BIOMARKER ASSESSMENT OF THE EFFECTS OF STRIP-MINE AND PETROLEUM REFINERY CONTAMINATION ON CHANNEL CATFISH

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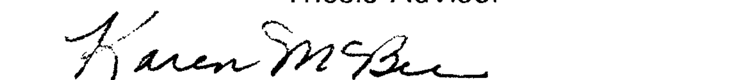

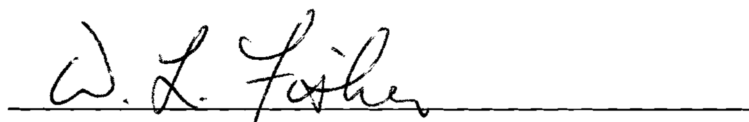

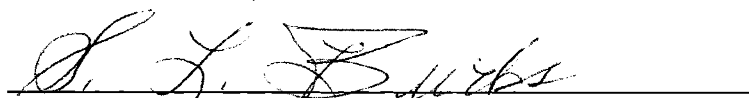

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CHAPTER I

Biomarkers: A Literature Review

Introduction

Since the establishment of the Environmental Protection Agency (EPA) in 1970, increasing significance has been placed on developing methods for establishing and measuring ecological risks due to exposure to toxic wastes (Bascietto et al., 1990). Specifically, an ecological risk assessment is the process of assigning levels and probabilities of occurrence of the adverse effects of human activities or natural catastrophes on the environment (Johnson, 1988; Suter, 1993). Two types of ecological risk assessments (ERA's), predictive and retrospective, are commonly used. Predictive assessments estimate nature, probability, and magnitude of adverse effects that currently may not be apparent, but could prove problematic in the future. Retrospective assessments differ from predictive assessments in that the environment is already affected and components such as sources, indicators of exposure, and indicators of effects can be observed and measured (Suter, 1990; Travis and Morris, 1992).

In order to conduct an ecological risk assessment, the presence, fate,

and toxicity of a chemical must be characterized (Stevens, 1989). Chemical analyses are conducted on matrices such as air, soil, and water to establish the presence/absence, concentration, and spatial variability of a contaminant (Shugart, 1990; Sprague, 1990). Biological analyses, primarily body burden tests, are conducted on exposed organisms to measure which contaminants are actually bioaccumulated by the organisms. Finally, toxicity tests are performed on organisms to determine the cause:effect or concentration:response relationships of contaminants. Toxicity tests can also be used to monitor the toxicity of effluent discharges and to evaluate the quality of surface waters (Sprague, 1990). From these data, recommendations can be made concerning the maximum concentrations of contaminants that do not adversely affect the general health of exposed organisms.

Unfortunately, predictions of the extent of exposure and the ecological effects that result from exposure to toxic wastes may be difficult to determine from chemical and biological analyses. Chemical analyses of soil and water samples do not determine whether a contaminant is biologically available. The contaminant may be bound to particulate or dissolved organic carbon in sediments or overlying waters, and thus be present in a non-bioavailable form. These analyses provide only a representative "snap shot" of a specific point in time. As such, they often do not adequately represent the chemical heterogeneity of an area, which

may result from environmental influences of storms or wind actions, or human interactions such as applications of a chemical or an effluent release over spatial and temporal gradients. In addition, chemical analyses are often difficult to relate to biological uptake or toxicity. The quantification of exposure is often cumbersome because many toxic chemicals are biotransformed within host tissues and eliminated rather than bioaccumulated. To further complicate matters, organisms can be exposed to toxicants through a wide variety of routes, including air, water, soil, and the food chain (McCarthy and Shugart, 1990). Toxicity tests usually are performed in the lab under controlled conditions and may not reflect true-life variabilities such as seasonal and diurnal temperature fluctuations, sediment loads, geographical variation in contaminant concentrations, and accumulation via the food chain (Adams et al., 1989; Sprague, 1990). Furthermore, toxicity tests usually measure endpoints such as lethality or reproductive failure and are limited by their inability to detect subtle changes or early indications of contaminant stress.

A current trend in ecotoxicology is to use responses of organisms as an indicator of environmental contamination (Theodorakis et al, 1992). This approach evaluates exposure and effects of environmental contaminants using biological markers or biomarkers. Biomarkers are defined as measurements of body fluids, cells, or tissues that indicate the presence of a xenobiotic or the magnitude at which the xenobiotic elicits a response in the

host (McCarthy and Shugart, 1990). Essentially, biomarkers are tools that clarify the relationship, if any, between exposure to a xenobiotic compound and impairment of the target organism's health (Suter, 1990).

Biomarkers are classified into two general types: those that measure exposure and those that measure organismal effects of the exposure (National Research Council, 1987). Exposure biomarkers are either used to identify foreign substances within the system, biological interactions between a xenobiotic and endogenous compounds, or other events in the biological system related to exposure rather than toxicity (e.g., inhibition of delta-aminolevulinic acid dehydratase indicating exposure to lead--Burch and Siegel, 1971; increased glutathione S-transferase activity--Kirby et al., 1990; and induction of mixed function oxidase enzymes--Payne et al., 1987). Biomarkers that measure effects provide evidence of damage to cells, organs, and systems (e.g., micro-nuclei formation--Metcalfe, 1988; Al-Sabti and Hardig, 1990; DNA strand breakage--Shugart, 1990; regenerated foci--Kent et al., 1988; steatosis--Deboyser et al., 1989; and carboxyhemoglobin formation following exposure to carbon monoxide--Smith, 1991).

The biomarker approach has several advantages over use of traditional chemical methods. Such an approach allows toxicological data to be monitored from the target organism, providing quick and accessible information that is not readily available with other methods. Biomarkers provide evidence that organisms have been exposed to toxic levels of

contaminants through physiological responses and indicate whether levels of contaminants have exceeded normal detoxification or repair capacities of exposed organisms. They provide evidence of exposure to compounds that do not bioaccumulate or are rapidly metabolized and eliminated from the organism's body. Biomarkers integrate an organism's response to varying concentrations of contaminants as bioavailability of the contaminant changes over temporal and spatial gradients. Biomarkers provide a biological measurement that is relevant to evaluation of ecological risks. Ultimately, a suite of biomarkers can provide evidence to statistically test hypotheses about linkages between exposure to toxic chemicals and ecologically relevant effects in target organisms.

Because most studies have focused on only one, or at most, a few biological parameters, a need exists for studies which examine multiple biological parameters in response to toxicant stress (Benson et al., 1990; Theodorakis et al., 1992). These studies would be beneficial in determining which organ systems or tissues are the most sensitive indicators of toxicity for certain chemical classes (Adams et al., 1989). Therefore, by using a suite of biomarkers, hypotheses concerning exposure to toxic chemicals, mechanisms of toxicity, target organs for toxic effects, and ecological effects can be tested.

Because many biomarker assays respond quickly to contaminant exposure, they can be a chief component of an environmental monitoring

program. In such a program, biomarker responses from an organism at a suspected contaminated site are compared to organism responses from a verified non-contaminated or reference site (McCarthy and Shugart, 1990). Reference organisms provide a baseline for comparison that includes biochemical changes resulting from the organisms' normal activities. Thus, a monitoring program utilizing biomarkers could aid in the early detection of environmental deterioration and help document recovery of affected ecosystems (McCarthy and Shugart, 1990).

By using an appropriate suite of biomarkers the health of exposed organisms can be assessed providing information for ecological risk assessments, and/or progress of site remediation. Biomarker assays are chosen based upon the mode of contamination or because of the specific contaminants known to be present at the site. For example, my research was conducted at two contaminated sites, a petroleum refinery and a coal strip-mine. Preliminary site analysis determined that both locations were contaminated with heavy metals, (cadmium, lead, zinc, and copper) and acidic water (pH 5 to 6) (Martin and Black, unpublished data). Thus, the suite of biomarkers were chosen to include a range of physiological responses to the stressors found at the study site.

Biomarker responses measured include indicators of metabolic, hematological, osmoregulatory, genotoxic, and organismal function and integrity. Hematocrit is the percentage of packed cell volume of

erythrocytes in blood. Decreased hematocrit has been observed following acute to semi-chronic exposures to cadmium (Koyma and Ozaki, 1984) and herbicides (Adamek et al., 1984) in carp (*Cyprinus carpio*), petroleum refinery effluent in Pacific staghorn sculpin (*Leptocottus armattus*) (Boese et al., 1982), and aflatoxin in channel catfish (*Ictalurus punctatus*) (Jantrarotai and Lovell, 1990). An increase in hematocrit resulted from acute to semi-chronic exposures to etomide, an anesthetic (Limsuwan et al., 1983), malachite green, used to treat fish fungus and protozoans (Grizzle, 1977), and high stocking densities (Klinger et al., 1983) in channel catfish, acidic water (pH $<$ 4.9) in rainbow trout (*Salmo gairdneri*) (Giles et al., 1984), and low oxygen levels in Amazon tambaqui (*Colossoma macropomum*) (Saint-Paul, 1984).

Measurement of hemoglobin is one of the simplest estimates used as an indicator of anemia, which frequently occurs as a result of infections, diseases (Larsen, 1964; Blaxhall, 1972), and following exposure to many environmental contaminants (Landrigan, 1982; Jagels, 1985). Decreased hemoglobin levels have resulted from acute to semi-chronic exposures to herbicides in carp (Adamek et al., 1984), malathion in Indian catfish (*Heteropneustes fossilis*) (Mishra and Srivastava, 1983), cadmium in carp (Koyma and Ozaki, 1984) and tilapia (*Sarotherodon mossambicus*) (Ruparelia et al., 1987), and chronic cadmium exposures in mice (Watanabe et al., 1986). Increased hemoglobin levels have resulted from acute exposures to

mercury in Mozambique tilapia (*Tilapia mossambica*) (Menezes and Qasim, 1984), nickel in Nile tilapia (*Tilapia nilotica*) (Ghazaly, 1992), acidic water in rainbow trout (Giles et al., 1984), and low oxygen concentrations in Amazon tambaqui (Saint-Paul, 1984).

Delta aminolevulinic acid dehydratase (ALAD) activity in whole blood has been accepted as an indirect measurement of exposure to lead (Mitchell et al., 1977). Aminolevulinic acid is a cystolic enzyme found in many tissues which acts in the synthesis of hemoglobin (Hammond and Belile, 1990). ALAD activity is advantageous over other indirect lead measurements because it is progressively inactivated by lead over a concentration range which corresponds to subclinical lead intoxication (Mitchell et al., 1977). In mallard ducks (*Anas platyrhynchos*) ALAD activity was rapidly inhibited after one month exposure to ingested lead shot (Dieter and Finley, 1978). In addition, ALAD inhibition has been correlated with lead exposure in many fish species (Heath, 1987).

Glucose levels are often indicative of metabolic and non-specific stress (Hunn and Greer, 1991) in which mobilization of glucose from storage sites occur in response to a stressor, causing elevated levels of plasma glucose. If exposure continues, glucose depletion may result. Decreased plasma glucose levels have been associated with acute to semi-chronic exposure to ambient oxygen reductions in red sea bream (*Pagrus major*) (Ishioka, 1982) and exposure to zinc in juvenile rainbow trout (Wagner and

McKeown, 1982). Elevation in glucose levels has resulted from acute to semi-chronic exposure to organo-chemicals such as diazinon in snake-headed murrell (*Ophiocephoelus puctatus*) (Sastry and Sharma, 1981) and malathion in catfish (*Clarias batrachus*) (Shobha-Rani et al., 1991).

Plasma chloride is an essential extracellular anion that counter-balances the cation, sodium, to help maintain neutrality of body fluids and to maintain osmotic regulation (Tietz, 1976). Elevation in plasma chloride concentrations has resulted from acute exposure to phenol in rainbow trout (Swift, 1982). Decreased plasma chloride has been measured in fish following chronic exposure to cadmium in rainbow trout (Giles, 1984), acute exposure to nitrites in channel catfish (Tomasso et al., 1979), DDT in flounder (*Platichthys flesus*) (Haux, 1979), and acidic water in rainbow trout (Giles et al., 1984; Witters, 1986), brook trout (*Salvelinus fontinalis*) (Tandjung, 1982), and white suckers (*Catostomus commersoni*) (Hobe and McMahon, 1982).

DNA strand breakage is a key parameter for assessing the genotoxic properties of environmental pollutants (Kohn, 1983). Many pollutants including organic compounds and heavy metals are chemical carcinogens and mutagens with the capacity to cause various types of DNA damage (Wogan and Gorelick, 1985). If the organism is unable to cope with DNA breakage and maintain DNA integrity through normal repair mechanisms, DNA strand breakage can be detected in several target tissues including liver

and nucleated red blood cells (McCarthy and Shugart, 1990; Theodorakis et al., 1992).

Condition factor (CF) is a useful index because the relation of length to weight may change as fish respond to stress (Barton et al., 1988). Changes in CF are usually interpreted as changes in fat storage or energy reserves (Brown et al., 1987). The CF value may also reflect changes in feeding activity or nutrient availability (MacKinnon, 1972; Tyler and Dunn, 1976). Organ somatic indexes, ratios of organ weight (liver, spleen, and kidney) to body weight, are used to assess individual organ stress levels. Increases in liver somatic index (LSI) have resulted from semi-chronic exposure to oiled sediments in flounder (Fletcher et al., 1982) and semi-chronic exposure in rainbow trout to pulp mill effluent (Oikari and Nakari, 1982). Increased incidence of fatty livers in rodents has been observed following semi-chronic exposure to zinc (Marrs et al., 1988). A reduction in LSI in fish has been correlated with semi-chronic exposure to acidic water in rainbow trout (Lee et al., 1983), heavy metals from a sulphur ore smelter in perch (*Perca flava*) (Larsson et al., 1984), and chronic exposure to petroleum hydrocarbons in Atlantic cod (*Gadus morpha*) (Kiceniuk and Khan, 1987). Increased relative weights of spleens have resulted from semi-chronic exposure to lead (Olgilvie and Martin, 1981), ethanol (Messiha, 1982), malaria (Oduola et al., 1982), and coal coprocessing fractions (Chu et al., 1992) in laboratory mice and rats. Reduction in relative weight of the spleen

has resulted from semi-chronic exposure to ethylene oxide in mice (Snellings et al., 1984).

An autopsy-based assessment described by Goede and Barton (1990) can be used to evaluate overall fish health and condition (Appendix A). Parameters include lesions on extremities which provide signs of external damage; fin erosion or disease; color of the bile in the gall bladder which provides a short term indicator of feeding activity and nutritional status of the fish (Love, 1975); and condition of the eyes, gills, kidney, liver, and spleen which provide general insight on the health of each fish (Goede and Barton 1990).

Objectives and Hypotheses

The previously mentioned suite of biomarkers was evaluated as indicators of heavy metal toxicity in caged channel catfish along a concentration gradient for a duration of up to three months. The objectives of the two studies were:

1) To evaluate the spatial relationships between metal accumulation by fish caged along a concentration gradient discharge and biomarkers measured in fish.

Hypotheses:

H_0: Heavy metal accumulation in caged fish is not inversely related to distance from source.

H_a: Heavy metal accumulation in caged fish is inversely related to distance from source.

2) To evaluate temporal relationships between biomarkers and length of exposure of fish to the contaminant source.

Hypotheses:

H_0: Biomarker responses of caged fish are linearly related to metal accumulation over time.

H_a: Biomarker responses of caged fish are not linearly related to metal accumulation over time.

3) To evaluate the utility of a suite of biomarkers in assessing stress responses of fish to heavy metal exposure resulting from different routes (i.e., water and sediment versus water only) of exposure.

Hypotheses:

H_0: Biomarker responses do not differ in fish exposed to contaminated water and sediments compared to those exposed to contaminated water only.

H_a: Biomarker responses differ in fish exposed to contaminated water and sediments compared to those exposed to contaminated water only.

Conclusions

The following chapters report results from two *in situ* heavy metal exposures. Chapter two describes results from a caging study at an abandoned coal strip-mine site, and chapter three details results obtained from a caging study at an abandoned petroleum refinery. By using a suite of biomarkers along spatial and temporal gradients, these studies will generate basic insight into the significance of exposure time and exposure concentration on the expression of the biomarkers assayed. The lack of such time concentration:response data has been a major criticism of the biomarker approach, especially concerning its potential use in ecological risk assessments.

These studies parallel an extensive research effort at both sites to study the ecological impacts of contaminants on terrestrial organisms by several investigators at Oklahoma State University. In the past, other aquatic projects focused on measurement of water chemistry and contaminant profiles in sediments and water at these sites. My studies, however, attempted to quantify the toxicological impacts of contaminants on physiological processes of aquatic organisms placed *in situ* at the two

sites. Coupled with data collected by other OSU researchers, this information will provide a quantitative measure of the current acute to semi-chronic toxicity of contaminants to aquatic and terrestrial biota at these sites. This information should be of interest to agencies responsible for tracking the remediation progress of these sites, as well as similar sites. According to Suter (1990), "If biomarkers are to become useful in ecological risk assessment, instead of suggestive risks, it must be shown that biomarker response increases at a predictable rate with increasing exposure."

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CHAPTER II

Biomarker Assessment of the Effects of Coal Strip-Mine Contamination on Channel Catfish.

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Abstract

A suite of biomarkers was used to evaluate acute (1 day) to semi-chronic (3 month) heavy metal-induced toxicity in channel catfish, *Ictalurus punctatus*, caged at an abandoned strip-mine and a non-contaminated reference site. Assays performed include indicators of metabolic, hematological, osmoregulatory, and genotoxic stress. Two cage designs were utilized to evaluate the importance of exposure routes: one excluding contact with the sediments and the other allowing contact with water and sediments. Significant DNA strand breakage was observed in catfish exposed to both exposure regimes, but evidence of DNA repair was

observed only in water-exposed catfish. Transient increases in hemoglobin, ALAD, and hematocrit levels were observed at 1 month's exposure for both exposure regimes, followed by a return to control levels for the duration of the study. Environmental conditions (i.e., weather related changes in water quality) may have contributed to the variable plasma chloride and glucose levels observed in all catfish exposed to strip-mine wastes. The transient changes in biomarkers followed by a return to reference values represent an initial stress and an acclimation to normal levels.

Keywords: biomarkers; channel catfish; coal strip-mine; ALAD; hemoglobin

Introduction

The coal mining industry began in Oklahoma around 1870 with underground mining practices. Surface or strip mining practices dominated the industry circa 1943, and are still the most widely used method of coal removal (Johnson et. al., 1982). These two methods of coal removal disturb large tracts of land exposing sequestered heavy metals, mainly lead (Pb), cadmium (Cd), and zinc (Zn) (Down, 1975; Kimber, 1978; and Johnson et. al., 1982). Heavy metals found in association with coal deposits may become available to wildlife and fish via mine tailings, wastewater runoff, or other related mining activities (Slack and Blumer, 1987; Adriano, 1986). Because toxicological research at coal mine sites typically does not focus on the metal toxicity aspects, little is known

concerning the impacts of heavy metal deposits associated with coal mining on terrestrial and aquatic biota (Hausbeck, 1995).

Eastern Oklahoma contains large deposits of medium to high volatile bituminous and subbituminous coal suitable as fuel for the generation of electricity and coke manufacture (Parkhurst, 1994). Even though many of Oklahoma's strip mines were abandoned prior to 1968 and have undergone some degree of reclamation, chemical analyses have determined detectable amounts of Cd, Pb, and Zn in soils at these sites (Hausbeck, 1995; Johnson et al., 1982).

In addition to using chemical analyses to assess environmental impacts, biomarkers are frequently used to assess organismal condition as a result of exposure to environmental contaminants. Biomarkers are measurements of physiological and organismal changes in body fluids, cells, or tissues and serve as indicators of the presence and/or effects of environmental contamination (Theodorakis et. al., 1992). Biomarkers also examine whether normal detoxification or repair capacities have been exceeded. The limiting factor to using biomarkers, however, is the lack of data correlating temporal responses, contaminant concentrations, and the expression of biomarkers.

A caging study was used to test the usefulness of a suite of biomarkers in assessing stress responses in channel catfish for an exposure duration of 1 day to 3 months. The objectives were to measure biomarker

extracellular anion balancing the cation, sodium, to help maintain electrical neutrality of body fluids and to maintain osmotic regulation (Tietz, 1976). DNA strand breakage is a key parameter for assessing genotoxic stress through various types of DNA strand damage caused by both organic compounds and heavy metals (Kohn, 1983; Wogan and Gorelick, 1985). Liver and spleen somatic indices (LSI, SSI), ratios of organ weight to body weight (minus viscera), are used to assess stress to individual organs. Condition factor was used to assess the condition of fish over a wide range of size because the relationship of length to weight may decline as fish respond to stress (Le Cren, 1951; Barton et. al., 1988). In addition, an autopsy-based assessment described by Goede and Barton (1990) provides evaluation of the overall health and condition of the catfish.

Materials and Methods

Study organisms

Juvenile channel catfish, *Ictalurus punctatus*, approximately 4 to 6 inches in length and averaging 20 to 30 g in weight, were obtained from the Experimental Fish Farming Station, Stuttgart, Arkansas. Channel catfish were selected because of their availability, survivability, and semi-benthic nature that provided greater interaction with the sediments.

Study cages

All plastic, cylindrical cages were constructed from two or three 0.5″ PVC hoops wrapped with 0.5″ polyethylene aquaculture mesh secured to the PVC framework using plastic cable ties. Two cage designs were used. The first design permitted channel catfish exposure to both the sediment and the water. The second design was modified to exclude contact with the sediment by raising the floor 18" from the sediment surface allowing only water exposure (Martin and Black, 1995) (Fig. 1). Demand feeders constructed from plastic components were secured to the top of each cage (McGinty and Rakocy, 1989). Feeders were filled with commercially available floating catfish chow and available *ad libitum*.

Study site

The Marler coal strip mine, Okmulgee Co., Oklahoma was abandoned around 1917 and has undergone some degree of reclamation. The site has two ponds. The smaller pond (pond 1) encompasses 1.02 acres and averages 1.5 m in depth; the larger pond (pond 2) encompasses 15.7 acres with an average depth ranging from 6 to 10 m (Fig. 2). The reference site was located at a man-made fisheries research pond (pond 11) with water supplied from Lake Carl Blackwell, Payne Co., Oklahoma. The reference pond averaged 1.5 m in depth, with a surface area of 0.261 acres (Fig. 3).

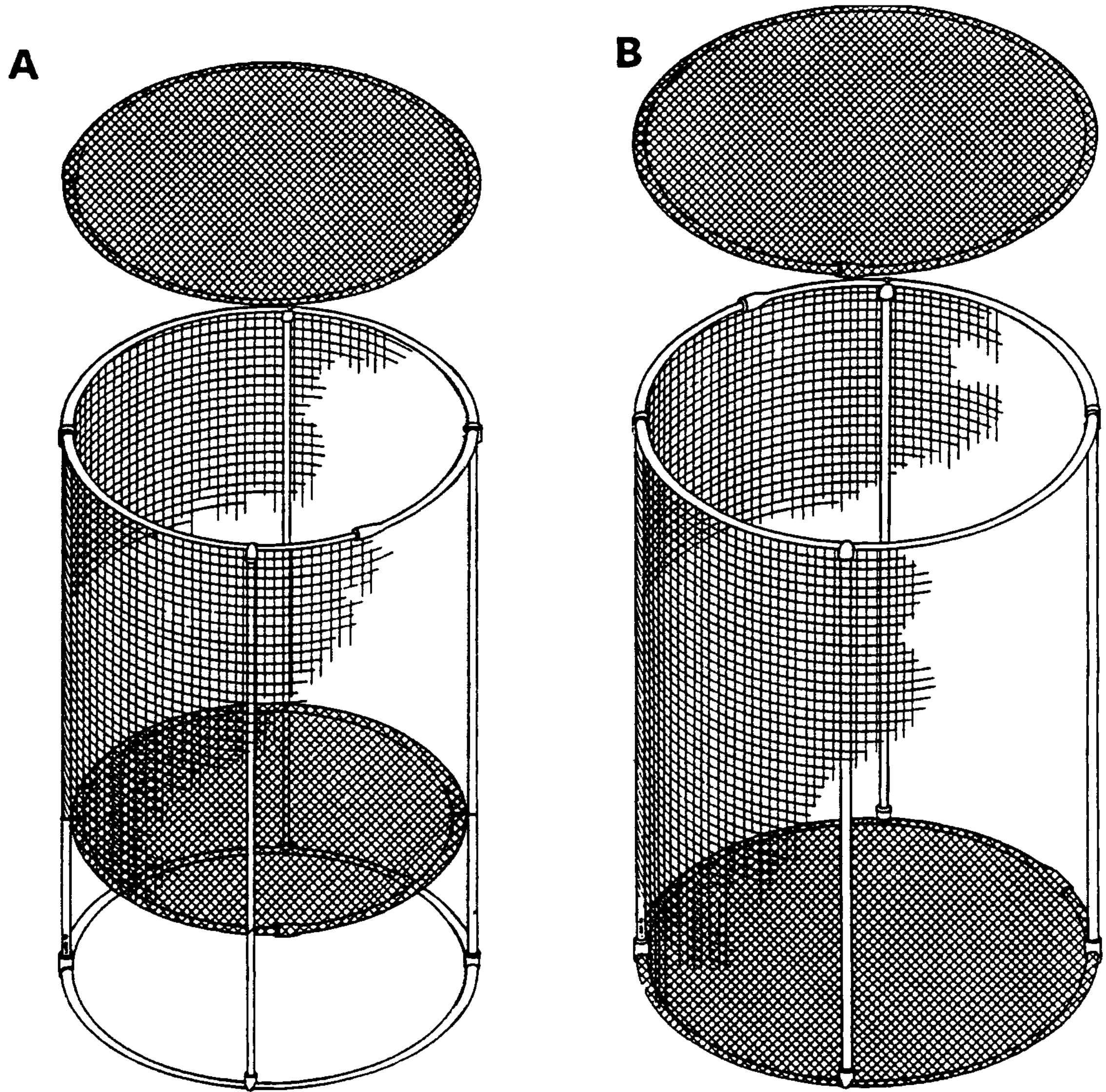

Figure 1. The two cage designs (A) water exposure cage and (B) water and sediment exposure cage.

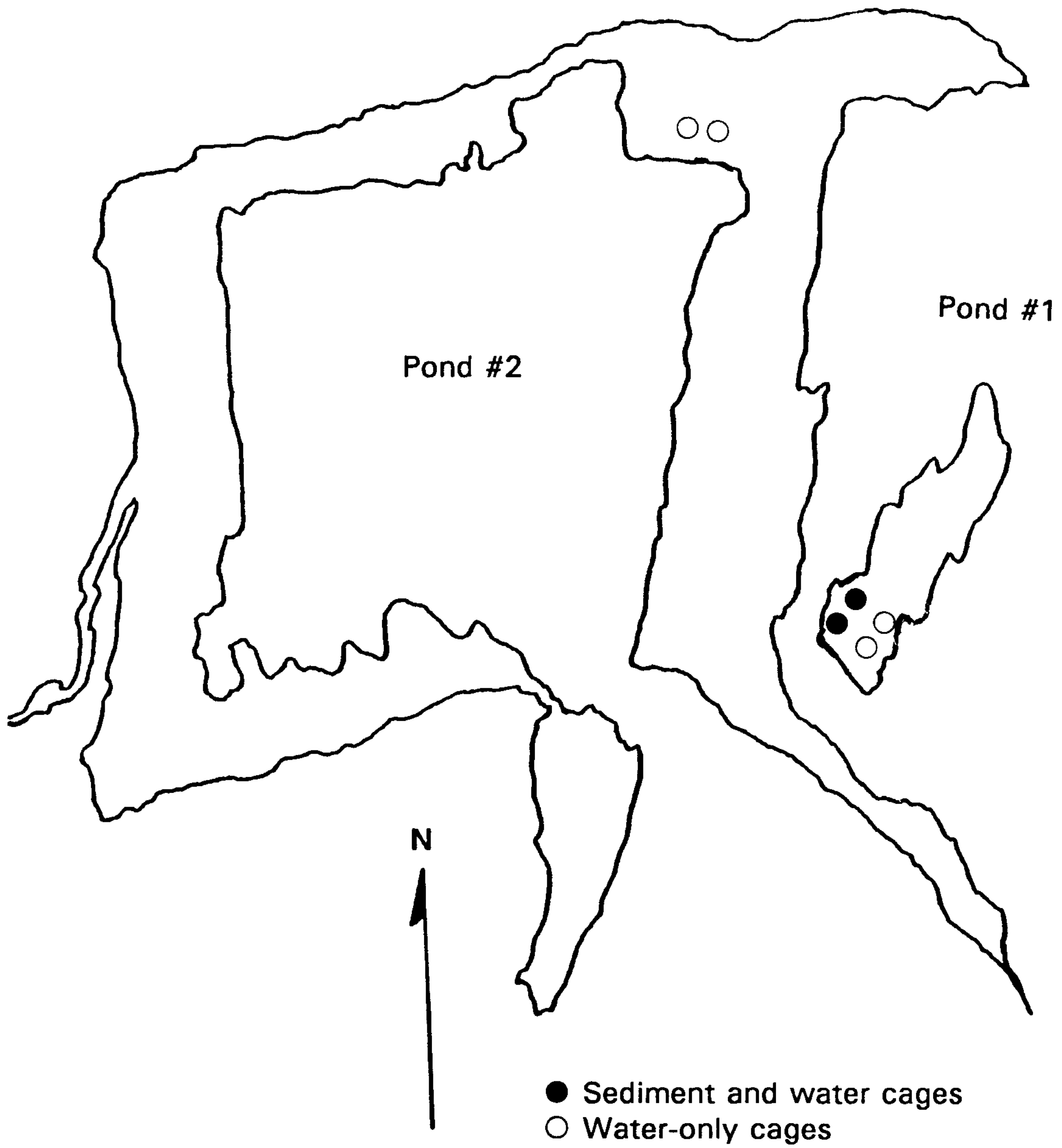

Figure 2. Cage locations and types at the abandoned strip-mine site located at Okmulgee Co., OK.

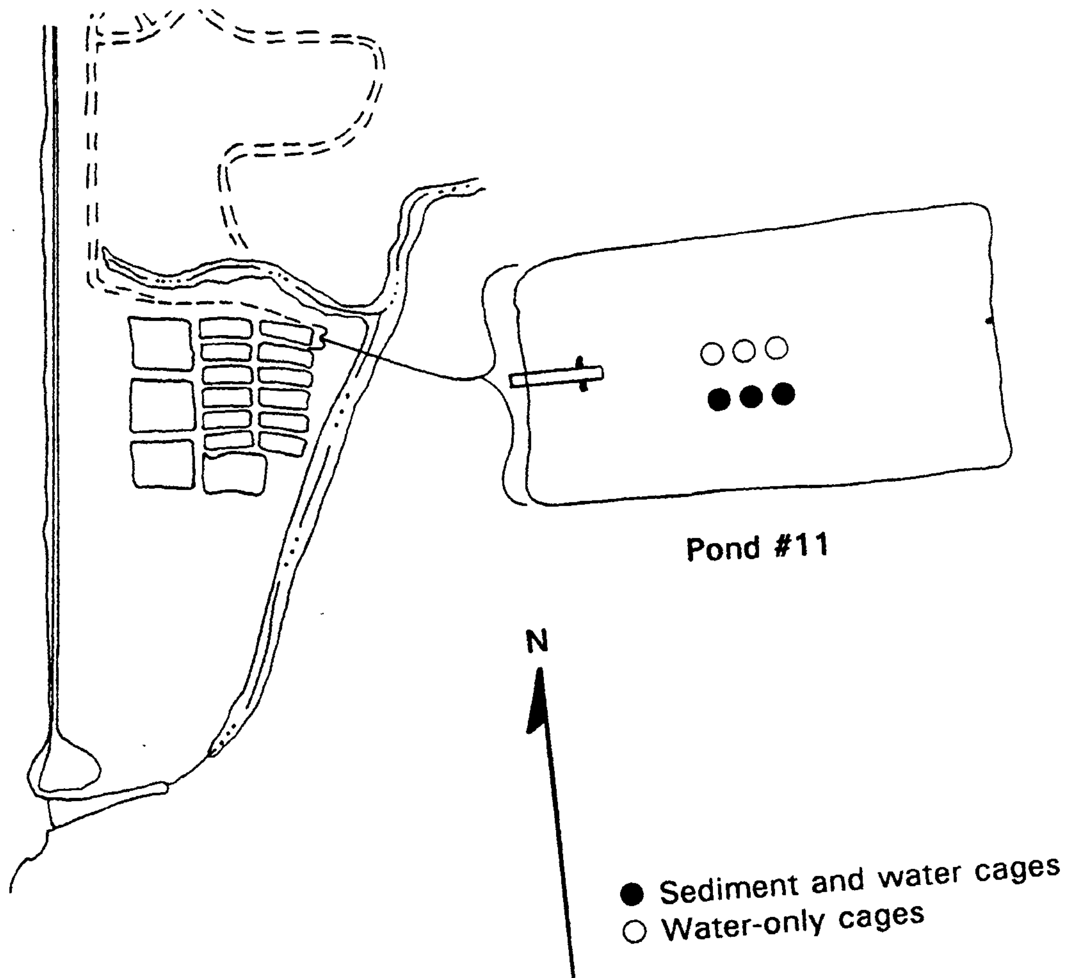

Figure 3. Cage locations and types at the reference site located at Payne Co., OK.

Caging locations

Four cages, two sediment exposure and two water exposure, were anchored using 1.5" PVC pipe in experimental pond 1. Only two water exposure cages rigged with floatation devices were used in pond 2, because the slope of the bank and depth would not permit placement of sediment exposure cages (Fig. 2). Six cages, three sediment and three water exposure cages, were anchored in the sediment at the reference site (Fig. 3).

Water chemistry

Data on water chemistry were measured prior to sampling the caged fish. Water pH, temperature, and dissolved oxygen (DO) were measured using a checkmate modular testing system (Corning, New York). Ammonia was measured using an ammonia test kit (Aquarium Pharmaceutical, Inc., Chalfont, PA). Water hardness was measured using a procedure described by Lind (1985). All measurements were taken approximately 6" below the surface.

Sampling protocol

Fifty channel catfish were placed in each cage at the beginning of the study. Six fish were removed from each cage at 1, 7, 28, 56, and 84 days post exposure. Immediately upon removal from cages, fish were anesthetized in 200 mg/l tricane methane sulphonate (MS-222; Cresent

Research Chemicals Phoenix, AZ). Blood was drawn from each fish into a heparinized plastic syringe using a venous puncture in the anal fin region (Rowley, 1990). Blood samples were divided into aliquots; one to be centrifuged for plasma collection and the other for whole-blood analyses. Blood samples were placed on ice to prevent lysing of red blood cells. To rule out sampling-induced stress, blood samples were taken within five minutes post-netting, a time period determined not to induce any significant impact on glucose levels due to stress from handling and capture (Chavin and Young, 1970). Immediately after bleeding, each fish was fin clipped for later identification, and placed in aerated buckets corresponding to individual cages. Fish and blood samples were transported to the laboratory for necropsy and centrifugation of blood samples. Blood samples were refrigerated and plasma was frozen until assays were performed. All blood and plasma assays were performed within 24 hours of the collection date.

Tissue collection

Upon arrival in the laboratory, fish were anesthetized, weighed, measured, and necropsied. Spleen and liver from each fish were exhumed and weighed. Liver was placed in a sterile eppendorf tube, frozen in liquid nitrogen, and stored at -80°C for the DNA electrophoresis assay. A sample of epaxial muscle was taken from each fish and stored at -20°C.

Metals analyses in tissue samples and sediments

Muscle samples of equal weights (approximately 2 g) were pooled per cage and sampling time, powdered in a cold mortar, and triplicate sub-samples were digested using the EPA Method 3050, Acid Digestion of Sediment, Sludges, and Soils modified for animal tissues (Tull-Singleton et. al., 1994). Sediment samples were digested using the standard EPA Method 3050, Acid Digestion of Sediment, Sludges, and Soils (EPA, 1986). Digested sediment and tissue samples were analyzed for zinc (Zn), lead (Pb), and cadmium (Cd) concentrations with a Model 5000 Perkin-Elmer Atomic Absorption Spectrophotometer using graphite furnace (Pb and Cd) or flame emission (Zn) techniques.

Biomarker assays

Hematocrit - Whole blood samples were collected in heparinized microcapillary tubes and centrifuged for three minutes in a microcapillary centrifuge. The capillary tubes were measured to determine percent packed red blood cell (%RBC) volume.

Hemoglobin - Hemoglobin was analyzed in whole blood using the standard cyanomethemoglobin method, in which ferricyanide is added to convert hemoglobin to form methemoglobin and combined with potassium cyanide to produce cyanmethemoglobin. The absorbance of the

cyanomethemoglobin solution is read on a UV-visible spectrophotometer at 540 nm. (Wintrobe, 1956).

Delta aminolevulinic acid dehydratase (ALAD) activity - ALAD activities in whole blood were assayed using a spectrophotometric assay developed by Burch and Siegel (1971). Erythrocytes in whole blood were lysed, incubated with a trichloroacetic acid reagent containing N-ethylmalimide, and mixed with a modified Erlich's reagent to develop a color reaction. Enzyme activity was determined by reading the absorbance of the solution using a UV-visible spectrophotometer at 553 nm.

Glucose - Plasma glucose was measured using a direct glucose colormetric test kit (Stanbio Laboratory, Inc., San Antonio, TX). A reagent solution consisting of *O*-toluidine and glacial acetic acid was used to form a colored mixture of glycosylamine and a Schiff base. The absorbance of the solution was measured on a UV-visible spectrophotometer at 630 nm and quantified using a standard curve (Hultman, 1959).

Plasma chloride - Plasma chloride was measured using a chloride test kit (Ref Lab Medical Analysis Systems, Inc., Camarillo, CA). This method is a modification of a technique described by Skeggs and Hochstrasser (1964), in which chloride and mercuric ions form a soluble, non-ionized compound. The solution was incubated in a water bath at 25°C and the absorbance was read on a UV-visible spectrophotometer at 520 nm.

DNA strand breakage - DNA was extracted from liver tissue using a modification of a standard DNA isolation protocol (Maniatis et. al., 1988). An extraction buffer with 10% sarcosyl was used to lyse the cells and extract DNA from a 25-mg liver sample. DNA was purified by initially extracting with CIP (50% chloroform/isoamyl alcohol (24:1):50% buffered phenol), and further purified by extracting with CI (100% chloroform/isoamyl alcohol) to remove any remaining traces of phenol. RNAse was added to degrade RNA and a 260/280 nm optical density analysis was performed using a UV-visible spectrophotometer to verify that the extract was free of RNA. Purified DNA and a DNA molecular weight standard (λ DNA Hind III fragments, BioRad Laboratories, Hercules, CA) were applied to two types of agarose gels: denaturing and nondenaturing gels. The nondenaturing gel consisted of 0.45% agarose in 0.5% TBE buffer (27 g Tris, 13.75 g Boric acid , 0.93 g EDTA adjusted to pH 8.0) with 40 μl ethidium bromide (10 μg/ml) added. The running buffer for the nondenaturing gel was 0.5% TBE. The denaturing gel was 1% agarose dissolved in a buffer containing 4 mM EDTA and 50 mM NaCl and was electrophoresed in a running buffer containing 15 mM NaOH and 0.5 mM EDTA. Both gels were electrophoresed at 17 volts for 12 hours using a wide mini sub-cell. Denaturing gels were neutralized with pH 8 tris buffer, and stained with ethidium bromide. Gels were photographed under UV light and the negative scanned on a laser densitometer (PDI, Model DNA 25). A

standard curve was plotted from the Hind III fragments, and the strand length of each sample fragment was determined from the standard curve. Average strand lengths were calculated by weighted average based on the relative band densities and strand lengths of fragments recorded in each sample.

Condition factor and organ somatic indices - Individual fish were weighed and measured, and the liver and spleen removed and weighed. The condition factor (CF) was calculated using the following formula: CF = total body length (mm)/wt $(g)^3$. Organ somatic indices were calculated using corrected body weights (excluding the GI tract and contents) according to the following formulas:

LSI(%) = liver wt. (g) x 100/total body weight (g)

SSI(%) = spleen wt. (g) x 100/total body weight (g)

Necropsy - Necropsy parameters measured include lesions on extremities, which provide signs of external damage, fin erosion, or disease; and conditions of the eyes, gills, kidney, liver, and spleen, which will provide general insight on the health of each fish (Goede and Barton, 1990) .

Statistics - DNA strand breakage and metal comparisons were made using Student's t-test. Data analyses for all other parameters were conducted using the general linear model procedure (GLM) on SAS

(Statistical Analysis System, Cary, NC), due to the amount of variability among individuals and several missing observations within the data sets. A significance level of ($p < 0.05$) was observed for all the statistical analyses.

Results and Discussion

Environmental conditions

Environmental conditions during the field exposure were highly variable. For the first three weeks of the study, weather conditions were favorable; however on day 26-27 of the exposure the experimental site was subjected to heavy rains (6"). On the 28 day post-exposure (PE) sampling, we found that the experimental site had water in pond 2 four feet higher than at the previous sampling time. The reference site in Payne Co. also received 5.5" of rain; however no fish were lost at either site. One week prior to the 84-d sampling more heavy rains and a tornado went through the experimental site. After these severe weather conditions cages 1, 2, 5, and 6 were destroyed. Cages 3 and 4 in pond 1 were tipped on their sides; however, each contained enough catfish for the final sampling effort. Reference cages were all intact with no disturbance observed.

Water chemistry - Dissolved oxygen, pH, and water temperature were similar in both experimental ponds, however the small pond (1) was observed to have a higher water hardness range than the larger pond (2).

The water temperature in the reference pond averaged 4°C cooler and one standard pH unit greater than the experimental site. The hardness value was more comparable to that found in pond 2 of the experimental site. No ammonia was detected at either reference or experimental sites for the duration of the study (Table 1).

Metal analyses in sediments and tissues - Sediments of pond 1 contained detectable concentrations of Zn, Cd, and Pb (Table 2). Only Pb was detected in water; Cd and Zn were below detection limits (Zn $\leq$ 0.05 μg/L and Cd $\leq$ 0.1 μg/L). Pond 2 sediments contained more Zn and Cd than pond 1. Only Pb and Zn were detected in water, whereas Cd was below detectable limits. The reference site at Lake Carl Blackwell contained comparable concentrations of Cd and Pb as detected in the experimental pond (1), however Zn was observed in concentrations approximately 4x below the amounts detected in the sediments of the experimental sites. Water samples for the reference site contained half the amount of Zn and twice the amount of Pb detected in pond 2, whereas Cd was below detection limits. No significant differences for Zn, Cd, and Pb in liver or epaxial muscle samples were observed between fish from the experimental cages and the reference cages. Muscle tissue samples for all cages contained trace concentrations of Zn and low concentrations of Cd and Pb (Table 3), possibly due to muscle not being a storage site for Pb or Cd. Metals were accumulated in livers by fish at all sites (Table 4). Fish from

Table 1. Water chemistry data for pH, dissolved oxygen, temperature, and hardness for experimental and reference sites.

	pH			DO (mg/L)			Temp (°C)			Hardness (mg/L)		
Day	P1	P2	Ref	P1	P2	Ref	P1	P2	Ref	P1	P2	Ref
1	5.5	6.0	6.5	6.7	7.2	7.3	31	31	27	224	128	144
7	5.5	5.5	7.0	5.1	5.2	6.5	31	31	26	240	126	150
28	5.5	6.0	6.0	4.4	4.5	3.7	33	33	24	220	122	140
56	6.0	6.0	7.0	7.2	6.6	8.3	20	18	19	180	130	156
84	5.0	5.5	8.0	5.6	5.8	5.0	21	20	18	162	128	148
Mean	5.5	5.8	6.9	5.8	5.8	6.1	27	26	23	205	126	147

Table 2. Concentrations of zinc, lead, and cadmium detected in sediments and water of the experimental and reference sites.

	Sediment (mg/kg)			Water (mg/L)		
Metals	P1	P2	Ref	P1	P2	Ref
Zinc	119.4	193.8	32.5	1.0	0.7	0.3
Lead	9.7	<1.0	8.6	0.1	0.2	0.5
Cadmium	0.3	1.0	0.3	<0.1	<0.1	<0.1

Table 3. Average Cd, Pb, and Zn concentrations (mg/kg) detected in muscle of channel catfish from experimental and reference sites. Values represent triplicate sub-samples pooled per sampling time at each site.

	Metal content in liver (mg/kg)					
Days of exposure	Sediment and water exposure			Water exposure		
Pond 1	Pb	Cd	Zn	Pb	Cd	Zn
1	0.46	0.19	17.88	0.049	0.081	27.28
7	0.012	ND	11.08	0.052	ND	13.21
28	0.052	0.071	22.23	0.051	ND	23.37
56	0.055	ND	26.17	0.048	ND	16.54
84	#	#	#	#	#	#
Pond 2						
1	@	@	@	0.076	0.072	24.21
7	@	@	@	0.052	0.072	22.43
28	@	@	@	0.069	ND	23.65
56	@	@	@	0.066	ND	24.14
84	@	@	@	#	#	#
Ref						
1	0.038	ND	19.69	#	#	#
7	0.037	ND	22.25	0.04	ND	22.5
28	0.035	ND	21.02	0.038	ND	21.69
56	0.041	ND	24.84	0.042	ND	22.15
84	#	#	#	#	#	#

\# No liver samples available
ND No metals detected
@ No sediment and water exposures in pond 2

Table 4. Average Cd, Pb, and Zn concentrations (mg/kg) detected in livers of channel catfish for experimental and reference sites. Values represent triplicate sub-samples pooled per sampling time at each site.

Days of exposure	Metal content in muscle (mg/kg)					
	Sediment and water exposure			Water exposure		
Pond 1	Pb	Cd	Zn	Pb	Cd	Zn
1	0.023	0.09	8.94	0.045	0.048	26.03
7	0.006	ND	5.54	0.017	ND	15.2
28	0.05	0.14	16.63	0.025	ND	11.68
56	0.027	ND	13.08	0.058	ND	18.68
84	#	#	#	#	#	#
Pond 2						
1	@	@	@	0.055	0.71	11.45
7	@	@	@	0.048	0.25	18.48
28	@	@	@	0.047	ND	22.6
56	@	@	@	0.063	ND	25.01
84	@	@	@	#	#	#
Ref						
1	0.031	ND	19.54	#	#	#
7	0.016	ND	14.8	0.016	ND	14.63
28	0.041	ND	23.52	0.041	ND	21.06
56	0.027	ND	16.56	0.039	ND	26.36
84	#	#	#	#	#	#

\# Not enough liver for sample analysis
ND No metals detected
@ No sediment and water exposures in pond 2

pond 2 had higher Cd and Pb concentrations than fish from pond 1; however, no significant differences were detected.

Hematological stress

Hematocrit - No significant differences in hematocrit (HCT) were observed between experimental and reference cages (Appendix B). As expected, due to the levels of dissolved oxygen found at the sites and the range of DO required by channel catfish, no indications of oxygen stress were observed. Increases in HCT have been linked with exposures to acidic water ($pH < 4.9$) in rainbow trout (*Salmo gairdneri*) (Giles et al., 1984). However, we saw no pH-induced changes in HCT, possibly because the pH was always ≥ 5 at all study sites.

Hematocrit ranges of both experimental and reference groups were comparable to nonstressed normal hematocrit ranges. Grizzle (1977) reported mean hematocrit values to range from 30.2 to 34% RBC for nonstressed catfish for similar exposure durations (3 to 28 day exposures). In two separate preliminary studies with channel catfish, hematocrit values averaged 33 ± 7.3 ($n=23$) and 28.2 ± 3.9 ($n=11$) %RBC, corresponding to the mid to low values in this study. Other researchers reported similar levels for non-stressed channel catfish ranging from 25.8 to 38.5 %RBC (Klinger et. al., 1983; Limsuwan et al., 1983; Sheehan and Lewis, 1986; Areechon and Plumb, 1990; Jantrarotai and Lovell, 1990). All of these

values fall within the range of values observed in our study.

Hemoglobin - Hemoglobin (Hb), an indicator of anemia, ranged from 41.7 to 84.1 g/l for both experimental and reference fish (Appendix B). Significantly lower Hb levels were observed at day 1 in one of the water exposure cages (cage 6, pond 2). Although an increase in Hb was observed in all experimental fish on day 28, the significance probably was due to an unexplainable 33% decrease in Hb levels in reference fish. No other significant differences were observed during the exposure (Fig. 4).

Decreased Hb concentrations have been reported for carp (*Cyprinus carpio*) exposed to Cd (Koyma and Ozaki, 1984), but in our study no Cd was detected in the water. A mean Hb value of 77.2 ± 10.8 g/L (n=23) was measured in preliminary experiments with catfish maintained in the laboratory under constant conditions. Other Hb values reported for nonstressed catfish are 79 ± 1.8 (Limsuwan et al., 1983), 68.9 ± 7.8 (Areechon and Plumb 1990), and 75.3 ± 1.2 g/l (Jantrarotai and Lovell, 1990). All of these correlate with Hb values observed in our study.

ALAD - The ALAD assay, a specific indicator for lead exposure, varied tremendously in both experimental and reference fish (Appendix B). ALAD activity measured in water-exposed fish (cage 6) from pond 2 was significantly higher at day 1 than the reference. ALAD values in fish

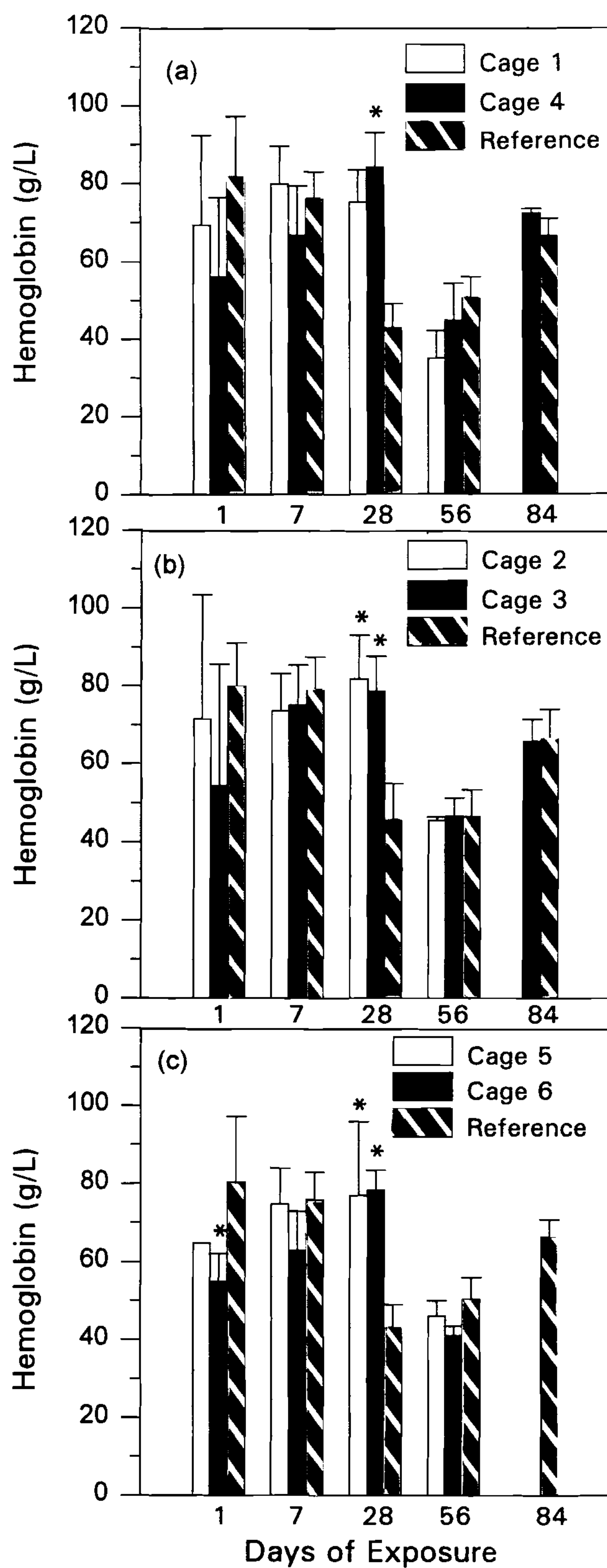

Figure 4. Hemoglobin concentrations (±SD) measured in catfish. Panels: (a) P1 water; (b) P1 sediment; and (c) P2 water. (* $P < 0.05$).

exposed to water and sediment in pond 1 (cage 2) were significantly higher at day 1 and both cages 2 and 3 were significantly higher at day 28 than the reference. No other significant differences were observed (Fig. 5).

We question the biological significance of these differences in ALAD activities in light of the extreme variation seen in the marker. Schmitt and colleagues (1993) also observed wide inter-individual variation over time in *Catostomids* exposed to lead-zinc mining activities. A cohort of 23 channel catfish held in the lab under constant conditions with no toxicant exposure were observed to have a mean of 461 $\pm$ 178 units of ALAD activity, suggestive of the same variation observed in the field. ALAD has been demonstrated to work well as a biomarker for lead exposure in fish (Johansson-Sjobeck and Larsson, 1978), *Daphnia* (Berglind et. al., 1985), and humans (Abdulla and Haeger-Aronsen, 1971), when Pb is the only metal present. However, when organisms are exposed to Pb in combination with Zn and Cd, such as we observed in the experimental site, interactions occur that mask the ALAD inhibition due to Pb exposure (Willoughby et. al., 1972; Schmitt et al., 1984; Berglind, 1986; Dwyer et. al., 1988; Schmitt et. al., 1993). Berglind (1986) observed a stimulation in ALAD activity in *Daphnia* exposed to Cd alone, but when daphnids were exposed to mixtures of either Cd or Zn with Pb no effects were observed. This may explain the non-inhibited levels observed in our study, because both experimental ponds contained zinc in the sediments, which may have masked the inhibitory

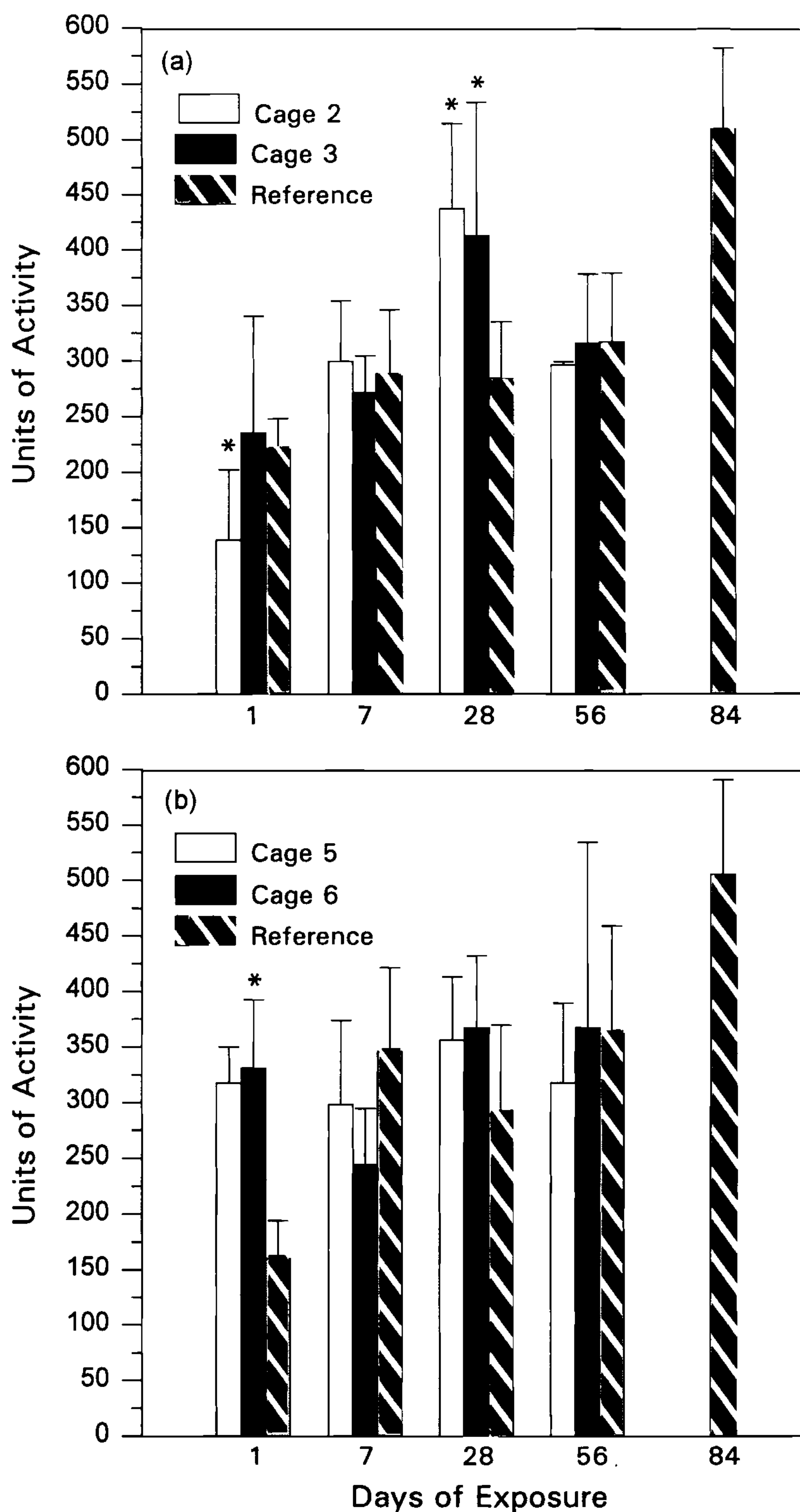

Figure 5. ALAD concentrations (±SD) measured in catfish. Panel (a) P1 sediment exposures and (b) P2 water exposures. (* $P < 0.05$)

effects of Pb on ALAD activity. This interaction becomes a problem when making measurements in the environment where organisms are rarely exposed to single metals and mixtures of heavy metals are not homogeneously distributed.

Metabolic stress

Plasma glucose - Using the metabolic stress indicator, plasma glucose, a classic stress response was observed in all cages for days 1-28 (Fig. 6) (Appendix B) . At day 1, glucose concentrations in fish for all exposure groups were higher than those measured in the reference fish, possibly indicating glucose mobilization due to environmental stressors. By day 7, apparent glucose depletion was observed in all experimental groups, except the water exposure cages in pond 2. By day 28, all glucose values had returned to levels similar to those in reference fish (water exposure) or significantly higher values (cages 3 and 6). No further changes were observed in water-exposed fish in pond 1 after day 28. However, further elevations in plasma glucose were observed in all fish in pond 2 at day 56. This may have occurred because pond 2 was fed by a stream and heavy rains fell just prior to day 56 which may have resuspended sediment-bound metals, causing elevated glucose levels in all fish. Other studies have mentioned similar effects of metal resuspension resulting from storm water runoff (MacIntosh, 1991) or in shallow lakes, such as the experimental pond

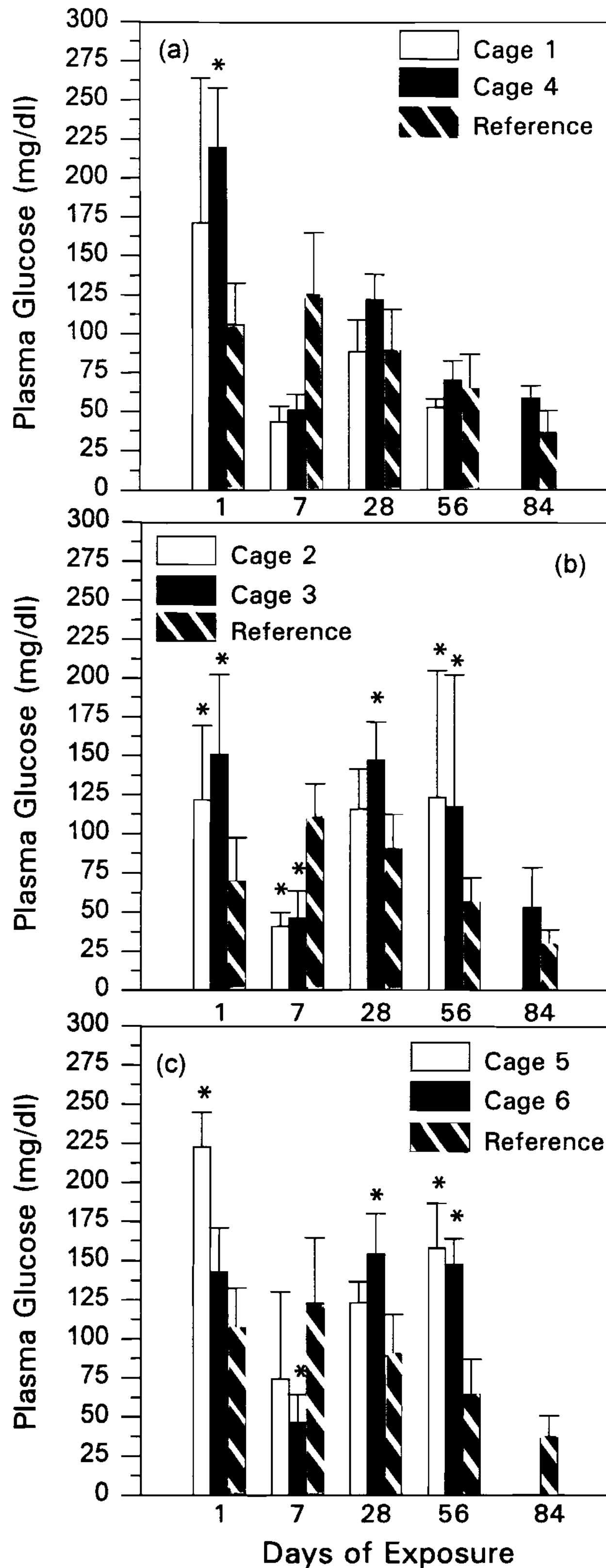

Figure 6. Glucose concentrations (±SD) measured in catfish. Panels: (a) P1 water; (b) P1 sediment; and (c) P2 water. (* $P < 0.05$).

1, due to physical disruption (Robbins et al., 1990).

Similar responses (glucose mobilization followed by depletion) were observed in channel catfish exposed to etomidate (Limsuwan et. al., 1983), bluegill sunfish (*Lepomis macrochirus*) and green sunfish (*Lepomis cyanellus*) exposed to low DO and high chlorine (0.4 mg/l) concentrations (Black and Martin, unpublished data). We found the assay to be a sensitive indicator; however, it is unclear whether alterations in plasma glucose levels were related to exposure xenobiotic changes or to environmental conditions. Furthermore, in concurrence with Lagadic and colleagues (1994), we also observed a considerable amount of individual variation in glucose values.

Osmoregulatory stress

Plasma Chloride - A transient decrease in plasma chloride was observed at day 1, followed by a compensatory increase at day 7 in fish from both sediment cages (pond 1). By day 28, a return to reference level was observed. This pattern correlated with observations in a study by Lewis and Lewis (1971) using juvenile channel catfish in separate exposures to zinc and copper sulfate. The low values observed on day 1 possibly resulted from acute metal exposure. An increase in plasma chloride observed in these fish at day 7 may have been an over compensation by these organisms in returning to normal osmoregulatory functions. By day 56, plasma chloride values become stable and approximate those of the

reference fish (Fig. 7). The averages values observed for plasma chloride depicted in Appendix B.

Zinc and other heavy metals disrupt the branchial respiratory epithelium of the gill influencing aspects of osmotic and ionic regulation in fish (Skidmore and Tovell, 1972). Holcombe et al. (1979) reported the gills of brook trout (*Salvelinus fontinalis*) readily accumulated zinc. Because cage 2 and 3 fish had access to the sediments containing high concentrations of Zn, exposures may have resulted in loss of chloride ions through the gills. Gills were not analyzed for metal content, thus it is not known if zinc was accumulated, leading to branchial chloride losses.

Genotoxic stress

DNA strand breakage: denaturing conditions - The denaturing assay is used to indicate genotoxic stress by monitoring average strand lengths in single-stranded DNA under alkaline conditions. DNA strand lengths were significantly longer in fish from pond 1 (water exposure) cage 1 and shorter in cage 4 than in reference cages at day 1 (Fig. 8) (Appendix B). A decrease in strand length was observed in cage 4 at day 7 and 28, followed by no change in strand length over the remainder of the exposure. Fish in sediment exposures in pond 1 (cage 3) had significantly shorter strand lengths at day 7 and 28. Fish from the water exposures in pond 2 had the

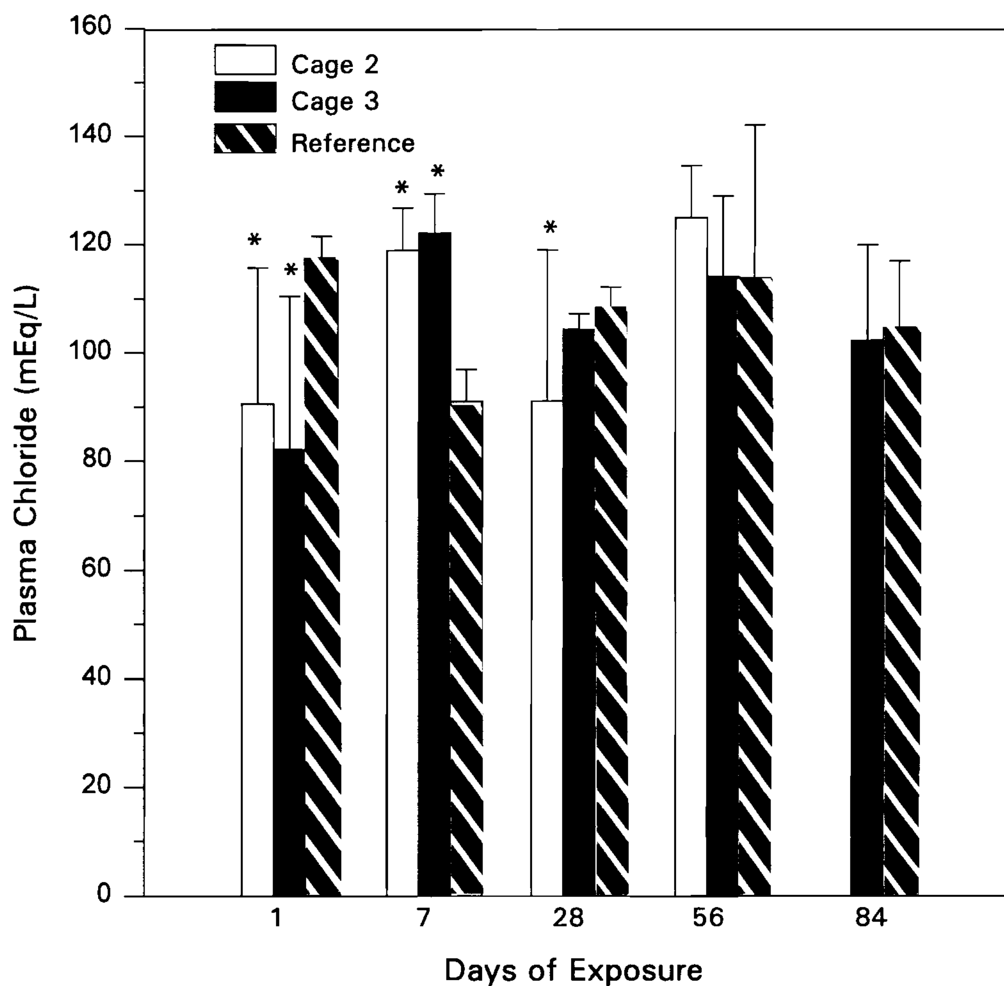

Figure 7. Plasma chloride concentrations (±SD) measured in catfish from sediment exposures. (* $P < 0.05$).

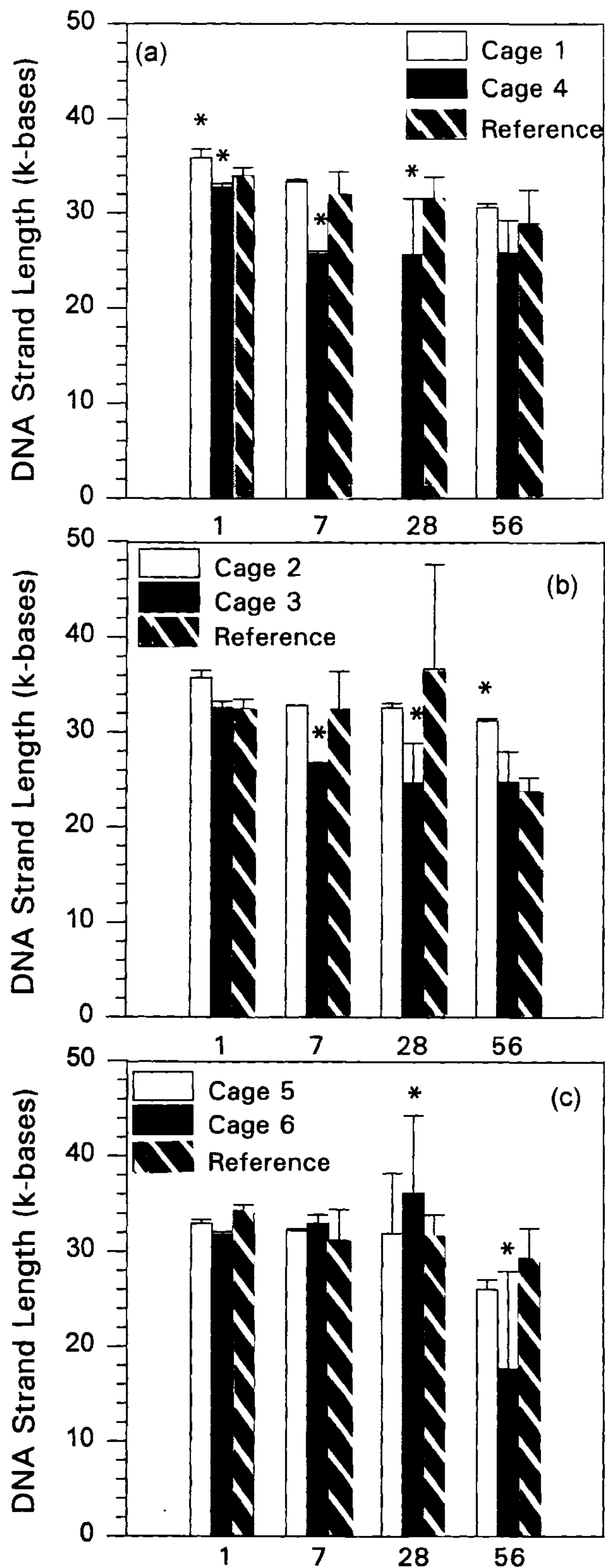

Figure 8. Denaturing DNA strand lengths (±SD) measured in catfish liver. Panels: (a) P1 water; (b) P1 sediment; and (c) P2 water. (* $P < 0.05$).

highest degree of single-stranded breaks. Fish in cage 6 of the pond 2 water exposures had significantly longer DNA strand lengths versus the reference at day 28. However, by day 56, a 50% reduction in DNA strand length (from a mean of 36.08 to 17.62 kilobases) was observed in fish from cage 6. No changes in DNA strand lengths in fish from cage 5 were observed during the entire exposure. Although cages 5 and 6 were located adjacent to each other, different degrees of strand breakage were observed. This difference may be explained by the feeding status of the catfish. The feeder on cage 5 was operational on day 56, however, the feeder on cage 6 would not dispense feed. Preliminary results imply non-fed channel catfish fish may be more prone to DNA strand breakage (Black and Martin, unpublished).

The advantage of the denaturing approach is that many agents promote 5- to 2000-fold more single-stranded breaks than double stranded breaks. Thus, alkaline conditions are more sensitive in the detection of strand breaks than neutral conditions. Furthermore, alkaline conditions promote the degradation of cellular RNA, which may interfere in the quantification of the ethidium bromide stained samples (Singh et. al., 1988). However, breaks in double-stranded DNA are considered to be of greater biological consequence because they can lead to chromosomal aberrations (Olive et. al., 1991; pers. comm. Steve D'Surney, Univ of MS).

DNA strand breakage: non-denaturing conditions - The non-denaturing assay is used to indicate genotoxic stress by monitoring strand breaks in

double-stranded DNA using non-denaturing gels. We observed significantly smaller double-stranded fragments at day 7 in the water exposure cages of pond 1 (Fig. 9) (Appendix B). Cage 3 in the sediment exposures of pond 1, had significantly shorter strand lengths than the reference at 28 and 56 days PE. Significantly smaller strand lengths were observed in experimental cages than reference cages on both day 7 and 28 in pond 2, followed by an increase in strand length at day 56, similar to that seen in the reference. An increase in strand length was observed in cage 5 between day 7 and 28. This may have resulted from repair mechanisms adding extra sequences to the damaged DNA. This pattern was similar to that reported by Shugart (1988) in which a rapid decline in double-stranded DNA was observed in bluegill sunfish, *Lepomis macrochirus*, and fathead minnows, *Pimephales promelas*, exposed to 1 μg/l benzo[a]pyrene, followed by an increase to control level at 30 days. Some strand breakage also occurred in the reference group in our study, further indicating that either the reference site was not “pristine” or normal background breakage was occurring.

Nonspecific stress indicators

Spleen somatic index - No changes were observed in spleen size from exposure to Pb, Cd, and Zn. Mean spleen somatic indices ranged from 0.06 to 0.12 for experimental fish and 0.04 to 0.21 for reference fish (Appendix B).

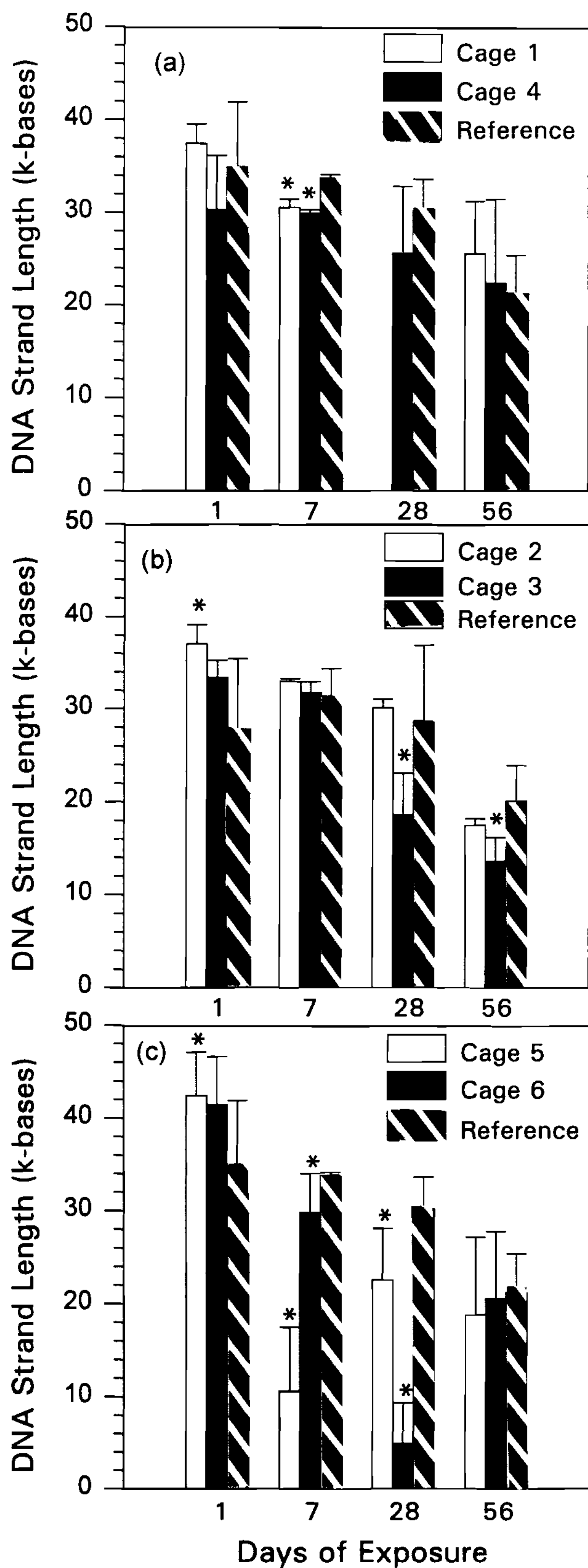

Figure 9. Non-denaturing DNA stand lengths (±SD) measured in catfish liver. Panels: (a) P1 water; (b) P1 sediment;and (c) P2 water. (* $P < 0.05$).

Liver somatic index - The LSI was significantly higher in fish from sediment cages 2 and 3 and water exposure cage 4 at day 7 in pond 1 compared with reference fish. Fish from water exposure cage 5 of pond 2 also had significantly elevated LSI at day 7 and 28 (Fig.10) (Appendix B).

Livers of fish with elevated LSI were a fatty, whitish-pink color nearly 2 to 3 times the size of a normal liver. This abnormality was always absent by the next sampling date. Increasing liver size through hyperplasia or hypertrophy may increase its capacity to biotransform xenobiotics through induction of the MFO system or synthesis in metallothionein production for sequestering heavy metals (Fletcher et al., 1982). This condition may have occurred at different time periods in the different experimental ponds due to differential metal accumulation or elimination from the liver.

Condition factor - No significant differences were observed in condition factor between experimental and reference sites (Appendix B). Although all of the catfish in the study were of the same cohort, fish size varied at the beginning of the study. By the end of the study experimental fish were slightly longer and heavier than reference fish, but the differences were not significant. However, because fish were sampled from the cage populations at each sampling date and not replaced into the cage, these data may not reflect true weight size differences for any exposure group.

Necropsy - Initial visual inspection of all catfish revealed no damage to the extremities (fins and skin) and normal eyes and gills. After necropsy, no

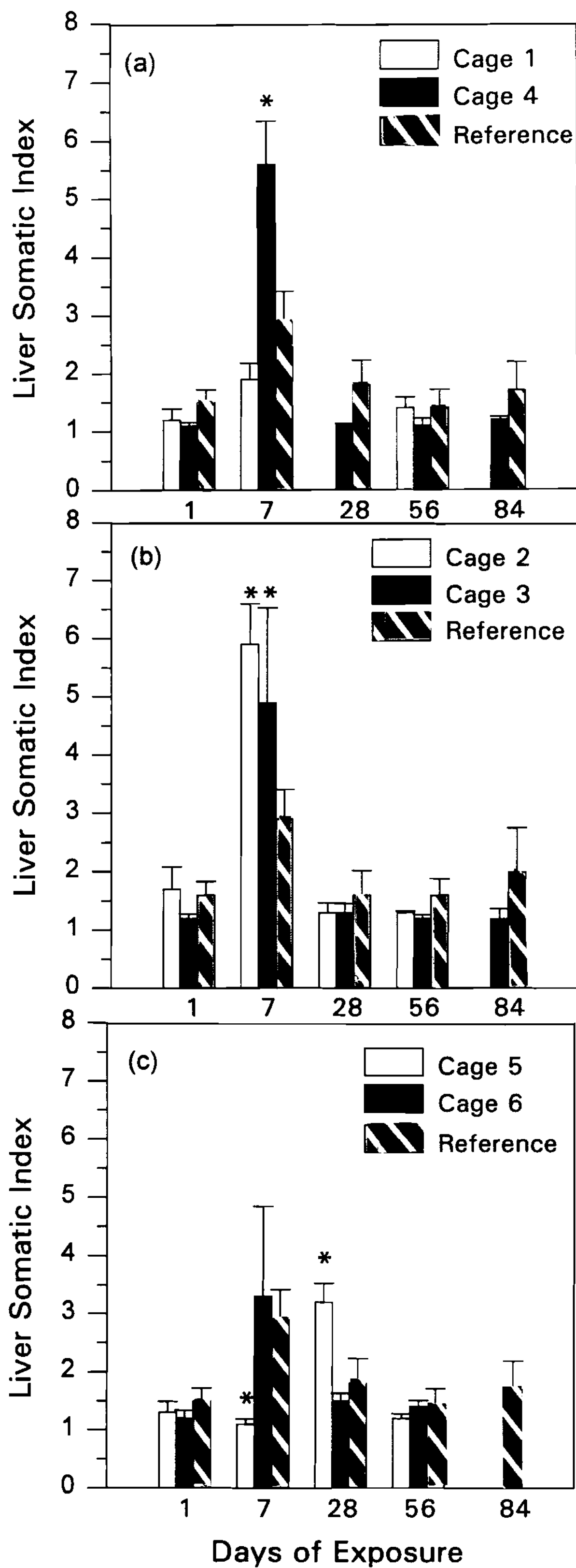

Figure 10. Liver Somatic Index (LSI) (±SD) in measured catfish. Panels: (a) P1 water; (b) P1 sediment; and (c) P2 water. (* $P < 0.05$).

deposits of visceral fat were observed, probably due to age of the catfish. In addition, no hind gut inflammation was visually apparent. A visual inspection of organs (i.e., spleen, liver, kidney, gut, and stomach), and the presence of parasites were noted (Appendix A; Table 5).

Spleens in all fish were found to be predominately red in color, with a few fish having pale red or black spleens. Although most livers were red in color, enlarged, fatty livers were also observed in cages of both experimental sites, primarily after 7- and 28-day's exposure (see LSI, previously discussed). Thirty to 50% of the catfish in all cages appeared to be actively feeding. The majority of fish were found to be devoid of parasites, however some fish (<15%) in both experimental and reference sites were infested with the yellow grub, *Clinostoma*.

Conclusions

In order for a biomarker assay to be an acceptable and reliable indicator of xenobiotic stress, the measured variable must be directly affected by the toxicant to the fish is exposed and should show significant change in the organism during exposure. Secondly, a baseline measurement for control conditions should be identified and be replicable. Finally, effects on the measured variable must not be affected by capture, handling, and sacrificial methods during the measurement process, so that it can be separated from toxicant exposure effects (Silbergeld, 1974). Inter-individual

Table 5. Necropsy data for channel catfish from experimental and reference sites. Values are the % occurence for each variable measured.

	Reference		Experimental		
	Sediment	Water	Sediment (P1)	Water (P1)	Water (P2)
Spleen					
Red	76.1	78.7	82.7	67.5	72.3
Pale	3.4	5.6	3.8	2.5	4.3
Black	3.4	2.2	3.8	5	10.6
Granular	17	13.5	9.6	25	12.8
Liver					
Red	96.6	84.9	76.9	74.4	66.7
Pale	3.4	15.1	15.4	20.5	13.3
Fatty	0	0	7.7	5.1	20
Stomach/ GI tract					
Full	4.6	14.6	30.8	22	17
1/4 - 1/2	51.7	58.5	44.2	34.1	29.8
Empty	43.7	26.9	25	43.9	53.2
Parasites					
Present	9.1	13.5	5.8	0	0
Absent	90.9	86.5	94.2	100	100

variation within the study population is one of the main problems in making predictions from environmentally-elicited biomarker responses. Some organisms will exhibit weak responses while others will have stronger responses. Transient fluctuations in some biomarker responses may occur and xenobiotic exposures can result in responses that last a matter of hours, or persist for weeks, months, or a lifetime (Depledge and Fossi, 1994). This variation in degree of response is thought to arise from a non-homogeneous exposure, predominantly in areas where environmental conditions change over small areas (Depledge, 1990). Due to this inherent variation, attempts to tease out xenobiotic-elicited responses from those that are environmentally induced become taxing, if not impossible, especially in areas with low levels of contamination.

We found both DNA strand breakage assays (non-denaturing and denaturing assays) to yield the best indications of acute to semi-chronic stress. Using these assays, we were able to detect decreases in strand lengths, and possible DNA repair in both the experimental and reference site over time. Unfortunately, the cause of the DNA strand breaks can not be determined with the methods used. Future research might include analyses for DNA adducts to determine whether metals actually cause adducts on DNA, which in turn have been proposed to decrease the integrity of the strand.

We observed significant differences in some biomarkers that indicate

differential response with exposure. Plasma chloride and hematocrit assays showed significant differences only in fish from sediment-exposed cages in pond 1. Concentration gradients also appear to affect organism responses to some biomarkers. Differences in plasma glucose and DNA integrity were observed in the water exposure cages in pond 2, a location with more available heavy metals than with pond 1.

Although we did observe metal accumulation over time in the organisms exposed to strip-mine effluents, the amount measured fluctuated, due either to metal elimination or mobilization to other storage sites within the organism. Because metal accumulations were not constant over time, we were unable to provide evidence of concentration-dependent responses of the biomarkers chosen. However, many biomarkers measured were useful in assessing stress responses over time. Temporal responses were observed for plasma chloride and DNA integrity assays. Other temporal responses might also be observed with a longer study duration.

Investigations using biomarker approaches need to continue to provide additional baseline data for both stressed and nonstressed organisms alike. Perhaps subtle differences in organismal response to stressors can be more easily identified once a database is established.

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CHAPTER III

Biomarker Assessment of the Effects of Petroleum Refinery Contamination on Channel Catfish.

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Abstract

A suite of biomarkers was used to evaluate acute (1 and 7 day) heavy metal induced toxicity in channel catfish, *Ictalurus punctatus*, caged at an abandoned petroleum refinery and a non-contaminated reference site. Assays performed include indicators of metabolic, hematological, osmoregulatory, and genotoxic stress. Two cage designs were utilized to evaluate the importance of exposure routes: one allowing exclusive contact with the water column and the other allowing contact with water and sediments.

Data collected at 1 day and 7 days post-exposure indicated that the

experimental fish were stressed, having significant increases in blood glucose. After 7 days of exposure, two hemodynamic parameters, hemoglobin and delta-aminolevulinic acid dehydratase, were significantly increased. There were no significant differences observed in other parameters measured.

Keywords: biomarkers; channel catfish; refinery wastes; ALAD; hemoglobin

Introduction

The Anderson-Pritchard Oil Corporation began refining oil at Cyril, Caddo Co., Oklahoma, in 1920. Production was in full swing in 1978 when the site was purchased by the Oklahoma Refining Company to produce gasoline, diesel, napthas, asphalt, and industrial solvents. In 1984, bankruptcy was declared and all refinery operations were shut down. In 1986, the site was placed on the Environmental Protection Agency list as a Superfund National Priority Site. The site encompasses approximately 240 acres, among which are a series of settling ponds (sludge traps) that allowed refinery wastes to settle out of the API separator discharge water. Many contaminant by-products associated with refinery wastes, including heavy metals and many residual aromatic hydrocarbons have become sequestered in the soils and sediments of these areas (Linda Lyhane, pers. comm., OK Dept. of Health, Waste Management Services). These chemicals may remain in deposition areas or migrate to surface or ground waters through

advective transportation or leaching processes. They may become biologically available to the inhabiting biota and possibly disrupt important ecosystem processes upon reaching natural waters beyond the boundaries of the retention areas.

In this study, we used a suite of biomarkers to evaluate acute heavy metal-induced stress in channel catfish caged in refinery settling ponds. Biomarkers assayed included indicators of metabolic stress (plasma glucose), hemodynamic stress (hematocrit, hemoglobin, and delta-aminolevulinic acid dehydratase--ALAD), genotoxic stress (DNA strand breakage), and osmoregulatory stress (plasma chloride); gross morphometric changes as indicated by a general necropsy; condition factor, and organo-somatic indices (kidney, liver, and spleen). These assays are designed to detect sub-lethal biochemical, physiological, and morphological changes in organisms exposed to toxicants. The utility of these methods lies in their ability to provide an"early warning" of toxicant stress in organisms before community and ecosystem responses can be detected.

The hypotheses we tested are: biomarker responses are correlated with increasing metal accumulation by fish; biomarker responses to heavy metal toxicity change over time as fish are exposed to contaminants; and responses are significantly different in organisms having contact with both the water and the sediments compared to those having contact with only the water column.

Materials and Methods

A series of seven oil sludge trap settling ponds for the API separator were selected at the Cyril refinery based on amounts of heavy metals present and depth of water at each pond. This series of traps was used to progressively settle contaminants (i.e., heavy metals and hydrocarbons) out of the effluent and into the sediments which were to be distributed on a land farm for contaminant degradation. Upon reaching the last trap, water was discharged into Gladys creek, thus leaving the site.

An on-site investigation resulted in use of the last two ponds (ST-6 and ST-7) of the series, because the first five ponds contained large amounts of oil present along the periphery as well as a prominent slick on the surface. In addition, ST-6 and ST-7 were found to have adequate dissolved oxygen concentrations (Table 1). The areas for ST-6 and ST-7 were 0.421 and 0.273 acres, respectively, and both ponds averaged 1.5 m in depth (Fig.1). The reference site was an aquaculture pond located adjacent to Lake Carl Blackwell in Payne Co., Oklahoma (Fig. 2). The reference pond averaged 1.5 m in depth, with a surface area of 0.261 acres.

Four plastic constructed cages (Martin and Black, 1995); two sediment exposure cages and two water exposure cages, were anchored in ST-6 and ST-7 to evaluate stress effects based upon modes of exposure (Fig. 3). Eight cages, four sediment and four water exposure, were anchored at the reference site.

Table 1. Water chemistry data for dissolved oxygen, pH, hardness, and temperature for experimental and reference sites.

	ST-6		ST-7		LCB-Pond 11	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
DO	3.6	5.0	9.3	6.1	6.6	4.6
pH	6.0	6.0	6.0	6.0	6.0	6.0
Hard	288	290	258	263	150	148
Temp	21	22	21	22	19.5	23

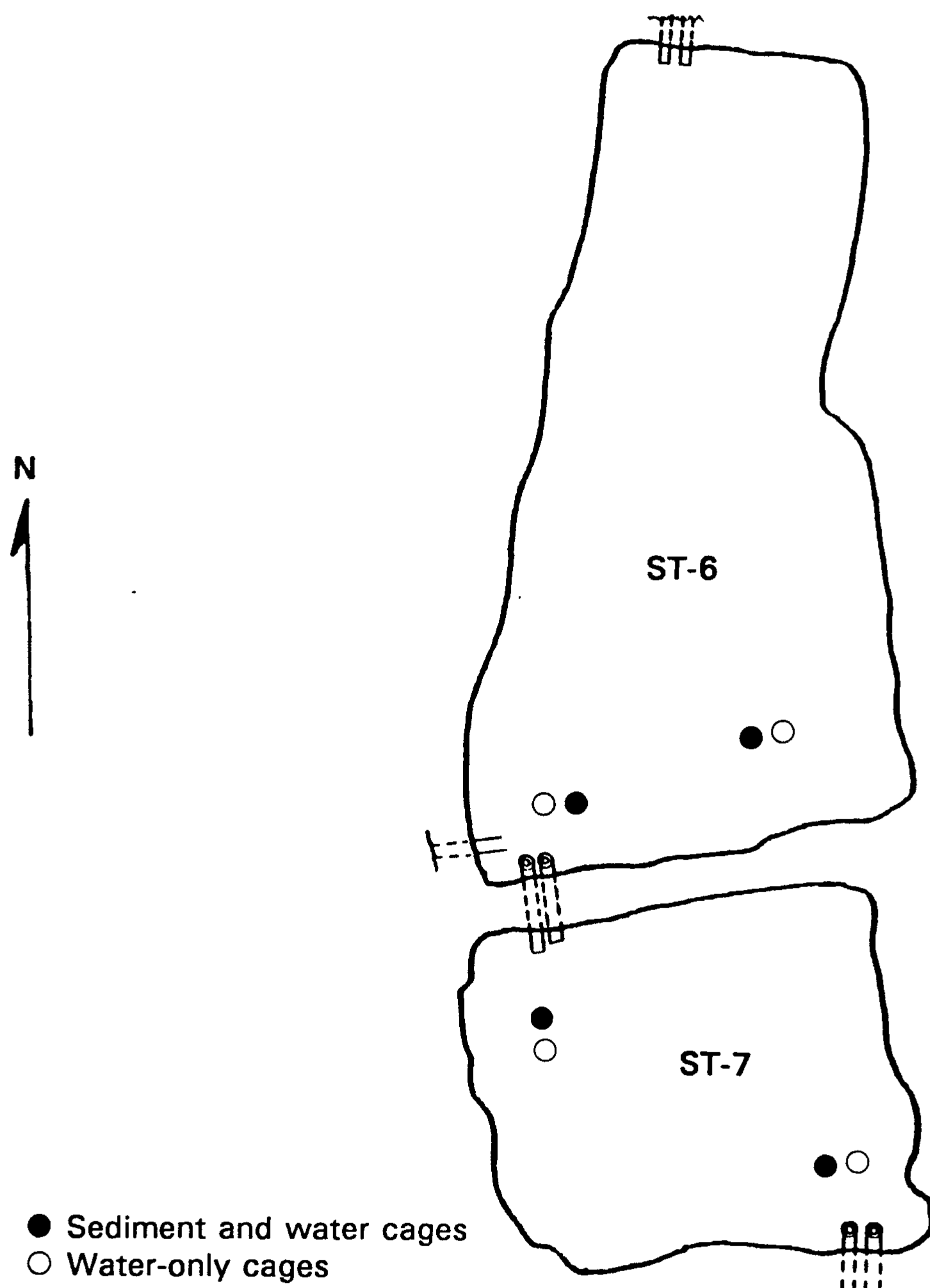

Figure 1. Exposure ponds (ST-6 and ST-7) at the abandoned petroleum refinery site located in Caddo Co., OK.

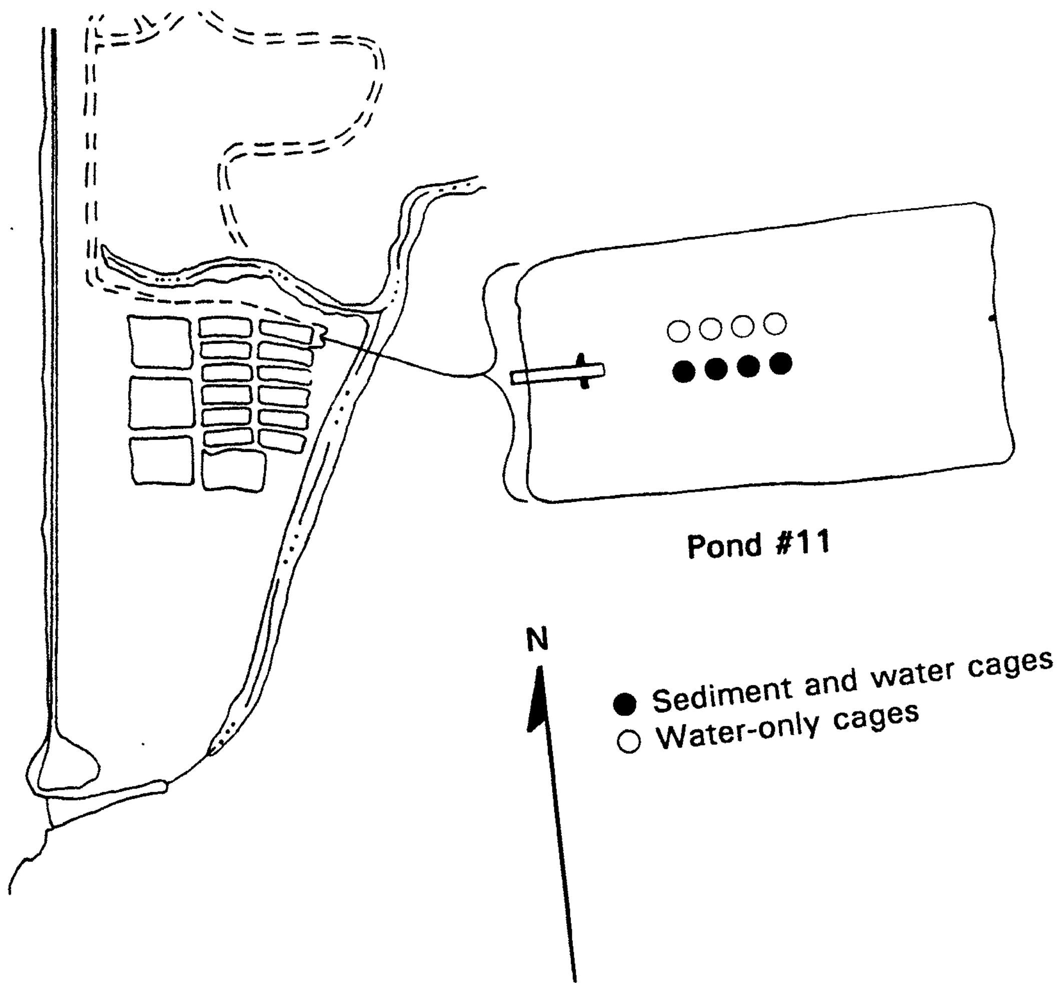

Figure 2. The references site, pond 11, located in Payne Co., OK.

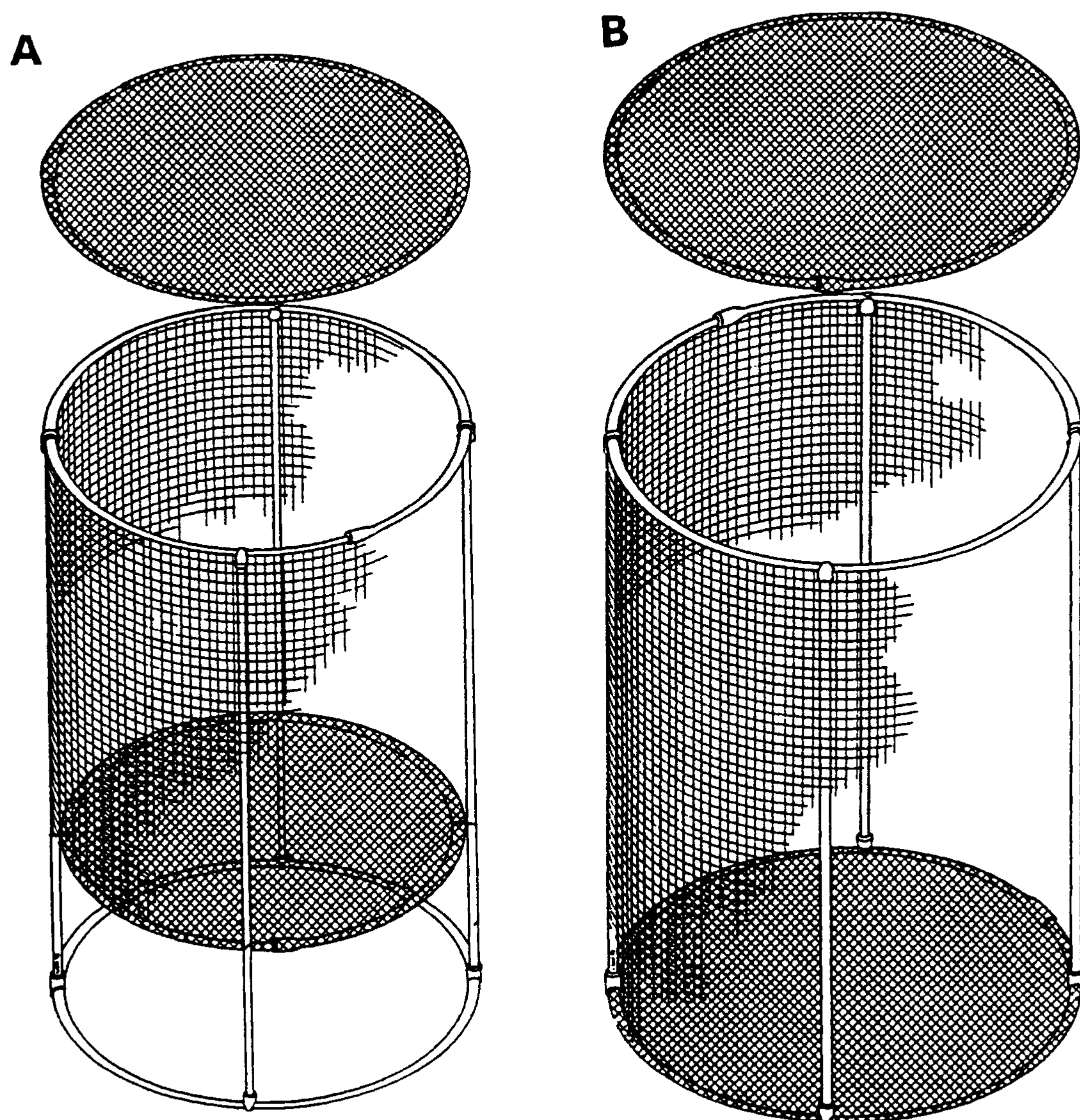

Figure 3. The two cage designs (A) water exposure cage and (B) sediment and water exposure cage.

Juvenile channel catfish, *Ictalurus punctatus*, 4 to 6 inches in length and averaging 18 to 32 g in weight, were obtained from the Blue River State Fish Hatchery (Tishomingo, Oklahoma). Channel catfish were selected due to their availability, survivability, and semi-benthic nature, which allows exposure through interactions with the sediments. Fifty catfish were stocked in each cage at the beginning of the study and on each sampling date. Originally, we intended to sample fish at 1, 7, 28, 56, and 84 days post-exposure, however 100% mortality occurred prior to the 28 day sampling at the contaminated site. No mortality was observed in reference fish. Therefore, data from the 1 and 7 day sampling times are presented. At each sampling time whole blood samples were taken for use in the following biomarker assays. Hematocrit, a percentage of packed red blood cells, was measured by centrifuging blood-filled capillary tubes as an indicator of oxygen related stress. Hemoglobin was measured using the cyanmethemoglobin method described by Wintrobe (1956). Delta-aminolevulinic acid dehydratase (ALAD), an indicator of lead exposure was measured using a method described by Burch and Siegel (1971). Plasma glucose concentrations, an indicator of metabolic stress, were measured using a direct glucose colormetric test kit (Stanbio Laboratory, Inc., San Antonio, TX). Plasma chloride concentrations, an indicator of osmoregulatory stress were measured using a chloride test kit (Ref Lab Medical Analysis Systems, Inc., Camarillo, CA). DNA strand breakage was

measured using denaturing and non-denaturing gels was measured in liver tissue as an indicator of genotoxic stress with a modification of a standard DNA isolation protocol (Maniatis et. al., 1982). Condition factor and organ somatic indices (liver and spleen) were measured as indicators of non-specific stress. A general necropsy was performed using a protocol similar to one described by Goede and Barton (1990) (Appendix A).

Water chemistry data were collected prior to sampling the caged fish. Water parameters (i.e., pH, temperature (°C), and dissolved oxygen (DO)) were measured with a Checkmate modular testing system (Corning, New York). Ammonia was measured using an ammonia test kit (Aquarium Pharmaceutical, Inc., Chalfont, PA). Water hardness was measured using a procedure described by Lind (1985). All parameters were measured approximately six inches below the surface.

Statistical comparisons were made using Student's t-test (DNA strand breakage and heavy metal analysis) and the general linear model procedure (GLM) on SAS (Statistical analysis System, Carey, NC). The general linear model was used because of the amount of variability among individuals and missing observations within the data sets. A significance level of ($P < 0.05$) was observed throughout all statistical analyses.

Results and Discussion

Water chemistry - Water chemistry values for pH, temperature, and

hardness were similar in both experimental ponds (Table 1). Pond ST-6 had lower dissolved oxygen (DO) concentrations than ST-7. The average water chemistry for the reference site closely approximated those averages observed at the experimental sites, varying primarily in lower water hardness. No ammonia was detected at the reference or experimental sites during the study.

Metal analyses in the sediments and livers - The sediments of ST-7 contained less than half as much zinc (Zn) and copper (Cu) than levels observed in ST-6 (Table 2). Lead (Pb) was comparable for both sites. No differences in metal concentrations were observed between the water samples for ST-7 and ST-6. The reference site located at Lake Carl Blackwell contained significantly lower trace amounts of Zn, Cu, and Pb in the sediments than the experimental sludge traps (Table 2). Metals in the water samples from the reference site were below detection limits (Zn 0.05 μg/L, Cu 0.02mg/L, and Pb 1.0 μg/L).

The livers of the individual fish were analyzed for Zn, Cu, and Pb concentrations. All livers from the Cyril exposures contained concentrations of Zn, Cu, and Pb (Table 3). The reference livers contained Zn and Cu at similar concentrations as found in the experimental sites, however no Pb was detected in the reference fish. Livers from the reference fish contained similar zinc concentrations of those observed at Cyril. These observed levels are also similar to those found in a similar heavy metal study at an

Table 2. Concentrations of zinc, lead, and copper detected in the sediments and water of the experimental and reference sites.

	Sediment (mg/kg)			Water (mg/L)		
Metals	ST-6	ST-7	Ref	ST-6	ST-7	Ref
Zinc	761.0	406.0	16.53	0.050	0.045	≤0.005
Copper	106.0	68.00	4.940	0.010	0.010	≤0.005
Lead	173.0	214.0	4.750	0.045	0.060	≤0.005

Table 3. Concentrations of zinc, lead, and cadmium detected in the livers of the catfish in the experimental and reference sites.

Days of exposure	Metal content in liver (mg/kg)					
	Sediment and water exposure			Water exposure		
ST-6	Zn	Pb	Cu	Zn	Pb	Cu
1	22.5	0.05	0.06	24.5	0.02	0.08
7	#	#	#	#	#	#
ST-7						
1	22.0	0.02	0.08	21.2	0.05	0.075
7	22.4	0.01	0.06	24.2	0.02	0.090
Ref						
1	19.4	ND	0.05	22.0	ND	0.06
7	26.0	ND	0.10	25.2	ND	0.11

No surviving fish
* No metals detected

abandoned coal strip-mine (Martin and Black, unpublished data), suggesting that the observed metal concentrations are probably normal levels. Metal accumulations in the livers did not differ significantly over time between the reference and ST-7 for both day 1 and 7, indicating that heavy metals were not acutely accumulated by the livers at these two sites. Lead was accumulated in experimental fish from ST-6 and ST-7, although accumulations were not significantly different. No lead was detected in the reference fish. Different metal accumulations in the liver based upon the mode of exposure (i.e., sediment versus water exposure) were also not observed for the acute durations of the study.

Biomarkers - Although the experiment was terminated after the 7 day post-exposure sampling date due to 100% mortality in the experimental groups, significant differences in several biomarkers were detected for the acute exposure.

The ALAD assay, a specific indicator for lead exposure, was observed to be significantly higher than the reference at all exposure times for all sediment exposure cages in both ponds ST-6 and ST-7 (Fig. 4). ALAD measured in fish in ST-7 water exposure cages did not differ statistically from the reference at day 1, however by day 7, ALAD concentrations were significantly higher than those measured in the fish.

Contrary to what was expected, we did not observe inhibition of

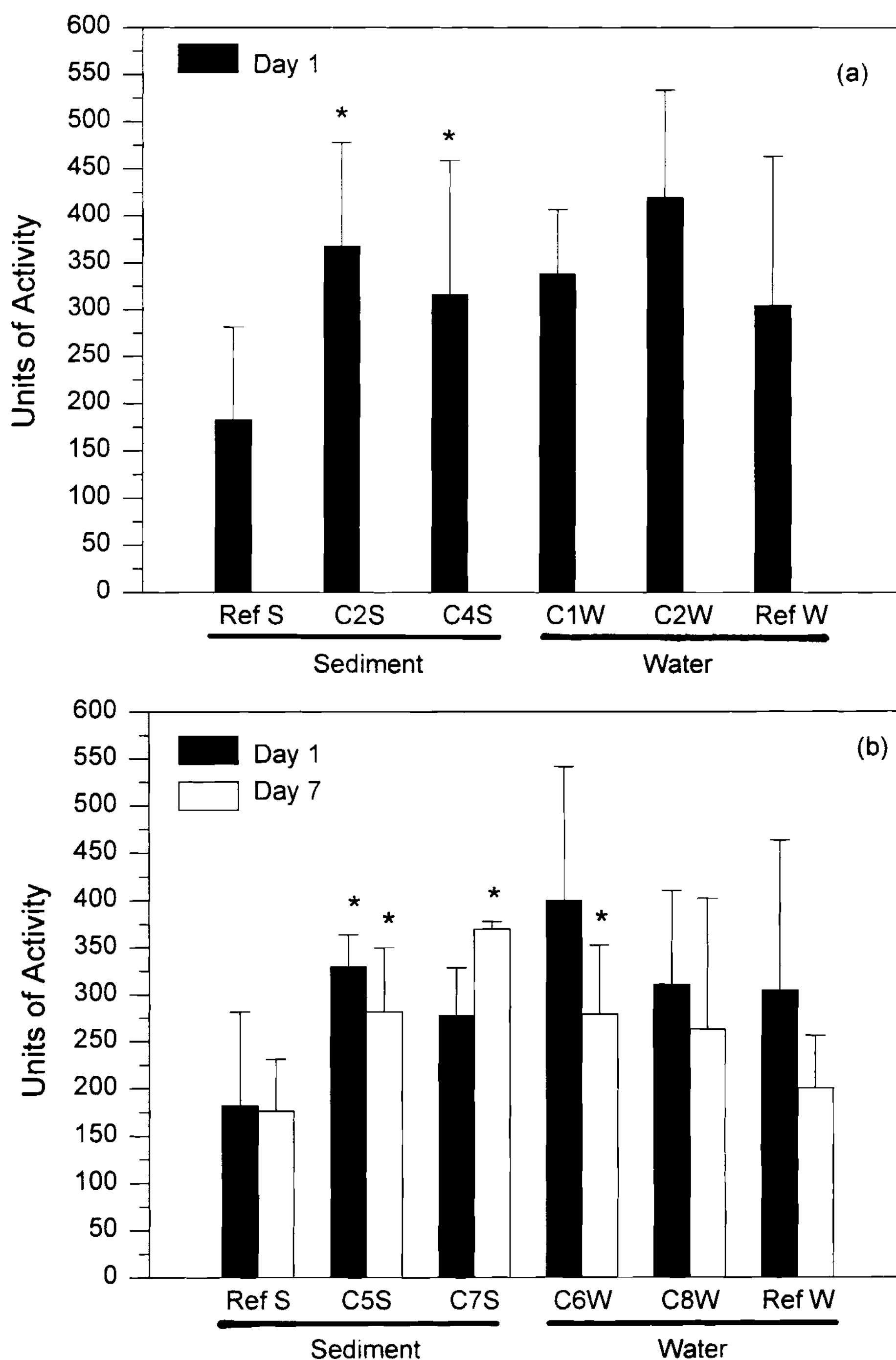

Figure 4. ALAD concentrations (±SD) measured in catfish at the reference site and (a) ST-6 and (b) ST-7. (* $P < 0.05$).

ALAD in the fish exposed at the contaminated site for either sampling date. The short exposure time of our study may have been a factor; however, Dieter and Finley (1978) reported the inhibition of the ALAD was rapidly induced in mallard ducks (*Anas platyrhynchos*) exposed to lead shot pellet. This inhibition did not return to normal levels for approximately four months. In addition, Berglind (1986) reported that when Cd and Zn are present as complex mixtures with Pb in the environment, the inhibitory effects of Pb on ALAD may be masked. Dwyer and colleagues (1988) measured Zn, Cd, and Pb concentrations in longear sunfish (*Lepomis megalotis*) exposed to mine tailings, and reported an inhibition of ALAD. The main difference between their results of ALAD inhibition and ours was that we measured 4 times the Zn in the liver samples as they detected, perhaps enough to mask the ALAD inhibition response. In addition, a stimulation of ALAD activity was observed in our study, similar to the stimulation of ALAD reported by Stirk (1973) in Japanese quail (*Coturnix c. japonica*) exposed to organic chemicals.

The formation of ALA is a rate limiting factor in the regulation of heme synthesis; as ALA increases, heme increases. Under normal conditions, the heme synthesis pathway is regulated by a feedback mechanism which will inhibit the formation of ALA when heme reaches a threshold. However, when an organism is exposed to certain environmental contaminants, control mechanisms break down, resulting in an increased accumulation of ALA,

which may cause increased levels of ALAD such as we observed in this study. The breakdown of control mechanisms has been observed with exposures to unsaturated hydrocarbons (DeMatteis and Lim, 1994). Many of these organic compounds are found in both ST-6 and ST-7 (Table 4).

At day 1, hemoglobin (Hb) levels were significantly higher in all water exposure fish, except cage 2, than the reference cages. No significant differences in Hb concentrations were observed among the sediment cages at day 1 (Fig. 5). However by day 7 post-exposure, hemoglobin levels were significantly elevated over the reference cages in fish from ST-7 sediment cages. The significant increase in levels of Hb were correlated with the increased ALAD activity, similar to reports by DeMatteis and Lim (1993).

Glucose concentrations for all fish in ST-6 were significantly higher than those of the reference fish (Fig. 6a). These levels were nearly 2 times those observed in ST-7, which implies increased stressful conditions in ST-6. This may be a factor contributing to acute lethality of fish by day 7 sampling in this pond. A classic stress response was observed in the fish from cages 5, 6, and 7 of ST-7 (Fig. 6b). At day 1, glucose concentrations were higher than those measured in the reference fish, possibly indicating glucose mobilization due to chemical (heavy metal or hydrocarbon) stressors. By day 7, decreases in concentrations of plasma glucose were observed. These responses correlate with similar responses seen by the authors at an abandoned coal stripmine site (Chapter II) and in channel catfish acutely

Table 3. Organic compounds detected in API separator sludge waste of ST-6 and ST-7 at Cyril (pers. comm. Linda Lyhane, Oklahoma State Dept. of Health).

Compound	Concentration (ppm)	
	ST-6	ST-7
Ethyl benzene	89	15
Toluene	10	41
Xylene	135	130
2-Methylnapthalene	210	53
Phenanthrene	4.5	7.6
Napthalene	9.9	4.3

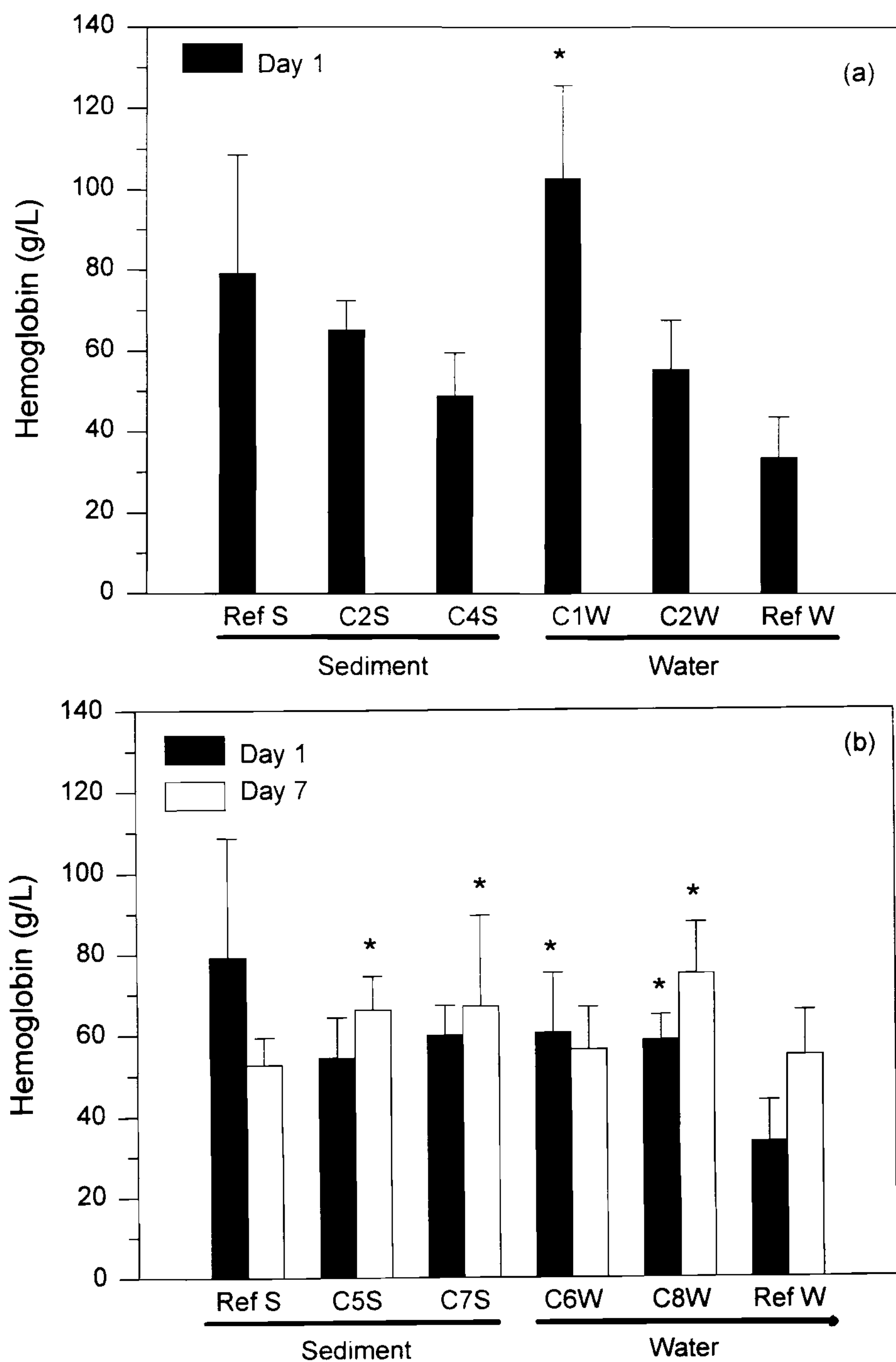

Figure 5. Hemoglobin concentrations (±SD) measured in catfish for reference and (a) ST-6 and (b) ST-7 sites. (* $P < 0.05$).

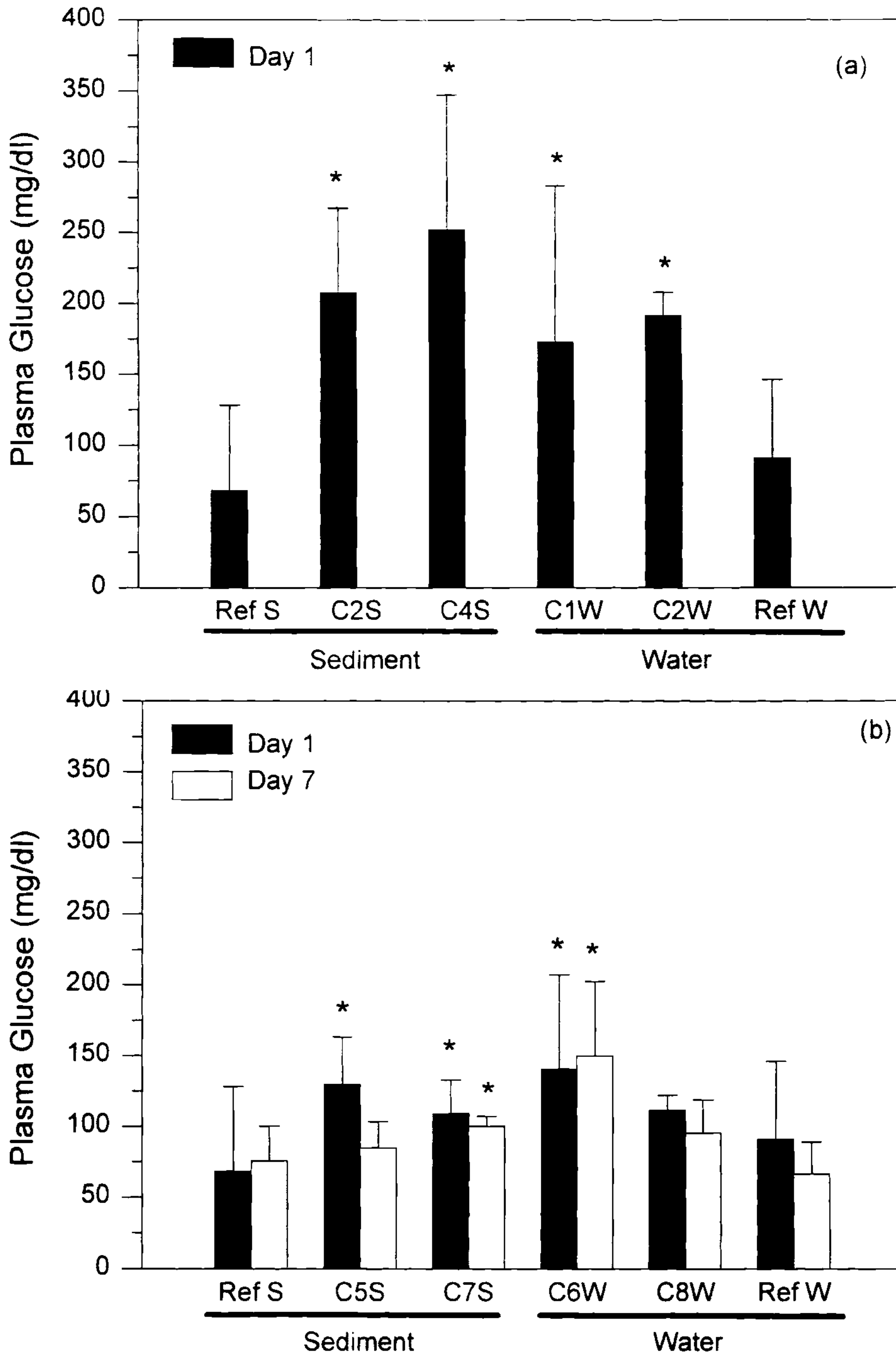

Figure 6. Glucose concentrations (±SD) measured in catfish for reference and (a) ST-6 and (b) ST-7 sites. (* $P < 0.05$).

exposed to etomidate (Limsuwan et al., 1983).

Plasma chloride concentrations were significantly lower in the fish from one water exposure cage from ST-7 (Fig. 7). No other significant differences were observed in plasma chlorides measured in fish from ST-6 or ST-7, compared to measurements from reference fish. The decreased chloride is thought to occur as a result of losses of chloride ions across heavy metal-damaged gill membranes (Heath, 1987).

No significant differences were observed among the suite of remaining assays: DNA strand breakage, hematocrit, condition factor, liver somatic index, and spleen somatic index. When performing the general necropsy, the only noticeable difference between the experimental and the reference fish was that the stomach and intestines of the experimental fish contained less food stuffs than those of the reference. This may be due to reduced food sources in the contaminated ponds or perhaps a reduced ability of stressed fish to successfully capture prey items.

Conclusions

In attempts to test the usefulness of various biomarkers as indicators of environmental stress in channel catfish, non-stressed baseline measurements need to be identified. This data is important when trying to compare biomarker data from the experimental to the reference sites, especially if the reference site contains low concentrations of a xenobiotic.

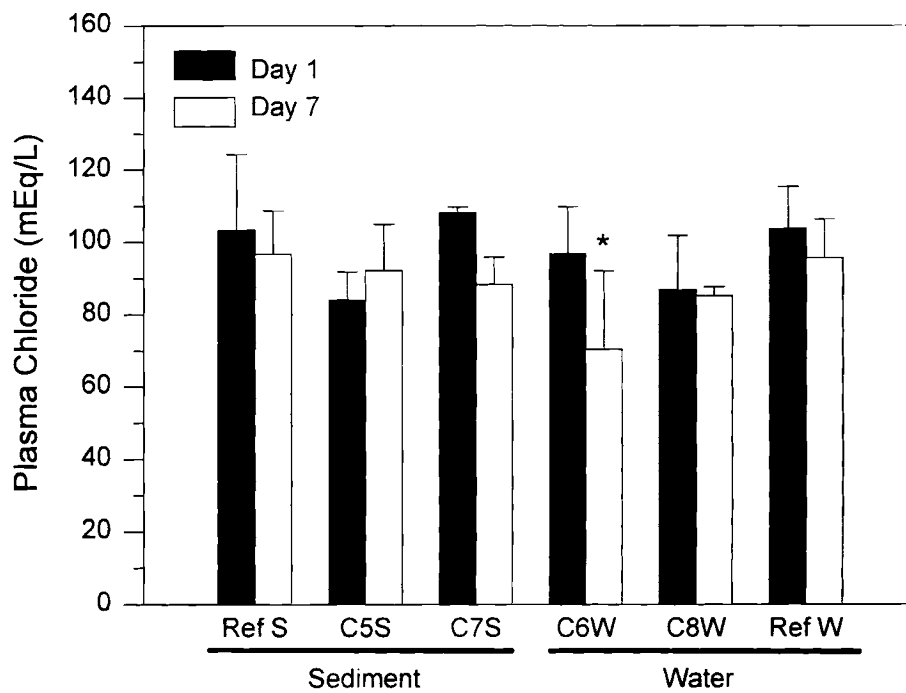

Figure 7. Plasma chloride concentrations (±SD) measured in catfish for reference and ST-7 sites. (* $P < 0.05$).

Once non-stressed baseline data have been established, these measurements can then be compared to marker measurements from *in situ* exposures.

Although data from exposure durations do not provide substantial proof, it appears that the Hb, plasma glucose, plasma chlorides, and ALAD may be suitable assays for measuring acute stress in fish exposed to refinery wastes. The remaining assays (hematocrit, DNA strand breakage, condition factor, and organo-somatic indices) may have shown significance if the study had continued for a longer time span. We did not feel that two time points were adequate to conclude whether biomarker responses correlate with increases in metal accumulations, therefore no correlation between concentration and response can be made. Additional studies conducted by the authors (Martin and Black, Chapter II) and others (Schmitt et. al., 1993; Depledge and Fossi, 1994) have revealed a tremendous amount of individual variability in biomarker responses to a stressor. Thus, two time points would not provide a true understanding of biomarker response trends. However, we did find limited evidence to support that exposure routes affect organismal stress levels, primarily in hematological responses (Hb and ALAD).

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APPENDIX A

CATFISH NECROPSY SHEET

DATE:________ CAGE DUR.______ CAGE #___

LENGTH_____mm WEIGHT___g FISH #_____ SEX M F

BLOOD PARAMETERS:
HCT________

GLUCOSE________

CHLORIDE________

HEMOGLOBIN______

CONDITION FACTORS:
LIVERSOMATIC INDEX_______

SPLENOSOMATIC INDEX______

GONADOSOMATIC INDEX_____

VISCEROSOMATIC INDEX_____

ORGAN WEIGHT (g):
LIVER_______

SPLEEN_______

VISCERA_______

GONAD_______

KIDNEY_______

HEART_______

VISCERAL FAT_______

DAMAGE TO EXTREMITIES:
- 0 - all fins and other extremities intact
- 1 - previous damage, healed over
- 2 - current damage/slight hemorrhaging
  location: fins; skin
- 3 - extensive tissue degradation;
  possibly with severe hemorrhaging and secondary infection

EYES:
- 0 - normal
- 1 - blind
- 2 - hemorrhagic
- 3 - exophthalmic
- 4 - missing

GILLS:
- 0 - normal
- 1 - frayed
- 2 - clubbed
- 3 - pale
- 4 - marginate

SPLEEN:
- 0 - red
- 1 - pale
- 2 - black
- 3 - granular
- 4 - enlarged

HIND GUT INFLAMMATION:
- 0 - no inflammation
- 1 - mild inflammation
- 2 - severe inflammation

KIDNEY:
- 0 - normal
- 1 - swollen
- 2 - mottled
- 3 - granular

LIVER:
- 0 - pale
- 1 - pale red
- 2 - fatty
- 3 - nodules present
- 4 - focal discoloration
- 5 - general discoloration

FEEDING STATUS:
- 0 - full stomach and intestines
- 1 - 1/2 or 1/4 full stomach and intestines
- 2 - empty stomach
- 3 - empty intestines

BILE:
- 0 - bile straw-yellow; gall bladder semi-full/empty
- 1 - bile yellow; bladder fully distended with bile
- 2 - bile light to grass-green bladder full
- 3 - bile dark green to blue-green

MESENTERIC (VISCERAL) FAT DEPOSITS:
- 0 - no fat deposits present
- 1 - 0 to 25% fat covering
- 2 - 25 to 50% pyloric caeca
- 3 - 50 to 75%
- 4 - caeca 100% fat covered

NOTES:____

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APPENDIX B

Mean hematocrit values (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Hematocrit (% RBC)				
EXPERIMENTAL		Days Post Exposure				
Pond 1		1	7	28	56	84
water	1	35.0 (11.9)	33.8 (4.8)	29.7 (6.2)	28.0 (2.0)	NA
sediment	2	35.3 (4.5)	35.0 (8.4)	32.5 (4.0)	31.0 (2.8)	NA
sediment	3	32.2 (5.9)	33.0 (2.7)	35.2 (4.0)	33.3 (4.9)	28.8 (1.5)
water	4	41.7 (4.7)	27.3 (2.9)	37.7 (3.0)	27.3 (1.4)	33.3 (1.2)
Pond 2						
water	5	30.0 (1.4)	30.5 (2.2)	27.0 (5.2)	31.0 (3.4)	NA
water	6	28.4 (4.2)	28.7 (1.5)	31.7 (4.5)	26.3 (8.2)	NA
REFERENCE						
LCB Pond 11						
water	1-3	35.4 (5.5)	35.7 (4.7)	28.6 (4.0)	30.6 (5.8)	30.9 (2.7)
sediment	4-6	40.1 (4.2)	32.9 (5.7)	30.6 (4.3)	25.8 (5.9)	32.2 (4.0)

NA No values for this time

Mean hemoglobin values (±SD) measured in fish fromfrom exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Hemoglobin (g/L)				
EXPERIMENTAL		Days Post Exposure				
Pond 1		1	7	28	56	84
water	1	69.4 (22.9)	79.9 (9.6)	75.2 (8.3)	34.9 (7.1)	NA
sediment	2	71.5 (31.9)	73.7 (9.5)	81.9 (11.3)	45.6 (0.9)	NA
sediment	3	54.5 (31.2)	75.2 (10.2)	78.8 (8.9)	46.7 (4.7)	65.9 (5.6)
water	4	56.2 (20.3)	66.7 (12.7)	84.1 (8.9)	44.7 (9.5)	72.2 (1.1)
Pond 2						
water	5	64.8 (0.0)	74.7 (9.3)	76.9 (19.0)	46.1 (3.9)	NA
water	6	54.9 (7.2)	62.9 (10.0)	78.3 (5.1)	41.1 (2.4)	NA
REFERENCE						
LCB Pond 11						
water	1-3	80.4 (16.8)	75.9 (7.0)	42.8 (6.2)	50.4 (5.6)	66.4 (4.4)
sediment	4-6	79.9 (11.2)	78.9 (8.6)	45.5 (9.6)	46.6 (6.8)	66.5 (7.6)

NA No values for this time

Mean aminolevulinic acid dehydratase values (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		ALAD (Units of Activity)				
EXPERIMENTAL		Days Post Exposure				
Pond 1		1	7	28	56	84
water	1	188.0 (107.3)	305.1 (125.1)	305.5 (108.9)	258.9 (8.6)	NA
sediment	2	139.1 (63.6)	299.5 (54.5)	437.0 (77.3)	296.1 (2.9)	NA
sediment	3	235.9 (104.3)	271.7 (32.9)	412.6 (120.8)	315.8 (34.9)	NA
water	4	216.9 (105.5)	321.4 (102.9)	361.5 (121.8)	305.0 (34.9)	NA
Pond 2						
water	5	317.6 (32.3)	298.1 (75.9)	356.1 (57.2)	317.9 (71.6)	NA
water	6	331.0 (61.5)	244.7 (49.9)	367.5 (64.6)	367.6 (166.9)	NA
REFERENCE						
LCB Pond 11						
water	1-3	160.4 (33.9)	345.8 (75.7)	289.2 (80.9)	362.2 (96.9)	506.0 (72.5)
sediment	4-6	221.4 (26.9)	286.2 (59.7)	284.3 (50.4)	316.9 (62.2)	509.0 (72.5)

NA No values for this time

Mean plasma glucose values (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site

Exposure/Cage#		Plasma Glucose (mg/dl)				
EXPERIMENTAL		Days Post Exposure				
Pond 1		1	7	28	56	84
water	1	171.1 (92.7)	43.2 (10.1)	88.4 (20.4)	52.6 (5.4)	NA
sediment	2	112.6 (47.5)	40.8 (9.8)	115.9 (25.7)	123.3 (81.5)	NA
sediment	3	150.7 (51.6)	46.1 (17.5)	147.2 (24.6)	117.3 (84.7)	53.2 (25.5)
water	4	219.5 (37.9)	50.8 (10.1)	121.8 (16.3)	70.3 (12.4)	58.5 (8.2)
Pond 2						
water	5	222.8 (22.1)	74.4 (55.9)	123.2 (13.4)	157.8 (28.9)	NA
water	6	142.8 (27.90	64.4 (18.0)	153.9 (26.3)	147.7 (16.2)	NA
REFERENCE						
LCB Pond 11						
water	1-3	106.0 (26.5)	122.7 (41.9)	89.1 (26.5)	64.2 (22.7)	36.4 (14.2)
sediment	4-6	69.5 (27.9)	109.02 (22.9)	90.5 (22.1)	56.3 (15.8)	28.7 (10.0)

NA No values for this time

Mean plasma chloride values (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Plasma Chloride (mEq/L)				
EXPERIMENTAL		Days Post Exposure				
Pond 1		1	7	28	56	84
water	1	103.9 (5.1)	109.9 (9.3)	106.1 (3.9)	133.3 (2.7)	NA
sediment	2	90.6 (25.1)	118.9 (7.9)	91.1 (27.9)	125.0 (9.6)	NA
sediment	3	82.2 (28.3)	122.1 (7.3)	104.4 (2.9)	114.2 (14.8)	102.4 (17.5)
water	4	103.5 (6.3)	108.9 (16.4)	106.1 (6.9)	109.1 (13.5)	96.6 (5.1)
Pond 2						
water	5	99.6 (1.2)	107.7 (7.7)	105.8 (0.0)	103.2 (16.6)	NA
water	6	96.1 (3.9)	116.0 (7.7)	105.8 (0.0)	103.2 (16.6)	NA
REFERENCE						
LCB Pond 11						
water	1-3	111.3 (13.5)	95.8 (8.5)	105.9 (7.3)	129.4 (32.4)	102.5 (10.7)
sediment	4-6	117.1 (4.5)	91.1 (5.9)	107.6 (4.6)	113.9 (28.2)	104.7 (12.3)

NA No values for this time

Mean liver DNA strand lengths (±SD) under denaturing (alkaline) conditions for fish livers from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Denaturing DNA Strand Length				
EXPERIMENTAL		Days Post Exposure				
Pond 1		1	7	28	56	84
water	1	35.9 (0.9)	33.4 (0.3)	NA	30.6 (0.4)	NA
sediment	2	35.8 (0.8)	32.8 (0.04)	32.6 (0.5)	31.2 (0.3)	NA
sediment	3	32.6 (0.7)	26.6 (0.3)	24.7 (4.2)	24.8 (3.2)	NA
water	4	32.8 (.04)	25.7 (.03)	25.7 (5.9)	25.8 (1.4)	NA
Pond 2						
water	5	32.9 (0.4)	32.2 (.02)	31.9 (6.3)	26.0 (1.0)	NA
water	6	31.7 (0.4)	32.9 (0.9)	31.6 (2.3)	28.9 (3.5)	NA
REFERENCE						
LCB Pond 11						
water	1-3	33.9 (0.8)	31.0 (3.3)	31.6 (2.3)	28.9 (3.5)	NA
sediment	4-6	32.4 (1.9)	32.1 (4.3)	36.7 (10.9)	23.8 (1.4)	NA

NA No values for this time

Mean liver DNA strand lengths (±SD) under non-denaturing (neutral) conditions for fish livers from experimental and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Non-Denaturing DNA Strand Length				
EXPERIMENTAL		Days Post Exposure				
Pond 1		1	7	28	56	84
water	1	37.45 (2.1)	30.6 (0.9)	NA	25.6 (5.7)	NA
sediment	2	37.0 (20.9)	33.0 (0.3)	30.2 (1.0)	17.5 (0.7)	NA
sediment	3	33.3 (1.9)	31.8 (1.2)	18.7 (4.4)	13.6 (2.5)	NA
water	4	30.4 (5.8)	29.9 (0.4)	25.6 (7.2)	22.4 (9.0)	NA
Pond 2						
water	5	42.4 (4.6)	10.6 (6.9)	22.6 (5.5)	18.8 (8.4)	NA
water	6	41.4 (5.2)	29.8 (4.2)	24.9 (4.4)	20.5 (7.3)	NA
REFERENCE						
LCB Pond 11						
water	1-3	34.9 (6.9)	33.8 (2.4)	30.2 (3.4)	21.2 (4.2)	NA
sediment	4-6	27.6 (7.8)	31.3 (3.1)	28.7 (8.2)	20.1 (3.9)	NA

NA No values for this time

Mean spleen somatic index (±SD) for fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Spleen Somatic Index				
EXPERIMENTAL		Days Post Exposure				
Pond 1		1	7	28	56	84
water	1	0.08 (0.02)	0.11 (0.06)	NA	0.08 (0.03)	NA
sediment	2	0.10 (0.01)	0.09 (0.02)	0.08 (0.02)	0.08 (0.02)	NA
sediment	3	0.07 (0.01)	0.10 (0.02)	0.07 (0.02)	0.08 (0.02)	0.09 (0.01)
water	4	0.09 (0.03)	0.09 (0.02)	0.07 (0.01)	0.09 (0.1)	0.07 (0.02)
Pond 2						
water	5	0.09 (0.03)	0.09 (0.02)	0.08 (0.03)	0.07 (0.02)	NA
water	6	0.09 (0.02)	0.09 (0.02)	0.06 (0.02)	0.09 (0.01)	NA
REFERENCE						
LCB Pond 11						
water	1-3	0.08 (0.04)	0.09 (0.02)	0.08 (0.04)	0.12 (0.06)	0.12 (0.09)
sediment	4-6	0.14 (0.19)	0.09 (0.02)	0.14 (0.21)	0.11 (0.07)	0.11 (0.03)

NA No values for this time

Mean liver somatic index (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Treatment/Cage#		Liver Somatic Index				
EXPERIMENTAL		Days Post Exposure				
Pond 1		1	7	28	56	84
water	1	1.2 (0.19)	1.9 (0.28)	NA	1.4 (0.19)	NA
sediment	2	1.7 (0.38)	5.9 (0.69)	1.3 (0.17)	1.3 (0.03)	NA
sediment	3	1.2 (0.08)	4.9 (1.63)	1.3 (0.16)	1.2 (0.07)	1.2 (0.17)
water	4	1.1 (0.06)	5.6 (0.75)	1.1 (0.03)	1.1 (0.12)	1.2 (0.05)
Pond 2						
water	5	1.3 (0.19)	1.1 (0.09)	3.2 (0.33)	1.2 (0.08)	NA
water	6	1.2 (0.14)	3.3 (1.54)	1.5 (0.13)	1.4 (0.11)	NA
REFERENCE						
LCB Pond 11						
water	1-3	1.5 (0.22)	2.9 (0.52)	1.8 (0.43)	1.4 (0.31)	1.7 (0.49)
sediment	4-6	1.6 (0.23)	2.9 (0.51)	1.6 (0.42)	1.6 (0.28)	2.0 (0.75)

NA No values for this time

Mean condition factor values (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Condition Factor				
EXPERIMENTAL		Days Post Exposure				
Pond 1		1	7	28	56	84
water	1	0.01 (0.001)	0.01 (0.001)	0.01 (0.002)	0.008 (0.005)	NA
sediment	2	0.028 (0.01)	0.003 (0.002)	0.002 (0.001)	0.001 (0.004)	NA
sediment	3	0.013 (0.01)	0.005 (0.002)	0.002 (0.001)	0.003 (0.001)	0.002 (0.001)
water	4	0.02 (0.01)	0.005 (0.002)	0.005 (0.002)	0.003 (0.002)	0.001 (0.0002)
Pond 2						
water	5	0.014 (0.01)	0.018 (0.01)	0.006 (0.004)	0.0012 (0.001)	NA
water	6	0.011 (0.01)	0.003 (0.001)	0.0011 (0.001)	0.0012 (0.001)	NA
REFERENCE						
LCB Pond 11						
water	1-3	0.02 (0.01)	0.012 (0.01)	0.012 (0.01)	0.009 (0.005	0.007 (0.003)
sediment	4-6	0.02 (0.01)	0.015 (0.01)	0.013 (0.01)	0.01 (0.004)	0.009 (0.006)

NA No values for this time

APPENDIX C

Mean hematocrit values (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Hematocrit (%RBC)	
EXPERIMENTAL		Days Post Exposure	
ST-6		1	7
water	1	27.3 (4.8)	NA
sediment	2	28.8 (2.3)	NA
water	3	26.5 (4.6)	NA
sediment	4	26.2 (3.2)	NA
ST-7			
sediment	5	26.3 (3.7)	29.6 (1.9)
water	6	26.7 (5.4)	30.0 (3.6)
sediment	7	27.7 (2.5)	29.3 (3.9)
water	8	27.0 (2.2)	32.4 (3.6)
REFERENCE			
LCB Pond 11			
water	1-4	27.0 (4.2)	28.7 (5.9)
sediment	5-8	26.5 (3.4)	29.5 (4.5)

NA No values for this time

Mean hemoglobin values ($\pm$SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Hemoglobin (g/L)	
EXPERIMENTAL		Days Post Exposure	
ST-6		1	7
water	1	102.6 (23.1)	NA
sediment	2	65.3 (7.2)	NA
water	3	55.5 (12.2)	NA
sediment	4	48.9 (10.7)	NA
ST-7			
sediment	5	54.3 (9.9)	65.9 (8.3)
water	6	60.5 (14.7)	56.2 (10.4)
sediment	7	59.8 (7.1)	66.8 (22.6)
water	8	58.5 (6.3)	74.8 (12.7)
REFERENCE			
LCB Pond 11			
water	1-4	33.4 (10.2)	54.7 (11.0)
sediment	5-8	79.3 (29.4)	52.7 (6.6)

NA No values for this time

Mean aminolevulinic acid dehydratase values (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		ALAD (Units of Activity)	
EXPERIMENTAL		Days Post Exposure	
ST-6		1	7
water	1	338.2 (68.4)	NA
sediment	2	368.1 (109.9)	NA
water	3	419.7 (113.9)	NA
sediment	4	315.8 (142.9)	NA
ST-7			
sediment	5	329.3 (34.3)	281.6 (68.4)
water	6	399.9 (158.7)	278.9 (73.6)
sediment	7	277.5 (50.9)	369.8 (98.1)
water	8	310.9 (99.2)	262.6 (139.2)
REFERENCE			
LCB Pond 11			
water	1-4	304.4 (158.7)	200.1 (55.7)
sediment	5-8	182.3 (99.3)	176.7 (54.9)

NA No values for this time

Mean plasma glucose values (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		GLUCOSE (mg/dl)	
EXPERIMENTAL		Days Post Exposure	
ST-6		1	7
water	1	172.7 (110.2)	NA
sediment	2	207.4 (59.7)	NA
water	3	191.2 (16.5)	NA
sediment	4	251.8 (95.7)	NA
ST-7			
sediment	5	129.8 (33.6)	84.8 (18.7)
water	6	140.8 (66.4)	149.8 (52.9)
sediment	7	109.1 (23.9)	100.2 (7.1)
water	8	111.7 (10.6)	95.4 (23.4)
REFERENCE			
LCB Pond 11			
water	1-4	91.2 (54.9)	66.3 (22.7)
sediment	5-8	68.4 (13.3)	75.6 (24.8)

NA No values for this time

Mean plasma chloride values (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Plasma Chloride (mEq/L)	
EXPERIMENTAL		Day Post Exposure	
ST-6		1	7
water	1	107.3 (21.8)	NA
sediment	2	84.7 (10.2)	NA
water	3	89.6 (24.7)	NA
sediment	4	86.6 (20.9)	NA
ST-7			
sediment	5	84.3 (7.7)	92.4 (12.6)
water	6	96.8 (13.2)	70.4 (21.8)
sediment	7	108.3 (1.5)	88.4 (7.5)
water	8	86.9 (14.9)	85.2 (2.5)
REFERENCE			
LCB Pond 11			
water	1-4	103.1 (11.8)	95.8 (10.7)
sediment	5-8	103.3 (21.1)	96.7 (12.1)

NA No values for this time

Mean denaturing DNA strand lengths (±SD) measured in fish livers from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		DNA Strand length	
EXPERIMENTAL		Days Post Exposure	
ST-6		1	7
water	1	22.1 (0.56)	NA
sediment	2	20.8 (1.13)	NA
water	3	20.4 (0.64)	NA
sediment	4	20.8 (1.09)	NA
ST-7			
sediment	5	26.7 (1.15)	22.6 (0.60)
water	6	26.2 (0.91)	23.0 (0.33)
sediment	7	24.6 (1.05)	NA
water	8	25.9 (0.51)	NA
REFERENCE			
LCB Pond 11			
water	1-4	22.5 (0.91)	22.6 (0.43)
sediment	5-8	25.0 (0.96)	22.4 (0.37)

NA No values for this time

Mean non-denaturing DNA strand lengths (±SD) measured in fish liversfrom exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		DNA Strand Length	
EXPERIMENTAL		Days Post Exposure	
ST-6		1	7
water	1	22.0 0.56)	NA
sediment	2	20.5 1.13)	NA
water	3	19.0 (0.64)	NA
sediment	4	19.0 (0.91)	NA
ST-7			
sediment	5	19.0 (1.15)	19.0 (0.60)
water	6	19.0 (0.91)	19.0 (0.33)
sediment	7	19.5 (1.05)	NA
water	8	19.5 (0.51)	NA
REFERENCE			
LCB Pond 11			
water	1-4	22.5 (0.91)	22.5 (0.43)
sediment	5-8	22.0 (0.96)	22.0 (0.37)

NA No values for this time

Mean spleen somatic index values (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Spleen Somatic Index	
EXPERIMENTAL		Days Post Exposure	
ST-6		1	7
water	1	0.06 (0.01)	NA
sediment	2	0.06 (0.01)	NA
water	3	0.07 (0.02)	NA
sediment	4	0.06 (0.02)	NA
ST-7			
sediment	5	0.06 (0.01)	0.08 (0.01)
water	6	0.06 (0.01)	0.09 (0.03)
sediment	7	0.06 (0.01)	0.08 (0.02)
water	8	0.07 (0.02)	0.08 (0.02)
REFERENCE			
LCB Pond 11			
water	1-4	0.07 (0.02)	0.12 (0.15)
sediment	5-8	0.07 (0.02)	0.09 (0.02)

NA No values for this time

Mean liver somatic index values (±SD) measured in fish from exposure and reference sites. Values represent six fish pooled per sampling time and site.

Exposure/Cage#		Liver Somatic Index	
EXPERIMENTAL		Days Post Exposure	
ST-6		1	7
water	1	2.03 (0.48)	NA
sediment	2	2.00 (0.45)	NA
water	3	1.80 (0.34)	NA
sediment	4	1.98 (0.55)	NA
ST-7			
sediment	5	2.26 (0.42)	1.97 (0.19)
water	6	2.23 (0.65)	1.92 (0.13)
sediment	7	2.06 (0.47)	2.07 (0.29)
water	8	2.10 (0.59)	1.52 (0.74)
REFERENCE			
LCB Pond 11			
water	1-4	2.26 (0.39)	1.48 (0.17)
sediment	5-8	2.54 (0.66)	1.60 (0.26)

NA No values for this time

VITA

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