried on at a contaminated site in Barataria Bay. According to Di Toro, et al. (1990), the AVS criterion is equally applicable in fresh and saline water bodies.

Active Chemical Remediation Strategies

Recent developments in the area of phosphazene polymers have aroused considerable interest in their potential technological applications. Cation exchange membranes are used in electrodialysis units for the desalination of brackish water and the removal and recovery of heavy metals from wastewater. Ion exchange membranes formed from phosphazene polymers (Allcock et al., 1977a and b) are particularly suitable for use in the electrodialysis cleanup of wastes containing hazardous metal ions and radionuclides due to their stability to thermal degradation and chemical attack. The ion exchange properties of polyphosphazene polymers are determined by the types of side groups attached to the phosphorus-nitrogen backbone. Because these side groups can be modified easily by nucleophilic substitution and exchange reactions, phosphazene polymers can be fabricated to yield materials with the desired properties. One of the goals of this subproject is to develop ion-exchange membranes made from phosphazene polymers suitable for use in selectively sequestering hazardous metal ions and radionuclides. A second goal of the first year's work was to establish the baseline performance for a commercially available Nafion cation exchange membrane from which we could eventually compare the performance of sulfonated or carboxylated polyphosphazene films.

Phosphazene Film Work

During the first year of the project, our efforts were focused on synthesizing phosphazene polymers with metal-binding functionalities. One of the most promising systems that was developed was a phosphazene polymer that contained sulfonic acid groups in a highly cross-linked membrane film supported on an inert matrix (O'Connor, et al., 1993). The key to the success of this system lies in the fact that the sulfonation (which renders the polymer soluble) was done after cross-linking the polymer on a glass support. We thus have developed an immobilized ion exchange polymer system capable, in principle, of separating metal ions from aqueous solutions. Other polymers and copolymers were synthesized. These include the functional groups shown in Fig 4. (structures marked with an asterisk have not been previously reported in the literature). A

summary of the experimental procedures used to synthesize the novel phosphazene polymers is given below.

Poly(di-4-ethylcarboxyphenoxyphosphazene)

Poly(dichlorophosphazene) (6.13g 0.053mol) was dissolved in 100mL THF, and added slowly (1 h) to a solution of sodium 4-ethylcarboxyphenoxide, prepared from 4-ethylcarboxyphenol (22.0g 0.13mol) and sodium (2.5g 0.11mol) in 100mL THF, and 0.1g TBAB. The reaction mixture was stirred at reflux for 96h, then cooled to room temperature. The polymer was precipitated by dropwise addition into water. The procedure was repeated and the product was dried *in vacuo* overnight, soxhlet extracted with ethanol for 4d to yield 10.0g polymer 1 (yield 42%).

Poly(3-methylphenoxy-4-ethylcarboxyphenoxyphosphazene)

Poly(dichlorophosphazene) (6.13g 0.053mol) was dissolved in 100mL THF, and added slowly (over 1h) to a solution of sodium 4-ethylcarboxyphenoxide, prepared from 4-ethylcarboxyphenol (6.6g 0.055mol) and sodium (2.5g 0.055mol) in 100mL THF, and 0.1g TBAB. The ratio of reactants was designed to bring about replacement of 50% of the chlorine atoms by phenoxy groups. The reaction mixture was stirred at reflux for 48h, then added slowly (over 1h) to a solution of sodium 3-ethylphenoxide (prepared from 3-ethylphenol (12.2g 0.10mol) and sodium (2.3g 0.10mol) in 50mL THF. The reaction mixture was stirred for another 48h, then cooled to room temperature. The polymer was precipitated by dropwise addition into water. The procedure was repeated and the product was dried *in vacuo* overnight, soxhlet extracted with ethanol for 4d to yield 13.0g polymer 2 (yield 61%).

Poly(4-nitrophenoxy-3-ethylphenoxyphosphazene)

Poly(dichlorophosphazene) (6.13g 0.053mol) was dissolved in 100mL THF, and added slowly (over 1h) to a solution of sodium 4-nitrophenoxide, prepared from 4-nitrophenol (7.7g 0.055mol) and sodium (1.3g 0.055mol) in 50mL THF, and 0.1g TBAB. The ratio of reactants was designed to bring about replacement of 50% of the chlorine atoms by 4-nitrophenoxy groups. The reaction mixture was stirred at reflux for 48h, then a solution of sodium 3-ethylphenoxide prepared from 3-ethylphenol (12.2g 0.10mol) and sodium (2.3g 0.10mol) in 50mL THF was added slowly (over 1h). The reaction mixture was stirred for another 48h, then cooled to room temperature. The polymer was filtered and washed extensively with water, dried *in vacuo* overnight, soxhlet extracted with hexane for 4d to yield 10.0g polymer 3 (yield 50%).

Poly(4-aminophenoxy-3-ethylphenoxyphosphazene)

To the solution of 5.0g of polymer 3 in 100mL THF, 2.0g (0.052mol) of lithium aluminium hydride in 10mL THF was added and the mixture was stirred under reflux for 8h. After the reaction mixture was allowed to cool to room temperature, 10% aqueous solution of sodium hydroxide was cautiously added to precipitate the product, which was collected and washed with limited amount of water. The yellow solid that was obtained was dissolved in 5% hydrochloric acid and filtered, the filtrate was neutralized with 20% aqueous sodium carbonate to give 3.9g (86%) white solid of polymer 4.

Poly(diazidophosphazene)

Poly(dichlorophosphazene) (6.13g 0.053mol) was dissolved in 100mL THF, and added slowly (over 1h) to a solution of sodium azide (10g, 0.15mol) in 100mL THF and 0.1g TBAB. The reaction mixture was stirred at reflux for 48h, then cooled to room temperature. The polymer was precipitated by dropwise addition into water, dried *in vacuo* overnight, to give 8.2g solid of polymer 5 (yield 78%).

Poly(di-nitromethylenephosphazene)

Poly(dichlorophosphazene) (6.13g 0.053mol) was dissolved in 100mL THF, and added slowly (over 1h) to a solution of sodium nitromethane prepared from nitromethane (7.0g 0.13mol) and sodium (2.5g 0.11mol) in 100mL THF and 0.1g TBAB. The reaction mixture was stirred at

reflux for 24h, then cooled to room temperature. The polymer was filtered and washed extensively with water, dried *in vacuo* overnight, to give 10.5g yellowish solid of polymer 6 (yield 84%).

Poly(di-malononitrilephosphazene)

Poly(dichlorophosphazene) (6.13g 0.053mol) was dissolved in 100mL THF, and added slowly (over 1h) to a solution of sodium dinitrilemethylen in 100mL THF, prepared from 7.8g (0.12mol) malononitrile, 2.5g sodium, and 0.1g TBAB. The reaction mixture was stirred at reflux for 48h, then cooled to room temperature. Dilute aqueous HCl was added under stirring until the solution turned to light brown (pH = 4-5), then worked up by filtration and washing with THF. The polymer was dried *in vacuo* overnight to give 8.9g solid of polymer 7 (yield 68%).

Absorption of Heavy Metal Ions in Nafion

The overall permselectivity of an ion exchange membrane is governed by equilibrium absorption/desorption processes at the upstream and downstream membrane solution interfaces and by the rate of ion movement through the membrane under the influence of concentration, electric potential, and/or pressure driving forces. Although ion partitioning at the membrane interface is a key contributor to the performance of an ion exchange membrane, very little is known regarding the absorption of divalent/monovalent salt mixtures into a cation exchange membrane.

The equilibrium uptake of two divalent heavy metal cations (Pb^{2+} and Cd^{2+}) in a Nafion cation exchange membrane was investigated. Nafion is a perfluorosulfonic acid membrane that is manufactured by E. I. Du Pont de Nemours and Co. It is currently being investigated for use in fuel cells, batteries, and electrodialysis separation processes. Equilibrium salt solubilities of Pb^{2+} and Cd^{2+} were measured in the presence and absence of monovalent alkali metal ions (K^+ and Li^+).

A consistent method was used to prepare membrane samples for the equilibrium uptake experiments. This was necessary to insure consistent membrane properties and reproducible membrane cation concentrations. 5cm X 2cm Nafion membrane strips were boiled in 7M HNO_3 for 90 minutes to remove impurities from the membrane films and to insure that the membranes were in the full acid form (i.e., a proton is associated with every SO_3^- ion exchange site). The membrane samples were then boiled in water twice to remove excess acid and stored in distilled water at room temperature until they were used in an uptake experiment.

The lead/potassium and lead/lithium experiments were performed by keeping the total bulk (external) salt concentration constant with the Pb/K and Pb/Li concentration ratios varying from 0 to 4:1. Three different bulk salt concentrations were chosen for examination: 0.05 M, 0.25 M and 0.5 M; in all experiments the anion species was NO_3^- . Standard absorption-, blotting-, and desorption-analysis procedures were employed in these experiments (Pintauro and Bennion, 1984). To insure complete equilibration of the membrane with a given salt solution, membrane samples were boiled in the salt water for 90 minutes, followed by a 24 hour soak at room temperature. The membrane samples were withdrawn from the salt solution and excess electrolyte was removed from the membrane surface by carefully wiping the films with filter paper. The swelling properties (change in membrane volume) and wet membrane density of each membrane strip were then measured. Salt was allowed to desorb from the membranes by soaking the films in deionized and distilled water. To remove cations bound to the membrane's ion exchange sites, the films were soaked in 2M HNO_3 . The total cation concentration in the water and acid soak solutions was determined by atomic absorption spectrophotometry.

Typical Pb^{2+} adsorption data in the presence of K^+ and Li^+ are shown in Tables 4 and 5 for a total external salt concentration of 0.05 M. The results show that lead is absorbed preferentially by Nafion when the external solution is a mixture of lead and potassium nitrate with a K^+/Pb^+

concentration ratio $< 3:1$. At $C_K/C_{Pb} \geq 4$, K^+ displaces Pb^{2+} in the membrane. In the lead/lithium system, Li^+ can not replace absorbed Pb^{2+} , even when the Li/Pb concentration ratio in the external solution is 4:1. The higher membrane-phase Pb^{2+} concentrations in the Pb/Li experiments are due to the fact that fewer lithium ions are absorbed into the membrane, as compared to K^+ . When the concentration of monovalent ions in the membrane is low, there are more ion exchange sites available for association with Pb^{2+} .

C_K/C_{Pb} in the external solution	Pb^{2+} concentration in Nafion (mol/l of wet membrane)	K^+ concentration in Nafion (mol/l of wet membrane)
0.25	0.570	0.079
0.667	0.553	0.202
1.5	0.470	0.309
3.0	0.428	0.447
4	0.350	0.477
5.7	0.361	0.504
7.5	0.328	0.543
9.0	0.311	0.592

Table 4. Equilibrium uptake of lead and potassium by a Nafion cation exchange membrane in a solution with a bulk salt concentration of 0.05M at 25°C.

Equilibrium absorption/desorption experiments were also performed with an external solution containing aqueous mixtures of $Cd(ClO_4)_2$ and $KClO_4$ salts. The results of one set of cation uptake experiments are listed in Table 6. Here the bulk concentration of Cd^{2+} was maintained constant at 0.1 M and the K^+ concentration was varied from 0 to 0.075 M. The results show that potassium ions can compete successfully with Cd^{2+} for ion exchange sites when the external solution contains 0.075 M $KClO_4$, as evidenced by the fact that essentially equimolar membrane-phase concentrations of K^+ and Cd^{2+} were measured when the external K/Cd concentration ratio was 0.75. Clearly, the data in Tables 4-6 show that Nafion cation exchange membranes exhibit an unusual absorption behavior for divalent/monovalent cation salt mixtures.

C_{Li}/C_{Pb} in the external solution	Pb^{2+} concentration in Nafion (mol/l of wet membrane)	Li^+ concentration in Nafion (mol/l of wet membrane)
0.25	0.645	0.0092
0.667	0.631	0.0197
1.5	0.632	0.0295
4	0.578	0.0511

Table 5. Equilibrium uptake of lead and lithium by a Nafion cation exchange membrane in a solution with a bulk salt concentration of 0.05M at 25°C.

C_K/C_{Cd} in the external solution	Cd^{2+} concentration in Nafion (mol/l of wet membrane)	K^+ concentration in Nafion (mol/l of wet membrane)
0.0	0.647	0.0
0.05	0.614	0.0415
0.1	0.571	0.0835
0.75	0.441	0.447

Table 6. Equilibrium uptake of cadmium and potassium by a Nafion cation exchange membrane in a solution with a bulk Cd^{2+} concentration of 0.1 M at 25°C.

Synthesis and Characterization of Phosphate-Based Microporous Solids

Microporous solids with large internal surface areas, such as the aluminosilicate zeolites, are known to be very useful in ion exchange and separation processes. One of the most important uses of zeolites as ion exchangers is for the treatment of liquid nuclear effluents (Dyer, 1989). Some of the natural zeolites such as phillipsite have been proved to be very efficient in removing some heavy metals such as Fe^{3+} , Zn^{2+} , and Cu^{2+} from the mining industry waste water. The purpose of this project is to develop new phosphate-based microporous solids with unique selectivities that would be useful in removing heavy metals from water. The synthesis process and characteristics of new synthetic materials are reported below.

Synthesis Method

The hydrothermal synthesis of $Co-PO_4$ and $Fe-PO_4$ was carried out between 200 and 230°C in a Teflon-lined stainless steel autoclave. All the solid materials formed from the hydrothermal synthesis were washed with deionized water and dried in air. The powder x-ray patterns of these materials were collected on the Scintag XDS 2000 Spectrometer at Tulane University. The magnetic susceptibility as a function of temperature for these materials was measured in a magnetic field of 1000 Gauss using a Quantum Design MPMS SQUID susceptometer. Preliminary thermal analysis was performed at a heating rate of 5.0°C per minute in a nitrogen atmosphere on the Shimadzu TGA-50H.

Discussion of Results

Hydrothermal treatment of $2CoCO_3 \cdot Co(OH)_2 \cdot H_2O$, H_3PO_4 , ethylenediamine, and H_2O in a mole ratio of 2.0:11.3:10.4:218.4 for 2 days at 220°C yields flaky crystals of a violet-colored solid. The XRD pattern of this sample indicates that the material is well crystallized (Fig. 5). Preliminary structure determinations reveal that the solid crystallizes in the space group $I4/a$ with $a = 14.711$ Å, $c = 17.81$ Å, and has a structural channel of around 5 Å. The susceptibility measurement in the temperature region between 1.7 and 300°K indicates that the material undergoes a long-range antiferromagnetic transition at 2.2°K as evidenced by the maximum in the temperature dependence of susceptibility (Fig. 6). The thermogravimetric analysis indicates that the solid undergoes a weight loss of 0.607% and 14.103% at 300.68°C and 392.66°C, respectively. The weight loss at 392.66°C may be the result of the loss of templating agent, 1,3-diaminopropane, which results in channels or voids in the solid.

Summary

The chemical remediation cluster has initiated a wide range of field and laboratory projects focused on active and natural remediation of heavy metals in aquatic environments. Active remediation methods using novel zeolites and ion exchange membranes are being studied in the laboratory to determine their metal sequestering capacity as a function of the molality of cations in the aqueous solutions. Competitive adsorption of heavy metals on clays and metal ion exchange reactions in

sediments affect the bioavailability of heavy metals in the Mississippi delta. Field and laboratory projects to determine the importance of these processes in controlling the concentration of dissolved heavy metals are currently underway. Ultimately, we seek to find ways to enhance the metal scavenging capacity of synthetic exchange media and sediments, thereby reducing the bioavailability of toxic heavy metals. The successful completion of these studies should lead to the development of remediation technology and provide an improved understanding of how pollutants are distributed and become bioavailable.

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Expert Geographical Information System For Assessing Hazardous Materials In Aquatic Environments

J.L. Regens, L. White, J.D. Wright, A. Rene, H. Mielke, R. Bakeer, B. Belkhouche, M. Barber

Hazardous substances, including radionuclides, heavy metals, chlorinated hydrocarbons, and industrial solvents, pose unique challenges in terms of environmental restoration and waste management, especially in aquatic environments. When stored, used or disposed of improperly, hazardous materials including transuranic wastes, high level wastes, low level wastes, greater than class C wastes, mixed wastes or chemical wastes can contaminate an array of environmental receptors ranging from soils, sediments, groundwater to surface water. Depending on the specific hazardous substance and site attributes, environmental restoration and waste management can be a complex, problematic activity. This is particularly true for the major Defense Programs facilities managed by the Department of Energy (DOE).

This research cluster consists of two discrete elements. Project Element #1 develops and applies GIS-based approaches to decision support for environmental restoration by delineating potential exposures and health risks at the Rocky Flats Plant and profiling contemporary and historical demographic/land use patterns at Sandia National Laboratories. Project Element #2 develops ESS software for surface water and ground water contaminants in the Mississippi River Basin.

Geographical Information System-Based Approaches to Decision Support for Environmental Restoration at Rocky Flats and Sandia

Because the DOE complex is the focus of large-scale remedial action efforts, growing concerns about the efficacy of remediation technologies, especially bioremediation of sediments and soils, underscores the need for valid, reliable data. Project Element #1 responds to the need to establish a quantitative baseline to evaluate remediation alternatives on a site-specific basis for major DOE nuclear weapons complex facilities. The development of geographic information system-based (GIS) approach to decision support for Solid Waste Management Units (SWMUs), or Individual Hazardous Substance Sites (IHSSs) in the case of Rocky Flats, within discrete Operable Units (OUs) is an essential element in meeting that need. The GIS-based approaches used in this project provide database management and analysis capability for spatial analysis, transport and fate modeling, exposure assessment, effects characterization, and statistical analysis. The project offers a way to integrate on a spatial-temporal basis available pollutant concentration, exposure, health risk, ecological attribute, and demographic/land data to characterize sites for remedial action at major DOE weapons complex facilities. Field applications are conducted at the Rocky Flats Plant and Sandia National Laboratories.

The set of activities comprising Project Element #1 are structured to provide an integrated database management and analysis system for decision support. The GIS-based approaches for environmental restoration assessment developed by this Project Element will establish a working UNIX-based, ARCHInfo GIS operating on a SUN platform. The system will be capable of accommodating vector and raster format data. Data elements will be entered with site-specific geographic identifier codes, observations will be spatially disaggregated, and all data will be subject to quality control/quality assurance procedures to insure reliability and validity prior to utilization for analytical purposes. The databases will be both descriptive and spatial in nature, provide a basis for tracking relational concepts, and enable detailed spatial comparisons as well as graphical representation of data arrays in conjunction with statistical analysis (Ripple, 1989; Ardalan, 1988). By creating an integrated GIS, Project Element #1 will aid research and decision-making about opportunities for innovative remediation strategies that can produce positive changes in concentrations of hazardous materials (Regens et al, 1994; Albers et al, 1993). It also will foster the continued development of a strong interdisciplinary capability and analytical infrastructure to sustain ongoing R&D efforts conducted through innovative partnerships at Tulane and Xavier.

Moreover, Project Element #1 is building a solid basis for mission-oriented, working relationships with DOE, its national laboratories, and other key Federal agencies active in environmental restoration and waste management.

The scope of work under Project Element #1 outlined below will focus on a two-pronged effort demonstrating the utility of GIS-based approaches as decision support aids for environmental restoration at major DOE facilities. Using IHSS 199 and IHSS 200 from OU3 as a study area, we will delineate exposure and health risk potential associated with resuspension of radionuclides in soils and sediments (Rocky Flats Plant). In addition, using baseline data, we will profile contemporary and historical demographic/land use patterns (Sandia National Laboratories). The DOE's Rocky Flats Plant and Sandia National Laboratories are ideal sites for the field applications conducted by Program Element #1 of this project. Because the sources of contamination are reasonably well-defined within a limited geographical area, the application of GIS-based approaches to those study locales will permit the systematic modeling of contaminant transport and fate processes, analysis of exposure and effects probabilities, and evaluation of demographic/land use patterns as a decision aid for identifying alternative bioremediation strategies for selected radioactive elements, especially Pu. The integrated GIS database and applications created by Program Element #1 will provide the information necessary for a systems study of the critical environmental pathways which result in human and ecological exposure to contaminants from the air or aquatic media.

The major objective of Project Element #1 is to provide spatially and temporally integrated, GIS-based approaches to decision support for environmental restoration. More specifically, this component of the project will:

develop and maintain a state-of-the-art GIS R&D infrastructure with a spatial analysis, modeling, and statistical analysis capability to integrate on a spatial-temporal basis pollutant concentration, exposure, health risk, ecological attribute, and demographic/land use data to characterize sites for remedial action at major DOE weapons complex or other Federal facilities.

The DOE nuclear weapons complex is composed of a number of major facilities located in 12 states, covering 3,350 square miles of land, and employing over 150,000 people (U.S. Office of Technology Assessment, 1991). Approximately forty-five years of operation of these large industrial facilities dedicated to metal fabrication, chemical separation processes, and electronic assembly has resulted in the release of enormous quantities of metals, radionuclides, and other chemical compounds. Thousands of SWMUs/IHSSs have been identified as a result of CERCLA/RCRA compliance activities currently underway throughout the weapons complex. The majority of the sites have contaminated soil, sediments, and/or groundwater. Several major statutory authorities including CERCLA, RCRA, and the Federal Facilities Compliance Act mandate risk-based remediation based on site characterization. As a result, DOE has identified the development of methodologies appropriate for conducting retrospective health and ecological risk assessments as well as the development of cost-efficient bioremediation technologies as priorities for R&D (U.S. Department of Energy, 1991).

Assessing the applicability of a particular, discrete remedial action similarly requires the systematic appraisal of historical baseline and contemporary monitoring data delineated for individual SWMUs or other appropriate spatial domains such as OUs. Those efforts need to estimate accurately risks, their potential residuals, and the attendant uncertainties in the appraisal (Krey et al, 1990; Thompson, 1990; Simon, 1990; Ng, 1990). This underscores the need for site-specific data for inclusion in chemical transport and fate models, exposure assessment, and the identification of effects (Loar and Suter, 1992).

Individual SWMUs or SWMUs sharing similar contaminants and/or spatial proximity are obvious units for analysis. They also may constitute reasonably functional ecosystems in a physical, if not

necessarily truly biological, sense. In addition, soils and sediments offer potential sinks for pollutant accumulation while the air and aquatic media offers pathways for pollutant transport which may affect human and ecosystem exposure levels. As a result, the integrated GIS database applications created by this project will provide the information necessary for a systematic study of exposure and effects potential as well as alternative land use regimes (Regens et al, 1994; Regens, 1983).

As noted above, the critical element in developing a successful environmental restoration program to remediate hazardous wastes in soils and sediments involves determining the concentrations, transport and fate, exposures, and effects of specific pollutants. And, in contrast to actions aimed at prospective risk analysis, this requires the use of site-specific data. This underscores the need for spatially and temporally compatible data. Analytic mapping procedures using GIS databases are particularly suited for addressing this problem (Ripple, 1989; Ardalan, 1988). Moreover, when used as a decision support aid, the use of integrated GIS-based approaches provides a powerful tool for assessing environmental restoration R&D priorities and remediation options.

The emphasis in Project Year 1 (April 1, 1993-March 31, 1994) was placed on (1) the start-up of the Entergy Spatial Analysis Research Laboratory located in the J. Bennett Johnston Health and Environmental research Building at Tulane University Medical Center to provide a central facility for data management and analysis and (2) establishment of working relationships with major facilities in the DOE complex to conduct a meaningful ER research program. Accomplishments include the initial acquisition and installation of computer equipment, GIS hardware and software, ORACLE database manager, and related software. The research team also conducted a thorough literature review that resulted in the compilation of an annotated bibliography on GIS applications of potential relevance to environmental restoration and waste management. Based on a June 1993 meeting with Dr. Schutte and Dr. Purdy at DOE Headquarters, the initial work plan was modified to reflect external reviewer and EM-50 guidance to emphasize development of GIS-approaches to decision support for environmental restoration focusing on "real world," field applications in order to test their utility and potential generalizability at actual DOE facilities. Presentations of the conceptual approach and capabilities were made at Los Alamos National Laboratory, Remote Sensing Laboratory/Nevada Test Site, Oak Ridge National Laboratory, Hanford, Rocky Flats, Savannah River Site, and Sandia National Laboratories following establishment of site liaison coordinated through EM-50. Using selection criteria jointly developed by EM-50 and Tulane/Xavier (ARCHInfo GIS at DOE site, ER-based driver for application, facility willing to partner for demonstration, data access protocols), site agreements with local ER and GIS managers, the DOE field office, and EM-50 to conduct the field applications at the Rocky Flats Plant and Sandia National Laboratories were formulated. Contacts also were established with the U.S. Army Corps of Engineers, National Biological Survey, Agency for Toxic Substances Disease Registry, DOE Comprehensive Epidemiological Data program, and U.S. Environmental Protection Agency. Dr. Regens was successful in obtaining a commitment from Entergy Corporation to make a gift to Tulane University designating the spatial analysis laboratory as the Entergy Spatial Analysis Research Laboratory. One technical paper drawing on the Project Element #1 efforts was accepted for presentation at Waste Management '94.

Project Year 2 builds on these accomplishments. For that time period, Project Element #1 will be devoted to completing the following series of tasks. Each of the tasks is associated with applying GIS-based approaches to environmental restoration at Rocky Flats and Sandia and were approved by ER and GIS managers at each site in consultation with EM-50. Each task included in the program plan and technical approach for this Project Element is a necessary step in developing state-of-the-art GIS-based applications to decision support. These tasks include:

- Completion of the conceptual model for GIS-based approaches to decision support for environmental restoration.

- Selection of appropriate transport and fate to estimate Pu resuspension potential in soils and sediments for IHSS 199 and IHSS 200 in OU3 at Rocky Flats.
- Acquisition of validated sampling data for IHSS 199 and IHSS 200 and estimation of contaminant concentrations with transport and fate modeling.
- Mapping isopleths for pollutant concentration estimates from transport and fate output to provide spatial comparison with initial concentrations from sampling data.
- Conduct exposure assessment and map exposure potential.
- Link exposure potential to human health risk estimates and map isopleths bounding probabilities.
- Compile available data and summarize documentation for contemporary (1990) and historical (1940-1980) demographic and land use data for the area within a 4-mile buffer of Sandia National Laboratories.
- Prepare GIS coverages with lineage reports for contemporary data.
- Generate maps displaying contemporary demographic and land use data.
- Prepare GIS coverages with lineage reports for historical data.
- Generate maps displaying historical demographic and land use data.

An Expert Geographical Information System for Contaminants Fate and Transport Modeling

A thorough literature review was conducted during Year 1 of the cluster project on published and on-going research on GIS, fate and transport models, expert systems (ES), database management, and ES applications in hydrology, water resources, environmental engineering and geotechnical engineering. Information was solicited from different software and hardware vendors regarding their products' costs, features and capabilities for both personal computer (PC) and UNIX based GIS software. A reference list was compiled containing collected and other available articles. The objective of the engineering faculty research in Years 2 and 3 of the project was identified as to:

- Establish a satellite GIS facility on Tulane Uptown campus at the Department of Civil and Environmental Engineering.
- Develop an Expert Geographical Information System (EGIS) platform for contaminant fate and transport studies.
- Use the EGIS to study the geographical areas of Los Alamos and Sandia.
- Implement the data collected by other DOE clusters for the Devil Swamp area in the GIS.

The literature review enabled the researchers to assess capabilities and requirements of currently available software and hardware. The research group developed a set of guidelines regarding the required capabilities and features of the GIS facility to be established on the Tulane Uptown campus.

The EGIS will be designed for use by fate and transport modelers with little or no experience in GIS, database management, and computer graphics. It is a user-friendly menu-driven platform that integrates databases, statistical inference mechanisms, tools to identify modeling features for simulation, graphics and scientific visualization of input/output data, and algorithmic tools for analysis and simulation. The tools to be integrated are the contaminant transport and fate models MODFLOW, MT3D, WASP and HSPF, the GIS software ARC/INFO, and database management software ORACLE. A unified smart interface between the different software components will be developed by the research team utilizing their expertise in fate and transport modeling, soil technology, and computer science. The shell NEXPERT will be used to develop the necessary interface. The smart EGIS will be a generic platform independent of the geographical region or type of data used in the study, and could be applied to other areas with different data sets.

After examining the various research aspects in the initial phase of the project (Year 1), a decision was made to focus on two specific areas of flow and transport modeling:

- (1) saturated ground water flow and single phase transport, and
- (2) dynamic surface water flow of soluble compounds.

The programs MODFLOW and MT3D were selected as the ground water flow and dissolved contaminant transport models to be implemented in the EGIS package to be developed in Years 2 and 3. Copies of the programs were obtained and compiled. Drawing on the experience of the principal investigators, a cursory review of publicly available flow and transport models resulted in a two-level approach. When data is sufficient to justify the expense and complexity of three-dimensional analysis a combination of MODFLOW and MT3D will be used to determine the fate of single phase transport within a saturated aquifer. If data set is sparse, or the area is too large, then a two-dimensional flow and transport model called GWTRANS will be used. Copies of the programs were obtained and compiled on personal computers. All three models have been obtained and are currently running on computer facility of the Department of Civil and Environmental Engineering (CEE) at Tulane.

EPA's Water Quality Analysis Simulation Program (WASP) was chosen as the dynamic surface water transport model. The latest version of the program was obtained from EPA and has also been loaded on the CEE computer facility. The program was selected because of its flexibility and its wide-spread use. Another model, called Hydrologic Simulation Program--FORTRAN (HSPF), is currently being evaluated to determine if it would more easily, or more accurately, predict tributary and non-point source loads for the WASP model. A dynamic flow model called DAFLOW is operating as the flow model. The link between the flow and transport model is still under development.

The EGIS will be used to study the geographical areas of two DOE national research sites at Los Alamos and Sandia. Both sites are located in the Rio Grande Valley, which encompasses parts of Colorado, New Mexico and Texas, along with several major metropolitan areas and large industrial complexes. Recent studies have revealed relatively higher levels of contamination in parts of the river region. Data will be obtained from government agencies including DOE, USGS, EPA and USACOE.

Some preliminary work has been accomplished on the Uptown GIS personal computer facility. Several software packages, including the expert system shell CLIPS by NASA, FORTRAN and C compilers by Microsoft Corp., and Autocad by Autodesk Corp., were acquired through departmental resources and installed on the Uptown GIS personal computers. The expert system shell LEVEL5 by Information Builders, Inc. and FoxPro database management software by Microsoft Corp. were also added to the software library through a separate grant from the US Army Corps of Engineers. Researchers from the CEE and the Geology Departments at Tulane have received as a grant from Landmark Graphics Corp. a comprehensive graphics package for geophysical, seismic, cartographic, reservoir and geo-data management valued at over \$250,000. This package will be loaded on the Sun server of the Uptown facility for use in the future GIS research by the two departments.

A PC based GIS system for East Jefferson Parish was developed using the available equipment and software. An electronic map of the greater New Orleans area, which includes Jefferson and Orleans Parishes, was digitized from a paper copy using a desktop Summagraphics MM series digitizer and the CAD software Autocad. The map contained several layers each displaying similar features. The Mississippi River, lake Pontchartrain, runoff drains and navigation canals were all included in one layer. A second layer displayed the locations of 750 soil boring logs obtained by the researchers from the US Army COE and two local geotechnical engineering firms. Other layers contained city streets, major water crossings and other geographical features. Soil data from the boring logs was stored in database file. Programs were developed to enter, sort, retrieve, and display soil data based on location on the map through menu-driven interfaces. This prototype system is similar in concept to the proposed EGIS.

Contacts have been established with several organizations involved GIS research such as the DOE, New Orleans District (NOD) of the Corps of Engineers (COE), COE Waterways Experiment

Station in Vicksburg (WES), Orleans Parish, and the Geography Department at the University of New Orleans (UNO). Several visits were made by the engineering faculty in Year 1 to these facilities which contributed to the formulation of the work plan for Years 2 and 3.

- Advantages and disadvantages of different hardware and software used in these facilities were recognized in the selection of equipment and software for the Tulane facilities.
- Some "hands-on" experience was gained by the engineering faculty by using the GIS facility at UNO. The UNO UNIX Sun station based facility has been recognized by ESRI as a formal training and research center for ARC/INFO since 1990. Demonstrations of some of the locally developed GIS applications at UNO indicated the need for an integrated software interface between the different components to enhance the user interface with the GIS.
- Agreement was reached to share geotechnical information currently available in the COE database of soil Boring Logs Data Management (BLDM) software developed for the Computer Applications in Geotechnical Engineering (CAGE) program at WES. The PC-based BLDM is used by the COE to maintain the geotechnical data collected nationwide. Geotechnical data for the Rio Grande Valley region will be obtained from earlier soil investigations available in the CAGE system.

Two technical papers were submitted for publication in the ASCE journal (under review) and an international conference in France (accepted) on use of expert systems in developing soil site investigation, and on reliability of soil properties obtained from laboratory and field tests. A third paper is under preparation. Future research proposals will be submitted to outside sources to extend the study to other parts of the Rio Grande Valley.

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Education Project

Enhancement Of Environmental Education At Tulane And Xavier Universities

Sr. M. Loughlin, S. E. O'Connor, L. A. White, S. Bhattacharya, J. Bennett, M. Zimmerman

The overall goal of the environmental education initiative of Tulane University (TU) and Xavier University (XU) is to develop a comprehensive environmental education program involving at least four schools in the two universities, in an effort to produce graduates who are competent to carry out DOE's mission of environmental restoration and waste management. The educational activities will work toward providing some of the additional 12,000 scientists, engineers and technologists DOE estimates it will need during the next five years to carry out its new mission. The first year of this project focused on the development of a master plan and an infrastructure which will facilitate the coordination of programs, build upon existing strengths and resources, and promote the maximal utilization and efficiency of resources. The implementation of coordinated curricula enables Tulane and Xavier to combine their strengths in a synergistic manner and to utilize common resources and faculty. The master plan provides an overall blueprint of a comprehensive program which will allow funding to be obtained for individual components. Indeed, the planning activities have already resulted in leveraged funding of the overall program, as evidenced by the awarding of a contract by the Health Resources Services Administration, Bureau of Health Professions, to conduct a project *To Determine Model Internship Standards for Environmental Health* and by the DOE to implement a *Hazardous Materials Management and Emergency Response (HAMMER)* training and education project.

Master Plan

The focus for Year 1 was the development of a master plan to establish a coordinated program in environmental education. The activities included establishing the necessary institutional infrastructure to carry out its program, inviting guests from academia to share information on their environmental education programs, traveling to meetings and conferences to enable Xavier and Tulane personnel to learn more about DOE-sponsored programs, and initiating the development of curricula, courses, etc which will form the foundation for the environmental education initiative.

Establishing Institutional Infrastructure

- Dr. Sally O'Connor was named the environmental education coordinator at Xavier University. The Center for Environmental Programs at Xavier was reorganized under her direction to further facilitate the coordination of environmental education, to prevent duplication of efforts and to ensure effective and efficient implementation of activities. Members of the Xavier Environmental Education Committee were named and included faculty members who represent various disciplines.
- At Tulane, Dr. LuAnn White was designated coordinator of the environmental education programs. In addition, she served as coordinator for the School of Public Health and Tropical Medicine (SPHTM), Drs. Joan Bennett and Michael Zimmerman were named coordinators for the College of Liberal Arts and Sciences (LAS) and Dr. Sanjoy Bhattacharya was named the coordinator for Engineering. This group met several times to address issues such as mechanisms for cross registration, limits on the number of courses which can be taken outside of a school, number of credits for courses and other operational issues. Administrative barriers have been identified and are being addressed.

- Dr. LuAnn White chaired the joint Tulane/Xavier Environmental Education Committee whose members included Joan Bennett, Michael Zimmerman, and Sanjoy Bhattacharya from Tulane and Sr. Stephanie Henry, Robert Fulginiti, Harold Vincent, Steven Duplantier and David Eatman from Xavier. The committee met several times to institute a mechanism for the coordination of activities, including efficient communication and implementation of new courses and new programs. Procedures for implementing relevant administrative tasks such as cross registration and student recruitment have been defined and implemented.

Workshops and Conferences

- To assist in their planning efforts, Xavier and Tulane Universities conducted two workshops focusing on Environmental Science Education curriculum development. The first was held on June 30, 1993 and was led by Dr. Robert Ford, Director of the Center for Energy and Environmental Studies at Southern University in Baton Rouge. The second workshop was led by Dr. Dilip Shah, Professor of Industrial Hygiene and Safety at North Carolina A&T University. Dr. Herbert Allen, Professor of Environmental Engineering at University of Delaware visited Tulane to provide input on the newly developed Environmental Engineering program on January 18-19, 1994.
- To become familiar with DOE's other educational programs, Tulane and Xavier faculty traveled to DOE-sponsored conferences. Mr. John Pecoul of Xavier and Dr. LuAnn White of Tulane attended the Outreach Program Review sponsored by DOE Environmental Education and Development in Denver, Colorado on May 26-27, 1993. Each made a presentation about institutional capabilities. Dr. White presented the objectives of this grant. Drs. O'Connor and White attended the Academic Partnership meeting held by DOE Environmental Education and Development in Rockville, Maryland on June 17-18, 1993. These meetings provided a forum for project personnel to become informed of other DOE sponsored educational activities.

Curriculum Development

- Xavier University's master plan identified new curricula which best utilize the resources of both universities. These included an Environmental Studies minor, an *Environmental Science* track within the Science Disciplines, a dual degree program in Environmental Engineering with Tulane's Engineering School, and a BS/MS combined degree program with the Tulane's SPHTM, which offers degrees in public health, industrial hygiene, and waste management. Xavier's master plan also included the infusion of environmental topics into its curriculum, to produce graduates in all fields who are environmentally literate. A comprehensive survey of the extent of on-going environmental course offerings, environmental research and summer programs is underway.
- Key Xavier faculty have been approached regarding the development of new courses which will make up the new academic programs. To ensure quality of program, proposals for development of new courses or infusion of environmental awareness in existing courses are reviewed by an independent committee (Faculty Mini-Grants) which approves only those mini-grants which are feasible and within the guidelines of the overall grant. The committee has approved five of the eleven mini-proposals it reviewed during the Fall Semester, 1993.
- At Tulane, the LAS committee has outlined a curriculum for an environmental studies coordinate major. The current environmental curriculum was reviewed and a revised set of requirements for the coordinate major in environmental studies was developed for all appropriate departments in LAS. Curriculum gaps were identified in the existing program and new courses were proposed to fill these gaps. Each department was contacted individually for suggestions and recommendations. Meetings were held with selected admissions officers, key faculty members and deans as well as with other relevant personnel to solicit their input and support for the new coordinate major. The coordinate major is described in document, *A Vision for Environmental Studies at Tulane University*, drafted in late Fall 1993.
- An in-depth survey of Environmental Studies Programs at 25 other universities was completed. Among the different schools, core curriculum content, different tracks of study and any unique features were identified. All well-developed environmental studies programs stressed a strong science foundation. In addition, a questionnaire was sent to former graduates of the

existing Tulane program. The questionnaire provided valuable student feedback. Overall, the students comments were positive and some suggested that the program develop more focus.

- A recruiting brochure was created introducing the Environmental Studies Program to potential applicants and in-coming freshmen. The brochure was distributed at the Tulane Newcomb freshmen reception. A brochure describing the new programs at Xavier is being prepared for distribution to students matriculating in the Fall semester, 1994.

- The Department of Civil and Environmental Engineering has developed a new curriculum leading to a BS degree in Environmental Engineering. The curriculum was offered to the sophomore class of 1993. Currently at least 10 students have joined the Environmental Engineering program with high interest among women and African-American students. This program is available to Xavier students who follow the 3 + 2 track to obtain a BS degree from Xavier in physics and a BS in environmental engineering from Tulane.

New Course Development

New courses were developed for the undergraduate programs and are open to all undergraduate students of Tulane and Xavier:

- Xavier faculty developed several new courses for the environmental science curriculum including: *Survey of Environmental Chemistry* (Sr. Henry), *Environmental Ecology* (Martinat), *Communication of Environmental Risk* (Allan) and *Environmental Toxicology* (Mielke). The *Survey of Environmental Chemistry* is offered during the Spring Semester, 1994, and includes a lab component.

- Dr. Bhattacharya developed a new undergraduate course, CVEN 207 *Introduction to Environmental Studies*. The course, open to all Tulane and Xavier freshman and sophomore students, has been scheduled for offering in the Spring Semester, 1994. Covering basic aspects of water and air pollution and ecosystems, this introductory course (with no prerequisites) is open to both technical (science and engineering) and non-technical students.

- Dr. Allen Apblett developed a new undergraduate course, CHEM 250 *Environmental Chemistry* and has been scheduled for offering in the Fall Semester, 1994.

Xavier ER/WM Scholars Program

The Xavier University Environmental Education Committee met several times to discuss issues regarding the DOE sponsored scholarship program. A brochure has been prepared describing the program, its guidelines and the selection process. The committee decided to call the program *The LIFE Scholars Program*, LIFE being an acronym for *Living Intelligently to Foster Earthcare*. A brochure describing the availability of scholarships to those students interested in a career in environmental restoration and waste management was distributed to the students during the Fall semester registration. The guidelines for the scholarship were published in the Xavier University *EnviroNews* publication, including the eligibility requirements, the benefits, the requirements of the program, and the selection process. Out of a total of 35 applicants, four were selected to receive LIFE scholarships. The LIFE scholars (Lawrence Carter, III, Camille Fouche, Temperance Smiley and Tamara Mosby) were placed under the mentorship of Xavier Faculty who have on-going research projects in environmental areas. The scholars will be encouraged to apply for summer internships at DOE labs to further enhance their education.

Initiation Projects

Heavy Metal Immobilization In Mineral Phases

A. Apblett

A successful waste form for toxic or radioactive metals must not only have the ability to chemically incorporate the elements but it must also be extremely stable in the geological environment. Therefore, many scientists have adopted a "naturalistic" approach to waste disposal materials whereby the choice of waste form is based upon the natural geological repository for these elements. Thus, ceramic wasteforms are sought which mimic those minerals that have sequestered the hazardous metals for billions of years. Such an approach has culminated in the development highly successful materials such as Synroc and monazite for nuclear waste disposal (Lutz, 1988). The usual approach to the incorporation of waste into a ceramic is to slurry waste solutions with the powdered ceramic matrix, evaporate the water, and then calcine at high temperatures. The problem with this method is that it tends to produce an inhomogeneous waste form that may contain intermediate leachable phases. Also, it does not allow separation of the metals into chemically-distinct classes that could be subsequently immobilized in individual better-suited mineral phases. As well, the traditional approach leads to considerable amounts of oxo-anions in the preceramic. These are strong oxidants at elevated temperatures and therefore lead to the necessity of a redox buffer to prevent the formation of highly-oxidized water-soluble compounds.

A practical alternative for the preparation of these materials is metal organic deposition (MOD) (Mantese 1989). Of the many methods of producing ceramics, MOD, is outstanding in its simplicity, versatility, and inexpensiveness. MOD utilizes metal organic precursors which upon pyrolysis are converted into their constituent metallic elements, oxides, nitrides, or other compounds. Suitable metal organic precursors are covalent compounds with the metal atom bonded to an organic group via an oxygen, sulfur, nitrogen, or phosphorus atom. The versatility of the MOD process arises from two factors; firstly, the ligands can be easily varied to adjust their physical and chemical properties to suit a particular purpose (e.g. this study has used long chain organic residues which allow extraction of the metals from water and their dissolution in organic solvents). Secondly, one or more metalorganics or other additives can be mixed and combined in any proportion prior to pyrolysis in order to produce hybrid materials with superior properties. Thus the production and testing of a large range of materials for use as sequestering matrices for toxic metals can easily be achieved.

The major contribution that the MOD process can make to ceramic waste forms is the ability to mix the toxic metals at a molecular level with the elements which form the ceramic matrix. With proper choice of organic ligands, the inclusion of significant amounts of alkali metals in the ceramic and, hence, their detrimental effect on durability may be avoided. As well, chemically distinct classes of metals (eg. oxophilic versus thiophilic) could be separated from each other using two separate complexants. Thus, they could be immobilized in separate, more appropriate, waste forms. The two waste forms that we have investigated in this study are Synroc (a titanate waste form used for high level radioactive waste) and sphalerite (ZnS). The latter mineral can form solid solutions with heavy metal sulfides (Duda, 1986), while Synroc should be suitable for oxophilic metals.

In the first stage of our research we identified thermally-unstable ligands which could fulfill the role of complexing toxic metal species and allowing their precipitation or extraction into non-

aqueous solvents. We had studied the complexation and thermal behavior of pyruvic acid oxime (PAO) with representative cations associated with nuclear reprocessing waste (Apblett, 1993). This ligand has the dual advantage that it precipitates most of the cations from solution and these complexes decompose at a very low temperature (ca. 200°C) to CO₂, acetonitrile, and, initially, the metal hydroxide. It was found that the introduction of the appropriate elements required for Synroc mineral phases to a simulated radwaste stream (Purex Waste B) followed by NaPAO and disodium fumurate leads to precipitation of a metalorganic Synroc precursor in which the components have been intimately mixed at the molecular level. This approach circumvents incorporation of alkali metals in the final ceramic and their detrimental effect on leach-resistance. As well, the high degree of mixing of radwaste and ceramic matrix elements leads to lower sintering temperatures reducing the probability of volatilization of toxic elements. The problem with using PAO is the expense of the ligand and the fact that there is no commercial source for it. We therefore studied the possibility of using a long-chain carboxylic acid (2-ethylhexanoic acid) to complex the radwaste elements so that they could be removed from aqueous solution by extraction into methylene chloride. The extraction was performed by dissolving the ligand as the sodium salt in the simulated radioactive wastestream and then extracting with methylene chloride. This approach was particularly successful at removing the lanthanides and transition metals from the wastestream. The extract was then mixed with appropriate amounts of the 2-ethylhexanoate complexes of the metals required for the ceramic matrix (Ca, Ba, Ti, Zr, Al). The solvent was then distilled (and retained for reuse) leaving behind a homogeneous mixture of the metal complexes that was amorphous to X-rays. Pyrolysis of this mixture at 800°C produced the expected Synroc phases.

We have also developed a route to Synroc that avoids the use of organic solvents or the necessity of a filtration step. When synthesizing Synroc, it is important to keep the amount of reactive oxygen present in the system low to avoid the formation of water-soluble higher oxides of the transition metals or actinides (e.g. CaUO₄). Since, typically radwaste contains nitrate salts of the metals and this anion is a very strong oxidizing agent at elevated temperatures the avoidance of oxidation of the metals has usually required addition of a redox buffer (e.g. titanium metal) to the preceramic powder. Unfortunately, this adds further complexity to the final wasteform and leads to deviation from the ideal stoichiometry. To circumvent this problem, simulated PW-4b high level radioactive waste was mixed with nitrate salts of the elements required for the Synroc matrix (except for titanium) in aqueous solution. The water was removed from the system and the residue was suspended by stirring in glacial acetic acid. An excess of acetaldehyde and the required titanium [as Ti(OEt)₄] was added to the mixture which was then heated at reflux for 12 hours. During that time, the nitrate salts were converted to acetates due to oxidation of the acetaldehyde by nitrate. The removal of the organic solvents from the mixture by rotary evaporator yielded a deep-red glassy solid that was completely amorphous to X-rays indicating intimate mixing of the radwaste and ceramic-matrix metal ions. This material decomposed and sintered upon heating to the usual Synroc phases (hollandite, BaAl₂Ti₆O₁₆, perovskite, CaTiO₃, and zirconolite, CaZrTi₂O₇) without the necessity of a redox buffer since acetate provides a neutral (or slightly reducing) atmosphere upon its decomposition. Such a process could also be extremely useful for other concentrated wastestreams that contain oxoanions.

Another wastestream that we are investigating is from wet lime-gypsum flue gas desulfurization plants (Lefers, 1987). This contains mainly harmless metals (calcium, magnesium, sodium, iron, and aluminum) but does contain significant amounts of toxic metals (manganese, cadmium, chromium, copper, mercury, nickel, lead, zinc and tin). The challenge is to separate the toxic metals from the others in such a manner that they may be easily incorporated into the ceramic wasteform. We have reacted each of the above metals with potassium ethylxanthate and found that ethylxanthate is very efficient at precipitating the heavy metals while leaving calcium, magnesium, and sodium in solution. All the precipitates were characterized by X-ray diffraction and their solubilities in common organic solvents determined. Thermal gravimetric analysis indicated that the ethylxanthate complexes decompose at extremely low temperatures (ca. 120°C) to sulfide

materials. The only volatile species in the off-gasses that we could detect by GC/MS was carbonyl sulfide, COS. Presumably, the decomposition also produces hydrogen sulfide and ethylene but these were not detectable using our instrumental parameters.

The xanthate complexes may be used in two ways to accomplish entrapment of heavy metals in sphalerite;

- (1) zinc ions may be added to contaminated water and it and the heavy metals may be co-precipitated by addition of potassium ethylxanthate to the solution
- (2) separated xanthate precipitates and zinc bis(ethylxanthate) may be dissolved in a high-boiling organic solvent (e.g. xylene) and the subsequent solution refluxed to deposit a homogeneous sulfide ceramic.

We have used a single toxic metal in our initial investigation in order to simplify the chemistry. Cadmium is often found at up to two and a half percent in naturally-occurring zinc sulfide and we therefore chose this amount for the amount of waste-loading. A solution of cadmium chloride and zinc chloride in appropriate amounts was treated with potassium ethylxanthate and the precipitate thus obtained was filtered off and dried. X-Ray diffraction (XRD) indicated that the cadmium and zinc precipitate homogeneously i.e. as a solid-solution of zinc and cadmium ethylxanthates. As well, we have demonstrated that a refluxing solution of a mixture of zinc and cadmium bis-ethylxanthates in xylene rapidly precipitates the sulfides as a very fine powder. In both cases, XRD indicated that the only phase present was sphalerite and no separate CdS phase was observed. Furthermore, the crystallite size was found to be less than 1 nm, making them excellent for processing into a sintered body. We are currently pursuing the treatment of the wet lime-gypsum flue gas desulfurization wastestream using this methodology. At the same time, leaching experiments are being performed on the wasteforms using either the Department of Energy MCC-1 method (MCC-1, 1983) or the Environmental Protection Agency's Extraction Procedure Toxicity Test (EPA, 1980).

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A Pilot Study of the Applicability of Polarography to Exposure and Bioremediation Problems in Aquatic Systems

K. Bundy

Abstract

This project has demonstrated the feasibility of using polarographic techniques to study pollution in Louisiana waters and has developed methodology for this purpose. Water and sediment samples from Devil's Swamp have been fractionated and analyzed for heavy metal concentration using differential pulse polarography. Lead has been found to be quite inhomogeneously distributed in the environment, with much higher concentrations being found in sediments and in suspended particulate form in the water, as opposed to being chemically dissolved in the aqueous phase. A laboratory study is planned in which an animal model will be used in controlled experiments to investigate internal organ uptake of heavy metals. The research proposed for next year includes collaborative efforts with two of the cluster groups involved with the DOE/EM project.

Introduction

This initiation project is a pilot study in which polarography, a trace level electroanalytical method, is being investigated with respect to its suitability for research involving certain heavy metal pollution problems. There is both a field and laboratory aspect to this study. The field study involves sampling and analysis of heavy metal levels in waters and sediments from Devil's Swamp and in internal organs of frogs which live in contact with these materials. Based upon the levels of pollutants found, a laboratory study will be conducted in which frogs will be subjected to controlled amounts of these heavy metal pollutants. Various organs will be monitored to observe bioaccumulation of these metals over time. It is hoped that these studies will lead to the identification of a sensitive biomarker for the degree of pollution present. The field study is currently well under way. The laboratory phase of the research should begin in about a month. This work is being conducted by the principal investigator and his graduate student David Berzins.

The metals being investigated are lead and chromium. These were selected for the following reasons. Our study was restricted to only two metals because of its limited pilot project nature. Although our original approach was to conduct a multi-element analysis and use this as a guideline for metal selection, the animal care and use committee approval for the laboratory phase of this project was contingent on identifying specific metals for testing. Lead and chromium were selected based upon the fact that we knew they were at the site, that they represent significant toxic hazards in certain situations, and, in the case of chromium, that it can exist in different valence forms with significantly different toxicity. The latter fact is significant to our investigation since polarographic methods are optimal for conducting speciation studies.

Our approach in these studies is patterned after previous polarographic experiments related to biomaterials and surgical implants in which trace levels of metals in animal and human tissues and bovine blood have been conducted. As is the case for materials released from implants into body tissues, once a metal contaminant is environmentally released, many possibilities exist as to its chemical form and the compartment where it is sequestered. In natural waters, metals may be found as free ions, or as various complexes with both inorganic and organic ligands(1). In sediments, metal ions may be adsorbed onto the surfaces of mineral and organic constituents. Alternatively metal may be retained in sediments in mineral or salt precipitates. The consequence for biota in aqueous environments, and the mechanisms by which they become polluted, can be quite varied because of the complexities of this inhomogeneous heavy metal distribution. This

research project is in part concerned with ascertaining the applicability of polarographic techniques for aiding in the understanding of these metal speciation and distribution questions.

The rationale for the studies with frogs is that observations have been made that various organs can accumulate heavy metals. For example, Ferguson et al(2), with tests in rabbits, have shown that cobalt (originally released into the blood stream due to corrosion of surgical implant material's) is stored in the spleen at 45 times the concentration in the liver. It is stored in the kidney at 9 times the liver concentration. Black (3) has shown that Cr in the rabbit liver can be as much as 85% above control levels due to severe corrosion of stainless steel implant materials. Although we are not aware of any organ studies involving metal accumulation in frogs, experiments with toads (*Xenopus laevis*) have shown that bone, skin, muscle, kidney, and liver samples from animals fed worms from lead contaminated soils have elevated concentrations compared to control toads fed uncontaminated worms(4).

Methodology

General considerations

An effective analysis of a polluted environment requires that attention be paid to a number of issues. Since contaminants may be inhomogeneously distributed, a sample taken for analysis must be capable of being separated into its various constituent fractions or moieties. The pollutant of interest must be capable of being extracted from each moiety, if this is necessary to render the pollutant into a form amenable for chemical analysis. Finally, a sensitive means for analysis must be available.

Samples Taken for Analysis

The following environmental materials have been analyzed in this project: Mississippi River water, Devil's Swamp water, bull frogs from Devil's Swamp, and water and sediment from a St. Charles Parish site. The St. Charles samples were used to validate our separation technology, while the other samples were taken for chemical analysis. Beef liver has also been used for developing analytical methodology for the frog tissue tests.

Separation

In order to understand possible mechanisms of animal exposure to pollutants, it is important to know what environmental moieties tend to sequester the contaminants. Initially, with Mississippi River water we experimented with a stirring and settling out process but later found that filtration was more effective in isolating individual fractions. At present a Spectrum Regulated Lab Vacuum Filtration Center is employed in our research. The sequential filters used are 290, 105, 20, and 1 μ m. This process allows the definition of various environmental compartments where pollutants could be accumulated. Coarse particulates (sand and rock fragments), fine particulates (clay particles), and the aqueous phase are separated out. For water samples, this procedure can be used directly. With sediments, initial suspension in distilled water is necessary.

Besides the above definitions of the compartments, in this report we have found a number of definitions of concentration to be useful. Parts per million (ppm) or parts per billion (ppb), mg/kg (or mg/l) and μ g/kg (or μ g/l) respectively, may be variously used to describe concentration of heavy metals in the polarographic cell, in a particular compartment or moiety, or in reference to the water or sediment as a whole. Weight percent (w%) is useful for describing the fractional amounts the various compartments or moieties represent of the whole.

Extraction

We have investigated both ashing and acid digestion procedures. The advantages of ashing are that it is possible to obtain more full recovery of material and it concentrates rather than dilutes the material being analyzed. On the other hand, acid digestion may provide a more realistic measure of the danger posed by a heavy metal pollutant. This is because it more closely simulates how pollutants could become bioavailable by contact with gastric juices. This would minimize

confusion between trace elements put in the environment by pollution and those which are naturally present. Although in principle either method will work to extract heavy metal elements from either tissue or environmental samples, in practice we use ashing for tissue specimens and acid digestion for environmental samples. This is because the amount of available biological material is limited, in contrast to sediment or water samples, which can yield adequate amounts of material for analysis by concentration (e.g. boiling of water) or by starting with a sufficiently large sample at the beginning of the separation procedure.

The process we have found effective for ashing of frog tissue is to use an electric multiple unit furnace type 051-PT (Heavy Duty Electric Co.) for 1-2 hr. at its maximum setting. A literature review revealed that standards have been developed by the American Society for Testing and Materials (ASTM) for extraction of trace elements from sediments. These are Standards D3974-81 and D4698-87 (5,6). The process we employ follows D3974-81 and involves specimen treatment with a nitric acid/hydrochloric acid mixture and heating to 95 °C. The other standard involves a more drastic treatment with a perchloric, hydrofluoric, and nitric acid mixture. Although D4698 would probably give a higher recovery yield, our rationale for using D3974 is as follows. Besides via the food chain, probably one of the main pathways for frogs to become exposed to heavy metal pollutants would be through ingestion of water containing particulate matter. Heavy metals would be leached from the particulate material as it passed through the digestive system and contacted acidic gastric juices. Although D3974 is a more drastic treatment than is contact with digestive fluids, it would probably give a better estimate of the proportion of heavy metal pollutants potentially bioavailable for intestinal absorption than would D4698.

Polarographic Analysis

Our polarographic analyses are conducted with an EG&G PARC Model 384B Polarographic Analyzer, a 303A Static Mercury Drop Electrode, and a Houston Instruments DMP-40 Digital Plotter. Polarography is a trace level electroanalytical method developed by J. Heyrovsky(7), who received the 1959 Nobel Prize in chemistry for his research. The basic principle of polarography is as follows. The sample to be analyzed is put into a cell containing a much larger volume of solution known as the supporting electrolyte. The electrical potential of the mercury drop is changed. At a given value of potential, known as the half wave potential, $E_{1/2}$, the substance being assayed will be electrochemically reduced. The current associated with this reduction is measured. The reduction takes place under electrochemical conditions known as diffusion control in which the rate of reduction is subjected to mass transport limitations. This means the measured current is directly proportional to the concentration of the substance being analyzed. Since currents can be measured at low (nanoampere) levels, concentration can similarly be measured at trace levels (on the order of part per billion [ppb] sensitivity). In a given supporting electrolyte, each chemical species capable of undergoing reduction has a unique value of $E_{1/2}$. This is a very useful feature in environmental investigations where speciation is an issue. For example, hexavalent chromium has a different $E_{1/2}$ than does trivalent chromium.

The supporting electrolytes (8) we are using in this investigation are 1) for Pb detection- 0.1 M citric acid with ammonium hydroxide added to adjust pH to 3, 2) for Cr+6- 0.1 M tartaric acid with NH₄OH added to achieve pH 9, and for trivalent chromium- 0.2 M acetic acid plus 0.2 M KSCN, pH 3.2.

The specific type of analytical method we employ is known as differential pulse polarography. In this method a potential in the form of a staircase ramp is applied to a mercury drop electrode. From the tip of the electrode, a series of mercury drops fall off at constant time intervals. The height of each step in the ramp is termed the scan increment. Modulation pulses with identical offset voltages are superimposed on the ramp every time a drop dislodges. The current is measured just before the pulse is applied and just before the pulse ends. The currents are compared, with the change in current being the signal to be processed. The resulting current I versus potential E curve (known as a polarogram) will show a current peak at $E_{1/2}$ which is

directly proportional to the concentration in the polarographic cell of the element being analyzed. In the tests that we have conducted in this project we have used a 20 mV pulse height, a 1 sec drop time, and a 2 mV scan increment. Since oxygen is a ubiquitous electroactive element which can act as an interference for the measurement, the polarographic cell is purged with oxygen-free nitrogen for 4 minutes prior to measurement.

Table 1 shows the half wave potentials and concentration detection limits in various tissues and environmental moieties using the supporting electrolytes previously mentioned (except where indicated). The values for lead are based upon our tests this year. The detection limits for lead are considered in more detail in the discussion of table 3 which is presented later. The values for chromium are based on the extensive experience in our laboratory with chromium analyses of blood and other tissues which have been previously described, e. g. in references 9-12.

TABLE 1
Half Wave Potentials and Detection Limits in Polarographic Cell of Various Ions
in Different Tissue Moieties, Fluids, and Environmental Compartments

Ion	E _{1/2} (mV vs. Ag/AgCl)	Moiety	Detection Limit (ppb)
Cr+6	-0.38	Aqueous fraction of bovine blood	5
Cr+6 (in 1M KCl)	-0.36	Aqueous fraction of bovine blood	40
Cr+3 (in 1M KCl)	-0.90	Aqueous fraction of bovine blood	--
Cr+6 (pH 7.8*)	-0.36	Bovine blood cells or serum proteins	100
Cr+6	-0.40	Bovine blood cells	4
Cr+6	-0.40	Bovine serum proteins	7
Cr+3 (pH 7.8*)	-1.05	Bovine blood cells and serum proteins	--
Pb+2	-0.53	Sediments	17
Pb+2	-0.47	Ashed bull frog femur	63
Pb+2	-0.47	Standard solutions	2.6

* - 0.4 M H₂O₂ + 0.3 M H₂SO₄ + 1 M NaOH to pH 7.8

Although in theory the concentration from a polarographic measurement could be computed from measurement of I and knowledge of various physical parameters related to the polarographic cell, the supporting electrolyte, and the ion being detected, in practice polarographic measurements are universally done with reference to calibration curves. These are graphs of measured I versus known standard concentrations. There are two methods for obtaining these curves- "blank subtraction" and "standard addition." Using blank subtraction, a measurement is made of a sample solution, known as the blank, which is chemically identical with the solution to be analyzed, with the exception that it does not contain the element being assayed. The sample containing the pollutant of interest is then measured, and the I versus E curve from the blank is subtracted from it. This leaves the response of the element of interest, eliminates background currents, and minimizes interferences. Similar tests are conducted in the same manner with known concentrations of standards placed in a polarographic cell separate from the test sample.

Blank subtraction works well when the carrier solution is very well characterized, e.g. distilled water or physiological saline solution.

However, for an environmental site such as Devil's Swamp it is essentially an impossibility to obtain control samples which are chemically identical to the test samples with the exception of their Pb and Cr content. A similar though lesser difficulty presents itself when analyzing animal tissue. Thus, how can one be sure that the environmental sample or tissue used to prepare the blank is identical to the sample with the only exception being the heavy metal content?

The standard addition method minimizes these concerns. Here the sample of interest is first polarographically measured, and the current peak associated with the unknown concentration is observed. Then, known amounts of the assayed substance are successively added and their polarograms determined. The concentration of the unknown is then mathematically calculated from this data. Although interferences are still present in this method, since the unknown and standards are measured in the same cell with the same solution, the interferences are the same in each case. The differences in current result from the different amount of the heavy metal. This method thus minimizes the influence of contaminants present, even when the exact chemical nature of the carrier solution is unknown (in contrast to the blank subtraction method).

Further details regarding our experimental approach were presented at the 1993 International Conference of the Society for Environmental Geochemistry and Health and will be published in the conference proceedings(13).

Results

The first tests conducted in the course of this initiation project involved the study of Mississippi River water. These were useful to acquaint us with some of the issues involved in the separation and analysis of polluted Louisiana water samples before we received materials from Devil's Swamp. A 3 liter sample was boiled to yield the solid residue, re-suspended in distilled water to allow settling out of coarse particulates, and boiled again to obtain the residue (mainly clay particles and salts). The sample obtained with this procedure weighed 0.17 g. It was subjected to multi-element analysis, using energy dispersive analysis via x-rays (EDAX), which was found to be insufficiently sensitive. X-ray fluorescence measurements were then conducted. Several heavy metal trace elements including some with significant toxicity were found, as given in Table 2.

TABLE 2

Metallic Trace Elements Found in the Fine Particulate/Dissolved Salt Residue of Mississippi River Water Sampled Near Belle Chasse, LA ($\mu\text{g/L}$)

<u>Fe</u>	<u>Mn</u>	<u>V</u>	<u>Cr</u>	<u>Ni</u>	<u>Cu</u>	<u>Other</u>
246.1	12.2	6.9	3.4 - 6.1	1.1	1.1	Sr

Since these trace elements are present in the water at ppb levels or above, which is the approximate resolution limit in the polarographic cell, their detection would require only a modest concentration step (to compensate for the dilution associated with placing the sample in the supporting electrolyte) if they are present as salts. If they are present in a form adsorbed on fine particulates, then additional concentration would be needed to account for dilution if acid digestion rather than ashing is used for extraction. In either case, these results indicate that detection of heavy metal pollutant levels in the river could be readily accomplished using polarographic methods. As a first study of extraction procedures, beef liver was soaked in a 200 ppm Pb solution. This was chosen since the frog liver is one of the target organs of interest to us in which pollutants might accumulate. 3.5 g was ashed as previously described, and the absorbed lead concentration in the tissue was found to be 74.9 ppm. To compare ashing and acid digestion extraction procedures, the acid digestion process that we have previously used

for measurements of chromium in animal tissue was employed. In this process, 0.33 g of liver was placed in 5 ml of H₂SO₄ and heated for 2 hrs. at 100 °C. After cooling, 5 mL of hydrogen peroxide was added, and a similar heating cycle was repeated. This process gave a 65.5 ppm Pb concentration. The similarity in results enhances confidence for both procedures. Although this acid digestion procedure is more drastic than the one we are using for our environmental sediment samples (ASTM D3974-81), it is an appropriate alternative to ashing for tissue samples (as is ASTM D4698-87), if their pollutant concentration levels permit, because complete extraction is possible.

After the above studies were completed, Devil's Swamp samples were obtained. Tests were conducted to determine the concentration detection thresholds in the polarographic cell when testing samples from the various environmental compartments. The results of these tests are given in Table 3.

TABLE 3
Concentration Detection Thresholds in the Polarographic Cell for Lead Analyses of Various Environmental Sample Types

<u>Sample Type</u>	<u>Detection Threshold (ppb)</u>
Lead Standards	2.6
Sediment Moieties (Acid Digested)	17
Ashed Frog Bone	63
Water (after 1 µm filtration)	78

Often in our previous testing we have observed lower thresholds in the polarographic cell when a heavy metal is added in a form where it is contained in a chemically simple carrier than when in a more complex form, such as an extract in an acid digestion solution. Table 3 seems to also demonstrate this effect. The minimum detection threshold for the sediment was about 6.5 times that observed for lead standard solutions. This may be due to a pH shift as the sample is added to the supporting electrolyte. An even higher threshold was found for the ashed bone. Possibly, adsorption of residual organic material on the mercury drop could interfere with the polarographic measurement at low concentrations. The highest value was observable for the filtered water. Since the presence of organic macromolecules as well as pH shifts both are possible here, perhaps the 78 ppb value compared to 2.6 ppb for standards reflects the superposition of these two effects.

It should be pointed out that these limits above represent those in the polarographic cell rather than the minimum detectable concentration in the environment. For example, the pollutants in water samples can be concentrated by boiling and evaporation to produce a detectable polarographic signal even though the original water concentration to be ascertained is well below 78 ppb. On the other hand, for ashed tissue samples the minimum detectable concentration in the cell might translate into a rather high tissue concentration if the amount of starting material is limited.

The concentrations of lead measured in the various compartments of Devil's Swamp water are shown in Table 4. The procedure for analysis of the aqueous phase involves starting with a 4 liter sample, filtration of particulate as discussed before, and boiling of the liquid phase to concentrate the dissolved pollutants. The table gives the classification of the solid moieties obtained, the percentages that they represent of the total particulate fraction, the concentrations of lead

observed in the particulate and aqueous moieties, the concentrations in the moieties referenced to the water phase as a whole, and the total concentration of lead in the water from all moieties.

TABLE 4
Lead Concentration in Various Compartments of Devil's Swamp Water

<u>Moiety</u>	<u>Size (μm)</u>	<u>Percentage of Total Particulate</u>	<u>Concentration (in moiety)</u>	<u>Concentration (in water)</u>
Coarse Particulate	>105	1.3 w%	1245 ppm	3.11 $\mu\text{g/L}$
Fine Particulate	20-105	97.4 w%	38.4 ppm	7.31 $\mu\text{g/L}$
Very Fine Particulate	1-20	1.3 w%	257 ppm	0.62 $\mu\text{g/L}$
Aqueous	<1	NA	3.67 ppb	3.67 $\mu\text{g/L}$
Total Lead	--	--	--	14.71 $\mu\text{g/L}$

It should be noted that, although the lead concentration in the aqueous phase is much smaller than in the particulate, because this is the dominant moiety by volume, it contains about 25% of the total lead present in the water.

For the analysis of sediment the procedure was to suspend a 35 g sample in 1 liter of distilled water and then filter as described. Table 5 shows the concentration of lead measured in various moieties, the fractional percentage the solid moieties represent of the total sediment, and the total lead in the sediment.

TABLE 5
Lead Concentrations in Various Compartments of Devil's Swamp Sediment

<u>Moiety</u>	<u>Size (μm)</u>	<u>Percentage of Total Particulate</u>	<u>Concentration (in moiety)</u>
Very Coarse Particulate	>290	16.8 w%	17.7 ppm
Coarse Particulate	105-290	3.9 w%	27.5 ppm
Fine Particulate	20-105	78.1 w%	19.6 ppm
Very Fine Particulate	1-20	1.2 w%	33.8 ppm
Total Lead in Particulate	--	--	19.8 ppm
Aqueous	<1	NA	11.8 $\mu\text{g/L}$

Note that more lead was found in the water of the sediment sample than in the aqueous moiety of the swamp water. This was not due to pH differences. The distilled water added to the sediment had a pH of 7.2, while that of the swamp water was measured to be 7.4. One possible explanation is a hardness difference in the two water samples. Another possibility is that the water from the sediment sample contained more particulates (in the size range smaller than 1 μm) which leached out lead ions when contacting the acidic supporting electrolyte.

With respect to testing the target organs from bull frogs taken from Devil's Swamp, based upon the measurements previously mentioned with *Xenopus laevis*, it was decided to test bone first. Two femurs were removed and ashed as described previously. No detectable lead was found in either case. The threshold of detection in this case was 521 ppb in the bone. A larger amount of bone sample will be used in our next round of testing of the Devil's Swamp bull frogs to lower our detection limit. The *Xenopus laevis* measurements(4), though, do report a 5 ppm Pb concentration in fresh bone from animals fed uncontaminated worms and 24 ppm in the bone of those toads fed a Pb contaminated worm diet.

Discussion

The experiments conducted in this pilot project have been successful in achieving our main objective- demonstration of the applicability and utility of polarography for study of pollution problems in aquatic environments of Louisiana and developing the methodology to do so. We have demonstrated the capability of this method for measuring lead levels in water, particulate particles, and tissues from animals taken from polluted environments. The heavy metal pollutant products have been seen to be quite inhomogeneously distributed. Much higher lead concentrations have been found in particulates rather than dissolved in the water. This is consistent with the relative insolubility of many lead salts (14). However, it is conceivable that if the anion composition of the site were to change by release of other pollutants this could cause the amount of soluble lead to increase. Lead solubility is reported to vary considerably in terms of water characteristics(4)- from 30 ppb in hard basic waters to 500 ppb in soft acidic water. Our soluble lead values are somewhat lower than these limits. An explanation for our relatively low concentration of lead in the aqueous phase would be that some portion of the dissolved lead in Devil's Swamp is not present as an ionic form capable of reduction. For example, it might be present complexed with a low molecular weight organic. We will shortly examine this possibility using AAS or ICP testing (which are sensitive to the total amount of lead present) and comparing the values with polarographic results. In a study of Rivers in Greece using atomic absorption spectrophotometry (15), the percentages of lead found in the liquid phase (25-50% of the total) compared to the particulates, were similar to those we have found in this study (25%). Soluble Pb in that work was reported to be 7-13 ppb, about what we have observed here.

As can be seen by comparison of tables 4 and 5, similar concentrations of Pb were found in the fine particulate fraction (20-105 μm) of the Devil's Swamp water and sediment, 38.4 and 19.6 ppm respectively. In both the sediment and water particulate, this fraction accounts for more than 75% of the solid matter. In the sediment a reasonably uniform lead concentration was observed- 17.7, 27.5, 19.6 and 33.8 ppm in the very coarse particulate (>290 μm), coarse particulate (105-290 μm), fine particulate, and extremely fine particulate (1-20 μm) moieties, respectively. The coarse particulate and the extremely fine particulate fractions were enriched in the water phase compared to the sediment, showing 1245 and 257 ppm respectively. There are several possible explanations for these discrepancies. One is that the findings are preliminary and that a more extensive series of measurements might not show these differences to be as great as they first appear. Another is that the relatively small amount of material yielded in the separation for these fractions could create artifacts. However, another explanation would be perhaps there is some stratification in the lead distribution in the sediment sample. For example, if the pollution were relatively recent and had not had much time to percolate through the sediment, then a hypothetical core drilled sample through the bottom of the swamp would show most lead at the surface with lesser amounts at a depth sufficient to reveal the baseline amount. The material which has become suspended in the water has most likely resulted from direct effluent or the stirring of surface or near surface sediment by hydrodynamic action. This mechanism would explain why enriched amounts of lead were found in two of the three compartments (assuming that the sediment sample analyzed originally was slightly subsurface), although would not illuminate why there is a relatively similar (although somewhat elevated) concentration in fine particulates.

These considerations bring up, indirectly, another issue which is of concern to pollution monitoring of Devil's Swamp. Were the heavy metal elements being measured put there by pollution or are they those naturally present? Lead is reported(4) to be present at a 16 ppm level in the earth's crust. Another aspect of this question is what kind of sample can serve as a control for lead concentration in Devil's Swamp? Possibly, use of a core drill to take a relatively deep subsurface sample could provide a definitive answer of the background lead concentration at the site.

It will be of interest to compare our results of lead concentration in Devil's Swamp with others who have tested the site. The only information we have so far is a communication of a prior test

result(16) which showed a water lead concentration of 150 ppb. This is higher than our value of 14.7 ppb, but there are several possible reasons for this. One could be that the site previously tested was more polluted than the one we examined. Another possibility is that our polarographic procedure, as mentioned earlier, only measures that portion of material capable of electrochemical reduction after extraction from its carrier. This could thus provide a smaller value than would AAS or ICP measurements, especially if more drastic extraction procedures are employed. It will be of interest to compare our results with other measurements in the DOE project of specimens taken at sites similar to those we have measured. With regard to our finding of 1245 ppm in the coarse particulate of Devil's Swamp, although the value does seem high, comparable values have been reported in Bayou St. Johns in New Orleans, a non-Super Fund site(17).

At present the implications of our measurements with frog femurs are not clear. Several hypotheses to explain our finding of undetectably low amounts of lead in the bull frog bones are as follows: 1) the animals were taken at locations in the swamp which were less polluted than the areas where the sediment and water were taken and thus the frogs suffered minimal exposure to lead; 2) the frogs were exposed to lead in the environment, but little of it was intestinally absorbed; or 3) the literature cited regarding *Xenopus laevis* is inapplicable because different species could behave differently regarding bioaccumulation or different lead-bearing pollutants could be differently metabolized. The last part of hypothesis 3 would mean lead could be stored in other organs in greater amounts than in bone. We will be examining this possibility shortly. If no lead is found in Devil's Swamp bull frogs, we will rethink which animal species should be used in our laboratory studies planned for the future. A prime candidate that will be investigated for suitability as an alternative, if need be, is the cray fish. This choice is based upon the preliminary finding of the Reproductive Toxicity cluster (18) that changes in reproductive organ weight are observable in crayfish exposed for 4 wks. to 150 ppb of Pb.

The studies we have conducted here demonstrate the importance of mechanistic studies regarding the pollution of aquatic environments and thus of the wildlife which inhabits them. In studies of heavy metal concentrations in animals, metal to metal variability may be related to differing tendencies of metals to collect in different organs as well as differential rates of absorption through the skin and digestive tract. These in turn depend upon understanding of the moiety in which the heavy metals are sequestered. For example, the relative lack of solubility of lead in Devil's Swamp water conceivably precludes skin absorption as a major pollution pathway, but with other pH or dissolved salt conditions this could be otherwise.

Once ingested, the bioavailability of elements is a prime consideration. For example, age, sex, and diet play major roles in absorption and retention of lead in the gastrointestinal tract(4). It is also known that lead from smaller particulates is absorbed up to 7 times more rapidly than from larger particulates(4). Thus, studies of the compartmental distribution such as those we have conducted here with lead are important features of understanding pollution problems. Such considerations also could affect heavy metal appearance in the food chain, with organisms living in or feeding on sediments of Devil's Swamp at relatively larger risk for lead accumulation, along with those organisms which feed on these animals.

A further interesting question, although not one which has been directly addressed in this research project, is the influence that flora have on the heavy metal pollution process. It is possible that, as plants absorb even sparingly soluble ionically dissolved metals, this would, via principles of thermodynamic equilibrium, enhance further dissolution from sediment. This is a question which should be further investigated in the future.

Future Plans

Several areas will be emphasized in our future testing program. Repeated concentration measurements of the environmental moieties reported on here will be conducted for purposes of

statistical analysis. Our major effort will be concerned with the laboratory animal study. As previously pointed out, we will need to select an animal species, preferably one which has been shown capable of bioaccumulation of heavy metals in Devil's Swamp. Our original plan was to use *Xenopus* frogs. The laboratory experiment was to involve an artificial environment in which the frogs would be subjected to 10%, 100% and 20 times the concentration of heavy metal pollutants in Devil's Swamp for 2, 6, and 10 weeks, after which the pollutant concentrations in various organs would be analyzed. Considering our findings of the inhomogeneous distribution of lead in waters and sediments of the swamp's environment, we will have to give serious consideration to the physical and chemical form of the heavy metal to which the frogs (or an alternative species) are exposed.

A major proportion of our work planned for the next project year involves collaboration with two cluster groups. Cooperative research with the "Natural and Active Remediation of Toxic Metals, Organics, and Radionuclides in the Aquatic Environment" cluster will involve applying the separation and polarographic methods described in this report to problems of mutual interest. One emphasis of this research will be to develop technology, including polarographic sensors, which are useful for in situ field assessments of pollution severity. Another dominant thread of the proposed research will be to consider several of the areas revealed as important in our activities on the present project. These concern the physical/chemical mechanisms which determine chemical forms and amounts of pollutants released into the environment which mediate uptake of released material by plant and animal life. The research with this cluster thus will have an applied as well as a more fundamental aspect.

Additional testing is planned in collaboration with the "Assessment of Mechanisms of Metal-Induced Reproductive Toxicity in Aquatic Species as a Biomarker of Exposure" cluster group. In this work polarographic methods, used along with ICP and AAS analysis, will be extended to analyze water, sediments, and wildlife from Bayou Trepagnier for heavy metals in cases where the speciation capability of polarography will be helpful. Cray fish, sun fish, and cat fish whose feeding habits will bring them in closest contact with different environmental moieties will be studied. Various tissues and organs of interest to the cluster regarding reproductive toxicity will be investigated (hepatopancreas, hemolymph, gills, and gonads) and assayed to determine the degree to which they accumulate various chemical species present at the site. Other tests will involve the use of polarography for measurement of concentrations of organic pollutants.

Additionally, we plan to submit a research proposal to the National Institute of Environmental Health Sciences (NIEHS) for the spring NIH deadline (June 1, 1994). This study will be an extension of the present work and possibly will be conducted in collaboration with other researchers on the DOE/EM project.

Conclusion

The polarographic method has been shown to be useful for assessment of pollution in Devil's Swamp. Effective methods for separation of aqueous and particulate moieties and for heavy metal extraction have been validated. Lead is extremely inhomogeneously distributed in the swamp and is mainly associated with sediments or suspended particulates. The manner in which wildlife would be exposed to this pollutant is thus quite complex and dependent on many factors. The mechanisms by which different chemical forms are dispersed in this environment and are accumulated in animals are planned to be investigated in the future via a controlled animal model and collaborative research with the "Assessment of Mechanisms of Metal-Induced Reproductive Toxicity in Aquatic Species as a Biomarker of Exposure" and the "Natural and Active Remediation of Toxic and Radioactive Metals in Aquatic Environments" cluster groups.

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An Interactive, Hypermedia Cultural Ecology Model of Risk Communication about Hazardous Waste Remediation for Scientists, Administrators and Students

S. Duplantier

Summary: The principal investigator collected information on cultural, archaeological, historical, biological, ecological, toxicological, risk communication, and socioeconomic processes in the Mississippi River basin study area. Emphasis was placed on St. James and St. John civil parishes in the first iteration of the educational multimedia software product. The material collected was in the form of reports, printed information, photographs, maps, drawings, and video tape. The information was scanned or digitized into a Macintosh computer and authored into an interactive series of HyperCard® "stacks" which can be accessed interactively by a user of the software. The model software project can be considered for publishing in CD-ROM format once the beta testing is completed by users and stakeholders from the region.

The processes of perceiving, discovering and understanding information about environmental, risk-based issues, and then negotiating and implementing just policies about such issues and dilemmas are complex and daunting.

Excellent risk communication and subsequent environmental equity demands that there be no unequal voices in the continuing dialogue among people and institutions which constitutes democracy-at-work. Indeed, both successful remediation of problems with hazardous materials and environmental equity can be by-products of excellent processes of communication among all the parties in disputes and conflicts.

Achieving high levels of understanding among participants in environmental disputes means not only that publics and the advocacy groups which represent them understand the ways of knowledge construction in the professional scientific community, but also that scientists understand how knowledge in general is socially and historically constructed. Science, as a knowledge practice, is embedded in history and culture. The debates among the philosophers of science about the social and cultural construction of science are not merely academic. Inequities arise in the misunderstandings between practitioners of different knowledge communities.

The educational products and processes which have resulted from this Initiation Grant project can serve as the "messenger RNA" between the science communities and the publics. The role is emphatically not one of a shallow brand of pseudo-public relations which is distrusted by angry and baffled communities. Rather, what this multimedia risk communication software can do is catalyze a truer, deeper, dialogic communication which demands understanding from corporate, political and scientific communities about cultural, social and historic construction of the public's knowledge.

This Project needs development and ongoing production time by the continued addition of more information over wider swaths of the study area. This will help to achieve its goals of using the latest multimedia communication technologies to make complex historical, social and cultural information available to the stakeholders in the New Orleans-Baton Rouge river corridor. The information is presently available in many scattered, inaccessible sources and repositories. When this data, images, accounts and interpretations of the massive cultural and historical dynamics of the river region are available in one interactive, hyperlinked source, then the stakeholders can communicate in a qualitatively different way. The CD-ROM containing the multimedia collection of information about the study area which may eventually be pressed could serve as an important tool

in the generation of a phase change amount of information complexity. Prior to this kind of multimedia software, it has not been possible to see/hear/understand the full complexity of "everything at once" which is required for the building of the consensus essential for a true democracy and participation by communities in economic and environmental decisions which affect their lives and health.

Complexly available information about the full cultural and natural ecology of the study area will allow people to communicate among themselves to increase understanding and preserve their natural and cultural environment. Historical perspective is a key dynamic in the resolution of the survival and health of the people, biota and general social and cultural ecology of the region. The private corporations who are presently the dominant economic force in the river parishes between Baton Rouge and New Orleans operate manufacturing and processing plants in order to produce products which make profits. The human and ecological health of the people and natural environment of the region where their plants are located are of concern only while the company is operating the plant. Should the board of directors in the corporate headquarters decide to shut the plant or move the operation, the ultimate survivability of the former workers, the rest of the local population and the ecological fate of the land are of no official corporate concern. There may be expressions of concern by individual corporate officers or by the corporate human relations departments and public affairs staffers, but corporations simply do not take the long term bioregional view (200-1000 years). No corporation makes fiscal or technical plans to deal with situations and problems created today which will have long term effects. Yet the current environment in the study area is a Pleistocene ecology largely unchanged in major physical and biotic parameters since the last 10,000 years. A richly varied archaeological human occupation in the area is co-terminous with the Pleistocene habitats. The last 300 years have seen rapid human population growth in the study area with concomitant severe ecological impact. The long view provided by archaeology and history can aid to bringing the realization to local representatives of anonymous and absentee corporate entities that the people of the river region care about the future for their children as least as much as they care about the overall economic health of the region today.

A community group could use the educational media products of this project to show to public officials, administrators and corporate boards of directors the meaning and importance of their expressive culture and folklife practices. Health and salubrity issues require the most exquisitely balanced communication and dialogue among different interests. The nature of interactive multimedia, non-linear educational and presentation media should aid in this process. These media permit the complexities of real world issues to be modeled vividly and represent the simultaneity and ambiguity of real life events.

Critical and Self-Reflexive Component of Risk Communication Processes

Risk communication often fails as much as it succeeds. This shows the need for a self-critical component for practitioners of risk communication. Not only must the full interactivity and complexity of the risk communication processes must continually be monitored and evaluated, but also the "bias" of the media used for communication must not be left to common sense presuppositions about effectiveness or effect across cultural, axiological, and epistemological boundaries. Dialogue among publics, organizations and governments about matters of ecological health take place through electronic media, in addition to traditional face to face forums and public gatherings. The nature of our democratic processes demands that people represent other people and that not everyone can attend a "town meeting." Clearly, new media are seen as aids to communication effectiveness. Yet, though new technologies of communication promise to make the processes of education more effective, it does not always work. The most technically proficient communicator knows that the new wonder machines can help in the always-difficult job of communicating effectively. The model risk communication software educational products of this DOE initiation grant can help make publics, business and government into active, critical users of technology and media in order to improve communication about hazardous wastes in the study area

in the light of larger cultural and societal goals.

Suggestions for Future Funding Directions. Research and Multimedia Production

Additional work with the goals of this project could help develop criteria, procedures and instruments to assess media of risk communication technologies and effects. An immediate result which could be expected is that the stakeholders will have the critical concepts and skills to put them in control of evaluating media, technologies and the societies these technologies tend to produce.

Another result of this project is the call for allowing people to understand and criticize current tools, methods and theories of risk communication. This could mean the establishment of a facility and a safe ground for envisioning humanly and naturally-sustainable cultures.

The complexity of the site and the bioregion specific geo-physiographic, cultural ecological data and semiotic systems makes even simple problems intractable if they are forced into procrustean databases. However, if a hybrid representation system based on multimedia and digital video was coupled with an analogical neural network system to process the non-linear information, then the chances of greater insight and intelligence being found in the cybernetic information user/supplier system is likely. More elaborate data definition of all facets of complex "objects" (information and semiotic structures) would allow better modeling of real world systems and processes.

The users of the system (publics, government and industry) would be connected on e-mail and have access to on-line documents, especially time-based documents (digital video and animations of processes). The multimedia delivery of this information would be the basis for interactive virtual reality displays of user consensus models of hazmat processes.

Bioenvironmental Analytical Support Services for DOE Clusters

W. George, J. Preslan

The DOE Analytical Support Laboratory, an extension of the Tulane Analytical Laboratories of the Division of Toxicology, has been actively collecting field specimens, initiating field exposure experiments on catfish, as well as performing analyses of several hundred specimens by Atomic Absorption Spectrometry, Inductively Coupled Plasma Spectrometry, and GC Mass Spectrometry between October 1993 and January 1994.

Analyses of 50 water, soil and biological specimens collected by our field staff from specific locations in Bayou Trepagnier have been performed for the cluster project, Assessment of Metal-Induced Reproductive Toxicity in Aquatic Species Cluster. The results have been used to characterize the Bayou, identifying lead, chromium and hydrocarbons as abundant contaminants and examining the distribution of these metals and organics in the sediments and in vertebrate and invertebrate organisms from the region. Of the more than 300 crawfish tissue specimens subsequently generated in laboratory experiments on the bioaccumulation of lead and chromium, about 80% have been digested, and more than 200 are presently in the final stages of analysis. Results of completed work are presented in the Reports submitted by Drs. Anderson and George.

In addition, containment pens placed at various locations in Bayou Trepagnier have been stocked with catfish fingerlings in a study designed to monitor biological uptake of hydrocarbons from the sediments. Although to date, survival of the fish has been poor, efforts are continuing in this work.

The training of laboratory staff which has occurred during these initial months is evident in the increased speed and proficiency with which samples are presently being processed. Analyses rest on a strong foundation of Quality Control and Quality Assurance governing all aspects of wet chemistry and analytical procedures established by the Laboratory. While initial analyses were performed on instrumentation in the Environmental Health Sciences Department at Tulane Medical Center, our laboratory personnel have also been responsible placing into service a newly purchased atomic absorption spectrometer at the Tulane Central Instrumentation Facility on the Tulane Uptown Campus.

Contacts with Drs. McPherson, Flowers, and Koplitz from the Chemical and Natural Remediation Cluster and Drs. Thiyagarajah and Thien from the Bioremediation Cluster have also been mutually beneficial, and increased interaction with these groups is anticipated in the future.

Evaluation of the Carcinogenic, Reproductive, and Developmental Effects of Mixtures of Contaminants on the Medaka Fish (*Oryzias latipes*)

W. Hartley and C. Roy

Introduction

This paper details the initial results of a study to evaluate the effects of mixtures. The study when completed will include cadmium in combination with other chemicals. The primary mechanism of cadmium toxicity on adult fishes is gill tissue necrosis, but this was observed at very high laboratory concentrations or special circumstances. (Hoar and Randall, 1988) attributed the toxicity of cadmium to inhibition of acetylcholinesterase and death by paralysis of the respiratory control system. The uptake of cadmium compromises the uptake of available calcium, which leads to the onset of hypocalcemia. Rosenthal et al. (1976) defined sublethal effects as any hinderance of normal development upon the embryonic or juvenile growth of an aquatic organism. These effects included histological, morphological, physiological, or ethological (behavioral) changes which may be induced in one stage of development but expressed in a later stage of development in terms of reduced survival potential. Rosenthal et al. (1976) observed Atlantic Herring eggs contained much more cadmium in the jelly coat of the egg than the embryonic interior. The major effector point of the aquatic embryo during fertilization was the protective membrane (chorion). The medaka (*Oryzias latipes*) is used as laboratory animal in various fields in biology, especially in developmental biology and genetics. Its relatively short life cycle, capacity to reproduce, sensitivity and ease of breeding are in part responsible for its utility in these fields (Yamamoto, 1976). The medaka is a very hardy fish in that it may withstand a wide temperature flux, and a range of salinities.

Michibata (1981, 1984, and 1986) provided very good information from which to base further, focused developmental studies involving medaka and cadmium toxicity. The *Oryzias latipes* embryos were exposed to a variety of diluted sea water concentrations which all contained the same concentration (10.0 mg/L) of cadmium chloride (Michibata, 1981). The mortality increased because of the reduced hardness in the rearing medium. Michibata provided a historical control for reduced hardness mortality in the absence of cadmium of 3.7%. A linear relationship was found between the hardness and cadmium concentrations over a 96 hour exposure. These results reflect the dependance of cadmium toxicity on the hardness of the medium (Michibata, 1981). There was a direct and proportional inverse relationship between the cadmium content of the embryos and the hardness of the medium. The amount of cadmium in the embryos decreased markedly with the rise in hardness (Michibata, 1981). Michibata (1984) investigated the ability of specific ions which constitute water hardness to have a protective or neutral effect on the medaka embryo in the presence of cadmium. The mortality of groups of embryos was different at varying ionic concentrations over a 120 hour exposure. He concluded that the calcium ion had the most protective effect on the medaka embryo. The stage susceptibility of the medaka egg was studied under a constant hardness (Ringer's Saline Solution) of approximately 130 mg/L CaCO_3 (Michibata, 1986). Michibata (1986) exposed medaka embryo at an early stage of life (blastula) to 6 cadmium chloride concentrations, ranging from 10.0 mg/L to 300.0 mg/L and concluded that the resistance of the medaka embryo to cadmium toxicity increases as the embryo matures up to stage 14 (mid gastrulation). He notes that the exposed embryos could not be distinguished from the control embryos if the exposed embryo had survived up until this stage.

There is a lack of aquatic toxicological research involving specific developmental effects of cadmium in the medaka embryo. There is a need to investigate the sublethal effects of cadmium in low concentration where mortality of the exposed biological organism is not the endpoint of toxicity. The toxicity of cadmium to embryonic, juvenile, and adult fish is variable according to species and stage of life. The object of the following experiments was observe the various physiological, morphological, and limited behavioral effects of cadmium on the medaka embryo.

A fuller knowledge of sublethal (developmental) effects produced by cadmium will provide valuable information in which to base further experimentation with mixtures of environmental chemicals which may cause developmental toxicity.

Materials & Methods

The embryos used were from the medaka colony at the Tulane University School of Public Health and Tropical Medicine. The breeding colony of medaka, which were originally obtained from Carolina Biological Supply (Burlington, N.C.), are mostly the varietal orange-red strain. A natural environment was procured using underground filters in the tanks to assure low water velocity. The ramshorn snail (*Planorbis corneus*) was introduced to the breeding colony, as suggested in Yamamoto, (1976) as a scavenger of excrement and excess food. Additionally, Cabomba grass and duckweed were added to the tanks. The air temperature in this laboratory ranges from 18-26 degrees celsius. Light is cycled mechanically in 12 hour intervals and was provided by standard fluorescent aquarium tubes. The colony was replenished regularly with embryos harvested at appropriate intervals. Fish health was maintained through biweekly volume changes in all tanks. The rock bed of the tanks was vacuumed and 80-90% of the water was siphoned and replenished with 50% dechlorinated tap water and 50% deionized water. Water chemistry was performed biweekly to insure proper water quality (pH, nitrite (N), ammonia (NH₃), and hardness (as CaCO₃ in mg/L)). The colony was fed TetraMin (W. Germany) staple tropical fish food three times daily. This diet was supplemented with feeding of live brine shrimp (*Artemia salina*) 3 times weekly to insure proper protein intake. The toxicity of cadmium to freshwater fishes and their embryos are wholly dependant on the hardness. Calcium ions present in the embryo rearing solution used to rear the eggs facilitated a protective effect on the embryos in the presence of cadmium. An experiment was performed to investigate the ability of the *O. latipes* embryo to grow and hatch in extremely low water hardness environments. The effect on virtually no water hardness in the water may effect the eggs chorion integrity and osmotic pressure. Researchers using the medaka fish for various studies have commented on the hardness of the rearing solution as having an effect on the embryo growth and development.

Retrieval of viable embryos from the breeding tanks was standardized to reduce the variation and insure the health of the embryos during experimentation. Embryo collection took place early in the morning immediately after the artificial light source in the laboratory cycles on. The females contained in the breeding tanks that are carrying eggs were visually identified and are collected via a small silken net. The females were then transferred to a large glass finger bowl containing either 50/50 dechlorinated tap water/deionized (type II laboratory grade) water or decanted water from the specific breeding tank from which the female was retrieved. After collection of enough embryo bearing females is completed to satisfy embryo number, all excess vegetal matter from the finger bowl was removed. The female is chosen, one at a time, from a finger bowl and held between the index finger and thumb with the silken material of the net covering the surface area between the skin of the hand and the scales of the fish. The female was kept either below water surface or touching the water surface to avoid excess stress. The female was held inverted with the anterior portion of the fish away from the body. The exposed eggs were gently brushed off of the protruding abdomen with the curved bend of microdissecting forceps. All of the unfertilized embryo are removed and disposed from the collective group of eggs. This was accomplished by visual identification of a cloudy yolk sack or broken chorion. A healthy, viable embryo was identified as having a clear yolk sac with no obvious signs of defects of the chorion of the interior of the embryo. Each embryo was inspected for prior stage development. The parameters which defined prior stage development were visual identification of eye formation (pigmented retina) which clearly shows through the chorion. All embryos accepted for experimentation are usually consistently in the range of stage 2-6 of development as outlined by Kirchen and West (1976). At magnification (X36), stage 2 is characterized by a yolk which is transparent and yellowish, and the perivitelline space present beneath the clear shell. Stage 3 is characterized by the beginnings of the germinal disc, which is physically made up of a disc-shaped cap at the animal pole. The viable

embryos were then denuded of filaments using a technique described in Marty et al. (1990). The removing of the filaments provides for clearer photomicroscopic documentation and reduction of possible infection and death by mold or fungus. The stock rearing medium in which the cadmium concentrations would be prepared is a mixture of salts which provide a healthy environment for the embryo to grow. The rearing salts produced a hardness of ≈ 131 mg CaCO_3/L . The chemical composition of the embryo rearing solution, purchased from Carolina Biological Supply (Burlington, N.C.), contains: NaCl (7.50g), KCl (0.20g), CaCl_2 (0.20g), NaHCO_3 (0.1 M). This solution was diluted with 480 ml of laboratory grade distilled water. The approximate hardness of the embryo rearing solution and the deionized water alone was determined by EDTA titration technique using the Hach Freshwater Fish Farming Field Kit FF-2 (Hach Corporation).

The following experiment was designed to determine the hatch rate of the medaka embryo in varying hardness regimes. The 120 embryos were separated into 6 groups (20 embryo per group) and reared in different aquatic conditions to observe the effects of rearing in distilled water as opposed to embryo rearing solution. Additionally, methylene blue was also added to two of the finger bowls containing distilled water only to control fungus growth. Embryos were collected according to the methods and materials previously discussed. The solutions were mixed in clean volumetric flasks and transferred to one liter finger bowls. The pH of all of the finger bowls were approximately 7.0. Both nitrite (NO_3^-) and ammonia (NH_4^-) were not present in any of the solutions used. A glass plate was seated on the top of the finger bowls to prevent evaporation or vector attack. The finger bowls containing the embryos were kept under ambient laboratory conditions where temperatures ranged from 23-25 degrees celsius. The light source was cycled in twelve hour intervals. The hatched embryos were immediately placed into 10% buffered formalin for histopathological and other studies.

The results of the different harnesses and the addition of methylene blue to distilled water were greatly varying hatch rates. The group duplicates were pooled and totals were calculated into percentages of hatch for each different rearing medium, the results showed that distilled water (with and without methylene blue) greatly affects the hatch rate of embryos reared in that environment. The finger bowls containing the embryo rearing solution resulted in a very high (94.5%) hatch rate. Upon visual inspection, the newly hatched embryos were lively and viable, showing no signs of debilitation. The hatched embryos from the distilled water finger bowls were lethargic and patsy white in color. The groups which were in distilled water with methylene blue added to control for possible mold growth, were colored a deep blue upon hatch. It was concluded that the presence of no calcium or magnesium ions in the water may have interrupted the osmotic pressure of the chorion, allowing for the methylene blue to penetrate and stain the developing embryo. Two of the embryos in this group were dead upon hatch. Based upon the results from this experiment, distilled water was not used as an alternative for the embryo rearing solution in the exposure experiments with cadmium. Further, it was concluded that methylene blue would be omitted from future experimentation to avoid the risk of osmotic pressure interruption by cadmium which may lead to high mortalities due to either cadmium or the methylene blue penetration. It is desirable to lower the hardness level in the exposure water to achieve full effect of the xenobiotic.

An experiment was designed that considers the structural and physiological state of the embryo at certain times and corresponding stages during the first 96 hours of life while continuously exposed to certain levels of cadmium solution. In the first exposure, cadmium chloride in a powder form ($\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$) was prepared in an aqueous solution of embryo rearing solution diluted to approximately 100 milligrams CaCO_3 hardness. The nominal values for the first cadmium exposure ($n=120$) were 8.0, 4.0, and 2.0 mg Cd/L. The nominal concentrations for the second exposure ($n=25$) were 1.0 g Cd/L, 60.0, 30.0, and 15.0 mg Cd/L. The samples are awaiting analysis by GBC 908 direct air-acetylene flame (graphite furnace) atomic absorption spectrometry. Before transferral of embryo to each vial, each embryo was visually examined microscopically (X 36) for any outward signs of developmental problems or general necrosis of tissue. Also, the

approximate stage was determined to make positive that none of the embryo being used in the experimentation were advanced past stage 7, as described by Kirchen and West (1976). The selected embryos were then transferred singly to approximately 10 milliliter borosilicate glass vials with hard plastic screw caps lined with aluminum foil. Before transferral of embryos, the vials were properly marked and decanted with approximately 7 milliliters of the respective concentrations. Transferral of each embryo was accomplished by disposable glass pipette with a 2.0 mm bore. After the vials were sealed, they were placed in racks at 28 degree celsius in a constant temperature swirling water bath. The embryos were observed by simply placing the vial horizontally against a dark field stage at low power magnification (X 36). For closer examination and photomicroscopy, the embryo was removed from its vial and placed in a well slide by glass pipette. Each embryo was observed at approximately 24, 48, 72, and 96 hours. Preceding the exposure, the corresponding stage at which the embryo should have attained was identified using the stage development guidelines of Kirchen and West (1976). The stage of development was noted at the specific time of observation. Physiological, morphological, or behavioral effects were noted. Additionally, the heartbeat of 30 percent of each experimental group was measured at 48, 72, and 96 hour observations.

In the first exposure, 120 medaka embryos from the Tulane University School of Public Health breeding colony were collected according to the procedure explained earlier in this section. There were 30 embryos per concentration. After completion of the second exposure experiment, it was realized that to make specific observations of each embryo, 30 per experimental group was too large because of the nature of the experiment. Therefore, the second experiment consisted of a smaller experimental group (n=25). Five embryos per concentration were employed. This allowed for us to make more detailed of observations of embryos. The embryos were prepared and processed according the procedures. Observation of the embryos development took place at 24, 48, 72 and 96 hours. In the second exposure (n=25), heartbeats were recorded for all embryos at each observation. In the first exposure (n=120), heartbeat was taken using a stopwatch for 30 seconds. The number attained was multiplied by two which resulted in a beat per minute count. In the second exposure (n=25), the heartrate was recorded for a full minute. An unavoidable problem with recording heartbeat of the embryo is the thrashing of the advanced embryo which may hide the heart during a count. This was especially evident in the 96 hour observations of otherwise healthy embryo in the low and control groups of both exposure experiments. The caps and aluminum liners were removed for a short period during each observation to insure proper oxygen transfer within the vial. The embryos which were still viable after 96 hours were allowed to remained in the cadmium solutions until hatch. The embryos were not transferred to normal rearing solution after the 96 hour exposure. The embryos were exposed to the respective cadmium concentrations until hatch. Upon hatch, the embryos were removed from the exposure vial and transferred to 10% neutral buffered formalin for histopathological and other studies.

Results

In the first exposure (n=120) death rates did not differ from the controls. There was no dose-response relationship of death as a result of increasing concentration. The average cumulative mortality of the exposed groups was $19 \pm 7.2\%$ as compared to control group cumulative mortality of 17% after 96 hours. The level of mortality in the second exposure (n=25) varied greatly. Both high groups (c=1.0 g/L, c=60 mg/L) had all members all die after 24 hours, Medium (c= 30 mg/L) 60%; Low (15 mg/L) 20%; and Control (c=0) 20%. Three embryos in the first exposure developed past stage 15 before death ensued. The stage in which the embryos died was generally relatively early. In the second exposure (n=25), deaths were seen as a result of cadmium exposure at the 24 hour and instant death upon exposure was seen at the two high concentrations(c=1.0 g/L, c=60 mg/L). One embryo did survive past stage 20 before death.

In the first exposure (n=120), the high group (c=8 mg/L) experienced 13% mortality at stages 11-13. These stages are characterized as arrested development from late blastula to early gastrulation (formation of dorsal lip). The medium concentration group (c= 4 mg/L) had the same results as the

high group. The only difference was that death in the medium group ($c=4$ mg/L) was characterized by arrested development in the 13th stage (early gastrulation) only. The low group ($c=2$ mg/L) only showed one embryo with arrested development in stage 13. The total mortality for the low group ($c=2$ mg/L) was 20%, but the stage development was more advanced before death in many of the effected embryo. One embryo in the low group ($c=2$ mg/L) advanced to stage 23 before death. Stage 23 is characterized by formation of an enlarged arterial vessel (early heart) and formation of optic lens. The full trunk was formed in this particular embryo. No formation of pericardial cavity or enlarged arterial vessel was observed. Necrosis of cells was seen along the trunk and anterior portion of the embryo. Similarly, one embryo in the low group ($c=2$ mg/L) advanced to stage 18 before death. Stage 18 is characterized by the progression of the neurula and formation of the embryonic axis (length=1 mm). The embryo had undergone partial development of the embryonic axis. Necrosis of cells along the trunk and forming forebrain was evident. The stage mortality in the controls of this exposure were variable, ranging from late high blastula (stage 11) to late gastrula. No deaths were recorded in the control group in which an embryo had advanced to a late stage before dying.

In the second exposure ($n=25$), the stage progression of selected embryo which survived showed lack of proper parameters to classify their development as normal. Mortality in the high groups ($c=1.0$ g/L, $c=60$ mg/L) showed arrested development at stage 4-6. These stages involve the development of cleavage planes. General necrosis of the cells was observed. This was characterized by the cell(s) darkened and shrunken. In the medium group ($c=30$ mg/L), 3 of the 5 embryo had arrested development at stage 11 (late high blastula). The remaining two embryo (Embryo No. 8 and 10) exhibited structural and physiological damage throughout development. At approximately 24-27 hours, the two embryos had both progressed to stage 20 by all other parameters, but no anterior somite formation was evident on either embryo. Progression of the optic vesicle was normal. By 48 hours, embryo No. 8 remained at stage 23 because of the lack of blood circulation. Similarly, embryo No. 10 had reached stage 25, but circulation was not at the rate in which the control group was developing. Additionally, No. 10 had body movement, but was very lethargic and infrequent. In the 72 and 96 hour observations, it was clear that both of the surviving embryo in the medium concentration group ($c=30$ mg/L) were having difficulty in development. Embryo No. 8 and 10 had only reached stage 26 in the 72 hour observation, whereas the low ($c=15$ mg/L) and control group were in stage 28-29. This trend of delayed development remained constant in medium concentration group ($c=30$ mg/L) up until hatch. Structural and functional defects were determined by the use of Kirchen and West (1976) and Rosenthal and Alderdice (1976).

Physiological Changes. The predominant physiological effect in the first exposure ($n=120$) was a unusual progression of the heartbeat of the developing exposed embryos. Heartbeat progression of all embryos remained normal up until the 72 hour observation. The heartbeat rate of the medium ($c=4$ mg/L) and high group ($c=8$ mg/L) failed to continue to progress to a faster rate, as compared to the control group. The mean heartbeat in the high ($c=8$ mg/L) exposures was 75 ± 10 BPM; the corresponding control group was 70 ± 4 BPM at the 48 hour observation. The mean heartbeat of the high exposure group ($c=8$ mg/L) taken in the 72 hour observation was 108 ± 10 ; the corresponding control group sample was 103 ± 9 . These rates correspond fairly close to each other. At the 96 hours observation, the high group ($c=8$ mg/L) mean heart rate was 87 ± 10 ; the control group average rate was 105 ± 14 .

In the second exposure ($n=25$), heartbeat rate also varied among exposed and nonexposed embryos. At 48 hours the two remaining embryos in the medium concentration ($c=30$ mg/L) group had a heartrate of 81 and 77 BPM. The mean heartrate of the 4 control group embryos was 81 ± 5 . Similarly at the 72 hour observation, normal progression of the two exposed embryos corresponded to the control group heartrate. But at the 96 hour observation, the medium group ($c=30$ mg/L) heartrates were 94 and 81 BPM; the control group BPM mean was 118 ± 3 .

Morphological Changes. Two early development defects observed in both exposures, which also usually preceded to the death of the embryo, were unusual and retarded growth and development of the blastoderm and early gastrula. In the first exposure (n=120), arrested development of embryos at stage 11-12 was characterized by darkened and dying cells of the blastoderm. Retarded gastrulation of the exposed embryo (stage 13-16) was observed as a failure of the gastrula to expand to the equator of the yolk. In the second exposure (n=25), embryonic development was terminated as early as stage 4-5 in the very high group (c=1.0 gm/L). This was observed as the cells in the development of cleavage planes appearing dark and condensed at the animal pole. In the high exposure group (c= 60 mg/L) in second exposure, development was halted at stage 11 (late high blastula). The blastula was dark rather than translucent and was spherical in shape rather than elliptical and flat.

Morphological defects were also seen in advanced embryonic development. The formation of somites which compose the backbone is a major parameter of proper development. In the first exposure (n=120), retarded somite formation was observed in two of the high concentration group (c=8 mg/L) embryo. This defect was also noted in the second (n=25) exposure. Embryo No. 8 in the medium concentration (c=30 mg/L) exposure group was observed at the 72 hour observation with retarded and structurally deficient somite formation. This was characterized by a heterogenous mass along the dorsal section of the trunk. This resulted in irregular spinal flexure in the advanced embryo. One embryo of the medium exposure group (c=30 mg/L) had no sign of any somite formation throughout the 96 hour observation period. Lack of proper coloration of the blood within the exposed embryos of the second exposure (n=25) was observed. Normal blood development was observed in all of the control embryos and in 3 embryos in the low concentration (c= 15 mg/L) group. The 2 surviving embryo in the medium exposure group (c=30 mg/L) and the remaining one of the low concentration embryo experienced improper blood circulation and coloration. This was characterized by retarded formation of the heart muscle, weak circulation of colorless blood containing no cells, and small clot formations in the aortae and vitelline vessels.

There were developmental effects in the experimentation in which only one case of a particular response to the cadmium solution was observed. In the first exposure (n=120), bursting of the chorion was observed by one egg in the high exposure group (c=8 mg/L). A small hole in the chorion could be observed which was visible under the microscope (X36). In the second exposure, (n=25), an exposed embryo at 30 mg Cd/L, showed an abnormal bifurcation of the embryonic axis at the 30 hour observation. This affect dissipated at the 72 hour observation. In the second exposure, a embryo in the high concentration group (60 mg/L) had a unusually small yolk sac and a row of dying cells which adjoined to the yolk sac. Death of this embryo at stage 19 ensued, showing full trunk and optic cup development encased in a mass of necrotic tissue. One embryo in the low concentration group (15 mg/L) of the second exposure (n=25) exhibited a small, definable cleft on the posterior lobe of the forebrain. The cleft dissipated and was not observed at the 96 hour observation. In the second exposure (n=25), at 30 mg Cd/L, severe macroencephaly was observed at the 72 hours. Additionally, unequal eye size and irregular texture of the perimeter of the larger eye was observed. Premature hatch-out was observed in both exposure experiments. In the first exposure (n=120), all remaining viable embryos (25) in the high concentration group (c= 8 mg/L) were hatched by day 16, whereas only 44% of the controls had hatched by day 16. The high group (c=8 mg/L) and medium group (c=4 mg/L) had 4 and 5 dead embryos upon hatch, respectively. Premature hatch-out was also seen in the second exposure (n=25). All of the remaining viable embryo from the medium (c=30 mg/L) and low (c=15 mg/L) groups hatched from a period of day 15-17 of the experiment. None of the control group had hatched before or on day 17 of the experiment. Control group embryos hatched from day 19-29. Both of the medium group (30 mg/L) embryos were dead upon hatch. Additionally, 2 of the low group (15 mg/L) embryos were dead upon hatch.

Discussion and Conclusions

The toxic effects of cadmium on aquatic organisms and embryos have been well documented previously by many researchers (Michibata, 1981; Marty, 1991; Carrier, 1988; Michibata, 1981). The results of the stage progression observations according to time of each exposure shows that cadmium produces an inhibitory effect on the proper progression of normal embryonic development. This was seen most profoundly in the medium concentration group (30 mg/L) of the second exposure (n=25). Normal progression of this groups physiological and morphological development was delayed as a result of cadmium exposure. The retardation of essential biological functions delayed the stage classification of the embryos. Similar physiological and morphological abnormalities have been observed in Pacific herring eggs exposed to varying levels of benzene. Sorenson, (1991) observed lower survival at hatch, increased incidence of abnormalities, delayed rate of development, abnormal progression of heart rate, and abnormal formation of the vertebral column.

Changes in the progression of the exposed medaka embryos heartrate were seen at the 72 and 96 hour observation times in both experiments. The decrease of the heartrate trend in exposed embryos (n=120, c=8, 4 mg Cd/L), (n=25, c=30, 15 mg Cd/L) compared to the continued normal advancement of the controls suggests that cadmium impedes on the normal development of the cardiac muscle and/or normal physiologic function. Changes in embryonic heart rate have been noted experimentally as a response to a variety of other pollution stressors. These include dinitrophenol (DNP) (Rosenthal (1976), sulfuric acid from titanium dioxide production (Rosenthal 1976); red mud from production of aluminum (Hoar and Randall, 1988), and cadmium (Sorenson, 1991). Lack of proper somite formation was a developmental effect of cadmium at the higher concentration of both of our exposure experiments. No studies showing this developmental effect as a result of cadmium exposure were located. Vertebral alterations in juvenile and adult fishes have been observed in previous studies. Benneison et al. (1974) reported spinal deformities (vertebral fractures) in mature minnows (*Phoxinus phoxinus*) exposed for 70 days to aqueous Cd as low as 7.5 ppb (Sorenson, 1991). The lack of structural integrity of the formed backbone, although observed in one exposed embryo, was not the major abnormality seen. The inability of the somites to properly develop in an ordered, homogenous matter was observed in both of the medium concentration group (c=30 mg/L) in the second exposure (n=25). This effect may be a result of initial formation of the skeletal system being compromised due to cadmium competition for developing calcium binding proteins. The lack of circulating colored blood has been observed in other teleost embryo studies. Pickering and Gast (1972) observed circulatory problems among newly hatched fathead minnows in a chronic exposure to zinc (Sorenson, 1991). In this study, many of the minnow embryos which were exposed had a normal beating heart, but red blood cells were not circulating, and many blood clots appeared throughout the vascular system. Cadmium may have the same effect on the hemopoietic system as zinc because of their similar chemical and toxicological properties. The inhibition of colored blood may be a result of cadmium restricting iron uptake, and/or proper heme production. Goldfish in soft water (21 mg CaCO₃/L) showed distinct reduction in ability to form hemoglobin and red blood cells after a two week exposure to 18 µg Cd/L (Hoar and Randall, 1988). Other studies found some of the particular structural effects seen in only one exposed embryo. It is at the blastodisc stage that zinc-treated eggs of zebrafish (*B. rerio*) produce protoplasmic protrusions projecting abnormally from the sides of the embryo (Sorenson, 1991). This was seen in the medium concentration group (c=30 mg/L) of the second exposure (n=25) and this effect dissipated after 48 hours.

The experimentation presented in this report is not complete. Further histopathological and other evaluations of the hatched and unhatched embryos, which are presently in 10% neutral buffered formalin, will be performed. Therefore, without having full knowledge of all of the developmental effects which have taken place during or as a result of the experimentation which may have not been observed or manifest fully during the observation period, general conclusive statements are

difficult. The only conclusions which may be made are summations of the qualitative observations regarding the sublethal toxicity of cadmium on the medaka embryos. Retarded or delayed stage development of the *Oryzias latipes* embryos were observed in cadmium exposed groups. Approximately 90% of the embryo which experienced delayed stage development in the first exposure (n=120, c= 8, 4, 2, mg Cd/L) died shortly after delayed stage development was observed. The second exposure (n=25, c= 1.0 gm Cd/L, 60, 30, 15 mg Cd/L) showed a slight increase in survival of embryos with delayed stage development due to aqueous cadmium exposure. Exposure of *Oryzias latipes* embryos to aqueous cadmium inhibited the proper progression of many early development parameters including improper or abnormally shaped cleavage planes, retarded development of the blastula, failure of blastula to flatten and advance into gastrulation, and improper formation of early and late gastrula. Physiological development observations revealed a decrease of heartrate during development of the exposed embryos. The trend of reduction of heartrate at the third day of development was apparent in both exposure ranges. Morphological abnormalities which were observed included improper formation of the vascular system, improper coloration of blood, macroencephaly, irregular spinal flexure, unusual clefts in yolk sac and early cranium, improper pigmentation of trunk, failure of embryo trunk to expand to full embryonic axis, and abnormal bifurcation of embryonic axis.

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A Combined Chemical + Enzymatic Method to Remove Selected Aromatics from Aqueous Streams.

X. Xu, V. John

Abstract

Aromatics are major pollutants found in aqueous environments and in sediments. While there are many chemical and biochemical processes to remove and/or destroy these contaminants, they have to be considered in light of the economics and the time-scales for treatment. We describe our initial work on a hybrid chemical+enzymatic technique to remove aromatics from aqueous stream. The aromatic is first converted to the corresponding phenol through classical Fenton type chemistry involving catalysis by Fe(II). The phenol is subsequently polymerized through an enzymatic mechanism, using horseradish peroxidase as the oxidative enzyme. The polymer is insoluble in water and can be easily recovered. In addition, such phenolic polymers are useful products with varied applications in coatings and resins technologies. Thus, the pollutants can be eventually converted to useful products.

Introduction

Aromatics are major organic pollutants found in aqueous waste streams. Phenols, for example are prevalent in waste streams from coal conversion processes, and are generated during coal pretreatment steps prior to combustion. Physical methods to remove such contaminants include air stripping of volatile organics, but the technique does not result in destruction of the contaminant compounds. The compounds can be degraded biologically, but the extensive times for complete destruction is a detrimental factor to be considered. Chemical methods include ozonation, peroxidation, photocatalysis, and hybrid versions of these techniques¹.

In 1983, Klibanov and coworkers² developed a novel enzymatic approach to remove phenolics from waste streams. Here, an oxidative enzyme, horseradish peroxidase, was used to couple phenolics resulting in a polymer that is water-insoluble. The polymerization mechanism is shown in Figure 1; polymer formation follows oxidative coupling where the addition of H₂O₂ leads to the initial formation of phenoxy type radicals and final linkage at positions ortho to the hydroxyl, to form an insoluble polymer chain. The polymer is thus precipitated out of solution and is easily recovered. The polymer is relatively nontoxic compared to the monomer, and can be easily compacted for landfill or incineration. Phenolic polymers are also used in resins and coatings and a viable option is the use of these recovered polymers for such materials applications.

Our approach expands on this technique to include not just phenolics, but a variety of other aromatics. Essentially, the method consists of chemically hydroxylating aromatics to the corresponding phenols and subsequently using the enzyme to couple the phenols and remove them from solution.

The development of the idea was somewhat by chance, and occurred while we were exploring the effect of added surfactant on the enzymatic polymerization of aromatic amines, through the research initiation grant. We found that the polymerization of anilines by horseradish peroxidase was relatively poor, and as a consequence added Fe(II) as an enzyme promotor. The resulting

¹ Ollis, D.F., Turchi, C. (1990) Environmental Progress. 9: 229.

² Klibanov, A.M., Tu, T., Scott, K.P., (1983) Science, 221: 259.

reaction was very vigorous with significant formation of polymer. From a study of the literature, we realized that the primary role of Fe(II) was not to promote enzyme activity, but rather to hydroxylate the aromatic amine to the corresponding phenol, which is then readily polymerized by the enzyme. Such hydroxylations of aromatics follow a classical Fenton mechanism³ to be detailed in the next paragraph. Indeed, most of the techniques listed above for oxidative destruction of organics (e.g. ozonation, photolysis, peroxidation) involve the initial creation of a hydroxyl radical (OH[•]) and hydroxylations of the organic. Realizing the potential generality of the concept, that it may be possible to hydroxylate benzenes and substituted benzenes and subsequently polymerize the resulting phenols to insoluble species, we have continued our studies with benzene as a model contaminant.

The Fenton reaction⁴ involves the oxidation of Fe(II) to Fe(III) and the consequent formation of a very reactive hydroxyl radical (OH[•]) below

Reactions of the hydroxyl with the aromatic are complex. For benzene, a pathway involves the initial formation of the hydroxycyclohexadienyl radical followed by dimerization and dehydration to biphenyl, or further oxidation to phenol, as illustrated in Figure 2. Biphenyl is insoluble and is therefore removed from solution. However, the phenol remains in solution and our objective is to then polymerize the phenol to completely remove it from solution.

The novelty of our approach is the coupling of the Fenton reaction to enzymatic polymerization of the resulting phenolic species using horseradish peroxidase. Results of the combined process are the subject of this report.

Experimental

Materials:

All chemicals were purchased from Aldrich Chemicals (Milwaukee, WI) with a purity of at least 99.8% and were used as such. The enzyme, horseradish peroxidase (type II: molecular weight 40,000, activity 200 units/mg), hydrogen peroxide (30%), and HEPES buffer were purchased from Sigma Chemical Company (St. Louis, MO). Deionized and doubly distilled water was used in all preparations.

Methods

The reaction mixture containing benzene and FeSO₄·7H₂O/FeCl₃ was placed in 40 ml EPA standard vials with PTFE septa/phenolic screwed caps and magnetically stirred. The overhead dead space of the vials were minimized to avoid loss of the substrate through evaporation. Mixture pH was adjusted using 0.01 M HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) buffer, HCl and NaOH. The initial pH of the reaction mixture is 5.5 adjusted with 0.01 M HEPES; the solution acidity maintains Fe(II) solubility. The Fenton reactions were initiated with the injection of H₂O₂ using a microsyringe. At least three hours were allowed to complete the Fenton reaction, before initiation of enzymatic polymerizations. At the end of the Fenton reaction, the pH is about 4.0. For the enzymatic polymerization, the pH was usually adjusted to 8.2 using NaOH prior to reaction initiation.

Conversion of the substrate was monitored using a gas chromatograph (Varian 3410) equipped with a flame ionization detector. A 15 m fused silica capillary column with a phenylmethyl silicone

³Smith, J.R.L., and Norman, R.O.C. (1963) J. Chem. Soc., 2987.

⁴Walling, C., (1975) Acc. Chem. Res., 8: 125.

stationary phase (1.5 μ m film thickness) was used for analysis. Benzene and phenol concentrations were directly determined by injecting a 0.5 μ l aqueous phase sample into the column held at isothermal conditions of 160°C, maintaining a flow rate of 26 ml/min (splitless). Both peaks eluted within 1 minute and were well separated. Biphenyl which is insoluble in water was extracted subsequent to reaction using diethylether. Analysis of biphenyl involved injection at an initial column temperature of 115°C which was immediately ramped to 140°C (5°C/min).

Identification of products was accomplished by GC/MS. The GC/MS was performed on a Kratos Profile Mass Spectrometer outfitted with a Shimadzu 14A GC and operated with the Kratos Mach3 software. The GC column was a DB1 (J&W Scientific) 30 m x 0.25 mm with film thickness of 0.25 μ m. Split injection (30:1) was performed and carrier flow rate was about 30 ml/min. The following temperature program was used: initial temperature was 70°C for 1 minute, first ramp was 10°C/min to 190°C, held for 1 minute and second ramp was 20°C/min to 270°C, held for 2 min. The injector, MS reentrant line and interface were all at 270°C. The positive EI (electron ionization) spectra were acquired in the nominal mode with the magnet scanned over 50-500 amu at 0.3 sec/decade and with a mass resolution of 600.

Results and Discussion

Figure 3 illustrates the typical rates of the Fenton reaction. The control experiment (without addition of Fe(II)) indicates no reaction. Upon addition of Fe(II), the Fenton reaction proceeds very rapidly with over 50% of the final conversion level being reached within 3 minutes; the reaction is essentially complete within the first hour with a final benzene conversion level of 80%. The pseudo-first order rate constant for benzene conversion through the Fenton reaction at these conditions is 0.2 min⁻¹. The fast initial reaction rate indicates the viability of the Fenton reaction in initial conversion of the aromatic.

Parameter variation experiments are summarized in Table I. At constant H₂O₂ and benzene initial concentrations of 6.4 mM, we note that the conversion goes through a maximum with Fe(II) addition. The reactions of Fe(II)/Fe(III) are complicated, but the Fenton reaction involves competition for hydroxyl radicals by Fe(II) according to

and depletion of the hydroxyl radicals available for benzene oxidation. An equimolar stoichiometry of Fe(II) to benzene appears to maximize benzene conversion at a given H₂O₂ concentration.

A second observation is that the phenol and biphenyl products account for almost all conversion products at the lower concentrations of H₂O₂ (up to 6.4 mM). At higher concentrations, the conversion levels of benzene increase to almost total conversion, but phenol and biphenyl form a smaller component of the total product. We have not detected any additional species in the aqueous phase. At these conditions, the precipitate is not fully extractable into diethyl ether, and may therefore contain additional products such as the phenolic oligomers that are formed through oxidative pathways catalyzed by the Fenton reaction. We are continuing studies to determine these insoluble products.

Although high benzene conversions can be carried out through the Fenton reaction, we find that the reaction requires a significant excess of H₂O₂ to achieve such conversion levels. Besides there are still phenols left in solution that have to be removed. With this intention, the enzymatic reaction was carried out to further reduce phenol levels. To determine if the Fenton reaction and enzymatic polymerization could be combined, two experimental approaches were considered. The first approach involved introducing the enzyme into the reaction medium prior to the Fenton reaction, and the second involved introduction of the enzyme after completion of the Fenton reaction. Both approaches however, involved adjustment of the pH to 8.2 prior to initiation of the enzymatic reaction. In the first approach, we observed no polymerization and reduction of phenol content,

indicating that the enzyme was inactivated during the course of the Fenton reaction. The observation can be understood from the mechanism for peroxidase catalysis⁵.

Compounds I and II are intermediate protein structure; compound I involves an oxo-ligated iron(IV) protoporphyrin „-cation radical, (+ P)Fe^{IV}O, and compound II is an iron(IV)-oxoporphyrin IX species, (P)Fe^{IV}O. The substrate is the electron donating species SH₂ (phenol). The free radical species SH combine to form the chain linkage. In the presence of excess H₂O₂, compound I is intercepted into an inactive form of the protein according to

Thus, it is important that this shunt does not occur and that the substrate access to the enzyme is maintained. We believe that the enzyme is inactivated during the Fenton reaction during the addition of H₂O₂, when there is an insufficient amount of the phenolic substrate.

When the reaction is done following a two step approach, where the Fenton reaction is completed, the pH adjusted, the enzyme introduced, and H₂O₂ added in small increments to initiate polymerization, we find that the reaction is efficient. Over 95% of the phenol formed during the Fenton reaction is removed from solution by enzymatic polymerization. With an enzyme concentration of 0.001 mg/ml, the reaction is sufficiently rapid, with final conversion being reached in 3-4 hours. The pseudo-first order rate constant for phenol conversion under these conditions is 0.03 min⁻¹.

Conclusions

Our results indicate the feasibility of the chemo-enzymatic method to remove benzene from the aqueous phase. The process can be visualized as a two-stage process (Figure 4) where the Fenton reaction is carried out in the first stage, and enzymatic polymerization in the second. To attain higher conversions of the aromatic, a recycle type system, or a train of two-stage reactors can be employed. The rates of benzene conversion are sufficiently rapid enough to imply added process viability.

In continuing work, we intend to extend the concept to substituted benzenes, such as the anilines, the chloro and nitrobenzenes, etc. A complete analysis of the reaction mechanisms is difficult with these substrates, but it is very possible to simply follow the destruction of the substrate to evaluate process viability. Continuing work will also address questions on the continued viability of the enzyme, and extensions of the concepts to mixtures of contaminants.

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Table I: Effect of Parameter Variations on Benzene Conversion and Product Yields through the Fenton Reaction.

Run No.	Fe(II) Conc. (mM)	H ₂ O ₂ Conc. (mM)	Benzene (B) Conc. (mM)	% Benzene Conversion (Moles B reacted / Moles B in) x 100	%Conversion to Phenol (P) (Moles P formed / Moles B in) x 100	%Conversion to Biphenyl (BP) (Moles BP formed / Moles B in) x100
1	6.4	2.13	6.4	21	14	5
2	2.13	6.4	6.4	48	39	5
3	3.2	6.4	6.4	51	38	10
4	6.4	6.4	6.4	57	38	14
5	19.2	6.4	6.4	40	26	7
6	38.4	6.4	6.4	28	14	8
7	6.4	19.2	6.4	82	54	10
8	6.4	38.4	6.4	95	10	1

Figure 1: Schematic of Enzymatic Polymerization.

Figure 2: Schematic of Benzene Reaction through the Fenton Mechanism

Figure 3: Kinetic studies of benzene conversion. Initial benzene and Fe (II) concentrations are 1.28 mM each. Initial hydrogen peroxide concentration is 3.84 mM.

Figure 4: Schematic of the combined two-stage process for aromatic removal.

Genetically Engineered Micro-organisms: Aromatic Hydrocarbon Biodegradation Genes From *Rhodococcus*

K. Kendall

Summary

DNA known to encode toluene biodegradation genes in *Pseudomonas putida* was used in Southern Blots to identify homologous DNA in the unrelated toluene degrading Actinomycete, *Rhodococcus* sp. ATCC 19070. Two strongly hybridizing *EcoRI* fragments of 2.3kb and 2.7kb respectively were cloned into *E. coli*. Sequence analysis of a 400bp section of the 2.3kb fragment demonstrated that it encodes proteins with similar amino acid sequences to the *xyIX* and *xyIY* genes of *P. putida*. These proteins are components of toluate oxygenase, the enzyme catalyzing the first step in the metabolism of benzoic acid.

Background

The aim of this initiation project has been to follow up preliminary results indicating that two biodegrading strains of the common soil bacteria *Rhodococcus* (*R. erythropolis* ATCC 4277 and *Rhodococcus* sp. ATCC 19070) contained DNA with sequence similarity to DNA known to encode toluene biodegradation genes in *Pseudomonas putida*. This suggested that we may be able to use *Pseudomonas* DNA to isolate *Rhodococcus* genes involved in the biodegradation of aromatic hydrocarbons. Because *Rhodococci* are very different bacteria from the more commonly studied *Pseudomonas*, it is likely that their biodegradation genes are also very different and thus may have advantages that can be exploited in genetically engineered micro-organisms.

During the first nine months of this project, we have found additional similarities between DNA encoding *Pseudomonas* toluene biodegradation genes and the chromosome of *Rhodococcus* sp. AT 19070. The two segments of DNA from this strain with the most striking similarity to *Pseudomonas* DNA have been cloned into *Escherichia coli*. We have started to determine the sequence of these pieces of DNA. Although the analysis of these sequences is not yet complete, we have found that the DNA does indeed encode enzymes required for the metabolism of aromatic hydrocarbons. In particular, we have determined that the DNA encodes proteins with amino acid sequence similarity to subunits of Toluate Oxygenase, a multi-component enzyme required for the conversion of benzoic acid to 1,2 dihydroxy cyclohexadiene carboxylate. We are currently extending this sequence analysis to determine if the entire toluene biodegradation pathway is encoded by the cloned DNA.

Summary

Identification of DNA fragments from *Rhodococcus* with homology to TOL DNA. Various DNA fragments from the TOL plasmid pWW0 encoding toluene biodegradation genes were used as probes in Southern Blots against DNA isolated from the *Rhodococcus* strains ATCC 4277 and ATCC 19070. As shown in Fig. 1, the "SacI-D" fragment of pWW0 (encoding part of the "lower" toluene biodegradation pathway) hybridized very strongly to a number of *EcoRI* fragments from both ATCC4277 and ATCC19070. In particular, bands of 2.3kb and 2.7kb were extremely prominent in the *EcoRI* track of ATCC 19070. This strain is capable of growth on toluene as a sole source of carbon and energy.

Cloning of TOL-hybridizing *EcoRI* fragments from ATCC 19070. Chromosomal DNA from *Rhodococcus* sp. ATCC 19070 was digested with *EcoRI* and fragments ranging in size from 1.5kb to 4.5kb isolated from an agarose gel. These were then cloned into the *EcoRI* site of pEMBL8 and clones exhibiting homology to the SacI-D fragment were identified by colony hybridization and dot blots of isolated plasmid. Clones of pEMBL8 containing the 2.3kb and

2.7kb EcoRI fragments were obtained by this method and named "Eco2.3" and "Eco2.7" respectively.

Sequence analysis of clone "Eco2.3". We are currently determining the DNA sequence of the Eco2.3 clone using a "random" dideoxy method. We now have partial sequence information for approximately 1000bp of this DNA. Although the sequence of the clone is not yet finished, we have found that it is definitely related to the sequence of the TOL plasmid. Fig. 2. shows the sequence of a 400bp region of Eco2.3 and the predicted protein amino acid sequences that could be encoded. The amino acid sequences of the two open reading frames (OrfA and OrfB) were scanned against the NCBI protein database. The strongest homologies found were to subunits of toluate oxygenase from *Pseudomonas putida* and *Acinetobacter calcoaceticus*. Figs. 3 and 4 show that OrfA and OrfB exhibit extremely strong similarity the carboxy terminus of XylX (Fig. 3) and the amino terminus of XylY (Fig. 4.) respectively. The actual DNA sequence of this region of clone Eco2.3 exhibits approximately 54% identity with the XylX-XylY encoding region of pWWO (Fig. 5).

Discussion

The results presented here clearly show that we have successfully cloned DNA from *Rhodococcus* sp. ATCC 19070 that encodes genes used in the biodegradation of toluene. Suprisingly, the actual DNA identity between the *Rhodococcus* and *Pseudomonas* sequences was only 54%. Although this was enough to permit the identification of the appropriate DNA fragments, the *Rhodococcus* DNA must encode enzymes with considerable amino acid sequence divergence from their *Pseudomonas* counterparts. It will be of interest to determine if these differences are reflected in the efficiency with which the two species are capable of degrading toluene.

We are currently extending the sequence data to cover the entire Eco2.3 clone and also to determine the sequence of the Eco2.7 clone. The entire "lower pathway" toluene biodegradation operon in *Pseudomonas putida* covers approximately 12kb. We expect that the Eco2.7 clone will also contain genes from this region of the *Rhodococcus* chromosome (giving a total of 5kb of cloned DNA). Possibly, the additional fainter EcoRI fragments in the Southern Blot (Fig. 1) represent the remainder of this operon. We will identify additional overlapping clones of *Rhodococcus* DNA using the Eco2.3 and Eco2.7 clones as probes to clone and then sequence the entire pathway. This section of work should be completed before the termination of this initiation project.

Future Projects

Once the entire pathway has been cloned, we will introduce it into both *E. coli* and *Streptomyces lividans*; *S. lividans* is a well characterized Actinomycete that is closely related to *Rhodococcus*. We will then use genetic manipulation techniques to over-express the genes in an attempt to obtain bacteria with enhanced ability to degrade benzoic acid and other aromatic hydrocarbon metabolic intermediates. We will also use the cloned genes to pick out other genes from related species of *Rhodococcus*, and additional genes (e.g. the toluene biodegradation "upper" pathway) from *Rhodococcus* sp. ATCC 19070. The various genes can then be 'mixed and matched' to create genetically engineered microorganisms with altered substrate specificities and biodegradative abilities.

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Association for Information and Image Management

1100 Wayne Avenue, Suite 1100
Silver Spring, Maryland 20910

301/587-8202

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Laser Ablation/Ionization Studies Related to the Removal of Nuclear Materials from Metal Surfaces

B. Koplitz

The nuclear build-up over the past 50 years has left behind a formidable environmental problem. Not only are the nuclear and chemical waste products of this effort major targets for clean up, but the many containment and processing facilities themselves are contaminated with radioactive material at an unacceptably high level.¹ For example, gaseous diffusion and isotope separation facilities lying within the Mississippi River basin (e.g. in Oak Ridge, Tennessee and Paducah, Kentucky) as well as throughout the country constitute a nuclear weapons legacy which the Department of Energy (DOE) must deal with in one way or another. Prior to re-tooling, dismantling, or razing any of these facilities, the various structures must be cleaned of radioactive material to an acceptable level.

One approach that has received significant attention as an option for cleaning walls (e.g. metal, concrete, etc.) contaminated with nuclear waste involves the use of laser ablation methods.¹ Laser ablation with a pulsed source such as an excimer laser is essentially a surface-heating process whereby the top few monolayers of the material are rapidly heated resulting in the ejection of matter in either atomic, cluster, or particulate form. The ejected species can be either neutral or ionic. One major issue with respect to many laser ablation studies involves identification of the species being ejected. What are the elements? Are they in atomic or cluster form? Are they charged or neutral? With regard to the ablation of nuclear materials, a second major issue concerns containment of the ejected materials. If one cleans a surface by stripping off the top layers of material, it is imperative that these radioactive atoms, particles, or ions be properly contained. If not, the problem may in fact become worse and instead of removing the material, one is actually dispersing it. (Asbestos removal has long been plagued by these types of problems.)

We use ablation and ionization lasers to study the ablation of contaminated metal surfaces. Experimentally, laser ablation of the metal of interest is carried out physically below an electric field using either 308, 248, or 193 nm radiation. (See Fig. 1. Here, the pressure is $\sim 10^{-5}$ Torr.) One of the plates forming the electric field is actually a wire mesh that allows ions to pass. A second laser beam (193 nm; 6.4 eV) is positioned to actively ionize neutral species that are formed during the ablation pulse and ejected upward from the surface. Ionized species are directed down the flight tube of a time-of-flight mass spectrometer (TOFMS) so that their nature can be identified. The laser ablation and ionization properties of various metals under two general sets of conditions can be studied: (1) ablation laser alone with pulsed electric field and (2) ablation *and* ionization lasers with pulsed electric field. With bare Ni or Fe as the target surfaces, the photon energy of the ionization laser (6.4 eV) is below the ionization potential of the individual atoms, so only larger clusters (i.e. those whose ionization potential approaches that of the work function of the metal) can be identified. However, monitoring cluster distributions as a function of laser fluence will lend insight into the laser ablation process.

A new experimental apparatus for conducting ablation/ionization experiments has come on-line in our laboratory during this reporting period. Paul Barnes (physics undergraduate student), David Dennison (chemistry undergraduate student), and Valentin Panayotov (chemistry graduate student) have constructed a new time-of-flight apparatus for studying ablation processes. The system has been calibrated via the photoionization N_2O and CH_3I . Following is shown the laser ionization region for the system.

- 1.) Reitz, W. and Rawers, J. "A Review: Laser Ablation and Its Effect on Surface Removal" Report, 18 pgs.

Fig. 1. Drawing of a portion of the laser ablation (308 nm) and ionization (248 nm) setup. The laser interactions actually occur within the ionization region of the mass spectrometer. Here, the sample metal surface is Ni, but others can be accommodated.

Asymmetric PVDF Pervaporation Membranes for the Removal of Organic Contaminants from Waste Water

P. Pintauro, J. Taravella,

Introduction

Polyvinylidene fluoride (PVDF) pervaporation membranes have been fabricated using a new technique which combines controlled solvent evaporation with precipitant (H_2O) vapor adsorption. The membrane exhibits an unusual asymmetric structure with a dense layer at the membrane/casting surface interface and a microporous layer at the membrane/air interface. Due to the hydrophobic nature and chemical stability of PVDF polymer, these membranes are ideally suited for the pervaporation separation of organics (e.g., benzene, toluene, xylene, chloroform, ethyl acetate, and alcohol) from dilute aqueous solutions.

Experimental

Membrane were cast from a solution consisting of 12-14 wt% PVDF, 13-15 wt% dimethylacetamide (DMAc) and 71-75 wt% acetone. Before casting, the polymer solution is heated to 48-50°C for 0.5-1.0 hour and is allowed to stand at room temperature for about 24 hours to remove all air bubbles. The solution is then spread uniformly to a depth of 200-250 μm on a wetted glass plate and allowed to air dry at 23-25°C and a relative humidity of 45-55%. A PVDF membrane can be fabricated by completely air drying the polymer film (for 3 hours) or by partially air drying the film (12 minutes) and then immersing the membrane in a series of aqueous precipitation baths (15 minutes immersion in a solution of 50 vol% water, 40 vol% acetone and 10 vol% DMAc maintained at 15°C, 15 minutes immersion in a second bath of 60 vol% water and 40 vol% acetone at same temperature, and final immersion in a pure water bath at 15°C for 30 minutes). After the membranes is withdrawn from the last bath it is allowed to air dry at room temperature.

To test the performance of the PVDF membranes, a flat sheet membrane pervaporation apparatus was used to collect flux and separation factor data. The total membrane area exposed to the feed solution was 225 cm^2 . A vacuum was applied on the downstream side of the membrane to generate permeate fluxes. To eliminate concentration polarization effects, a nylon mesh turbulence promotor was placed on the feed side of a membrane, and the linear velocity of the feed solution was maintained greater than 14 cm/sec; under these conditions the measured organic separation factors and fluxes were always independent of the upstream fluid flow conditions. In a typical pervaporation experiment, the system was allowed to stabilize for about one hour before collecting a permeate sample in a liquid nitrogen cold trap (the collection time was usually 15-30 minutes). The membrane separation factor and organic flux were determined from the total weight of sample collected and the gas chromatograph analysis of the permeate. Experiments were repeated several times to insure reproducibility; separation factors and organic fluxes normally varied by no more than $\pm 5\%$.

Pervaporation separation experiments were performed with wet cast PVDF membranes and dilute aqueous feed solutions of o-dichlorobenzene (70 ppm) and styrene (100 ppm). Both organics have boiling points greater than that of water and trace quantities of these compounds cannot be removed from water by air stripping. The effects of feed temperature (30, 45, and 60°C) and downstream pressure (0.03, 0.05, and 0.08 atm.) on organic separation factor and transmembrane organic flux were determined. Here, the organic separation factor is defined as the ratio of the wt% organic to wt% water in the permeate divided by the ratio of the wt% organic to wt% water in the feed solution. Although the PVDF membranes exhibited an asymmetric microstructure (as determined by scanning electron microscopy), we found identical organic fluxes and separation factors when

either the dense or porous sides of the PVDF film faced the feed solution (so long as the linear velocity of fluid over the membrane surface was > 14 cm/s to insure that there was no concentration polarization). Typical experimental separation data for the selective removal of *o*-dichlorobenzene from water are listed in Table 1. The PVDF membranes work very well with both high organic separation factors and high transmembrane organic fluxes. For comparison purposes, the separation factor for a 20 ppm *o*-dichlorobenzene in water feed solution using a polyether block amide (PEBA) pervaporation membrane was found to 1020 and the separation factor for a 35 ppm styrene in water feed (with a PEBA membrane) was 741 [1].

Some work was also performed on the piezoelectric properties of asymmetric PVDF membranes. Recent studies have suggested that significant molecular-level changes occur in a polarized PVDF polymer. The C-F bonds in PVDF become permanently aligned by the application of a high electric field. the so-called "polar" phases of the polymer where there is specific and coordinated alignment of the C-F units may be more hydrophobic than non-polarized PVDF. Since the hydrophobic nature of the PVDF polymer is presumably playing a major role in the selective removal of organics from water in a pervaporation separation, we investigated the effects of polarizing a PVDF membrane on benzene/water separation. A wet cast PVDF membrane was placed between two copper plate electrodes and polarized (poled) for 15 minutes at an electric field strength of 100×10^6 V/m. From X-ray diffraction scans of poled and unpoled films we determined that only a small fraction of the C-F bonds in the polymer had become aligned. We also found that the performance of the poled membrane for benzene/water separation was essentially identical to that for a normal asymmetric PVDF film. Work is continuing on developing experimental methods for applying more intense electric fields to the PVDF films in order to increase the number of aligned C-F units.

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Table 1

Pervaporation Separation of *o*-Dichlorobenzene from Water Using Asymmetric PVDF Membranes
o-Dichlorobenzene Feed Concentration: 70 ppm

Feed Temperature and Downstream Pressure	Permeate Concentration ¹ (wt%)	Permeate flux (g/m ² -h)		Separation Factor
		Total ²	Organic	
T=30°C				
0.03 atm.	2.24	84.8	2.04	351
0.05 atm.	4.20	42.7	1.79	658
0.08 atm.	9.42	11.9	1.12	1254
T=45°C				
0.03 atm.	1.09	295	3.21	198
0.05 atm.	2.56	127	3.22	394
0.08 atm.	4.31	74.6	3.13	718
T=60°C				
0.03 atm.	0.96	593	5.67	155
0.05 atm.	0.94	394	3.69	184
0.08 atm.	0.31	151	0.46	80

¹ 0.01 wt% = 100 ppm

² Total flux refers to organic + water

Table 2

Pervaporation Separation of Styrene from Water Using Asymmetric PVDF Membranes
 Styrene Feed Concentration: 100 ppm

Feed Temperature and Downstream Pressure	Permeate Concentration (wt%) ¹	Permeate flux (g/m ² -h)		Separation Factor
		Total ²	Organic	
T=25°C				
0.03 atm.	6.61	42.7	2.82	808
0.05 atm.	8.59	18.6	1.60	839
0.08 atm.	9.39	6.70	0.42	862
T=45°C				
0.03 atm.	2.5	252	6.2	234
0.05 atm.	11.8	59	6.7	1089
0.08 atm.	28.6	8.9	2.5	3921

¹ 0.01 wt% = 100 ppm

² Total flux refers to organic + water

Initiation of Research Collaboration Between the Tulane/Xavier CBR and the Institute of Radioecological Problems in Minsk, Belarus

J. Bennett, S. Ramer

This grant was designed to lay the foundations for a program of collaborative research between the Tulane/Xavier Center for Bioenvironmental Research (CBR), which is studying Hazardous Materials in Aquatic Environments of the Mississippi River Basin, and the Institute of Radioecological Problems (IREP) of the Belarus Academy of Sciences in Minsk. We had already broached the possibility of such collaborative research with Dr. Georgii A. Sharovarov, the Director of IREP, during his visit to Tulane University during the fall semester of 1992, but our discussions were quite general in nature.

In order to determine the most fruitful areas for scientific collaboration, we visited IREP in Minsk from May 30 - June 13, 1993. We had several goals in the visit. The first was to get a more detailed understanding of IREP itself: the scope of its activities, the skill and training of its personnel, the level of instrumentation and equipment to which its personnel have access, and something of its overall character. The second was to provide IREP's representatives with fuller descriptions of the work on aquatic environments being done by the Tulane/Xavier CBR. The ultimate goal of the visit was to define at least the general parameters of a specific collaborative project.

The IREP is located at Sosny, a suburb of Minsk about twenty minutes' drive from the city, which was built to house the institute's employees. The IREP was placed at some distance from the city because of the research nuclear reactor that was originally on its grounds. Many of the institute's employees continue to live in Sosny itself, although the majority commute by bus or car from Minsk.

Almost all of the activities of the IREP today are directly related to the Chernobyl catastrophe and its impact upon Belarus. Only about twenty per cent (20%) of the IREP's budget comes from direct outlays from the Belarus Academy of Sciences, and this limits the possibilities for doing pure science within the institute. The bulk of the institute's budget comes from outside contracts, most of which come from the Belorussian government in connection with Chernobyl-related tasks. The IREP plays a central role in providing measurements of the levels and character of radioactive contamination in the country; in doing research on the fate and transport of radionuclides by wind and water; in doing research on the combined effects of radioactive and other hazardous wastes, particularly the country's extensive chemical wastes and other industrial pollution; in developing proposals for effective remediation; and finally in designing instrumentation devices that can be useful in any of these tasks.

The IREP is by no means the only institution in Belarus that is concerned with the consequences of the Chernobyl disaster. For example, it is only peripherally engaged in any activities related to epidemiological studies or more general public health problems. However, it does maintain close lateral ties with other institutes of the Academy of Sciences that are concerned with the consequences of Chernobyl. In the course of our meetings, it became clear that the institute's central task is to provide an intellectual and empirical basis for the rational remediation of Belorussian territory. This primary focus upon problems of remediation makes for a good match with the research activities of the Tulane/Xavier CBR.

The IREP encompasses eleven laboratories or working groups that are engaged in work on the following problems:

1. Radioecological problems of safety of nuclear technologies.
2. Mathematical modeling and forecasting of the radioecological situation.
3. Methods and technologies of radioecological protection.
4. Environmental diagnostics.
5. Radiochemical studies of the environment.
6. Assessing the behavior of radionuclides in the environment.
7. Radiation and toxicological ecology.
8. Radiation safety.
9. Means of decontamination.
10. Radiation and chemical problems.
11. Radioactive and non-radioactive discharges.

During the first week of our stay, we visited and talked with the staffs of these laboratories, usually meeting with one in the morning and one in the afternoon. Throughout our discussions at the IREP, we adopted the following division of labor. Professor Bennett, of the Department of Cell and Molecular Biology at Tulane University, served as the scientific consultant and scribe of the project. Professor Ramer of the History of Department served as translator and as a facilitator in the negotiations. In the evenings, after a day at the institute, we would share impressions and discuss what we had seen, whom we had met, and what kinds of questions still needed answers.

In most cases, there were only limited amounts of equipment and supplies at the IREP. Scientists were doing the best they could with antiquated tools. Those whom we met showed a pronounced awareness of the synergistic health effects of radionuclides with other industrial pollutants, and several laboratories were working with rodent models to quantify these effects. Assaying, tracking, and monitoring the levels of radionuclide contamination levels, transport and so forth are available based on the hundreds of thousands of assays that have been conducted since the disaster.

Drs. A. Gvozdev, A.O. Katanaev and their colleagues have developed a "Beta-Gamma Radiometer" to measure low levels of ^{90}Sr , ^{137}Cs , and ^{40}K . Members of the laboratory told us that it is an ultra-sensitive device capable of directly measuring low levels of radionuclides in foodstuffs and environmental samples without prior radiochemical extraction. They are hoping to market the radiometer under the name "Beta-91," available from "The Small Promoting Enterprise," in Sosny, Belarus.

We tried to estimate the collaboration potential not simply of laboratories, but of the people in them. Obviously, we were on the lookout for those laboratories in the IREP that were doing work that would be compatible with the Tulane/Xavier CBR project in remediating the Mississippi River's aquatic environment. We also sought to identify scientists whose overall approach to their seemed to hold out the greatest promise of success in collaborative work.

In both these regards we were particularly impressed with the mathematical and computer modeling laboratory, whose members have developed a variety of computer models concerning the fate and transfer of radionuclides in Belarus. This was the most "Western" laboratory in the institute, and we were particularly impressed by how quickly we were able to find a common scientific language with its members. Most of the scientists in this laboratory are relatively young. Most of them read English, and several speak English reasonably well. They were well informed about current developments in computer science in the West and clearly eager to engage in the kind of collaborative work that we were suggesting. When we asked them what work they had done on the fate and transfer of radionuclides in water, they showed us an interesting modeling project they had done on one heavily contaminated river system in southeastern Belarus. We asked them to come up with a description or what they would like to do in collaboration with us, and they completed a draft proposal for such collaborative work in a few days.

As is often the case in the former Soviet Union, the computer scientists that we met in this laboratory were far more sophisticated than the hardware to which they had access. There are only three IBM-type i386 PCs in the laboratory, which means that individual scientists have exclusive access to a computer for only a short period every day. Several 486/66Mhz PCs could radically enhance the overall productivity of this laboratory.

Our visit to Minsk and preliminary definition of a common research area was only the beginning of our tasks. Upon our return to Tulane in late June, we embarked upon a series of tasks that were necessary to implement a collaborative research project. The first was to establish a quick and reliable communication link between the Tulane/Xavier CBR in New Orleans and the IREP in Minsk. Our goal was to establish a connection on Internet. The first step achieving this goal was to establish our own computer connection at the IREP. Throughout the fall we enjoyed relatively easy communications using a fax modem on both ends. The Internet tie has been more difficult to get up and working, although we have made considerable progress in this direction and expect the Internet connection to be established before the end of the grant period.

The more substantive task that we faced upon returning to New Orleans was to talk with those members of the Tulane/Xavier CBR whose special area of research is the mathematical modeling of the fate and transport of hazardous materials, since these scientists would have to take the lead in the collaborative endeavor that we had envisioned. We met with Dr. E. Michaelides of the Department of Mechanical Engineering at Tulane, who is such a specialist, and he took the lead in writing a proposal for the kind of collaborative research that we had discussed in Minsk. We intend to submit an application for a three-year cluster grant. The topic of this proposal, which builds upon the discussions that we had in Minsk, is: "Collaborative Research with the Institute of Radioecological Problems (Belarus Academy of Sciences, Minsk): Fate and Transport of Radionuclides in Belarus After the Nuclear Explosion at Chernobyl." The project will focus upon the fate and transport of radionuclides in several river basins of southeastern Belarus and also the fate and transport of radionuclides resulting from forest fires. We are currently awaiting the results of the scholarly review process.

From the outset, we were interested in the possibility of bringing a graduate student from the IREP to Tulane. While in Minsk, we discussed the possibilities for graduate training in the United States with a number of the IREP's graduate students. We were particularly impressed by Mr. Oleg Pimenov, a graduate student who speaks fluent English. During the fall semester, we worked with Dr. E.E. Michaelides and Graduate Dean Susan Davis Allen of Tulane University to invite Mr. Pimenov to enter Tulane's graduate program in Mechanical Engineering. Tulane provided Mr. Pimenov with a tuition waiver and graduate stipend for the period January - September, 1994. He arrived in New Orleans on December 31, 1993 and will be enrolled in graduate classes at Tulane.

Our experience in defining the collaborative research described in this report has persuaded us that it is indeed possible to carry out fruitful scientific collaboration with the IREP, and has reinforced our original conviction that the kind of collaboration that we are seeking to realize has important larger implications for environmental scientists in the United States and the former Soviet Union. For those working on problems of remediation in the United States, Belarus and the entire former Soviet Union are a laboratory of environmental disaster. There is potentially much to be learned from the dedicated scientists of these countries who are working in the midst of this disaster. Conversely, they very much need to profit from whatever insights our own research on environmental remediation can yield. In a quite literal sense, any projects that would tend to bring these scientists together have the potential to improve the fate of mankind.

Risk, Stress, And Restructuring In The U.S. Petrochemical Industry: A Case Study From Louisiana

J. T. Roberts, J. Baugher

How do social factors at work affect one's perception of workplace hazards and mediate the relationship between physical conditions of the job and psychological distress? Understanding the factors that affect workers' and management's perception of risks is crucial for informing effective policies on occupational hazards and will assist in the creation of better stress alleviation programs at the firm and industry levels. Energy-related sites such as oil refineries and chemical processing plants are inherently dangerous workplaces, and recent reorganization in the industry in response to an economic crisis has increased perceived risks and worker anxiety. The purpose of our investigation has been to assess the factors that affect perceptions of risk by different categories of workers in the oil and chemical industry.

Our goal has been to develop and test a survey instrument which traces what workers are worried about, why, and how that worry affects them. Since beginning our research in April, 1993, we have constructed a questionnaire and completed data collection at a chemical facility near Baton Rouge, Louisiana. The survey on work content and job stress was developed from existing validated indices and supplemented with a series of new items on work in the chemical industry. The survey instrument was finalized with input from management and union representatives at the plant, and each provided a cover letter requesting participation by workers in the study.

The survey contained measures of physical risks and chemical exposures, how workers are organized and managed, psychometric measures of anxiety and depression, alcohol and tobacco use, union membership and job satisfaction, workers' methods of coping with risks, social support, and personality. Items on work organization measured workers' decision latitude, psychological and physical demands of the job, shiftwork, production demands as affecting safety, fear of reporting unsafe conditions and refusing unsafe work, threats of layoffs, bonuses for doing hazardous tasks, routinization of work, and poor communication between workers.

Several survey items were designed to assess the specific problems that arise during periodic plant shutdowns for maintenance, called "turnaround" or "outages." During those periods there is intense pressure to get production back "on line" as quickly as possible, and workers are often are on duty twelve hours a day, six or seven days a week. We lay out in greater detail the origins and justification of the survey instrument in a separate section below.

We distributed surveys at shift change times over a two week period in November, 1993, and collected them in sealed boxes at the plant gates. Two hundred and thirty-eight surveys of 356 distributed were returned, for a response rate of about 64 percent. The data has been input by a teaching assistant John Hall and graduate research assistant for this project John Baugher. We have also completed initial data analysis. We will first provide a basic descriptive statistics report to management of the plant for their review, then present them to union representatives. The firm's identity is being kept confidential in all publications. We expect to complete this initial report by mid-February, and further analysis later in the spring. We have also successfully negotiated with a large oil refinery to survey their workers in early February, 1994. Initial results of our study will be presented at the Eastern Sociological Society annual meetings in Baltimore March 17-20, 1994 (see section below).

We have also conducted empirical research utilizing secondary data on worker stress and perceived hazards. That work involved data from the International Social Survey Project (ISSP), including representative samples of eleven nations in North America and Europe, plus Israel. The

relationship between "unhealthy" and "stressful" work held up in eight of eleven countries (see below). We submitted an article from that study completed this summer under our DOE funding entitled "Hazardous Workplaces, Class and Stress: Evidence from an Eleven Nation Study" to two outlets. First, to the journal Social Forces in September and in December to the American Sociological Association (ASA) for its annual conference upcoming in August, 1994. The paper has been accepted for presentation at the ASA meetings in Los Angeles.

Finally, in October, 1993 we completed and submitted an application for two years of funding to complete this project to the National Institute for Occupational Safety and Health through their small grants program. That proposal, entitled "Perceived Hazards and Stress in Petrochemical Work," requested funding to support the P.I. and graduate research assistant John Baugher for two years to continue our current Department of Energy funded initiation research. That study would also employ our pre-tested self-administered questionnaire to workers at three or four more petrochemical facilities in Louisiana. We expect to hear from NIOSH in May or June, 1994, and funding would begin in July.

As just laid out, our research has proceeded in two stages: a secondary data analysis, and our own survey. These two stages are discussed in more detail below.

Stage 1: Evidence from an Eleven Nation Study on Workplace Hazards, Social Class, Gender, and Stress

Through secondary analysis of International Social Survey Program (ISSP) data, we examined an important question raised by Aneshensel: "Do the distribution of stressors vary meaningfully across strata?" (1992:19). We proposed that physical hazards at work may be among the most dramatic and unstudied ways in which they do. This claim is based on a series of recent studies which have supported the greater importance of long-term chronic rather than acute stressors (House et al. 1986; Liem and Liem 1978; Pearlin 1983, 1989). Sociological studies have since begun to examine the structural sources of stress, evaluating the hypothesis that gender, ethnicity, and economic class backgrounds are important predictors of exposure to stressful situations and that each of these groups bring different resources for coping (see Aneshensel 1992 for a review).

We examined responses to a nearly identical battery of questions administered in 1989 to representative samples in eleven nations through the International Social Survey Program (ISSP) to test the cross-cultural robustness of a series of variables representing both physical and social characteristics of the job as affecting occupational stress (see Roberts and Baugher, under review). The workplace characteristics we were able to examine from the ISSP data included both physical and social aspects of the job: responses to items on "dangerous" and "unhealthy" workplaces, union membership, work as a supervisor, relations with co-workers and boss or employees, and amount of control over planning one's workday. We also considered respondents' marital status, sex and age, and in a separate analysis, items on self-reported social class. The study documented that both social and physical aspects of the job, plus class membership and gender all make separate contributions to explaining self-reports of job stress.

During this stage we more fully elaborated our theoretical models and analysis techniques. The exercise provided support for the robustness of the relation between exposures and workplace stress, while weighing the importance of social categories such as class, gender, and cultural setting (nation). The full paper is available upon request.

Stage 2: Questionnaire Design, Data Collection, and Initial Findings

One major accomplishment during our first months of DOE funding was a review of the literature on measuring stressors and stress and the development of a 189-item questionnaire which operationalizes the concepts critical to the proposed study. We include here the justification for our conceptual and measurement variables.

Questionnaire Design

A considerable body of empirical research concerns the physical and psychological impact of occupational stress. However, Kohn (1990) recently argued that with few possible exceptions, no research "adequately tests the stress hypothesis" in its effects on psychological functioning outside the workplace. A complete model of job stress, Kohn maintains, must include three elements: objective job conditions, feelings of stress arising from those conditions, and indicators of off-the-job psychological functioning. We attempt here to construct a comprehensive model of occupational stress beyond Kohn's conceptualization, however, our model includes Kohn's suggested components.

While Kohn focuses on the objective conditions of work in his research on the effects of work on personality, we are most concerned here with the subjective conditions of work for both theoretical and methodological reasons. We do, however, objectively measure job complexity with data, people, and things. We use the Dictionary of Occupational Titles (DOT) codes to establish a base measure of workers' level of job complexity with data, people, and things and then modify these scores (if necessary) based on job descriptions by management to reduce error due to within occupation variance. While job complexity is most clearly operationalized objectively, we take issue with Kohn's exclusive focus on objective job conditions.

Research indicates, for example, that the perception of adequate social support is a consequential factor in attenuating the effects of potential stressors. Moreover, we argue that at least one other critical component of our model, job demands, implicitly requires subjective measurement. Conceptually we follow Karasek and his colleagues (Karasek & Thoerell 1990) including job demands in our model, and use their items to operationalize that variable. Kohn (1990:49), however, explicitly critiques Karasek and colleagues' subjective measure of job demands arguing that "it is therefore difficult to evaluate their findings." The job demands component of Karasek's JD-C model, however, concerns the "psychological demands of the job" which may necessitate subjective measurement. Additionally it is not feasible, nor advisable, to objectively measure other concepts in our model such as personality and coping resources: job demands and all other measures are self-reported survey items.

Undeniably, personality has an impact on the perception and report of stress, however "decades of research have still not reliably identified *the* personality variables" related to physical and psychological distress (Karasek & Theorell 1990:96). Brief and colleagues (1988) argue that negative affectivity (NA), a personality construct reflecting the tendency to focus on the negative aspects of the environment and making a person more disposed to distress, is a confounding variable in the job stress literature, "inflating" the relationship between job stress and job strain. They operationalize NA using the Taylor Manifest Anxiety Scale (TMAS, Taylor 1953), a supposed measure of "trait" anxiety, as opposed to a transient anxious state induced by a stressful situation. While we do not reject the possibility of "trait" anxiety confounding the relationship between job stress and job strain, we find using the TMAS to measure NA in a cross-sectional study problematic. A longitudinal research design would be the ideal, if not the only, method for disentangling manifest anxiety (a long-term personality trait) and situation-specific anxiety (a stress response), but such research remains beyond our current possibilities.

Bhagat and colleagues (1985:203) indicate that "stress is responsible for psychological outcomes, such as anxiety and depression," therefore anxiety has been conceptualized as both the cause and the effect of stressful situations. Everly et al. (1986) also indicate that "anxiety is a subset of stress," therefore they attempt to establish construct validity of their stress measure -- the Everly Behavioral Survey-Revised (EBS-R) -- by demonstrating a high correlation of the EBS-R with the TMAS ($r=.79$). The use of the TMAS to validate an outcome measure (Everly et al. 1986) as well as a causal variable (Brief et al. 1988) supports our assertion that it is problematic to distinguish transient and trait anxiety in cross-sectional research. An alternative personality construct that is likely to mediate the relationship between job stress and job strain is a *mastery orientation* (Karasek

& Theorell 1990:101). Following Pearlin and Schooler (1978), we include three measures of psychological resources: self-esteem, self-denigration, and mastery.

Attempts to assess overall levels of stress have led researchers to the study of life event change and its effects on physical and psychological health. However, the effects of life change on health are consistently modest (see review by Aneshensel 1992). Additionally, improvements in life event measurement have not significantly increased the association between life event change and psychological distress, therefore recent studies focus on social psychological mechanisms and coping resources that attenuate the effect of stress on mental and physical well-being. One such resource that has received much empirical support in its attenuating effect on stress is social support.

The social support literature has proliferated in the past two decades, however, measures of social support have increased in number almost as rapidly as empirical studies (for a systematic review of social support measures, see House & Kahn 1985). This notwithstanding, social support is typically operationalized in terms of the existence or quantity of social relationships, the structure of these relationships, the functional content of these relationships or some combination of these aspects (House & Kahn 1985). Following House and Kahn's (1985) recommendation to measure social support in terms of at least two of these aspects of social relationships, we measure the existence and functional content of social support.

The theoretical framework specifying the relationship between stress, social support, and health is crucial in measuring social support (House & Kahn 1985, Depner et al. 1984). Our causal model includes stress-buffering effects of social support (Wheaton 1985, LaRocco et al. 1980), therefore we do not measure the structure of social relationships, as research indicates that membership in social networks does not buffer the impact of stress on mental health (Aneshensel 1992). By stress-buffering we mean that as stressors increase, social support is mobilized. Additionally, the mapping of social networks in large samples is not cost-effective (House & Kahn 1985).

LaRocco et al. (1980) advise distinguishing among different sources of support (e.g. spouse, co-workers, supervisors) and House and Kahn (1985) suggest the effects of different types of support (e.g. emotional, instrumental, informational, and appraisal) be distinguished. We focus on the existence of support rather than the quantity of social relations, as this is most cost-effective. Moreover, it is the absence of *any* social support that is most detrimental to health, with gains beyond the first supportive relationship not carefully studied (House & Kahn 1985).

Lack of social support is typically associated with poor psychological health, therefore general measures of stress often are not conceptually distinct from measures of coping resources such as social support (e.g., University of Manchester Institute of Science and Technology 1987). Moreover, such stress scales often include items measuring distinct psychosocial states such as depression and anxiety (e.g., Everly et al. 1986). Therefore, we conceptualize stress as a multi-dimensional construct and attempt to measure three conceptually distinct components: depression, anomie and psychological distress.

No available survey instrument has operationalized the social relations of work as laid out by Dwyer (1991). Therefore we have developed a series of questions measuring authoritarian control, fear of layoffs, work with or for subcontractors, presence of bonuses for doing hazardous tasks, boredom due to routinization of work, poor communication between workers, and the respondent's work shift schedules.

Finally, we follow Karasek's Job Content Questionnaire (JCQ) to measure different aspects of hazardous and noisome workplaces and work in confined spaces (Karasek 1985). We modify Karasek's JCQ questions to measure both perception of hazards *and* subjective feelings concerning those conditions. For comparison of our case studies with national samples, we supplement our

questions on hazardous work with items from the International Social Survey Program asked to representative samples of workers in eleven nations in 1989.

Data Collection

We have chosen plants and gained access through their local trade organization, the Louisiana Chemical Association (LCA). Officials of the LCA have provided detailed descriptive information on the industry in the state and have passed information regarding our study to manufacturers. Some of these have in turn expressed interest in the project, and these make excellent study sites. At this time we have completed data collection at one chemical plant through our negotiations with the LCA.

As mentioned above, we distributed surveys at shift change times over a two week period in November, 1993, and collected them in sealed boxes at the plant gates. Two hundred and thirty-eight surveys of 356 distributed were returned, for a response rate of about 64 percent. As this plant self-selected into our study, we acknowledge the need to compare working conditions and employee stress levels at this facility with a disparate sample. We have, therefore, negotiated access and will begin data collection at a larger petrochemical facility in February 1994.

Initial Findings

We have just completed data entry for the first chemical plant where we conducted our survey. Our initial findings indicate that it is critical to measure both perception of exposure and subjective assessment of those risks (worry) in attempting to predict hazardous work's effects on stress. We analyzed cross-tabulations of perceived exposure to various workplace risks by respondents' level of worry concerning those conditions. We then tested the hypothesis of symmetry for these cross-classifications as to assess the level of correspondence between perception of exposure and worry. These results indicate that this correspondence between perceived risk and worry varies by the type of risk.

For example, there is strong correspondence between one's perceived level of exposure to carcinogens at work and one's level of worry about those exposures, as indicated by the fit of the model of symmetry ($L^2 = 4.30$, $p > .22$). The same can be said of exposure to dangerous tools, machinery, or equipment at work and one's level of worry about them ($L^2 = 6.11$, $p > .10$). That is, workers who report they are "always" or "often" exposed to these conditions are likely to "always" or "often" worry about them, respectively. Likewise those report they are "hardly-ever" or "never" exposed to these conditions "hardly-ever" or "never" worry about them. These findings, therefore, support a one-to-one relationship between levels of exposure and worry, wherein workers worry in congruence with their level of exposure.

Perhaps the most interesting finding so far, however, is that workers tend to worry disproportionately about other risks such as explosions, burns and fires; hazardous materials stored in the work area; and work with dangerous chemicals, as indicated by the misfit of the model of symmetry to the data ($L^2 = 54.02$, $p < .001$; $L^2 = 52.02$, $p < .001$; $L^2 = 13.41$, $p < .05$, respectively). For example, workers who report they are only "sometimes" exposed to these conditions are likely to worry "always" or "often" about them. This relationship holds up regardless of level of exposure for these risks, such that even low levels of exposures ("hardly-ever" exposed) leads to disproportionately higher levels of worry. The significance of this finding is the indication that workers worry about several workplace risks even at times of non-exposure. A critical next step in our analysis, therefore, is to understand why certain risks are more worrisome to workers than others, and to assess whether such worrying affects off-the-job psychological functioning.

Our program of research has been welcomed by some plant personnel managers who see the opportunity to gain insight into stressors affecting their workforces. Another long-term goal is to assist these plants in setting up or improving their employee assistance/stress management

programs, both by identifying worker stress and its sources and in moving to alleviate it. Towards those ends, our current study in a large refinery is specifically being tailored to evaluate the firm's social climate given the recent institution of a new employee participation program. The potential for industry buy-in or co-sponsorship of this kind of research is apparent: both firms have recently spent tens of thousands of dollars on much simpler studies of stress and work climate by private consulting firms. The current study therefore could provide the baseline data for evaluating future stress management programs and will inform more nuanced research in the future.

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Selective Complexation of the Uranyl Ion using Modified Polymeric Supports

D. M. Roundhill

We have recently examined the complexation properties of aminomethylenebis(phosphonic acids) towards selected trivalent metal ions, and have found that they form highly stable complexes with these oxophilic metal centers.^{1,2} These metal complexes were found resistant to metal ion dissociation in acidic media and present in aqueous solution as both 1:1 and 1:2 (metal ion:ligand, M:L) species. The uranyl ion, although structurally different from simple M^{3+} ions because of its linear geometry, possesses a similar +3 to +4 charge at the metal center due to participation of the uranium 5f atomic orbitals.³ Because of the high positive charge on uranium in the ion UO_2^{2+} , and because of the potential capacity of the axial oxygens on this ion to form intramolecular hydrogen bonds with a proton on the ligand, there is a good possibility that the uranyl ion will form stable complexes having 1:1, 1:2 and 1:3 (M:L) stoichiometries with aminomethylenebis(phosphonates) as ligands. There is considerable precedent that a phosphonate group will form strong complexes with the UO_2^{2+} ion, and there is the additional possibility that the amino moiety on these ligands will provide further selectivity for complexation *via* hydrogen bonding.⁴ Furthermore, these ligands are simple and inexpensive to synthesize, thus making them potentially useful for application in large scale batch separation processes.

To test these concepts, we have measured the stability and protonation constants of the different complexes formed in aqueous solution between the UO_2^{2+} ion and the compounds *N, N'*-dimethylaminomethylenebis(phosphonic acid) (MAMDP) and aminomethylenebis(phosphonic acid) (AMDP) (figure 1). From these data we can evaluate the potential for compounds of this type to be useful as uranyl ion sequestering agents.

Figure 1

Experimental Section

Uranyl nitrate, $UO_2(NO_3)_2 \cdot 6H_2O$ (99+%) was obtained from Strem Chemical Co. Tetramethylammonium hydroxide, $[(CH_3)_4N]OH$ was obtained as a 1.0 M solution from Aldrich Chemicals. Perchloric acid was obtained from CMS, and tetramethylammonium nitrate, $[(CH_3)_4N]NO_3$ was obtained from Johnson Matthey. The tetramethylammonium nitrate was dried in a vacuum oven prior to use. Reagents used in the ligand syntheses were standard reagent grade, and were used without prior purification. Microanalyses were carried out by Galbraith Inc., Knoxville, TN. Melting points were determined on a hot stage apparatus. 1H and $^{31}P\{^1H\}$ NMR spectra were obtained on a Bruker AC200 spectrometer as D_2O solutions. The compound MAMDP was synthesized by the Mannich-type reaction of dimethylformamide with phosphorus trichloride and phosphorous acid as reported in the literature.^{2,5} Standard base solutions were kept

free of carbonate as much as possible by diluting commercial solutions with degassed distilled water, followed by storage under nitrogen.

Ligand Synthesis

Aminomethylenediphosphonic acid (AMDP). $\text{H}_2\text{NCH}(\text{PO}_3\text{H}_2)(\text{PO}_3\text{H})$: Using formamide in place of dimethylformamide as solvent, AMDP was synthesized in a similar manner to MAMDP. One difference was that after the water had been added to the reaction mixture, the solution was refluxed for 5 h. After completion of the reaction, a colorless precipitate appeared. The solvent was then partially removed on a rotary evaporator, and the residue triturated completely with the addition of 95% ethanol. Filtration and recrystallization of this solid material in dilute hydrochloric acid yielded the product as colorless microcrystals. Yield 60%. Anal. Calcd. for $\text{CH}_7\text{NO}_6\text{P}_2$: C, 6.32; H, 3.72%. Found: C, 6.98; H, 4.02%. ^1H NMR: δ 3.60 t ($^2J(\text{PH}) = 18$ Hz). $^{31}\text{P}\{^1\text{H}\}$ NMR: δ 9.49s. mp 290° (dec).

Potentiometric Methods. Potentiometric titrations were carried out in an airtight 150 mL vessel fitted with a nitrogen inlet/outlet, a Ross combination electrode, an automatic temperature compensating probe, and a magnetic stirrer.⁶ Readings were taken using an Orion 720A digital pH/ISE meter, and acid/base aliquots were delivered through a 10 mL buret accurate to 0.01 mL. The system was single-point calibrated by measuring the p[H] of an accurately known strong acid solution. All titrations were carried out at $\mu = 0.10$ M with tetramethylammonium nitrate as supporting electrolyte. The pK_w of this solution was measured as 13.88. All solutions used were degassed prior to titration. Standardized solutions of 0.1 M tetramethylammonium hydroxide and perchloric acid were used as the base and acid, respectively. Metal and ligand solutions were gravimetrically standardized at concentrations of 0.01 M. Both ligands were titrated in the forward direction (acid to base) to determine the pK_a 's. The stability constant determination was achieved by back titrating, first by adding the volume of base necessary to bring the p[H] > 11.5 , then titrating with acid generally until a precipitate formed. Triplicate titrations were carried out at 1:1, 1:2, and 1:3 metal to ligand concentration ratios, with metal concentrations at ca. 1-2 mM and solution volume of 60-70 mL. Data from each titration were analyzed separately using the iterative least-squares fit programs PKAS and BEST, none to a σ fit (stand. dev.) of $> 3 \times 10^{-2}$. Values for metal hydrolysis were obtained from the literature.⁷

Immobilization of 5-(3-bromopropyl)-25, 26, 27, 28-tetrahydroxy calix[4]arene on Polyethyleneimine. A solution of 5-(3-bromopropyl)-25, 26, 27, 28-tetrahydroxy calix[4]arene (1.354 g, 2.48 mmol) in tetrahydrofuran (20 mL) was added dropwise to a stirred solution containing polyethyleneimine (3.716 g of a 50% solution in water) in water (10 mL). After the first few drops were added the solution turned cloudy, but further addition of the calixarene solution resulted in the formation of a clear brown solution. This solution was stirred for 5h at room temperature. The reaction mixture was then shaken two times with 500 mL aliquots of a mixed acetone : petroleum ether (1 : 4) solution. The aqueous tetrahydrofuran layer was heated at 80°C under reduced pressure to yield a constant weight residue. This brown solid was collected. Yield 3.062 g (95%). Anal. Found: C, 61.2; H, 9.29; Br, 6.40; N, 19.9%. IR (KBr pellet) : 2333 cm^{-1} and 2351 cm^{-1} $\nu(\text{R}_3\text{N}^+-\text{H})$. The TGA gives $T_{\text{glass}} = 75.5^\circ\text{C}$ (after extrapolation) and $T_{\text{decomp}} = 231.3^\circ\text{C}$ with the loss of 3.5% water until 93.8°C , and an additional 3.6% until 231.3°C .

Immobilization of 5, 11, 17, 23-tetrachloromethyl-25, 26, 27, 28-tetrahydroxycalix[4]arene on Polyethyleneimine. A solution of 5, 11, 17, 23, -tetrachloromethyl-25, 26, 27, 28-tetrahydroxycalix[4]arene (1.37 g, 2.22 mmol) in tetrahydrofuran (60 mL) was added dropwise to a stirred solution polyethyleneimine (9.84 g of a 50% solution in water) in water (100 mL). A precipitate was immediately formed. The mixture was stirred for 2h at ambient temperature and then poured into acetone (500 mL). The precipitate was filtered and then washed with acetone (2 x

50 mL). The yellow solid polymer was dried to constant weight at 60° C under reduced pressure. Yield 4.77 g (76%). Anal. Found: C, 56.7; H, 9.34; Cl, 3.8; N, 22.5%. The calixarene impregnated polymer was insoluble in water and common organic solvents.

Results and Discussion

Protonation Constants

Protonation constants for the diphosphonic acids MAMDP and AMDP were determined quantitatively under the same experimental conditions that were used for the stability constant determinations.⁸ The potentiometric curves obtained from these data were fitted using four protonation parameters. The protonation constants obtained from the titration of each of these diphosphonic acids with base are shown in Table 1. In each case the highest valued constant (log K₁) was assumed to involve protonation at the amine site. This first protonation is followed by protonations at three of the phosphonate sites (log K₂, K₃ and K₄). The unusually high values for log K₁ reflect the electrostatic stabilizing effect of protonation on the small tetraanion, and, in the case of MAMDP, the significant electron donating properties of the methyl groups. The low values found for log K₄ show the high potential of these compounds to act as bidentate ligands at very high acid concentrations. Indeed, studies on bis-phosphonate metal complexes have shown that this class of ligand display far more affinity for polyvalent metal ions at high acid concentrations than do most carboxylate analog ligands.⁹⁻¹¹

Table I **Logarithm of the Protonation Constants for the Ligands AMDP and MAMDP^a**

		MAMDP	AMDPA
log K ₁	L ⁴⁻ + H ⁺ → HL ³⁻	13.4(0.1)	11.72(0.04)
log K ₂	HL ³⁻ + H ⁺ → H ₂ L ²⁻	9.18(0.02)	8.42(0.02)
log K ₃	H ₂ L ²⁻ + H ⁺ → H ₃ L ⁻	5.01(0.02)	5.42(0.02)
log K ₄	H ₃ L ⁻ + H ⁺ → H ₄ L	1.3(0.1)	1.4(0.1)

a. Numbers in parentheses are mean standard deviations.

Stability Constants

Uranyl complexes with MAMDP and AMDP both share a characteristic insolubility at low solution pH. The titrations used to obtain the stability constant values have therefore been carried out in the reverse (base to acid) direction than those that were carried out to obtain the protonation constants. Dissolution to give a homogeneous solution is achieved by adding base to an aqueous solution of the diphosphonate and the UO₂²⁺ ion until a p[H] value of greater than 10 is obtained. In all cases bright yellow clear solutions are formed. The complexes remain soluble in aqueous solution, depending on the M:L ratio, down to a solution pH of 2.5 to 5. The AMDP/uranyl combination shows a somewhat lower solubility in aqueous media than does the MAMDP/uranyl combination.

Acceptable fitting¹² of a chemical model to the titration data requires the presence of both 1:1 and 1:2 (M:L) complexes in solution, with protonations occurring successively up to MLH₂ and ML₂H₄²⁻ species. Also required is the inclusion of both the mono- and the bis-hydroxylated derivatives of the mono-ligated complexes, ML(OH)³⁻ and ML(OH)₂⁴⁻. These species are present in solution at high p[H]. The stability constant data are obtained from titrations performed at M:L concentration ratios of both 1:1 and 1:2, which provided well-fit refinements based on this chemical model. Titrations of solutions containing a M:L ratio of 1:3 were also carried out, but attempts to include more complicated (ML₃) species and their respective protonated forms in the refinements lead to ambiguous results. The inclusion of polynuclear complexes in the model failed as well to give lower standard deviations. Uncomplexed uranyl ion and hydroxy uranyl species are estimated from the data fitting to be present at concentrations of less than 1 ppm throughout the

p[H] range of the titration. For $ML_2H_n^{(n-6)}$ complexes the overall results do not preclude the existence of protonated species beyond $n = 4$, the presence of which cannot be detected given the solubility constraints at the lower end of the pH range. The continued addition of strong acid to the solution after precipitation occurs does not noticeably dissolve any of the precipitate. The results of the iterative stability constant calculations for solutions containing the UO_2^{2+} ion and either MAMDP or AMDP are given in Table 2.

Table 2 Logarithm of the Protonation and Stability Constants for the Complexation of AMDP and MAMDP with UO_2^{2+} .^a

		AMDP	MAMDP
$\frac{[ML^{2+}]}{[M^{2+}][L^{4-}]}$	$\log K_{101}^b$	25.9(0.4)	24.8(0.0)
$\frac{[M(OH)L^3][H^+]}{[ML^{2+}]}$	$\log K_{1,11}^c$	-10.4(0.2)	-10.7(0.1)
$\frac{[M(OH)_2L^4][H^+]}{[M(OH)L^3]}$	$\log K_{1,21}$	-11.3(0.2)	-12.0(0.3)
$\frac{[MHL^-]}{[ML^{2+}][H^+]}$	$\log K_{111}$	6.7(1.2)	9.8(0.1)
$\frac{[MH_2L]}{[MHL^-][H^+]}$	$\log K_{121}$	6.0(1.0)	5.3(1.1)
$\frac{[ML_2^{6-}]}{[M^{2+}][L^{4-}]^2}$	$\log K_{102}$	30.5(0.3)	32.4(0.5)
$\frac{[MHL_2^{5-}]}{[ML_2^{6-}][H^+]}$	$\log K_{112}$	11.0(0.3)	21.8(0.5) ^d
$\frac{[MH_2L_2^{4-}]}{[MHL_2^{5-}][H^+]}$	$\log K_{122}$	10.3(0.2)	
$\frac{[MH_3L_2^{3-}]}{[MH_2L_2^{4-}][H^+]}$	$\log K_{132}$	6.6(0.1)	7.4(0.1)
$\frac{[MH_4L_2^{2-}]}{[MH_3L_2^{3-}][H^+]}$	$\log K_{142}$	5.0(0.4)	5.0(0.1)

^a The numbers in parenthesis indicate standard errors

^b The designation uses MHL as 111

^c The designation uses M(OH)L as 1-11

^d The composite value of $\log K_{112} + \log K_{122}$

The 1:1 (M:L) species distribution plots (figures 2 and 3) show that in the p[H] range of 5.5-8 the major solution species is MHL^- , with ML^{2-} and $M(OH)L^{3-}$ among others being formed at higher p[H] values. In the low p[H] range a precipitate forms. From the species distribution curves, the predominant complex present under these experimental conditions is MH_2L , although the precipitate has not been positively identified as having this stoichiometry. The major feature of the 1:2 plots is the predominance of the complex $MH_2L_2^{4-}$ over a wide p[H] range.

The results show exceptionally high values for 1:1 stability constants, with $\log K_{101}$ being 25.9 and 24.8 for complexation with AMDP and MAMDP respectively. This high stability is in agreement with other studies of phosphonate complexes of the uranyl ion, where it has been shown that these complexes are unusually stable in comparison to isostructural carboxylate complexes.¹¹ This high stability has been attributed to differences in the electrostatic net charge between carboxylate and phosphonate type ligands,¹⁰ as well as to a small entropic effect of phosphonate ligands having more potentially binding oxygen atoms than do carboxylates. In addition, bis-phosphonate ligands with a planar configuration possess more favorable coordination properties toward the restrictive geometry of the uranyl cation than do "wrap around" type ligands such as EDTA.

The values of our stability and successive protonation constants for AMDP and MAMDP with the uranyl ion are very consistent. Differences between the ligands are reflected in slightly higher stability constants for the combination of MAMDP and UO_2^{2+} , and, except for $\log K_{142}$, slightly more acidic ancillary protonation sites for the AMDP and UO_2^{2+} . These numbers appear to reflect macroscopically the trends of the uncomplexed ligand acid dissociation constants, yet they cannot unambiguously be assigned to the amine or to any particular phosphonate oxygen site. It is difficult therefore to ascertain the degree of hydrogen bonding between the protonated amine and the "yl" oxygens on UO_2^{2+} , although it is reasonable to assume that such an interaction would bestow added stability to these complexes at solutions of moderate to low p[H]. At higher p[H], deprotonation of the amino moiety should lessen this effect considerably for AMDP and, lacking any further potentially hydrogen-bonding hydrogens, eliminate it entirely in the case of MAMDP. Because the stability constant for AMDP is only slightly greater for the 1:1 complexes than those for MAMDP, a strong hydrogen bond interaction to the axial oxygen atoms of the uranyl ion is likely non-existent for both series of complexes.

Recently the stability constants for the complexation of UO_2^{2+} with the diphosphonates, methanediphosphonic acid (MDPA), 1-hydroxyethane-1,1-diphosphonic acid (HEDPA) and ethane-1,1-diphosphonic acid (EDPA) have been measured. The structures of these ligands are shown in their doubly deprotonated forms (H_2L^{2-}) in Figure 2. The respective $\log Q_{101}$ and $\log$

Figure 2

Q_{102} values for H_2L^{2-} as the ligand are 5.34 and 8.31 for EDPA.¹⁰ The log Q_{102} values for H_2L^{2-} are 11.67 and 11.76 for MDPA and HEDPA respectively. By comparison these respective log Q_{101} and log Q_{102} values, where Q is an equilibrium expression of the type $[MH_2L]/[M^{2+}][H_2L^{2-}]$,¹³ are 18.5 and 23.1 for AMDP, and 17.4 and 21.6 for MAMDP. These values reflect the higher basicity of these two ligands. Thus AMDP and MAMDP have significantly higher stability constants (Table 3). MDPA, EDPA and HEDPA differ from AMDP and MAMDP in that they do not have an appended amino group. The presence of this group leads to the formation of

Table 3 Comparative Stability Data for Binding of Phosphonate Ligands to UO_2^{2+}

Ligand	log Q_{101}	log Q_{102}
AMDP	18.5	23.1
MAMDP	17.4	21.6
EDPA	5.34	8.31
MDPA	----	11.67
HEDPA	----	11.76

zwitterions, which results in the H_2L^{2-} forms of AMDP and MAMDP being phosphonate trianions rather than dianions.

One approach to comparing ligating abilities relates stability constants to ligand total basicities. A linear regression fit based upon stability constants of a variety of carboxylate ligands proposed by Nash¹⁰ relates log K_{101} to the total free energy of ligand protonation represented by the sum of the ligand protonation constants, given by $\log K = 0.22 (\pm 0.27) + 0.68 (\pm 0.03)^\circ \Sigma pK_a$. Application of this formula to MAMDP and AMDP gives calculated log K_{101} values of 19.8 (± 1.1) and 18.6 (± 1.1) for MAMDP and AMDP respectively. The difference of $10^{3.9-8.4}$ between the regression line and the experimental data represents substantially enhanced stability as compared to carboxylate ligands. Several other geminal bis phosphonates likewise possess increased stability, showing differences in the range of $10^{1-4.3}$, somewhat lower than the differences for the structurally similar MAMDP and AMDP ligands. This correlation suggests that some added stability is conferred through the presence of the amino groups. This added stability may be the result of delocalization of the lone electron pair of the aminomethylenediphosphonate into the complex, or it may be due to more subtle factors.

Our data show that the compounds MAMDP and AMDP have extremely high binding constants for the UO_2^{2+} ion, and that the complexes remain in solution over a wide pH range. Under highly acidic conditions, however, the combination of a high stability constant coupled with a low solubility of the uranyl complexes makes these ligands good candidates for use as uranyl ion precipitating agents.

Preparation of Insoluble Cross-Linked Polymers

Polyethyleneimine is a useful polymer for functionalization studies because it can be readily obtained in water soluble form, and it also has evenly spaced amine functional groups along the chain that can be used to introduce chemical modifications. Our synthetic approach to functionalizing the polymer is to introduce a haloalkyl substituent onto the calixarene, and to then use this group as an alkylating agent for the amine groups that are spaced along the polyethyleneimine chain. Such a route is shown schematically in figure 3. By changing the relative stoichiometric ratio of the calixarene and the polyethyleneimine that is present in the solution, it is possible to quantitatively vary the loading of the calixarene that is bound onto the polymer chain up to the saturation limit of the polymer where all the amine groups are alkylated. For the preparation of insoluble cross-linked calixarene impregnated polymers it is possible to use

Figure 3

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 $\log Q_{101} = \log \beta_{121} - \log \beta_{021}$ and $\log Q_{102} = \log \beta_{142} - 2 \log \beta_{021}$
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**Hazardous Materials in Aquatic Environments
of the
Mississippi River Basin Project**

**List of Funded Projects
and
Participating Investigators**

Tulane/ Xavier Universities

P.I.	Title	Co-Investigators	Department	Type of Project
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Abdelghani, A.A. Environmental Health Sciences (TU)	Biological Fate, Transport, and Ecotoxicity of Toxic and Hazardous Waste in the Mississippi River Basin	Tulane: Bart, Henry Thien, Leonard Ellgaard, Erik Sherry, Thomas DeVall, Margaret Denslow, Julie Galina, F Akers, Thomas Anderson, Anne Englande, A Reimers, Robert Hartley, William Mizell, Merle Ide, Charles Tompkins, Robert Horner, W.E. <hr/> Xavier: Pramer, Yashoda Phadtare, Shashi Martinet, Peter Obih, Patience Hill, Craig Mandal, Tarun Howard, Brian Huang, Tien Thiagarajah, A	EEO Biology C&M Biology C&M Biology EEO Biology USDA EEO Biology EEO Biology Envir Health Sci Envir Health Sci Envir Health Sci Envir Health Sci Envir Health Sci C&M Biology C&M Biology C&M Biology Immunology <hr/> Pharmacy Pharmacy Biology Pharmacy Pharmacy Pharmacy Pharmacy Pharmacy Pharmacy	Collaborative Cluster
Anderson, Mary Anatomy (TU)	Assessment of Mechanisms of Metal -Induced Reproductive Toxicity in Aquatic Species as a Biomarker of Exposure	Tulane: George, William Agrawal, Krishna Preslan, Janet Rege, Arvind Sikka, Suresh <hr/> Xavier: Lopez-Anaya, A	Pharmacology Pharmacology Pharmacology Pharmacology Urology <hr/> Pharmacy	Collaborative Cluster
Apbett, Allen Chemistry (TU)	Heavy Metal Immobilization in Mineral Phases	None		Initiation
Barber, John T. * EEO Biology (TU) * has been integrated into Abdelghani project	A Multifaceted Study of Heavy Metal Pollution of Wetland Ecosystems	Tulane: Fink, Mark Ensley, H.E. Apblett, Allen Fingerman, Milton Thomas, Charles	Chemistry Chemistry Chemistry EEO Biology USDA	See Abdelghani Cluster

P.I. Title Co-Investigators Department Type of Project

Bhattacharya, Sanjoy Civil Engineering (TU)	Bioremediation of Selected Contaminants in Aquatic Environments of the Mississippi River Basin	Tulane: Bennett, Joan Englande, A. J. Law, Victor Mullin, David Xavier: Mielke, Howard Buckalew, David Echert, John Fulginiti, Robert Kamath, Burde Ross, Joseph	C&M Biology Env Health Sci Chemical Engr C&M Biology Pharmacy Biology Biology Biology Pharmacy Biology	Collaborative Cluster
Bundy, Kirk Biomedical Engineering (TU)	A Pilot Study of the Applicability of Polarography to Exposure and Biomediation Problems in Aquatic Systems	None		Initiation
Duplantier, S. Communications (XU)	An Interactive Hypermedia Model of Risk Communication About Hazardous Waste Remediation for Scientists, Administrators, and Students	Tulane: Kittie Watson Xavier: Calef, Scott	Communications Philosophy	Initiation
Fauci, Lisa Mathematics (TU)	Pore-level Flow, Transport, Agglomeration and Reaction Kinetics of Microorganisms	Tulane: Gaver, Donald Moore, Peter Papadopoulos, K. Xavier: Sharma, Bhu Dev	Biomed Engr Mathematics Chemical Engr Mathematics	Collaborative Cluster
George, William Pharmacology/ Toxicology (TU)	Bioenvironmental Analytical Support Services	None		Initiation

P.I.	Title	Co-Investigators	Department	Type of Project
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Hartley, William Environmental Health Sciences (TU)	Evaluation of the Carcinogenic, Reproductive and Developmental Effects of Mixtures of Contaminants on the Medaka Fish	None		Initiation
John, Vijay Chemical Engineering (TU)	The Removal of Phenolics and Aromatic Amines from Aqueous Streams Through Enzymatic Polymerization in the Presence of Surfactants	None		Initiation
Kendall, Kevin Cell and Molecular Biology (TU)	Genetically Engineered Microorganisms: Aromatic Hydrocarbon Biodegradation Genes from Rhodococcus	None		Initiation
Kopplitz, Brent Chemistry (TU)	Laser Ablation/Ioniza- tion Studies Related to the Removal of Nuclear Materials from Metal Surfaces	None		Initiation
Loughlin, Monica Dean, College of Arts and Sciences (XU)	Enhancement of Environmental Education at Tulane and Xavier Universities	Tulane: Bhattacharya, S Hassell, J Bennett, Joan McDowell Zimmerman White, L Xavier: O'Connor, Sally	Civil & Env Engr Computer Sci C&M Biology Geology Philosophy Env Health Sci Chemistry	Collaborative Cluster

P.I.	Title	Co-Investigators	Department	Type of Project
McPherson, Gary Chemistry (TU)	Natural and Active Chemical Remediation of Heavy Metals in Aquatic Environments	Tulane: Pintauro, Peter Gonzales, Richard Flowers, George Xavier: Zhang, Jian O'Connor, Sally	Chemical Engr Chemical Engr Geology Chemistry Chemistry	Collaborative Cluster
Pintauro, Peter Chemical Engineering (TU)	Asymemetric PVDF Pervaporation Membranes for the Removal fo Organic Contaminants from Waste Water	None		Initiation
Ramer, Samuel History (TU)	Initiation of Research Collaboration Between the Tulane/Xavier CBR and the Institute of Radioecological Problems in Minsk	Tulane: Bennett, Joan	C&M Biology	Initiation
Regens, James Center for Bioenviron-mental Re-search (TU)	Expert Geographical Information Systems for Assessing Hazardous Wastes in Aquatic Environments	Tulane: White, LuAnn Wright, James Hughes, Janet Rene, Antonio Bakeer, Reda Belkhouche, B Barber, Michael Xavier: Mielke, Howard	Env Health Sci Sociology Biostats & Epidem Env Health Sci Civil & Env Engr Computer Sci Civil & Env Engr Pharmacy	Collaborative Cluster
Roberts, J. Timmons Sociology (TU)	Risk, Stress, and Restructuring in the U.S. Petrochemical Industry: A Case Study from Louisiana	None		Initiation
Roundhill, David Chemistry (TU)	Synthesis of New Resins for the Absorption of Actinide Ions	None		Initiation

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