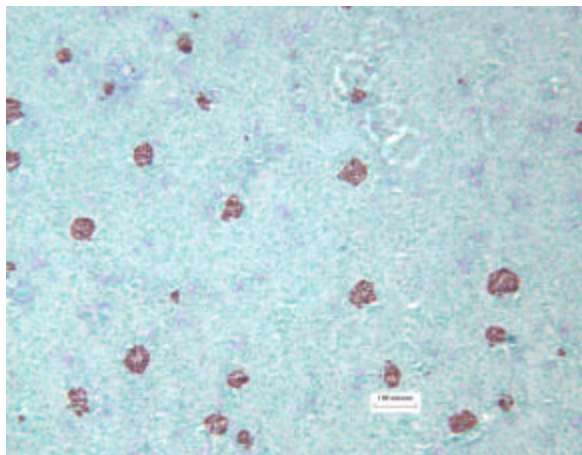![USGS logo](science for a changing world)

National Wetlands Research Center, USGS, Lafayette

Fish Bioindicators of Ecosystem Condition at the Calcasieu Estuary, Louisiana

by Jill A. Jenkins

Open-File Report 2004-1323

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Outside front cover photographs:

Histological section showing splenic macrophage aggregates from a black drum sampled in April 2000 from the Calcasieu Estuary, Louisiana. Magnification bar equals 100 microns. Photomicrograph was taken with a Leica Diaplan microscope using Image Pro image analysis. (left)

Menhaden with a tumor dorsally located near the head. Fish was sampled near the mouth of Bayou D'Inde in the Calcasieu Estuary.

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Preface

In late 1999 and 2000, the U.S. Environmental Protection Agency (EPA) was responsible for a Remedial Investigation/Feasibility Study (RI/FS) at the Calcasieu Estuary. The RI/FS involved the investigation and characterization of organic and inorganic chemical contamination, as well as assessment of human health and ecological risk and alternatives to mitigate unacceptable levels of environmental contaminants. This has been a cooperative effort involving EPA, Louisiana Department of Environmental Quality, National Oceanic and Atmospheric Administration, the Lafayette, Louisiana Ecological Services Field Office of the U.S. Fish and Wildlife Service (FWS), the National Wetlands Research Center (NWRC), Biological Resources Discipline, U.S. Geological Survey (USGS), and EPA contractors (CDM Federal Programs Corporation). Chemical contamination, primarily from local industrial activities, had been detected in the surface water, sediment, fish, and crustacea in previous studies. The goal of this study (Agreement No. 4-2000-BF04) was to assess fish and their biomarkers as indicators of possible adverse impacts that are due to contaminants in the environmental media from designated areas of concern.

This report describes and documents all biomarker datasets collected in the 2000 fish health monitoring effort performed by USGS and FWS personnel at the Calcasieu Estuary. Site designation and fish collection was in accord with the overall field sampling plan authored by EPA and their contractors. Fish and fish parts were processed in the field by FWS Fisheries Resource- and NWRC-associated personnel. Further analyses were conducted at NWRC and some subcontractors.

The author thanks Randy Pausina and Karen Foote of the Louisiana Department of Wildlife and Fisheries for sharing their data on normal fish condition from the Gulf region, Louise Stanley for estimating ages per otoliths, Dave Nieland and Andy Fisher of the Coastal Fisheries Institute of Louisiana State University for assistance in interpretation age data, Don Tillett of USGS in Columbia, MO for liver enzyme data, Connie Kersten of McNeese State University for gonad histology, the NWRC field crew and especially Jaquie Craig for efforts on this project.

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Introduction

Exposure of feral populations of animals to environmental contaminants is a global problem, and fish populations offer appropriate models for examining the effects of contamination (Jenner and others, 1990). Since the 1920s, the Calcasieu Estuary in southwest Louisiana (Fig. 1) has been heavily industrialized by both petrochemical and agrochemical plants, and it has been affected by physical alterations such as the construction of the Calcasieu Ship Channel (completed in 1941). The area is a prime location for these industries because of the close proximity of petroleum and gas reserves and is a direct transportation route via the estuary to the Gulf of Mexico. The entire Calcasieu Estuary has been the subject of environmental studies dating back to the early 1970s (MacDonald and others, 2001a), where previous investigations indicate that surface water and sediment have inorganic and organic contaminants. In collaboration with the EPA and other stakeholders, the Baseline Ecological Risk Assessment (BERA) (MacDonald and others, 2001a; 2001b) details the complete sampling design for the overall project and the analytical processes designed to support risk management decision-making in the Calcasieu Estuary Initiative. These documents provide information characterizing the nature and extent of the contamination and estimate the risks to human health and the environment posed by the contaminants, and they describe the numbers, types, and locations of industrial inputs (see report for the National Oceanic and Atmospheric Administration (NOAA) (Curry and others, 1996).

Industrial contaminants are too numerous for a detailed listing here, but they include several heavy metals, semi-volatile organic compounds, volatile organic compounds, petroleum hydrocarbons, and chlorinated hydrocarbons (Curry and others, 1996; MacDonald and others, 2001a). In 1999, a screening-level ecological risk assessment was conducted (CDM, 1999), whereby historic levels of contaminants were researched and compared to current levels (Goldberg 2001). The substances of concern from the areas of concern (Table 1) listed over 100 contaminants, including metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organochlorine and other pesticides, chlorophenols, chlorinated benzenes, chlorinated ethanes, phthalates, cyanide, and acetone. The compounds of greatest concern to aquatic-dependent wildlife are those that are persistent and bioaccumulative, such as these found in the estuary: PCBs, polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), hexachlorobenzene (HCB), hexachlorobutadiene (HCBD), and organochlorine pesticides. Animals can receive either direct or indirect exposure to contaminated environmental "media," including water, soil, sediment, air-water interface, or air.

The estuary provides important habitat for resident and migratory waterfowl, shorebirds, and wading birds. Nongame migratory birds species include least bittern (*Ixobrychus exilis*), white ibis (*Eudocimus albus*), yellow rail (*Coturnicops noveboracensis*), and stilt sandpiper (*Micropalama himantopus*). Extensive emergent wetlands provide valuable spawning, nursery, and feeding habitat for several species of sport and commercial importance, including spotted trout (*Cynoscion nebulosus*), southern flounder (*Paralichthys lethostigma*), red drum (*Sciaenops ocellatus*), black drum (*Pogonias cromis*), blue crab (*Callinectes sapidus*), brown shrimp species, and white shrimp species. To assist the FWS in an assessment of its trust resources in the Calcasieu Estuary (for a list of threatened and endangered animals in or near the estuary, see MacDonald and others, 2001a), the goal of this project, as reported herein, was to assess the condition of fishes (Table 2) in the estuary.

Many agencies have incorporated various biological measures into their bioassessment programs to evaluate the quality of surface water resources (Adams, 2002). Relying on chemical criteria alone rather than on animal biological responses to intermingled stressors, may inaccurately portray the biological and ecological condition of aquatic systems. Some advantages
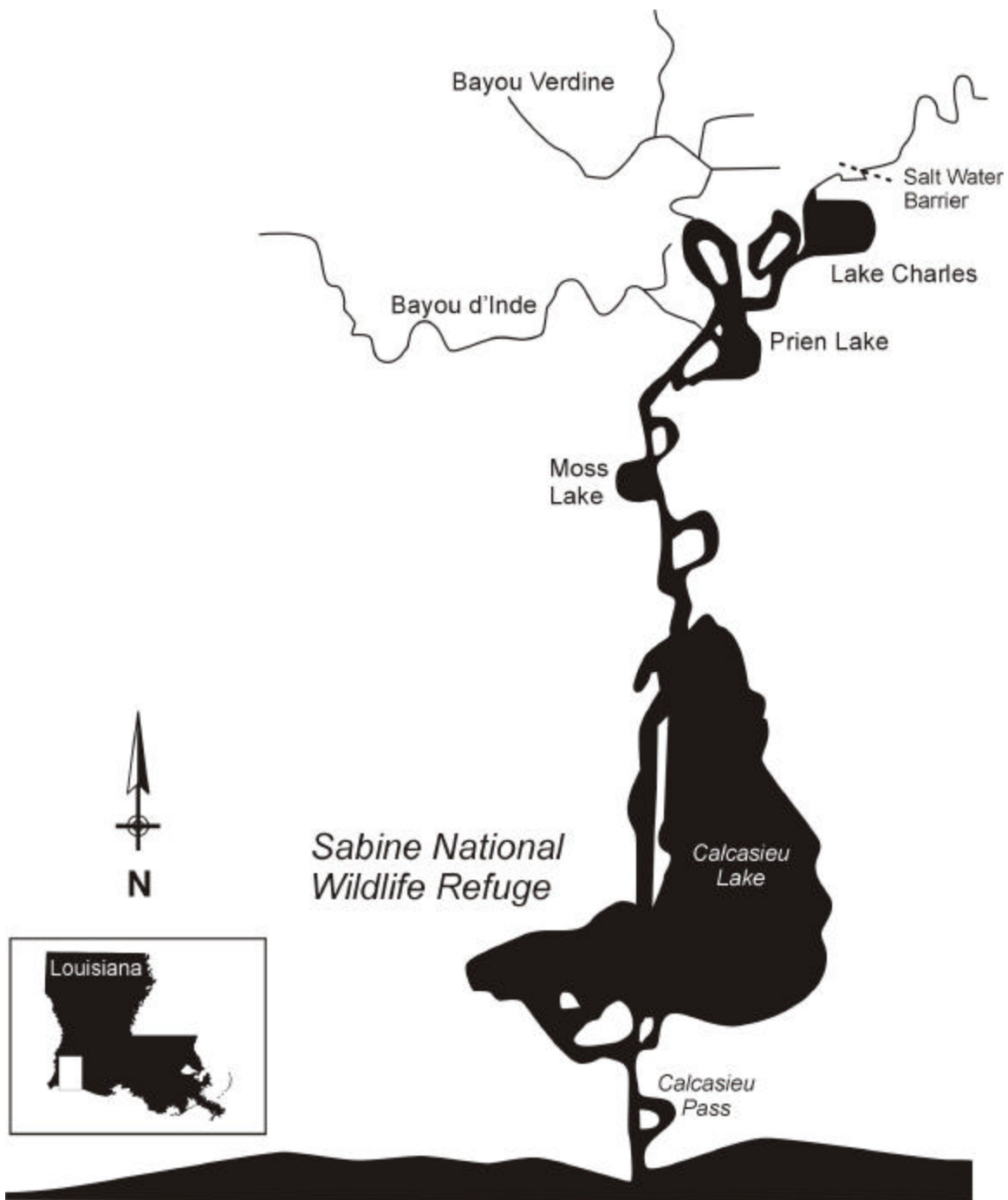
Figure 1. Site location map, Calcasieu Estuary, Louisiana.

Table 1. Biota sampling locations in the Calcasieu Estuary from north to south

Areas of Concern	Acronyms
Upper Calcasieu River (Lake Charles)	LACH
Clooney Island Loop	CLIL
Coon Island Loop (northeast)	CINE, C
Upper Bayou d'Inde	UBI
Middle Bayou d'Inde	MBI
Lower Bayou d'Inde (PPG and nearby wetlands)	LBI, D
Prien Lake (and old river channel)	PLRC
Middle Calcasieu River (Citgo surge pond)	MCR
Moss Lake (Bayou Olsen and Moss Lake)	ML, L
Choupique Bayou*	CHPQ
Bayou Bois Connine **	BBC
Grand Bayou**	GB
Sabine and Bayou Olsen	S

* This site was originally chosen as a reference site, but biomarker endpoints for this highly agricultural area disallowed this categorization.

**Reference sites also included Sabine National Wildlife Refuge (S). For more detailed location of sampling sites, see MacDonald and others, 2001a.

Table 2. Fish species collected during Phases I and II, forage, and life history* briefs.

Species	Forage	Spawning
Gulf Menhaden	Filter feeders; detritis, zooplankton.	Spawn Oct-Mar; fall migration to Gulf in fall; inshore Mar
Striped Mullet	Benthic and interface feeders; detritis, plants, crustaceans	Offshore spawn Nov-Dec
Sheepshead Minnow	Plants, invertebrates, vertebrates	Overwinter offshore; spring spawn
Speckled Gar	Insects, crustaceans, fish	Spawn April, in flowing water
Atlantic Croaker	Detritis, plants, invertebrates, fish	Estuarine-dependent; Spawn Oct-Mar, peak Nov; Gulf migratn Sep-Nov
Striped Bass	Primarily fish	Spring spawn, near-fresh water; anadromous
Gafftopsail Catfish	Detritis, plants, invertebrates, crustaceans, fish, garbage; bottom feeders	Spawn inshore mudflats May-Aug; males carry eggs 70 d; sediments neg.
Spotted Trout	Insects, crustaceans, fish	Estuarine spawn Apr-July
Red Drum	Crustaceans, shrimp, crab, fish	Spawn Sep-Nov; estuarine in summer
Black Drum	Zooplankton, crustaceans, crabs, oysters, molluscs, fish	Spawn Feb-Mar in estuaries; bimodal GSI
Southern Flounder	Invertebrates, shrimp, fish (mullet)	Spawn Nov-Mar (June)

* Life histories available in Lassuy, 1983.

of studying fishes in assessing ecosystem conditions include association of fish with the sediments and the water, association of each life stage with the environmental media, and their physiology integrates several biological response mechanisms. Fish are a crucial part of the food web, are generally less mobile than birds, and may serve as forage for waterfowl. The difficulties in studying fish are that they forage across a broad range, are nomadic, and may not be long-lived. As a comparative animal model, much data have been generated using fishes in aquatic toxicology and in environmental studies, so appropriate associations may often be drawn across species lines.

Many biological and physical stressors, in addition to chemical stressors, can impact aquatic organisms and aquatic-dependent wildlife. These stressors may be natural, such as daily or seasonal temperature fluctuations, or anthropogenic such as contaminants, thermal effluents, or physical habitat modifications. The Calcasieu Estuary serves as a host for several such stressors. Sublethal stress is generally first manifested at the suborganismal level, where effects can be measured via cellular components such as enzymes or functions, such as the immune system. Depending on its severity, sublethal stress may limit physiological systems, reduce growth, and impair reproduction. Biomarkers are functional measures of exposure to environmental stressors, which are usually expressed at the suborganismal level of biological organization (Adams, 2002). The magnitude of change is measurable and often related to the severity of the exposure. Evidence of biological exposures has been defined by the USEPA (1991) as those endpoints that measure the apparent effects of stressors, including chemical water quality criteria, tissue residues, and biomarkers.

The biomarker measurement strategy employed in this study was in keeping with the focus on the chemical stressors in Calcasieu Initiative, whereby risks to organisms in the estuary were compared to those of the reference sites. Animal responses to mixtures of chemicals are often different from those by individual chemicals (de Souza-Bueno and others, 2000). It is not possible to measure the impact by chemical stressors individually or the mixtures, without biological and physical stressor impacts; biomarkers that are sensitive and specific only to the source of exposure are rare (Sorensen and others, 2003). Therefore, the biomarker choice strategy was directed across several levels of biological organization (from animal condition to biomolecular responses) in the fishes, thereby assessing stressor impacts across a breadth of biological responses. Where lines of biomarker evidence reinforce each other, more confidence is placed in assessments of overall animal health and biological integrity of their ecosystems. In this study, biomarker data were collected on these biological levels of organization: genetic, biochemical, physiological, histopathological, immunological, and reproductive.

Genomic DNA alterations or fragmentations are widely used in physiological, genetic and toxicological studies. Many waterborne pollutants have cytogenetic properties which in fish cause enhanced frequency of chromosomal aberrations or the alteration of the structure of DNA. Alternations of DNA can be in the primary structure and is called "DNA damage," whereas the change in the base sequence is called "mutation." If alterations occur in germ cells, the DNA damage can be heritable. Measuring damage to DNA resulting from exposure to mutagens, carcinogens or cancer chemotherapy drugs is central to many studies in cancer biology, environmental health, and toxicology (Potter and others, 2002). There are strong correlations between DNA damage and the development of cancer, infertility, and poor sperm quality. Flow cytometry is a powerful tool for identifying aneugenic and clastogenic effects of environmental contaminants on the vertebrate (Al-Sabti, 1985; McBee and others, 1987; Jenner and others, 1990; Lamb and others, 1991; George and others, 1991; Lingenfelser and others, 1997; Lowcock and others, 1997; Whittier and McBee 1999; Choi and Meier 2000) and invertebrate (Klobucar and others, 2003; Gielazyn and others, 2003) genome.

Genotoxicants also can effect metabolic reactions, such as the cytochrome P450 induction, which detoxifies and can generate reactive oxygen intermediates, as well as generate metabolically activated products that may form adducts (another biomarker). Many PAHs are metabolized by cytochrome P450 monooxygenases (CYP) 1A1, which can result in metabolic activation with subsequent formation of PAH-adducts in DNA (Sorensen and others, 2003). Genotoxicants can induce Phase II enzymes which are responsible for glutathione conjugations and detoxifications. The mixed-function oxygenase enzyme system which plays roles in detoxification and molecular breakdown, is induced by contaminants. Reactive oxygen species can be generated by contaminants such as PAHs (Adams, 2002) and they are known to damage structures such as DNA (see Irvine and others, 2000).

The immunocompetence of fish depends on a complex set of coordinated events and on such factors as external temperature, sexual maturation (Saha and others, 2003), and anthropogenic disturbances (Sindermann, 1996). Blood cells are the mediators of defense mechanisms in animals, and WBCs are key components of innate immune defense (Jenkins and Ourth, 1993) where defense responses are measurable and influenced by stressors (Adams, 2002). In response to stressors in the aquatic environment, an overall drop in white blood cells (WBCs) could indicate immunosuppression. An overall increase could result in infection or reaction to stressors mediated by cortisol hormone response. In all teleosts, cortisol is the major corticosteroid produced under stress, and it has been indicated as the major factor that mediates the suppressive effects of stress on reproduction (Consten and others, 2002). Circulating levels of cortisol (another biomarker) are often used as an indicator of the degree of stress experienced by fish (Adams, 2002). Numbers of the neutrophil cell type, in response to increased circulating cortisol, are often indicative of stressful conditions or infectious disease (Ellsaesser and others, 1985; Ellsaesser and Clem, 1986).

Histopathological biomarkers, or cellular changes in tissues and organs, represent an integration of cumulative effects by physiological and biochemical stressors (Hinton and Lauren, 1990) and therefore can be linked to exposure (Myers and Fournie, 2002). The histopathological biomarker approach, linking lower to upper levels of biological organization, is most useful in multidisciplinary studies. Many lesions are well validated experimentally by exposure to specific stressors. Macrophage aggregates (MA) are reliable indicators of exposure to sediments contaminated with organics or low dissolved oxygen (Myers and Fournie 2002) and are more numerous in fish from contaminated sites (Fournie and others, 2001). MA are focal accumulations of macrophages found in the spleen, head kidney, liver, and sometimes testes. They change in number, size, and pigment, and they function to localize products of tissue destruction (see Wolke 1992). MA are areas of immune reactivity against foreign and dying cells that can wall off the area of infection. It has been established that many environmental chemicals are hormonally active and can impact reproduction (see Blazer, 2002) in terms of altered hormone levels, histopathology, and morphological changes such as gonadosomatic index (GSI). In this study, gonadal histology for determining sex (as per Patino, 1995) of representatives of the two predominant fish species in the Calcasieu Estuary sampling were compared with sex identification by visual morphology and their GSI from each species.

Because the nutritional status of fish can change according to season, related food availability and changes in feeding behavior, conducting sampling during the same season is preferable for control for such influences on studies involving organs (Myers and Fournie, 2002). Organosomatic indices are common approaches for assessing fish health (Adams and others, 1993), as ratios of organ weight to body weight, where the GSI is calculated according to gonad weight/total weight x 100. Depending on its severity, sublethal stress may limit physiological systems, reduce growth, and impair reproduction, where GSI is a convenient morphological index. The splenosomatic index is the ratio of the spleen weight to whole body weight. The spleen is an

organ through which blood is filtered, is involved with new blood cell development, and immunological interactions. Spleen swelling can indicate a pathobiological response. The liver is responsible for enzymatic decontamination processes, vitellogenin production, and stores glycogen as energy reserves. Stressors raise serum glucose, and liver glycogen stores can be depleted in adverse conditions.

Condition factor is an index of employed regularly in fisheries and is based on length and weight of the animal. Relative condition factor of fish (Kn) (Le Cren, 1951) assumes that heavier fish are in better condition than lighter fish of the same length (Sutton and others, 2000). Any stresses in the natural environment can have an effect on fish overall health and condition; therefore, Kn can be employed as an integative biomarker. Both anthropogenic and natural stressors are incorporated into Kn; however, there are natural fluctuations or differences in condition factor of fish due to species, sex, and season (e.g. temperature, spawning, photoperiod, prey quantity/quality) (Le Cren, 1951; Sutton and others, 2000; Björnsson and others, 1989), so special attention must be given to these variables when developing models.

Materials and Methods

Study Area

The Calcasieu Estuary is located in the vicinity of Lake Charles in Calcasieu Parish, Louisiana, through which the Calcasieu River flows to the Gulf of Mexico near Cameron, Louisiana. The areas of concern (AOC) are primarily the bayous in the northern part of the estuary, and according to the RI/FS, the primary study area was subdivided into the Upper Calcasieu River, Bayou d'Inde, and the Middle Calcasieu River, whereas the Lower Calcasieu River is less contaminated and was considered as a reference area (see Table 1 and Figure 1). An additional reference site was Sabine National Wildlife Refuge located to the west of the Calcasieu Estuary near the Texas border and connected to the estuary via an intracoastal waterway. For statistical purposes, to negate the possibility of confounding factors and to engender the power of species sample sizes, the data collected from sites designated as contaminated were pooled, as were data from the reference areas.

Water Quality and Other Environmental Parameters

Physicochemical parameters in surface waters were recorded daily by EPA and contractors. Such factors included temperature, pH, salinity, hardness, dissolved oxygen, specific conductivity, electronic potential, and total dissolved and suspended solids. Exemplary readings for Phase I (April 25 – May 1 2000) are: 7.73 pH, dissolved oxygen at 6.45 g/L, water temperature at 26.3 °C, salinity 0.8%, turbidity at 87 ntu, total dissolved solids at 9 g/L, and conductivity at 1.41 siemens/m. Latitude and longitude coordinates for samplings were recorded. Sediment samples and porewater were tested for chemistry and toxicity, the benthic community was surveyed, and other assessment endpoints were obtained (MacDonald and others, 2001a; 2001b).

Fish Collection

Fish were collected by angling, gill netting, and electrofishing in April 25 – May 1, 2000 (Phase I) and October 27 – November 10, 2000 (Phase II). Fish representing 13 species were collected (Tables 2 and 3). Health assessments were performed according to the USGS Biomonitoring of Environmental Status and Trends (BEST) protocol (Schmitt and others, 1995). Observations were made on length, weight, internal and external body appearance, parasite incidence, and organ color and condition.

Table 3. Fish species obtained for study during Phase I (spring) and Phase II (fall) of the Calcasieu Initiative.

Species	No. Phase I	No. Phase II	Total
Spotted gar	6	0	6
Lepisosteus oculatus			
Striped mullet	6	0	6
Mugil cephalus			
Spotted trout (speckled seatrout)	11	61	72
Cynoscion nebulosus			
Black drum	49	10	59
Pogonias cromis			
Red drum	2	20	16
Sciaenops ocellatus			
Southern flounder	6	10	16
Paralichthys lethostigma			
Gulf menhaden	3	0	3
Brevoortia patronus			
Gafftopsail catfish	4	0	4
Bagre marinus			
Atlantic croaker	7	3	7
Micropogonias undulatus			
Striped bass	0	1	1
Morone saxatilis			
Sheepshead	0	2	2
Archosargus probatocephalus			

Biomarkers

Data were collected at four levels of biological organization: organismal (condition factor), physiological (enzymatic, histological, stomach organic contents, and organosomatic indices), cellular (blood cell counts; histological), and molecular (DNA integrity).

Age Estimates

Otoliths were processed and analyzed by Louise Stanley, Consulting Fisheries Technologies, Montreal, Canada, in order to estimate ages of individuals. The process was based on the fact that opaque zones form during periods of colder weather (3-5 months), and translucent zones form during warmer water periods (7- 9 months) (Nieland and Wilson, 1993; Wilson and

Nieland, 1994; Nieland and others, 2002). Using these data, an algorithm employing hatch dates per species was used for estimating age.

Organosomatic Indices

Gonads, spleens, and livers were weighed, and each was divided by body weight to provide an index. All indices were calculated for black drum, red drum, and spotted trout from both Phases I and II.

Histology

Gonads

Testes and ovaries placed in Bouin's solution (Luna, 1992) were histologically processed and stained using haemotoxylin followed by eosin counterstain. Histological determination of sex and stage of development in females were interpreted by Connie Kersten, Department of Environmental Sciences, McNeese State University, Lake Charles, Louisiana. Interpretations were adapted from descriptions in reference documents including Blazer, 2002, Patino, 1995; and Macchi and others, 2002.

Spleens

Spleens were stored in buffered formalin (Luna, 1992), and histologically processed and stained by the Louisiana State University Veterinary School Pathology Laboratory. Sections were stained using periodic acid schiff protocol for optimal visualization of MA. MA were measured in square pixels and then converted to square microns. MA were scored at NWRC using brightfield microscopy and image analysis for frequency (mean number of MA per mm^2 tissue), area (or mean size in μm^2), and percent total area occupied per mm^2 of spleen tissue. Occurrence and size of MA were assessed by scoring three random 100x fields of view per spleen section using computer-based image analysis. Frequency and area data were log-transformed to achieve normal distributions.

If present in the spleen, metacercariae were scored. Representative micrographs documented parasite presence.

Stomach Contents

In attempts to distinguish inorganic from organic contents within the gut, stomach contents were collected, dried, and ashed in a muffle furnace according to NWRC in-house protocols. Because of mechanical difficulties, not all samples were analyzed.

Condition Factor

Black drum (*Pogonias cromis*), red drum (*Sciaenops ocellatus*), spotted seatrout (*Cynoscion nebulosus*), and southern flounder (*Paralichthys lethostigma*) were collected during Phase I and Phase II. Total length (mm), weight (g), sex, and month of capture were noted.

Historical Data

To generate regression equations with the data collected from normal fish captured from non-impacted sites in the Louisiana Gulf Coast region, historical length-weight data for the four species were obtained from the Louisiana Department of Wildlife and

Fisheries. These historical data had been collected from 1995-99 and included total length, weight, sex, and season of capture. Using this large database of 8,926 data points enabled the development of reliable length-weight regression equation models for Kn per species. The number of data points from diverse sample locations for red drum was 812, for black drum n=596, for spotted trout n=7,409, and for southern flounder n=109. In order to correspond with the sample dates in the Calcasieu Estuary collection, data obtained in March through May were considered "spring," while data collected from October and November were considered "fall." Least-squares regression methods were used on log-transformed length and weight measurements to determine length-weight relationships ($\log_{10}$ weight on $\log_{10}$ length) for each species. Significant differences between sex, season, and the interaction between the 2 were taken into account, and therefore for each species, there was a possibility of 1, 2, or 4 separate length-weight regression equations, depending on significant differences among subpopulations.

Calcasieu Estuary Data

The slope and y-intercept from the length-weight regression equations were used in the relative condition factor equation $Kn=W/aL^b$, where W is body weight (g), L is total length (mm), "a" is the slope, and "b" is the y-intercept. This equation, with the correct slope and intercept (per species, sex, and season), was then used with the Calcasieu data to calculate relative condition factor (Kn) for each fish. The Kn data were then used to assess the differences between reference and contaminated sites.

Analysis of variance (ANOVA) was used on Kn data to determine significant differences in Kn between species and between contaminated and reference sites. Because of low sample sizes from each site (n=1), data from the species were pooled before the ANOVA to assess site type differences.

Differential Blood Cell Count

Whole blood was collected into sodium heparin anticoagulant for smearing and fixation. Leucocytes included cells other than red blood cells (RBC), such as phagocytes (monocytes and neutrophils), thrombocytes, and lymphocytes. Two of the methanol-fixed blood smears were stained by Wright Giemsa (WG) (Luna, 1992), and 2 of the formaldehyde vapor-fixed blood smears were stained by Sudan Black B (SB) (Ellsaesser and others, 1984; Jenkins and Ourth, 1993), and counterstained with WG or methyl green. Sudan Black B stains the lipids in neutrophils. The WG staining allows differentiation between WBC and RBC. No attempts were made at further differentiation of WBC into categories such as eosinophils, thrombocytes, monocytes, or lymphocytes. In order to collect data for cell counts, at least 300 total cells (RBC + WBC) per slide were counted in duplicate.

Hepatic EROD Activity

Ethoxyresorufin-O-deethylase activities (EROD assay) from livers stored in liquid nitrogen were determined by Dr. Don Tillett, Columbia Environmental Research Center, BRD, USGS, Columbia, MO. The activities of the cytochrome P4501A enzyme system from microsomes of liver endoplasmic reticulum were determined for Phase I and Phase II fish.

Blood DNA Integrity

Because the susceptibility of DNA to damage and repair can differ between cell types, only blood cell nuclei were employed in the analysis for consistency. Blood is also a homogeneous cell population, so it lends itself to flow cytometry analyses, whereby supernumerary peaks have been attributed to more than one cell type (Tiersch and Wachtel 1993). Whole blood was collected into acid citrate dextrose anticoagulant for analysis of nuclear DNA integrity. Cells at one million nuclei per mL were stained using a modification of a standard propidium iodide nucleic acid staining dye (Crissman and Steinkamp, 1973), and measurements were made by flow cytometry using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA)(BDIS). Degradation of nuclear proteins occurs during tissue autolysis and this results in enhanced binding of fluorescent dyes to DNA (Alanen and others, 1989), and since subpopulations may show decreased size (or forward scatter) (Zbieranowski and others, 1993), such false aneuploidy was discounted in these analyses as per the following data collection methods. Data were collected in one-parameter histograms, dot plots with size versus DNA fluorescence, dot plots with nuclear fluorescence area versus width or doublet discrimination mode, and size of nuclei versus granularity. Data were analyzed using CellQuest software (BDIS). In addition to increased DNA coefficient of variation (CV), deviations from normal diploid histograms were noted and scored for severity, where a severity scale was developed: 1 represented a score as diploid or normal, 2 represented a slight deviation from normal, 3 represented more deviation from normal, and 4 was clear aneuploid. Aneuploidy may be defined as a chromosomal condition in which member chromosomes of the haploid set are present in an unequal number of copies (Guo and others, 1998). Data were analyzed by Chi Square.

Statistical Analyses

Appropriate analyses were run per biomarker. Data collected from sites designated as contaminated were pooled, as were data from the reference areas. Generally data were log transformed and ANOVA performed. Data for EROD and DNA integrity were ranked and analyzed with nonparametrics. Comparisons among sites and correlations among biomarkers were made.

Results

There were 98 fish caught in Phase I, 10 by hook and line, and 88 by gill net. There were 107 fish caught in Phase II, all by hook and line. Total fish caught in Phases I and II was 205, where 170 were obtained from 11 designated contaminated sites, and 35 were obtained from 3 reference sites. A total of 11 species were processed (See Table 2).

External and Internal Morphology

External examination results are reported only for fish captured by hook and line. The observances for the fins, opercles, gills, and pseudobranchs are reported. Observations on internal organs were obtained for all fish.

Fins

In Phase I, 4 (4.1%) fish exhibited fin damage, such as frayed, split, or eroded fins, while all 107 (100%) fish in Phase II had one or more types of damage.

Opercles, gills and pseudobranchs

Of the 102 fish from sites designated as contaminated, 79 (78%) exhibited abnormalities. Of the 19 fish from reference sites, 10 (53%) displayed abnormalities. Abnormalities included shortened opercles and gill arches, frayed and pale gills, and hemorrhagic pseudobranchs.

Head and body surface

Of the 115 fish examined from sites designated as contaminated, 21 (18%) displayed abnormalities, such as tumors, lesions, scars, or parasites on the head or body surface. Of the 27 fish examined from reference sites, 3 (11%) had abnormalities.

Liver and kidney appearance

Liver abnormalities included nodules, granular appearance, or parasites (see Adams and others, 1993). Of the 115 fish from contaminated sites, 6 (5%) displayed abnormal livers, whereas 1 of the 22 fish (4%) from reference sites showed abnormal livers. Of the 115 fish from contaminated sites, 7 (6%) showed deposits in the kidney, versus 0 of 22 fish (0%) from the reference sites.

Splenic parasites

Parasites in the spleen were not common. The majority of fish spleens had zero parasites, and if present, the number was less than 5, and generally no more than 1 or 2. However, D-33 had 62 parasites, and PLRC-5 had 18.
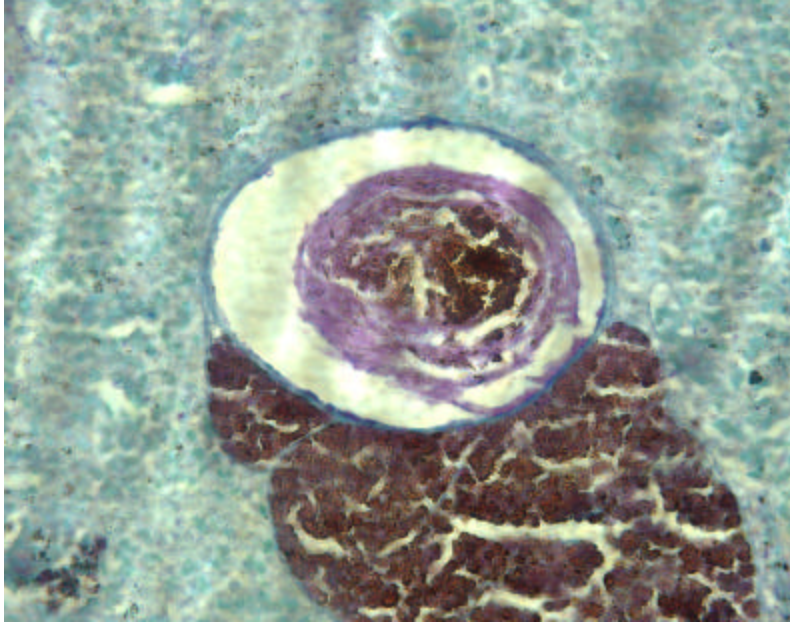
Figure 2. Metacercariae (center) in spleen tissue of fish D33, black drum from Bayou D'Inde, associated with a macrophage aggregate as imaged with light microscopy. Magnification was 400X.

Notable tumors

During Phase I, three Gulf menhaden, *Brevoortia patronus*, from one school of fish were collected in Coon Island NE and showed protruding tumors from their cranial regions (See Figure 3). Histopathological processing and analysis by Jack Fournie of Gulf Ecology Division of the National Health and Environmental Effects Research Laboratory at EPA, Gulf Breeze, FL reported the tumors as neural neoplasms, such as neurofibromas or schwannomas.


Figure 3. One of three Gulf menhaden from a single seine at Coon Island NE showed cranial tumors diagnosed as a neural neoplasm.

Age

All extracted otoliths were processed and the age of the animal was estimated, except for PLRC6 because of otolith malformation. In the data generated, 'Age' referred to the number of opaque zones observed within and at the edge (either partially or fully formed) of the specimen. 'Edge condition' referred to the appearance of the growing edge – either 1, 2, or 3 denoting an opaque edge, or 4, 5, or 6 denoting a translucent edge:

- Edge condition 1 – opaque zone at the edge, just forming to 1/3 complete
- Edge condition 2 – opaque zone at the edge, 1/3 to 2/3 complete
- Edge condition 3 – opaque zone at the edge, 2/3 to fully complete
- Edge condition 4 – translucent zone at the edge, just forming to 1/3 complete
- Edge condition 5 – translucent zone at the edge, 1/3 to 2/3 complete
- Edge condition 6 – translucent zone at the edge, 2/3 to fully complete

Using estimated hatch dates per species from the scientific literature, age was then established by using a developed algorithm (Dave Nieland, Louisiana State University, Coastal Fisheries Institute)(Nieland and Wilson, 1993; Wilson and Nieland, 1994; Nieland and others, 2002). Opaque zones form during periods of colder water temperatures (late fall to early or mid spring - a period of 3 to 5 months, with the translucent zones forming during warmer water periods - a period of 7 to 9 months). The timing of opaque zone formation varies with species and somewhat across the year depending on water temperatures. Species show an opaque zone at the core if they are hatched in the fall with first growth during the winter; those that hatch in the late spring or early summer with first growth in warmer periods will show a translucent zone at the core of the otolith.
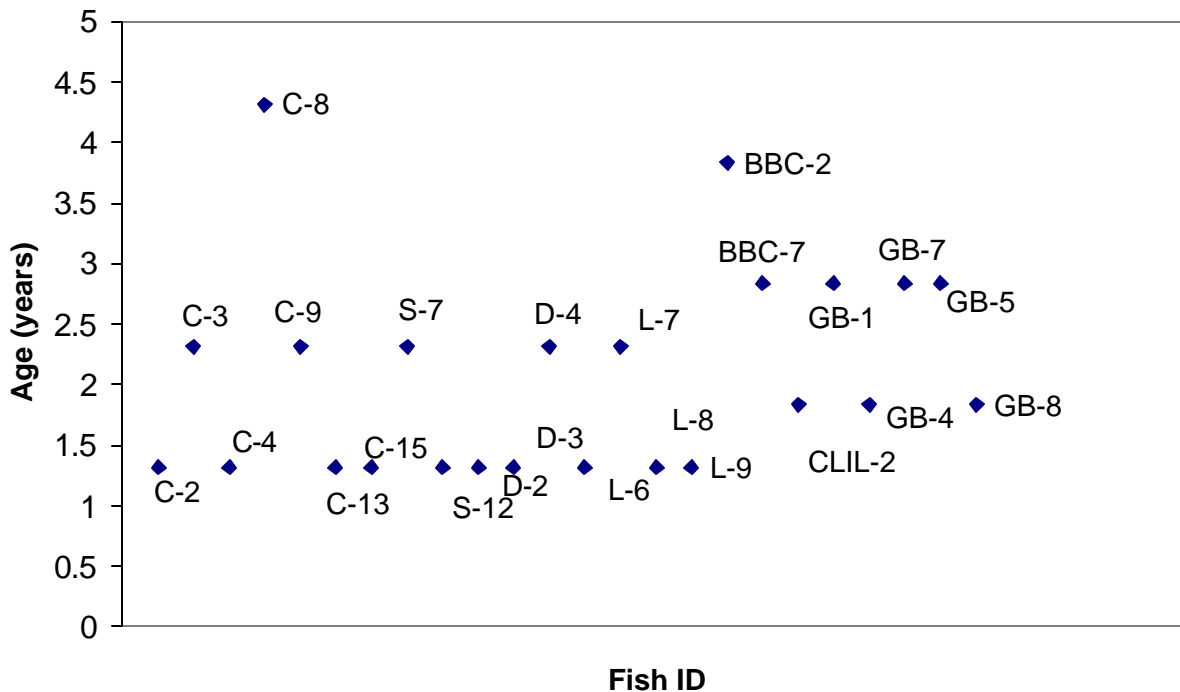
Figure 4. Estimated ages from otoliths of individual black drum.
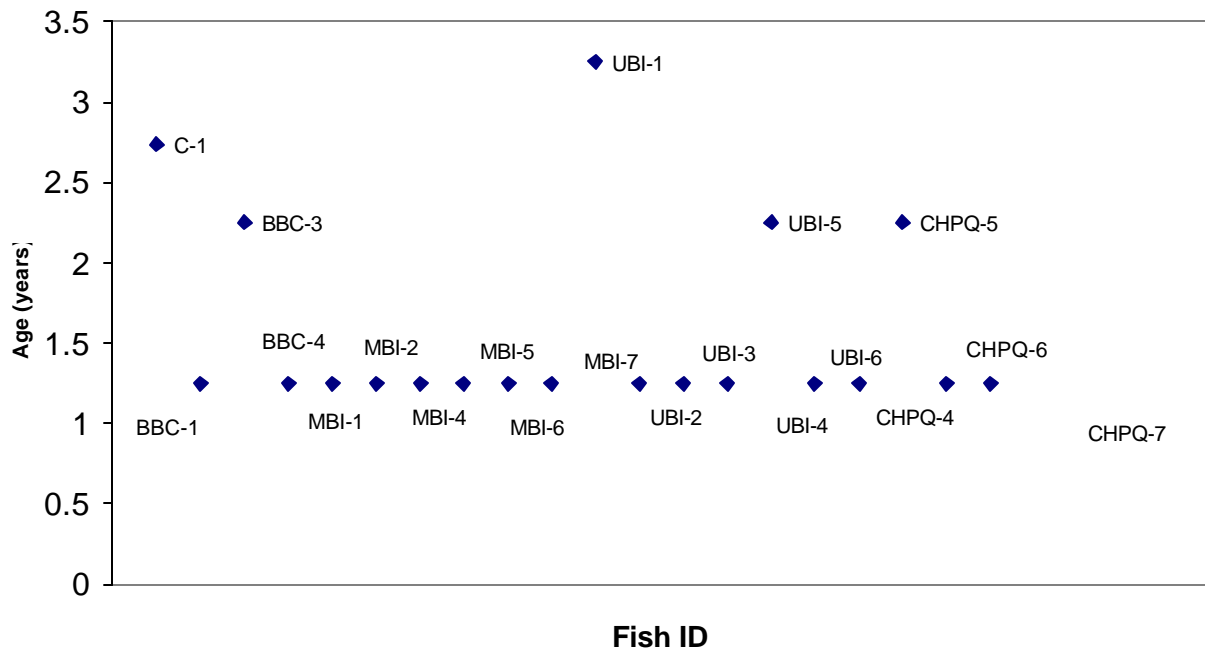
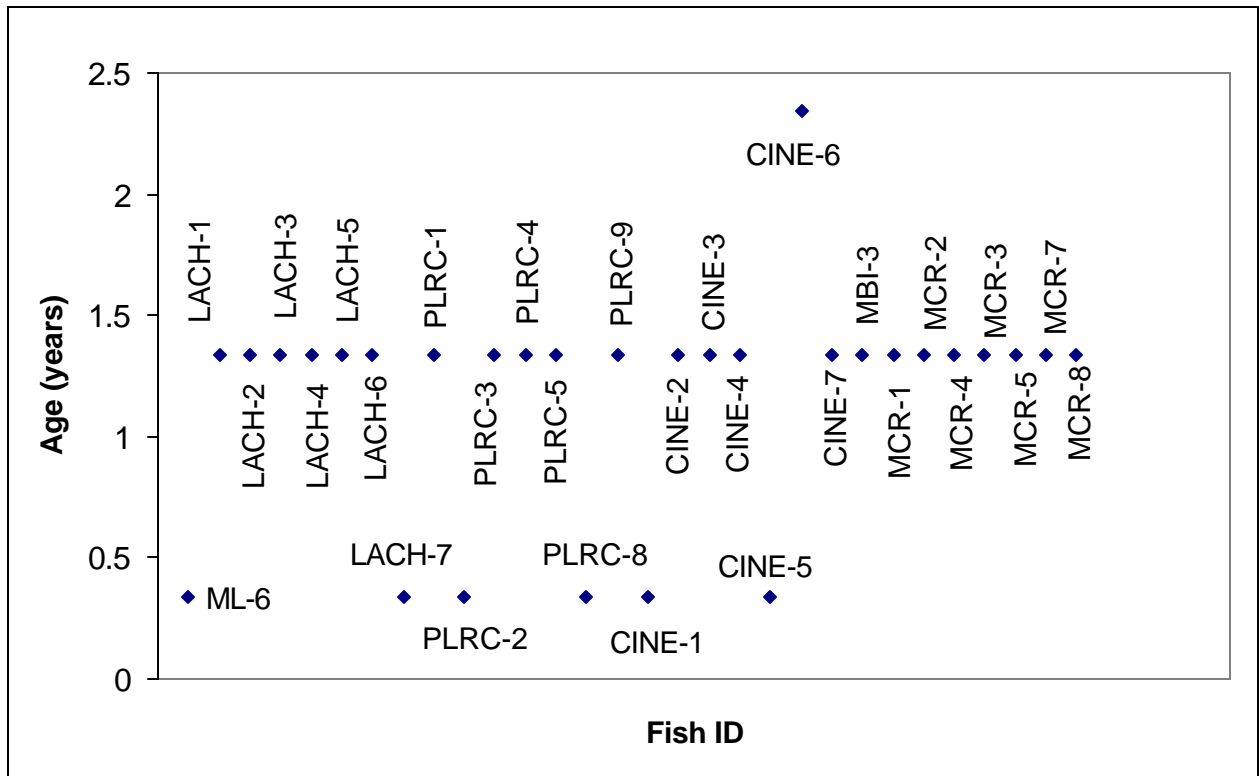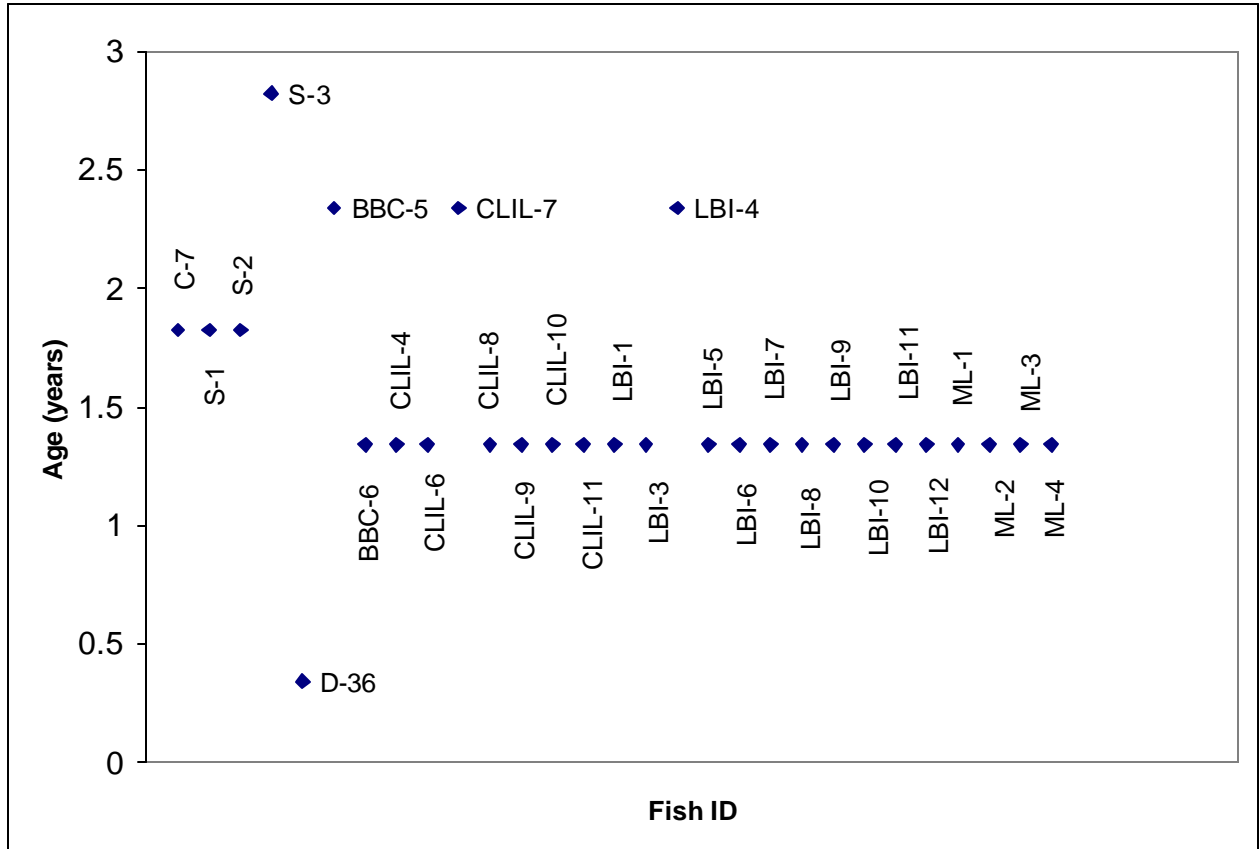**Figure 5**. Estimated ages from otoliths of individual red drum.

Figure 6. Estimated ages from otoliths of individual spotted trout.

Organosomatic Indices

GSI

An analysis of covariance was run on log-transformed data in order to remove the effect of spawning condition due to season on gonad weight. It was found that black drum had a significantly lower GSI at contaminated sites than in reference sites. There were no significant differences for either red drum or spotted trout.

Contaminants can induce changes at the cellular level in gonads. Intersex or ovotestis and atresia have been fairly well researched in terms of chemical exposures (Blazer 2002). In this study, gonadal histology of the two most predominant species pointed toward feminization in black drum from contaminated sites, and only slightly so in spotted trout (see Table 4).
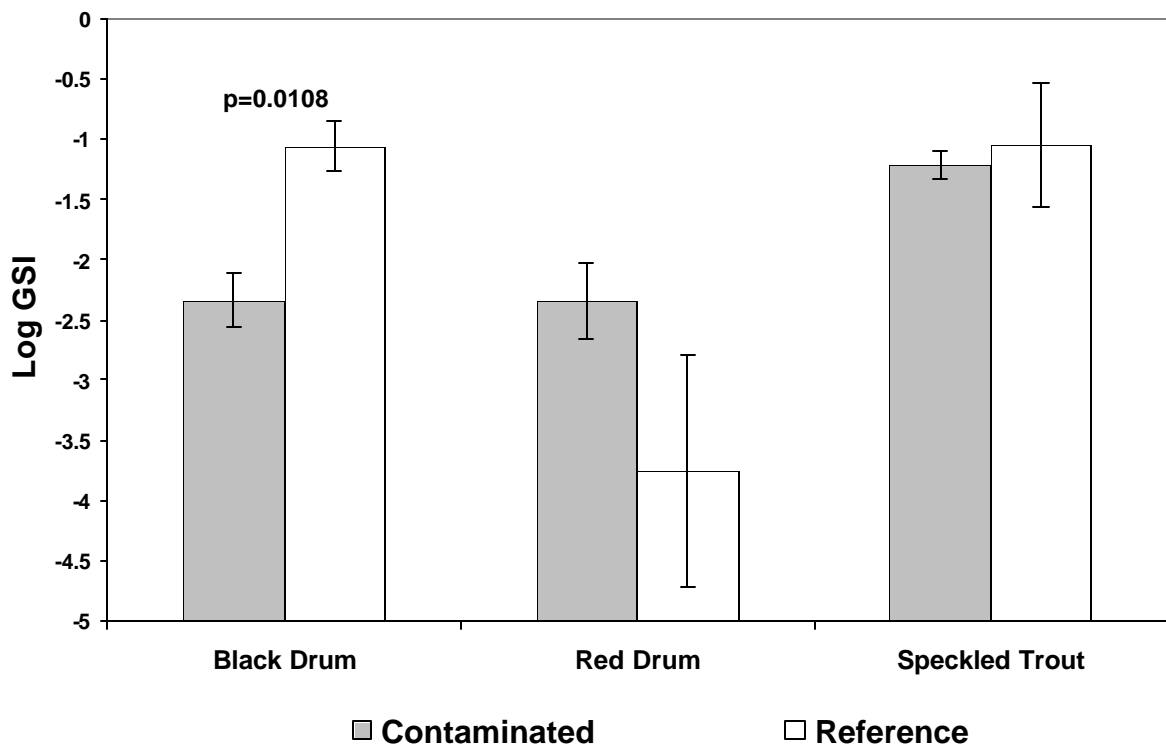
Figure 7. Gonadosomatic indices for three species of fish. Significant differences existed between the sites with black drum (p=0.0108).

Splenosomatic Index

For black drum, this index was significantly higher in fish from contaminated sites than from reference sites; however, there were no significant differences for red drum or spotted trout. Data were log transformed.

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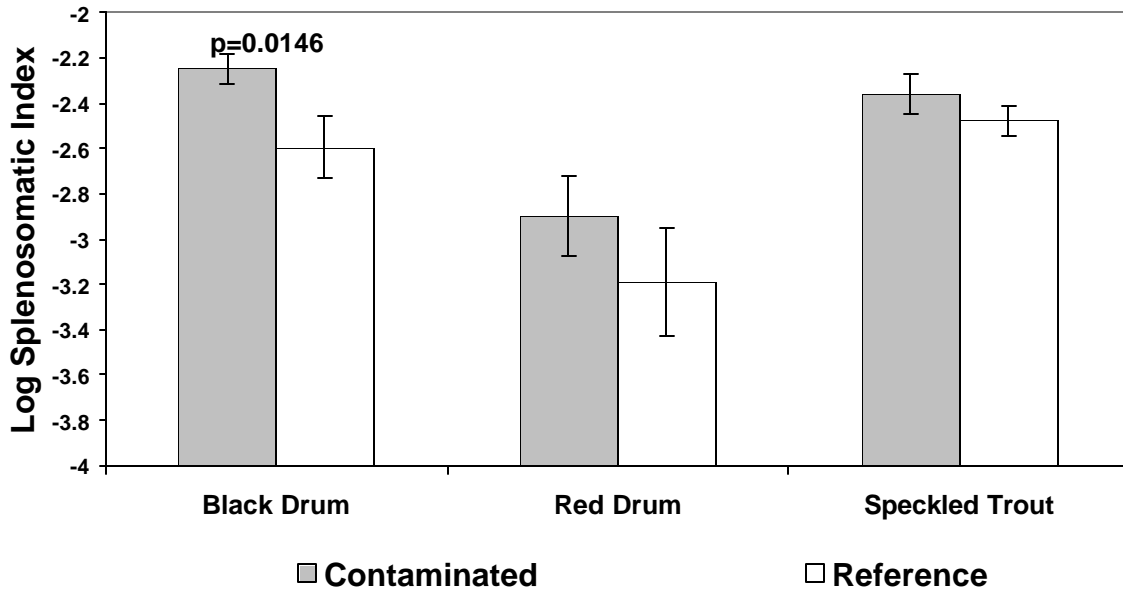
Figure 8. Splenosomatic indices for three species of fish, where significant differences were seen with black drum (p=0.0146).

Hepatosomatic Index

A significantly lower hepatosomatic index (HSI) was seen in spotted trout from contaminated sites compared to reference sites; however, there were no significant differences for either of the drum species. Data were log transformed.
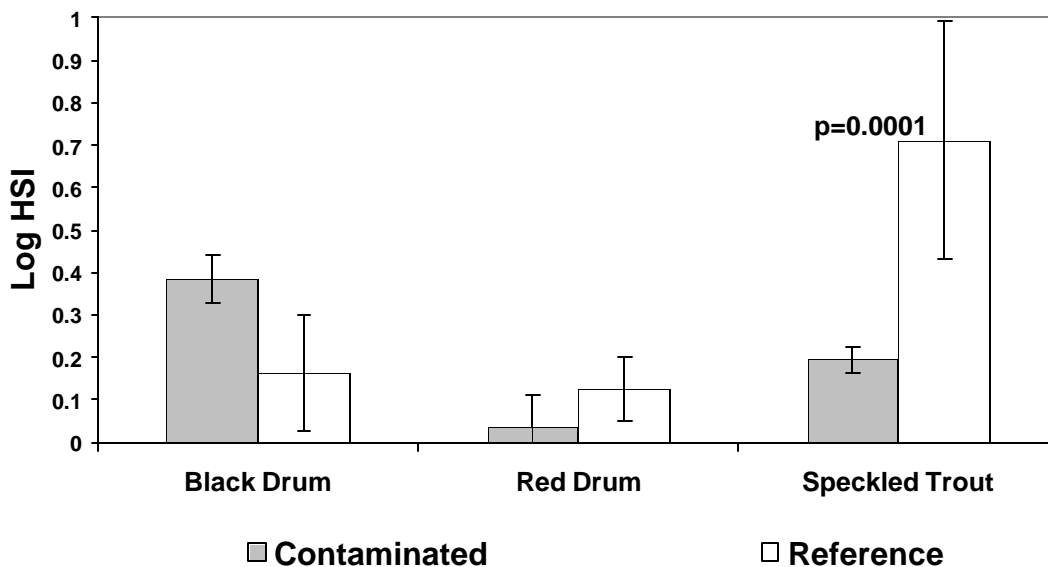
Figure 9. Hepatosomatic indices for three species of fish, where significant differences were seen with spotted trout (p=0.0001).

Table 4. Identification of sex by histology and by visual, morphological inspection compared with gonadosomatic index and age.
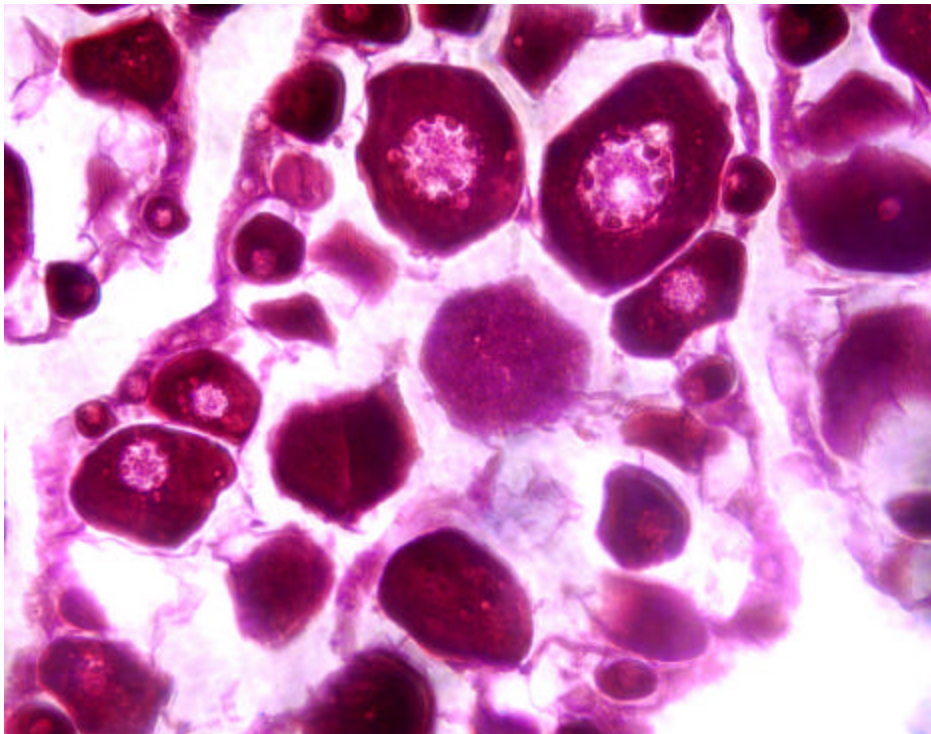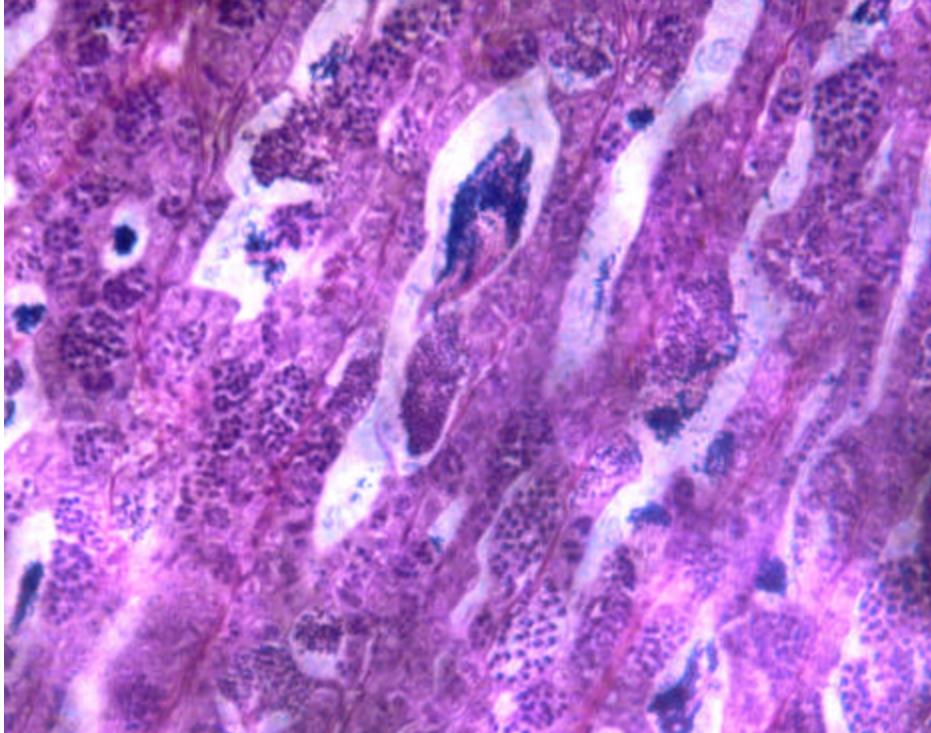
Black Drum	Site[a]	Histological Sex[b]	Morphological Sex	GSI[c]	Age (years)
GB 7	R	M	M (ripe)	0.319	2.83
GB 5	R	M	M (ripe)	0.092	2.83
S 7	R	M	M (spent)	0.090	2.37
L 10	R	M	Indeterminate	0.046	n.d.[d]
D 2	C	F	M	0.097	1.32
D 3	C	F	M	n.d.	1.32
D 4	C	F	M	0.189	2.32
D 11	C	F	M	0.079	n.d.
CLIL 2	C	F	M	4.010	1.83
Spotted Trout					
S 1	R	F	F	2.760	1.83
S 2	R	F	F	5.038	1.83
S 3	R	F	F	4.276	2.82
LBI 1	C	M	F	0.502	1.34
LBI 3	C	M	M (ripe)	0.172	1.34
LBI 4	C	M	M (ripe)	0.313	1.34
LBI 5	C	F	F (spent)	0.590	1.34
LBI 6	C	F	F (spent)	0.539	2.34
LBI 7	C	F	F (spent)	0.681	1.34
LBI 8	C	F	F (spent)	0.545	1.34
LBI 9	C	M	M	0.158	1.34
LBI 10	C	F	F	0.553	1.34
LBI 11	C	F	F	0.542	1.34
LBI 12	C	F	F	0.648	1.34
MBI 3	C	F	F	0.390	1.34
BBC 5	C	M	M	0.210	2.34
BBC 6	C	F	F	0.583	1.34

[a] Sites that were designated as contaminated are "C" and reference sites are "R."
[b] Males are "M" and females are "F."
[c] GSI is gonadosomatic index, or gonad weight divided by body weight times 100.
[d] No data is "n.d."

Figure 10. Histological sections of gonad from black drum GB 7 (Grand Bayou, reference site) (top). Sex identification was male by morphology, as was histological identification. Bottom section is from gonad from black drum D4 (Bayou D'Inde, contaminated site) that was morphologically identified as male and histologically identified as female. Magnification 400X.
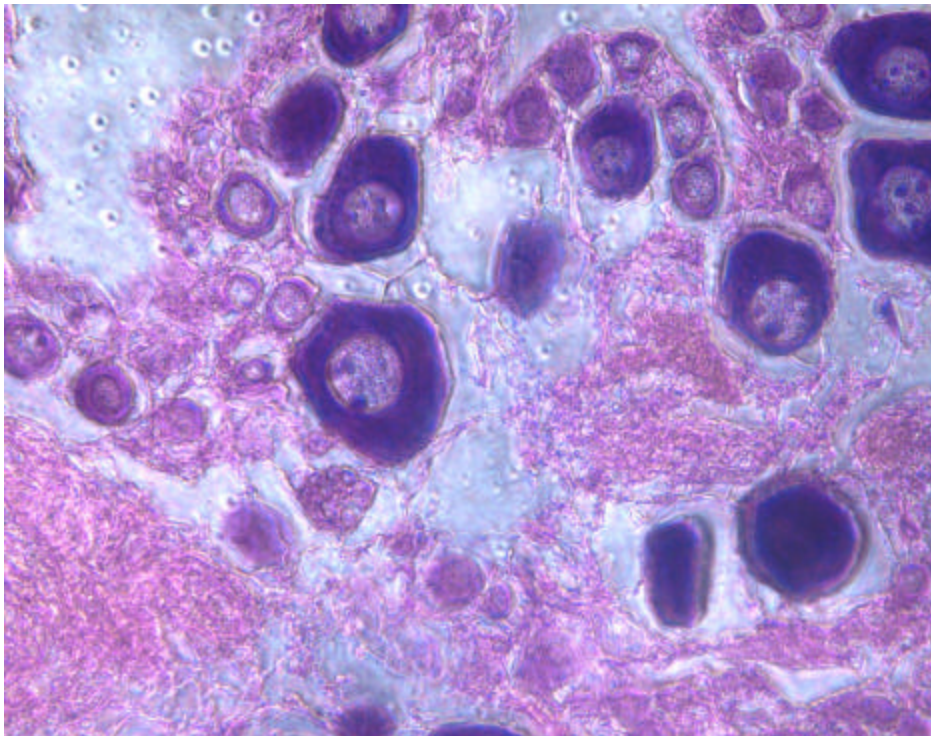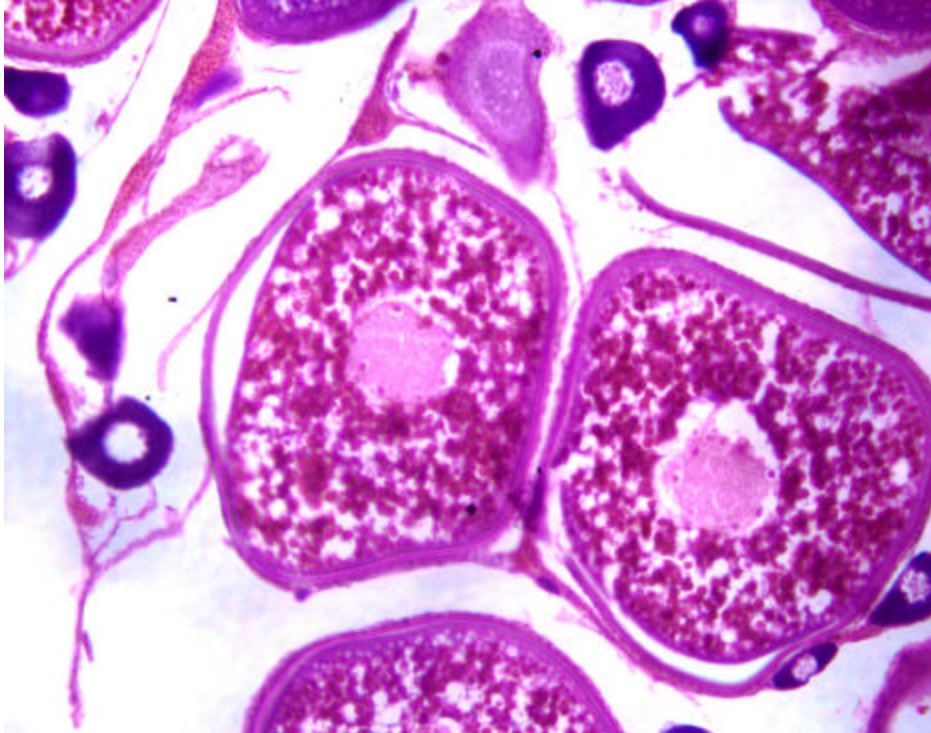
Figure 11. Histological sections of gonad from spotted trout S1 (Sabine, reference site) (top). Morphological sex identification was female, as was histological identification. Maturation level was estimated as Stage IV. Magnification was 200X. Bottom section is from gonad spotted trout LBI 1 (Lower Bayou d'Inde, contaminated site) that was morphologically identified as male and histologically identified as female. Maturation was estimated as Stage II. Magnification 400X.

Macrophage Aggregates

Splenic MA frequency and area data were log transformed to achieve normal distributions. MA frequency was significantly higher in spotted trout and red drum from contaminated sites than from reference sites ($p \leq 0.05$). Age was a significant covariate in red drum. On the other hand, there were no significant differences in MA area per μm^2 between sites for the three species. The MA frequency at contaminated sites was 17.9 ± 1.1 (n=110) per mm^2, and at the reference sites it averaged 9.5 ± 0.8 (n=23) for data pooled for the species. The MA area at contaminated sites averaged $22,216 \pm 2346$ (n=110) μm^2, and at the reference sites it averaged $12,810 \pm 2030$ μm^2 (n=23) for data pooled for the species.
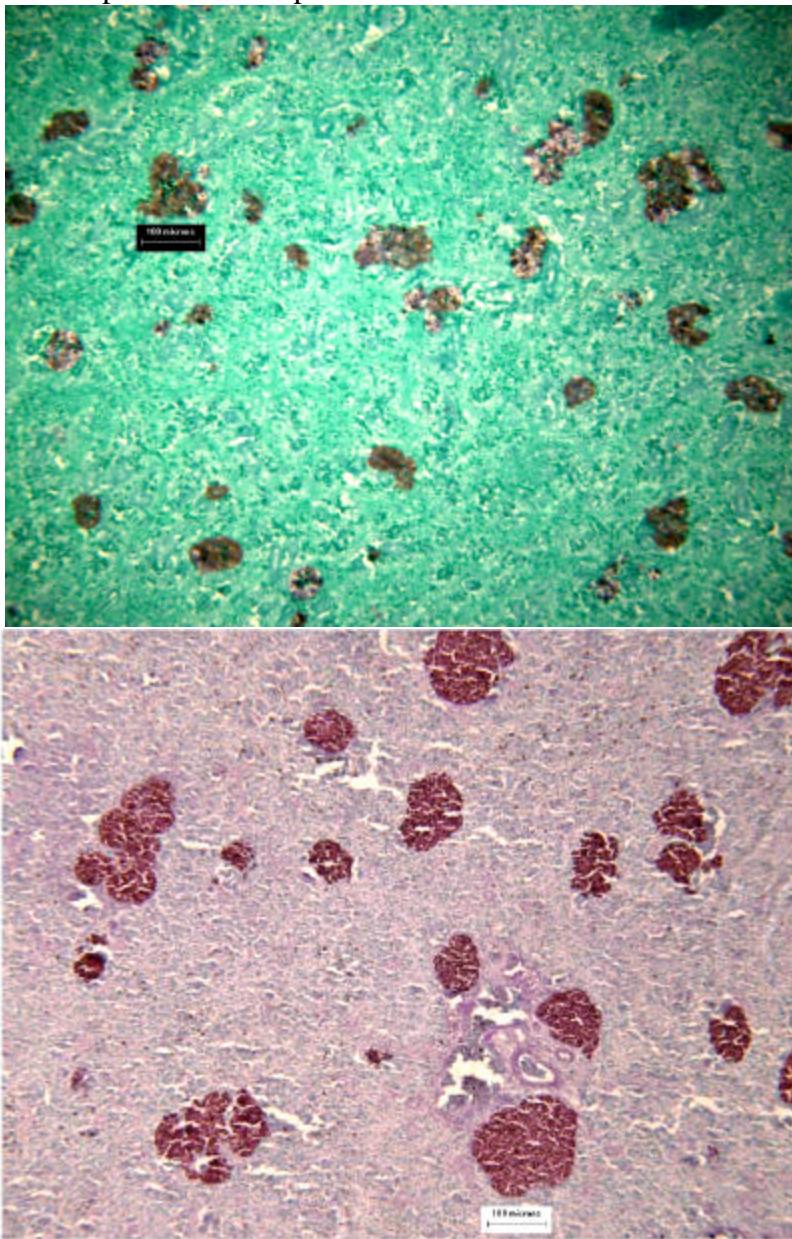
Figure 12. Photomicrographs of macrophage aggregates in a southern flounder from Choupique Bayou (CHPQ 1) (top) and black drum from Bayou D'Inde (D 33). Magnification bars are 100 µm.

Stomach Contents

Black drum as a group consumed comparatively more inorganic contents than did the other species (see Tables 5 and 6). Per species, a few individuals consumed notably more than others (see Figures 13-18), but overall, the relative percentages of inorganic to organic contents were similar.

Table 5. Dried and ashed stomach contents and percent organic and inorganic contents were determined by weight for individual red drum (RD), southern flounder (SF), spotted trout (ST), and black drum (BD).

Species	Fish ID	% Inorganic	% Organic	% Inorganic/%Organic
RD	BBC*-1	6.776	93.224	0.073
RD	BBC-3	13.777	86.223	0.160
RD	BBC-4	7.249	92.751	0.078
ST	BBC-5	6.468	93.532	0.069
ST	BBC-6	9.446	90.554	0.104
SF	CHPQ-1	6.117	93.883	0.065
SF	CHPQ-2	6.473	93.527	0.069
RD	CHPQ-4	25.642	74.358	0.345
RD	CHPQ-5	8.740	91.260	0.096
RD	CHPQ-6	22.687	77.313	0.293
RD	CHPQ-7	25.141	74.859	0.336
SF	CHPQ-8	7.982	92.018	0.087
SF	CHPQ-9	6.178	93.822	0.066
ST	CINE-1	6.169	93.831	0.066
ST	CINE-3	7.490	92.510	0.081
ST	CINE-4	7.570	92.430	0.082
ST	CINE-5	6.096	93.904	0.065
ST	CINE-6	7.277	92.723	0.078
ST	CINE-7	11.819	88.181	0.134
SF	CINE-8	13.874	86.126	0.161
SF	CINE-9	11.053	88.947	0.124
ST	CLIL-1	7.180	92.820	0.077
BD	CLIL-2	30.022	69.978	0.429
SF	CLIL-3	15.391	84.609	0.182
ST	CLIL-4	7.333	92.667	0.079
ST	CLIL-6	6.591	93.409	0.071
ST	CLIL-7	9.824	90.176	0.109
ST	CLIL-8	17.954	82.046	0.219
ST	CLIL-9	9.831	90.169	0.109
ST	CLIL-10	7.372	92.628	0.080
ST	CLIL-11	10.710	89.290	0.120
SF	CLIL-12	18.553	81.447	0.228
BD	D-33	53.777	46.223	1.163
RD	D-34	21.619	78.381	0.276
ST	D-36	11.659	88.341	0.132
BD	GB*-1	4.199	95.801	0.044
BD	GB-4	3.536	96.464	0.037
BD	GB-5	16.897	83.103	0.203

BD	GB-6	6.593	93.407	0.071
BD	GB-7	54.359	45.641	1.191
BD	GB-8	14.772	85.228	0.173
ST	LACH-1	6.176	93.824	0.066
ST	LACH-2	7.103	92.897	0.076
ST	LACH-3	6.083	93.917	0.065
ST	LACH-4	8.165	91.835	0.089
ST	LACH-5	9.649	90.351	0.107
ST	LACH-6	5.713	94.287	0.061
ST	LACH-7	10.999	89.001	0.124
ST	LBI-1	9.393	90.607	0.104
ST	LBI-3	8.181	91.819	0.089
ST	LBI-4	8.502	91.498	0.093
ST	LBI-5	7.726	92.274	0.084
ST	LBI-6	8.495	91.505	0.093
ST	LBI-7	7.207	92.793	0.078
ST	LBI-8	8.501	91.499	0.093
ST	LBI-9	7.292	92.708	0.079
ST	LBI-10	9.878	90.122	0.110
ST	LBI-11	8.818	91.182	0.097
RD	MBI-1	9.044	90.956	0.099
RD	MBI-2	11.480	88.520	0.130
RD	MBI-4	13.132	86.868	0.151
RD	MBI-5	16.015	83.985	0.191
ST	MCR-1	10.308	89.692	0.115
ST	MCR-2	6.147	93.853	0.065
ST	MCR-7	8.927	91.073	0.098
ST	MCR-8	9.238	90.762	0.102
ST	ML-2	10.343	89.657	0.115
ST	PLRC-1	8.244	91.756	0.090
ST	PLRC-2	11.598	88.402	0.131
ST	PLRC-9	6.529	93.471	0.070
RD	UBI-1	23.556	76.444	0.308
RD	UBI-2	10.307	89.693	0.115
RD	UBI-3	9.439	90.561	0.104
RD	UBI-4	22.225	77.775	0.286
RD	UBI-5	17.239	82.761	0.208
RD	UBI-6	7.024	92.976	0.076

* GB is Grand Bayou and BBC is Bayou Bois Connine, reference sites.

Table 6. Ranges of percent inorganic/percent organic stomach contents per species.

| Species	Minimum	Maximum
Southern flounder	0.065	0.228
Black drum	0.037	1.191
Red drum	0.066	0.345
Spotted trout	0.061	0.219
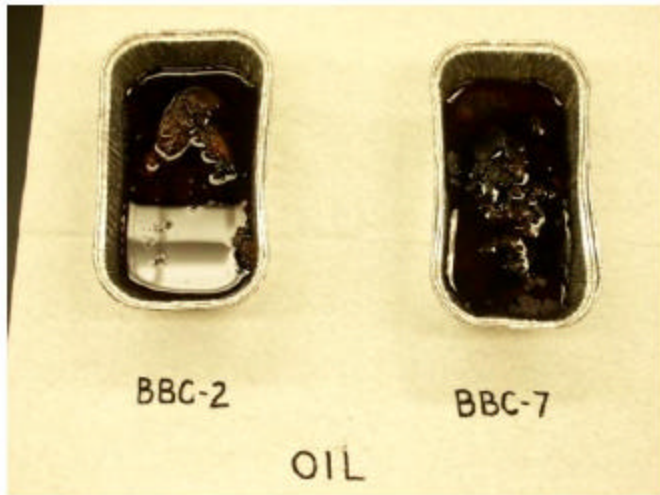
Black drum stomachs from Bayou Bois Coninne after drying.
Not ashed due to likely fire hazards.

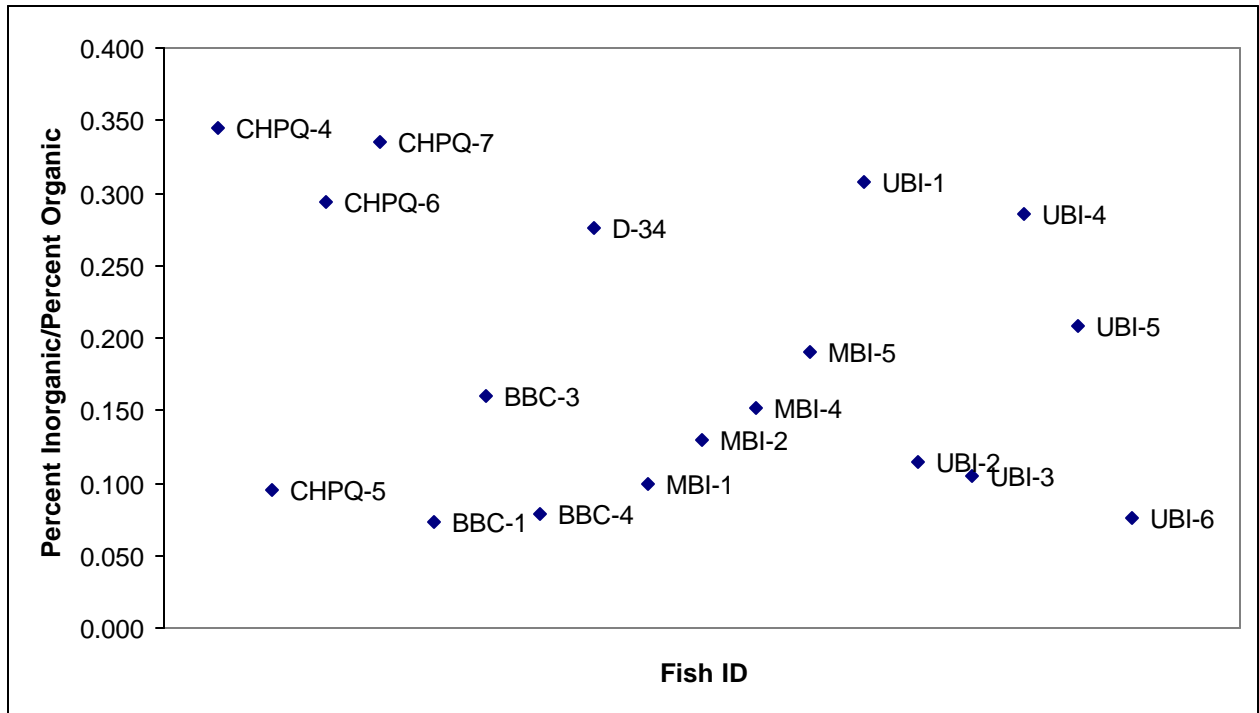**Figure 13**. Two stomach contents did not dry, so were not ashed.

Black drum stomach from Bayou d'Inde

Figure 14. A black drum from Bayou D'Inde had consumed many inorganic compounds, such as bivalve shell and fish bones.
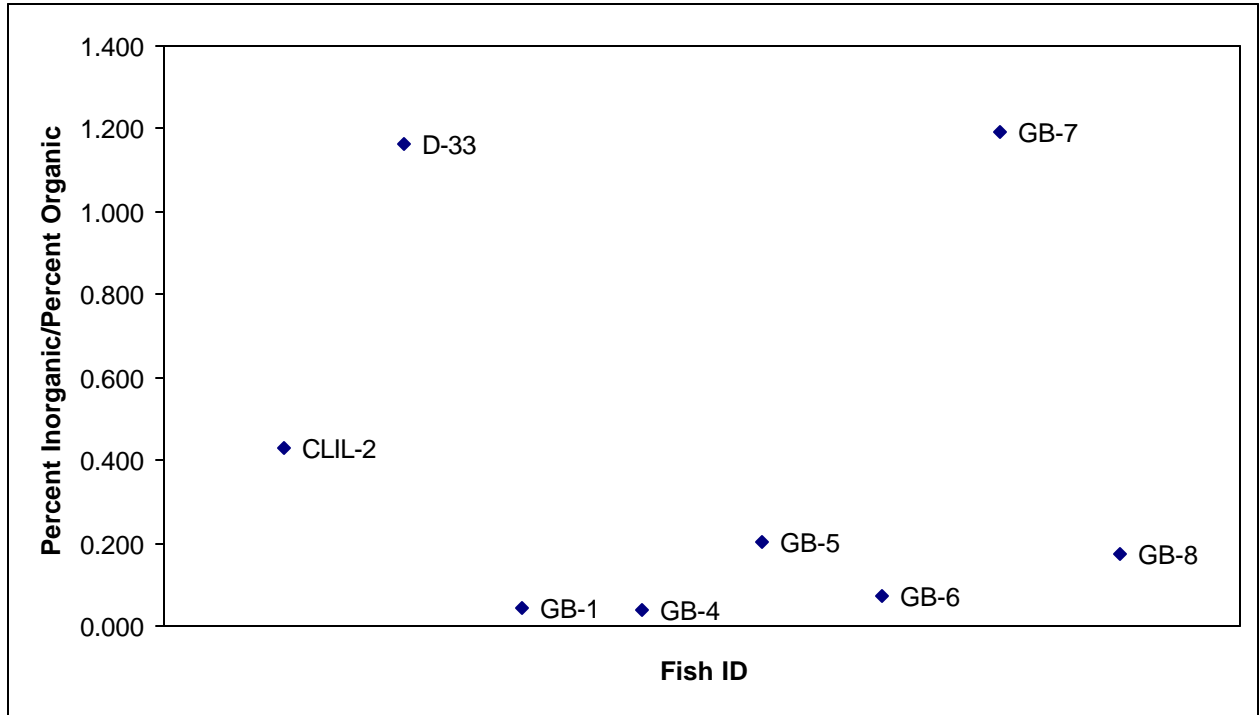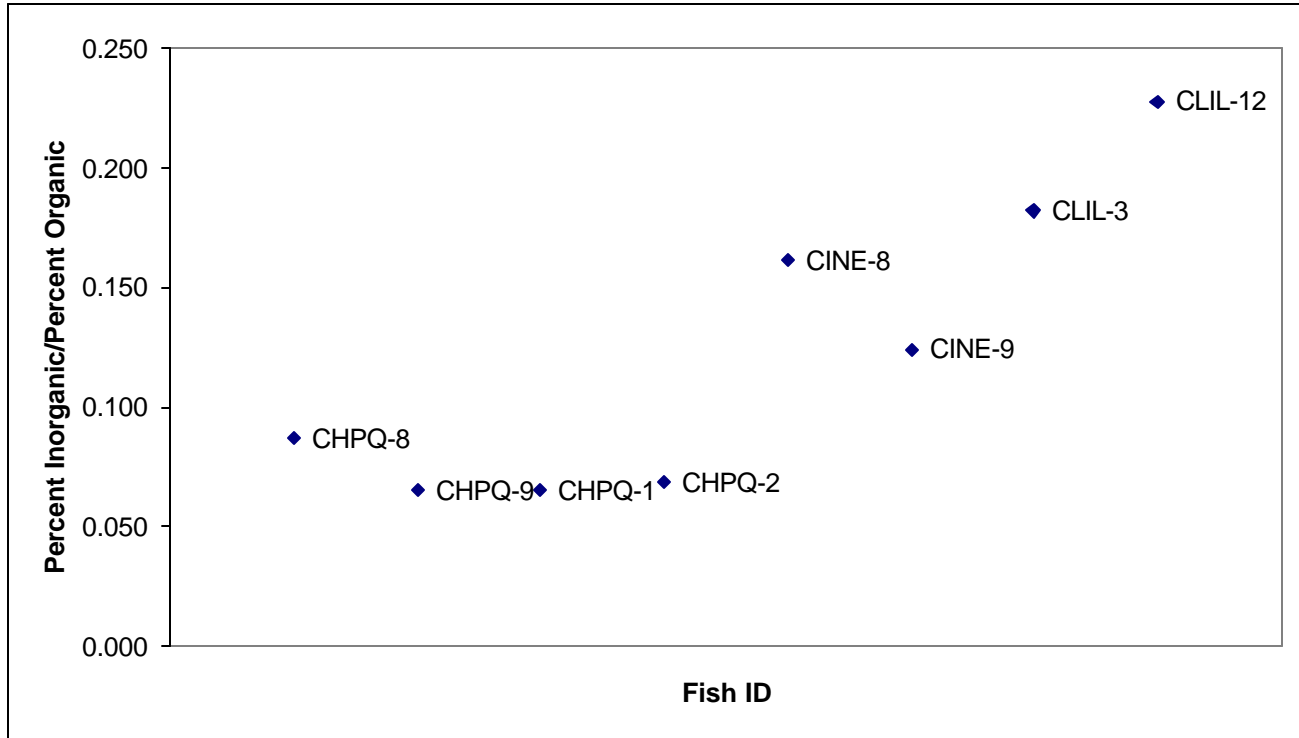
Figure 15. Percent inorganic stomach contents divided by organic contents for black drum.
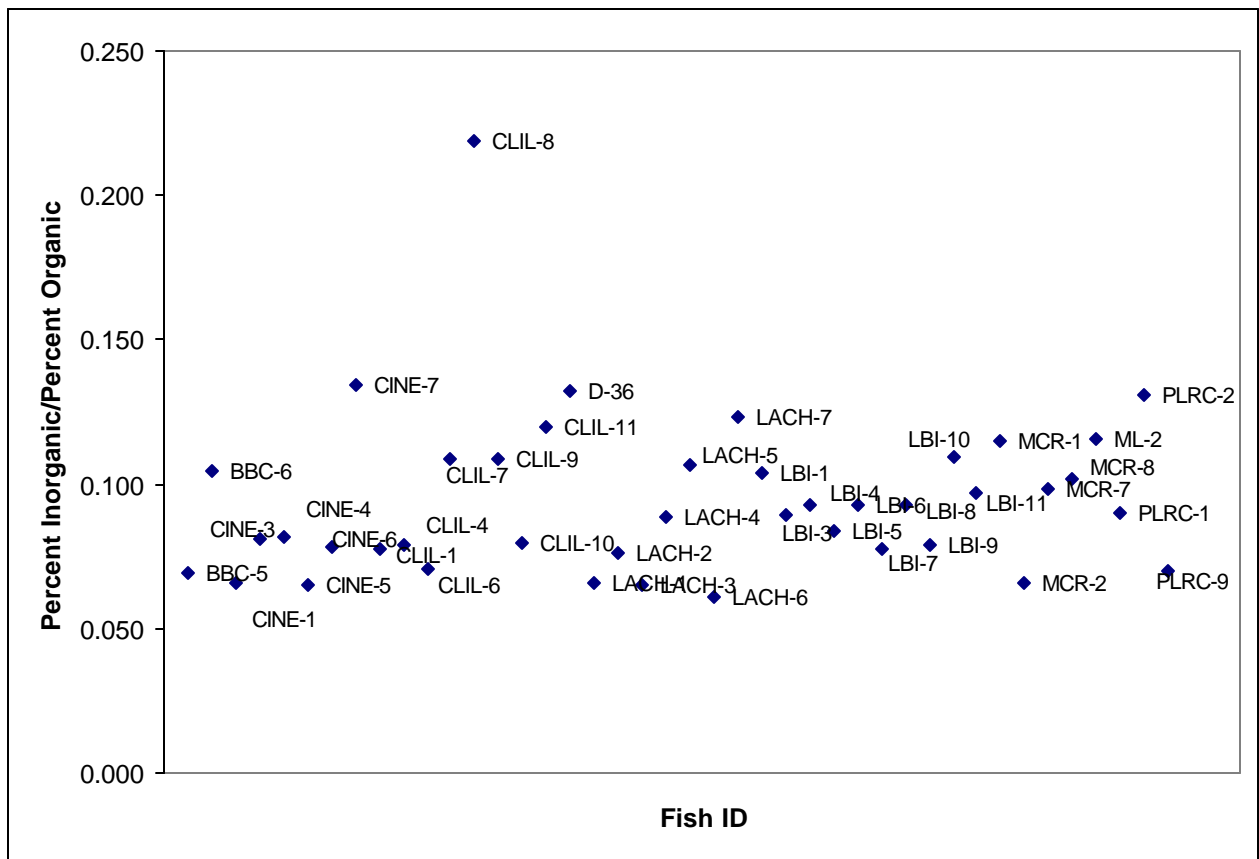

Figure 16. Percent inorganic stomach contents divided by organic contents for red drum.

Figure 17. Percent inorganic stomach contents divided by organic contents for southern flounder.

Figure 18. Percent inorganic stomach contents divided by organic contents for spotted trout.

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Differential Blood Cell Counts

The range of percent WBC was approximately 2-8 percent per species. A few fish were outliers, in particular from Prien Lake.
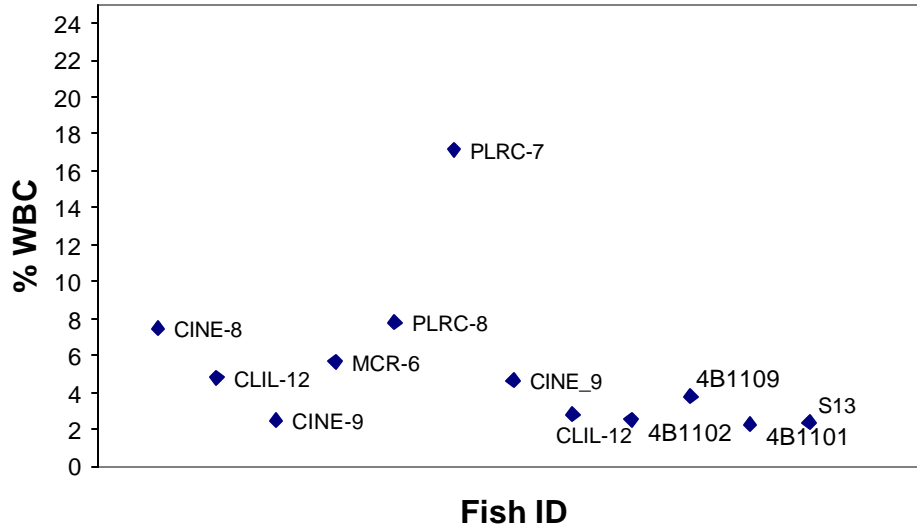
Figure 19. Southern flounder percent white blood cells. Fish identifications are noted. A few extra data points were collected from individuals from Choupique Bayou that were used for other analyses, and they are labeled as 4B series.
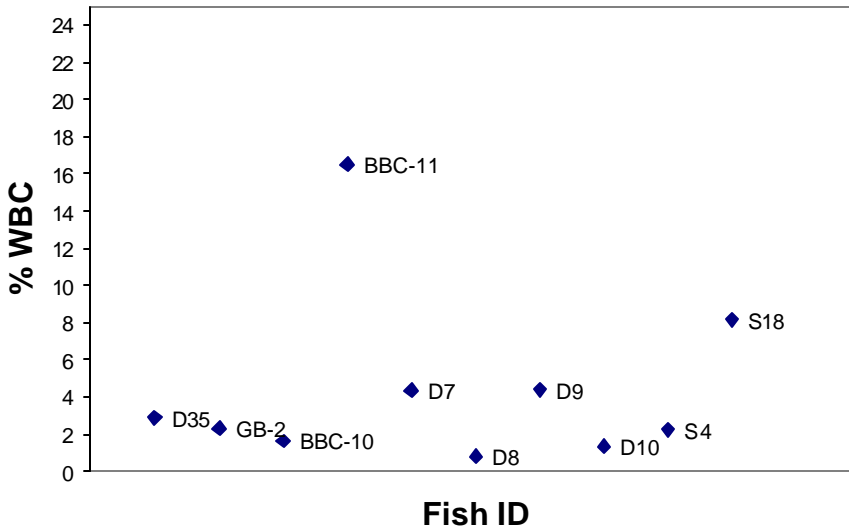
Figure 20. Atlantic croaker percent white blood cells. Fish identifications are noted.
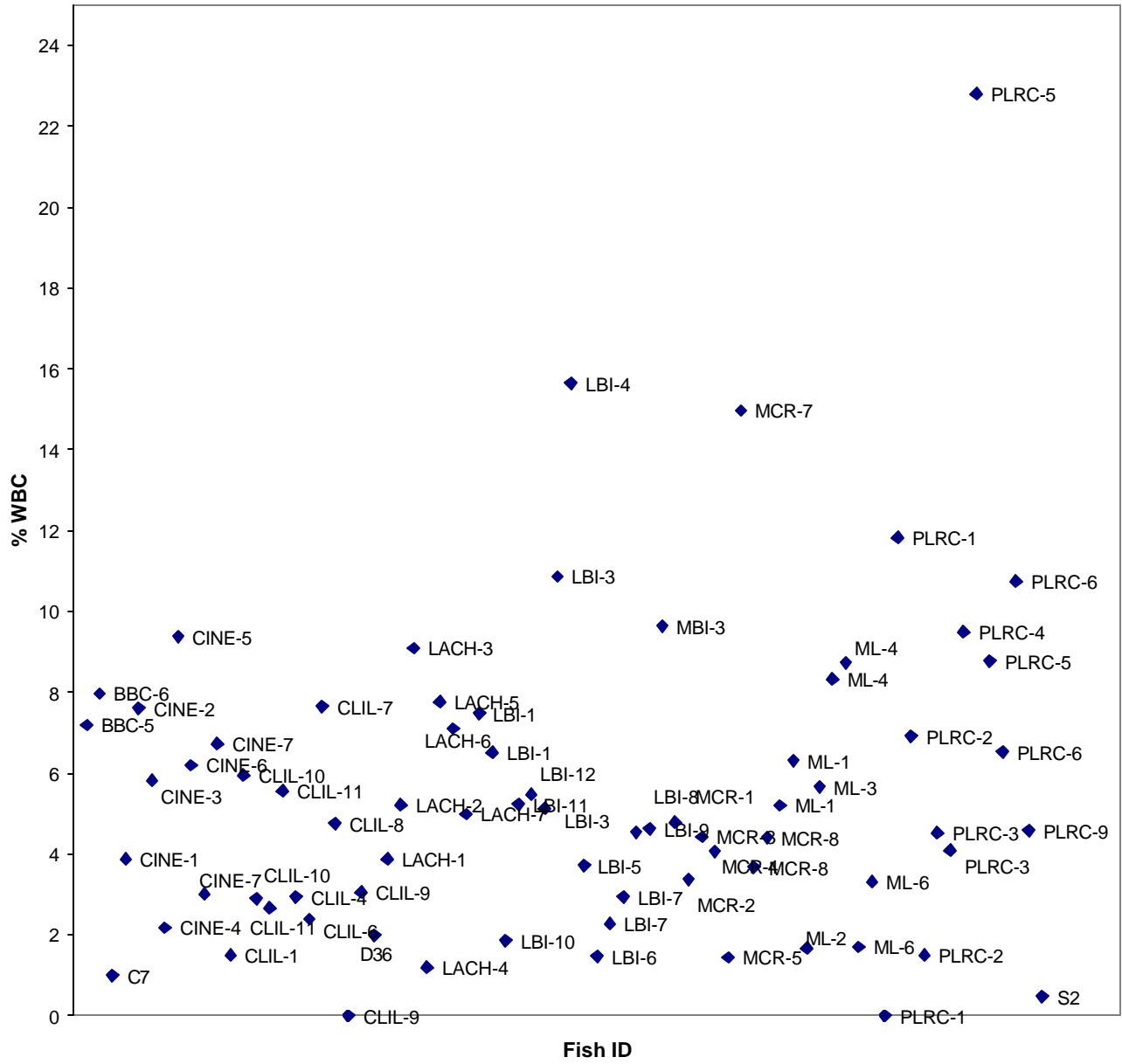
Figure 21. Spotted trout percent white blood cells. Fish identifications are noted.
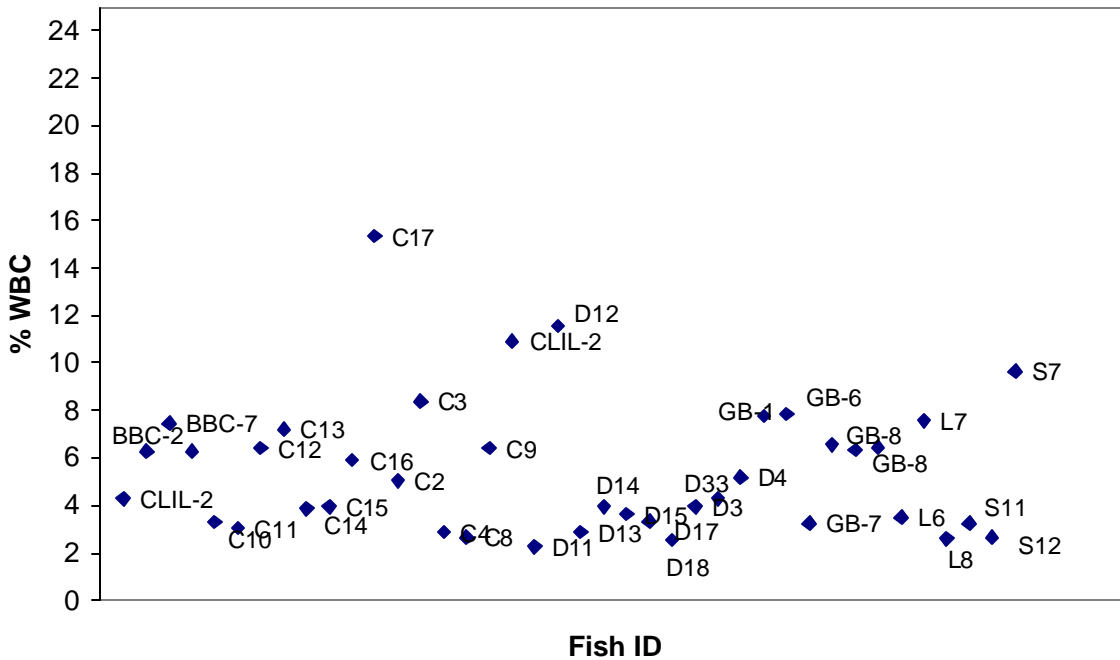
Figure 22. Black drum percent white blood cells. Fish identifications are noted.
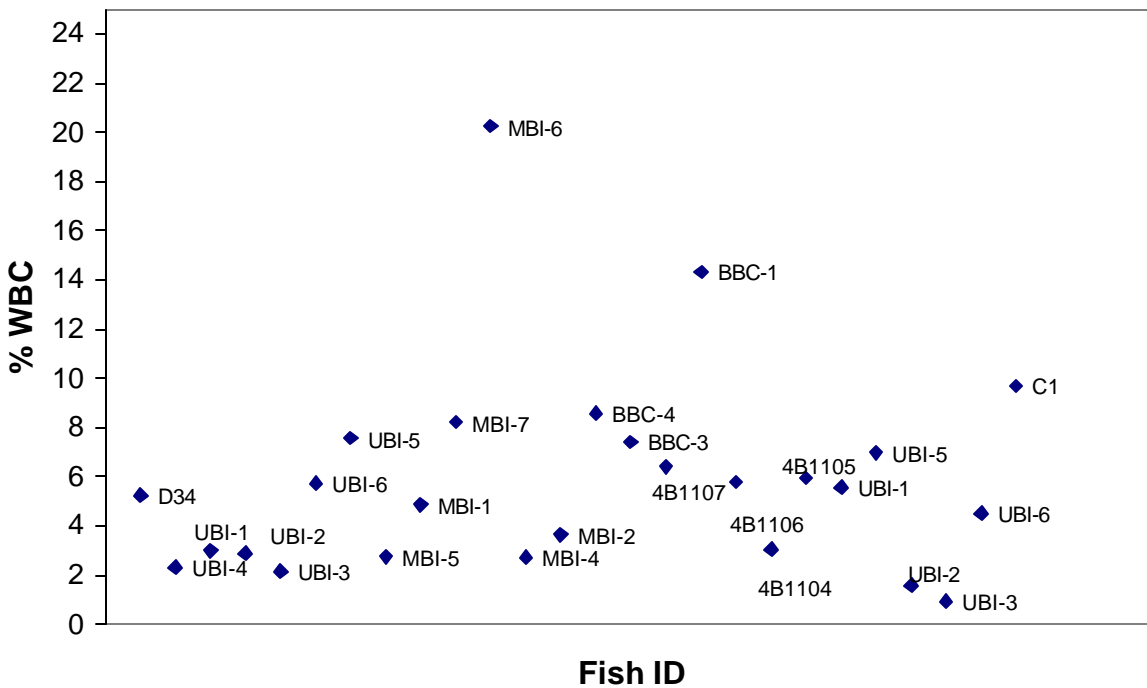
Figure 23. Red drum percent white blood cells. Fish identifications are noted. A few extra data points were collected from individuals used for other analyses from Choupique Bayou, hence the 4B series.
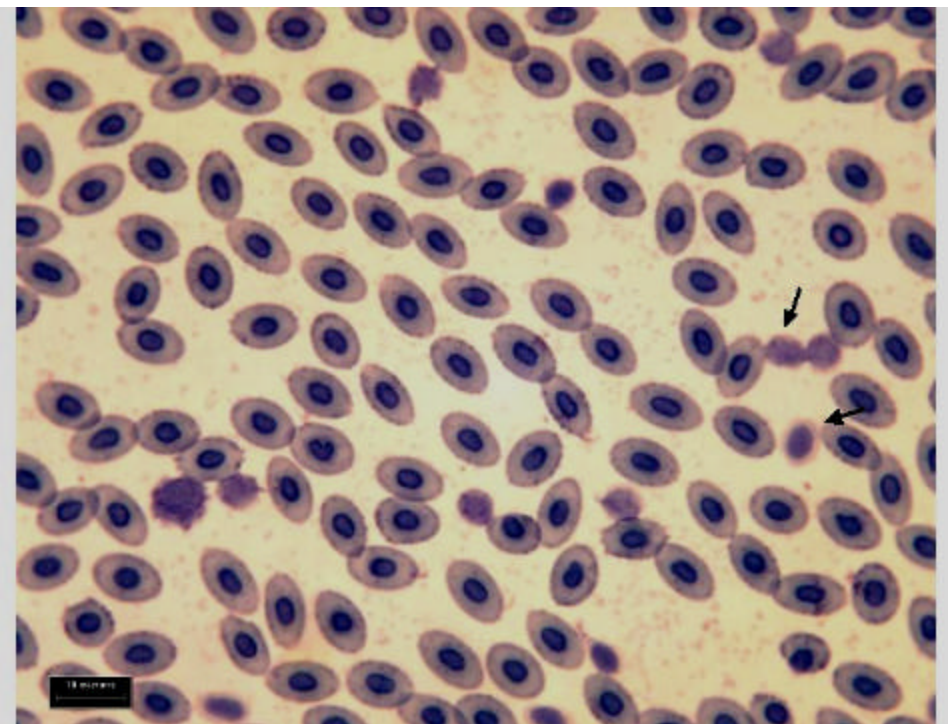
Figure 24. Wright Giemsa stain of whole blood from red drum. Arrows are pointing to white blood cell types, whereas the majority of cells are clearly nucleated and red blood cells. Magnification bar is 10 μm.
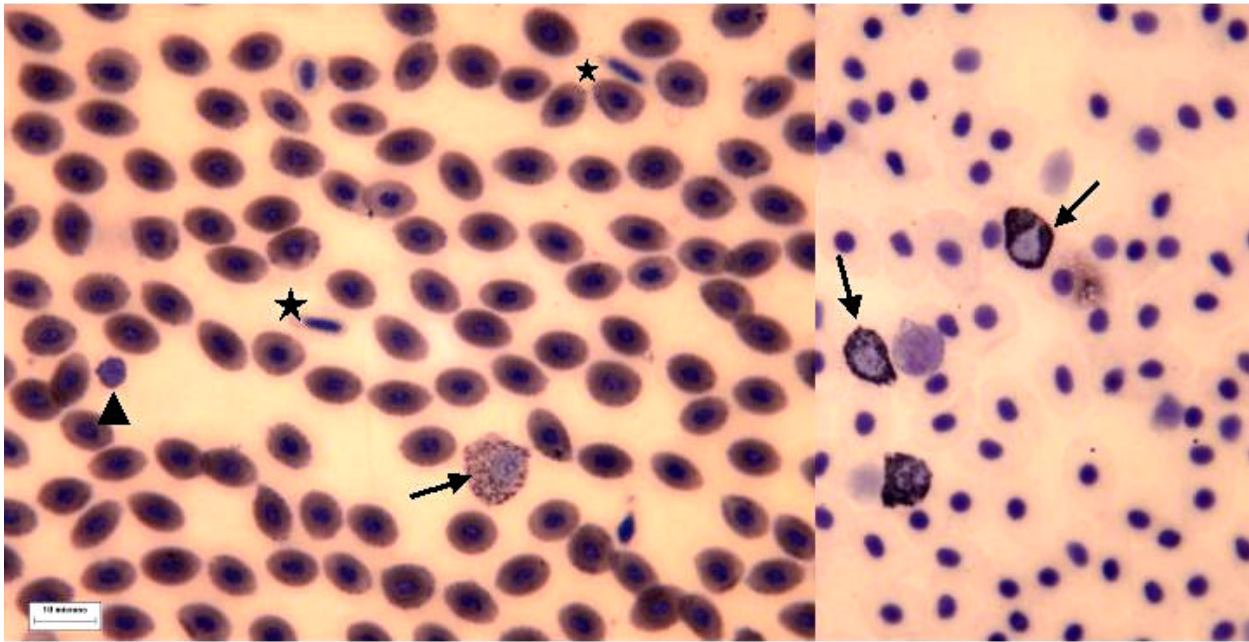
Figure 25. Southern flounder (left) and black drum whole blood stained with Sudan Black B and counterstained with Wright Giemsa. Arrows are pointing to neutrophils, stars indicate thrombocyte–type cells, and the arrowhead points to a lymphocyte type cell. Magnification bar is 10 μm.

Blood DNA Integrity

Significantly higher levels of abnormal DNA were found in fish from contaminated sites than those in reference sites for black drum, red drum, and spotted trout. No southern flounder were used in the analyses. For black drum, 64% of fish from the contaminated sites showed abnormal DNA, whereas 22% of fish from the reference sites showed abnormality (p=0.0003). For red drum, 53% of the fish from contaminated sites versus 0% from the reference sites showed abnormal DNA (p=0.0252). For spotted trout from the contaminated sites, 18% showed DNA abnormalities while no fish from the reference sites showed blood cell DNA abnormalities (p=0.0256).
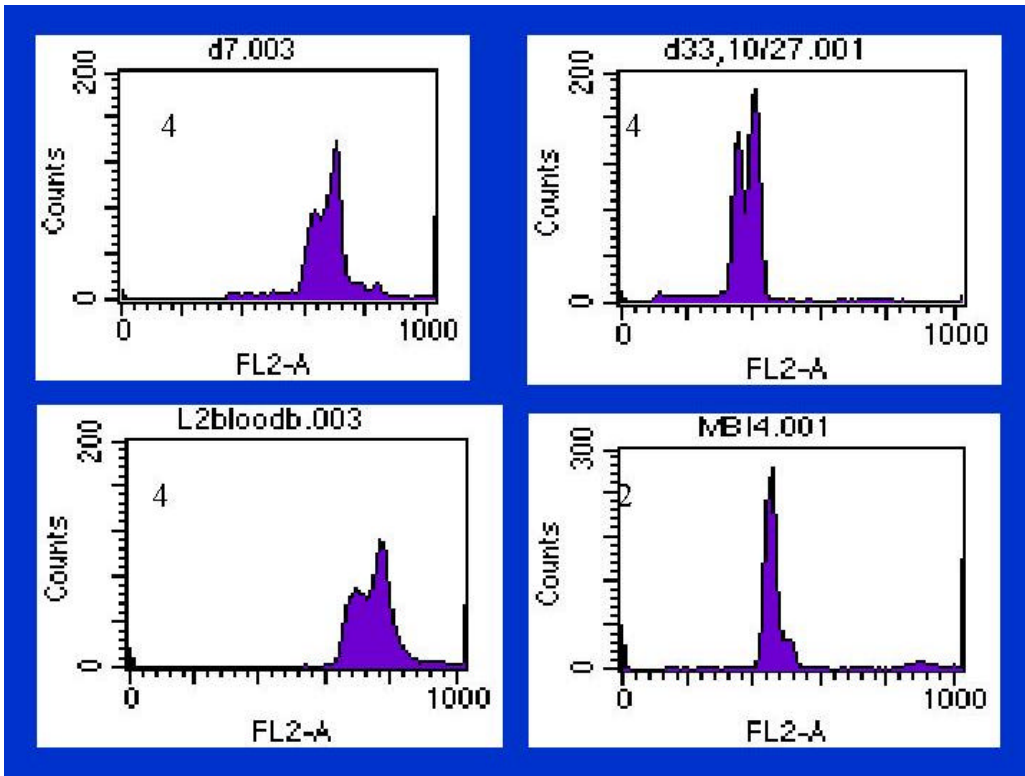
Figure 26. Representive flow cytometric histograms with DNA on the X axis showing blood cell DNA abnormalities. The more divided the peak, the more severe the abnormality. Normal histograms would reveal one peak.
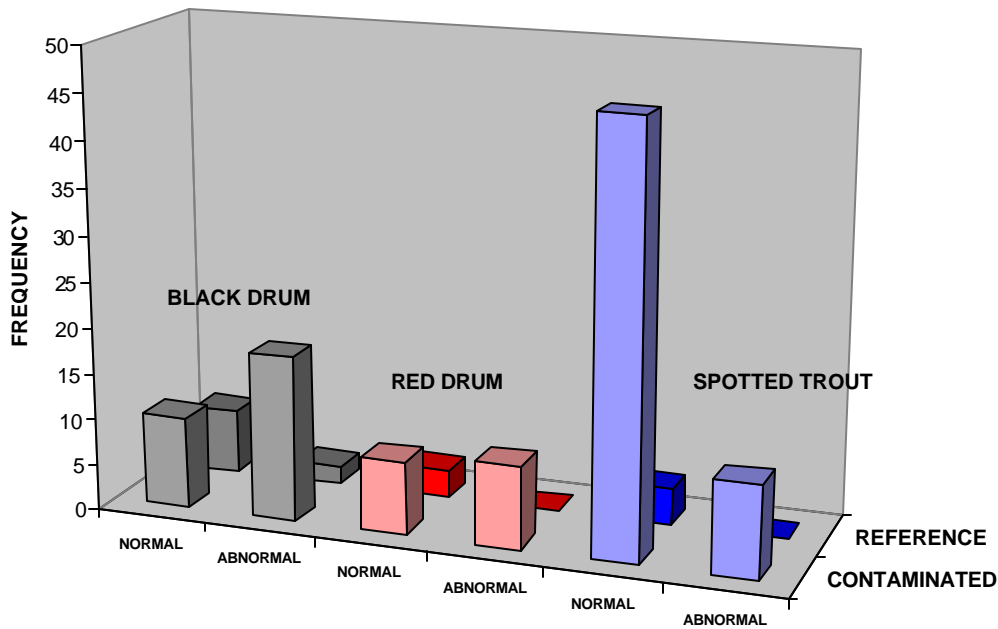
Figure 27. Frequency of blood cell DNA abnormalities categorized as normal or abnormal by site type.

Hepatic EROD

Data for enzyme activities (pmol/min*mg) were placed into ranges of low = 0 –10, medium = 11-99, and high = 100+. Differences in site type were noted for black drum (p=0.0034 and spotted trout (p=0.0084), but not for red drum, where $p \leq 0.05$.
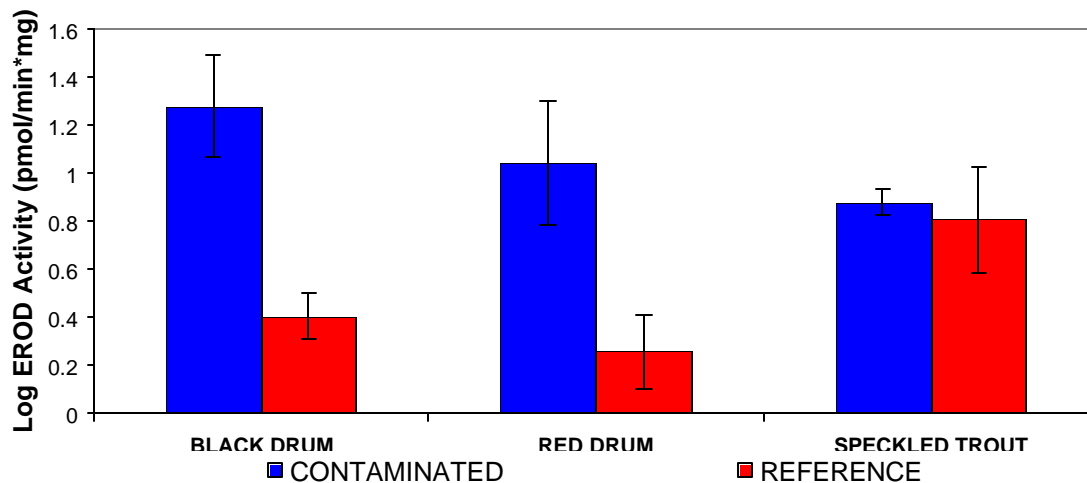
Figure 28. Log of average EROD activity per species.
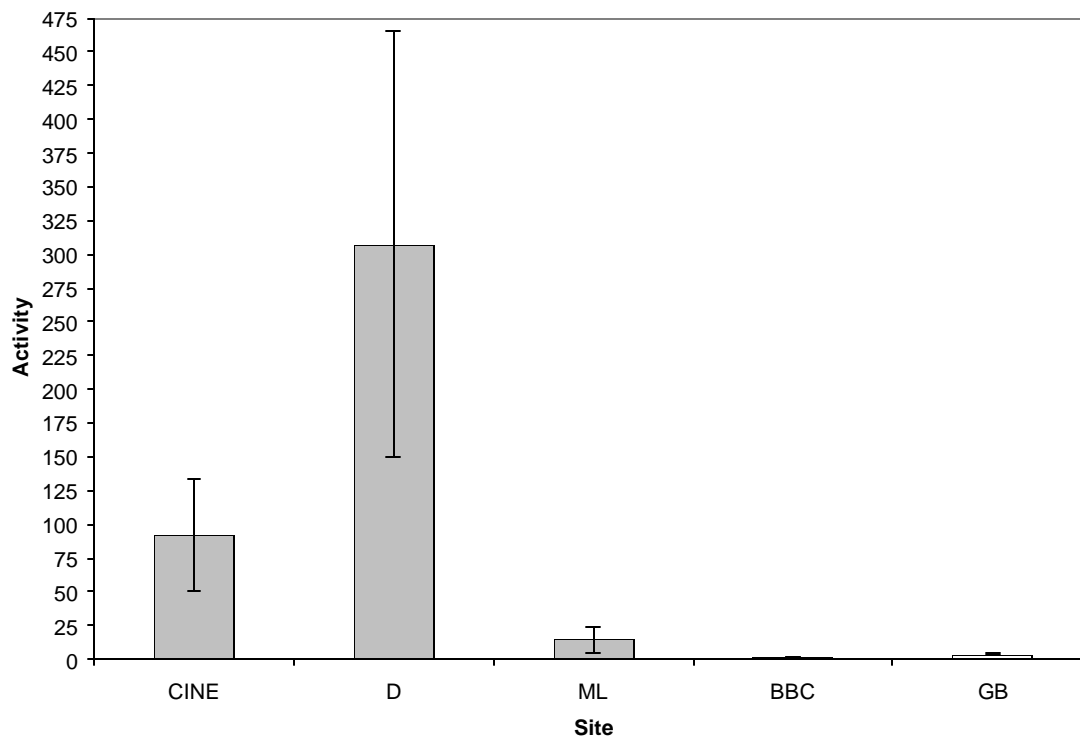
Figure 29. EROD activity (pmole/min*mg) for black drum by site.
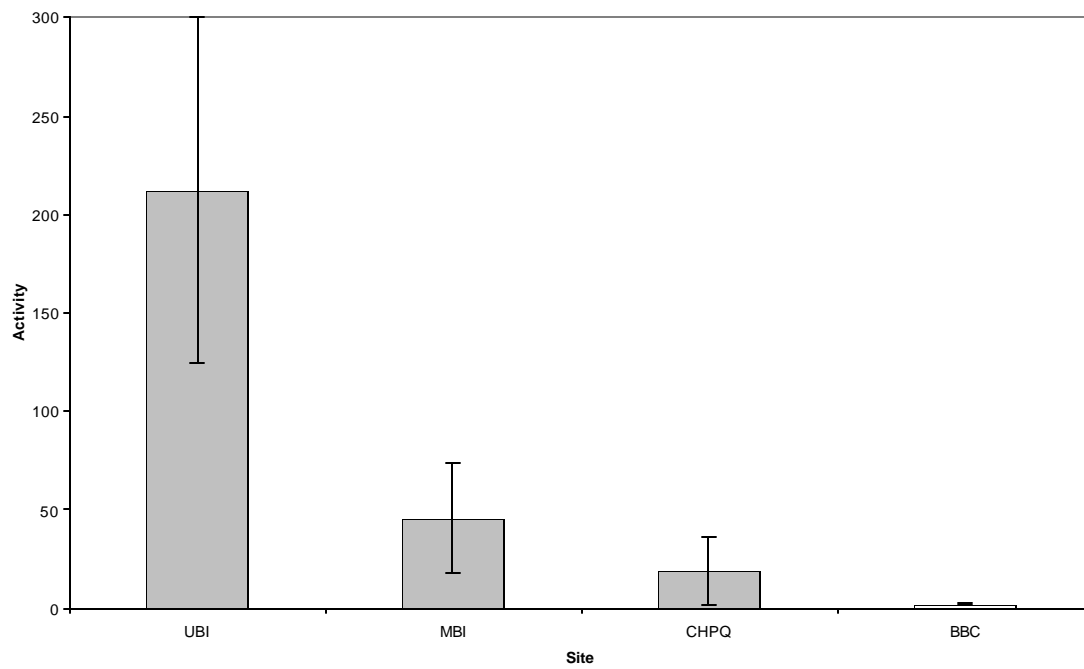
Figure 30. EROD activity (pmole/min*mg) for red drum by site.
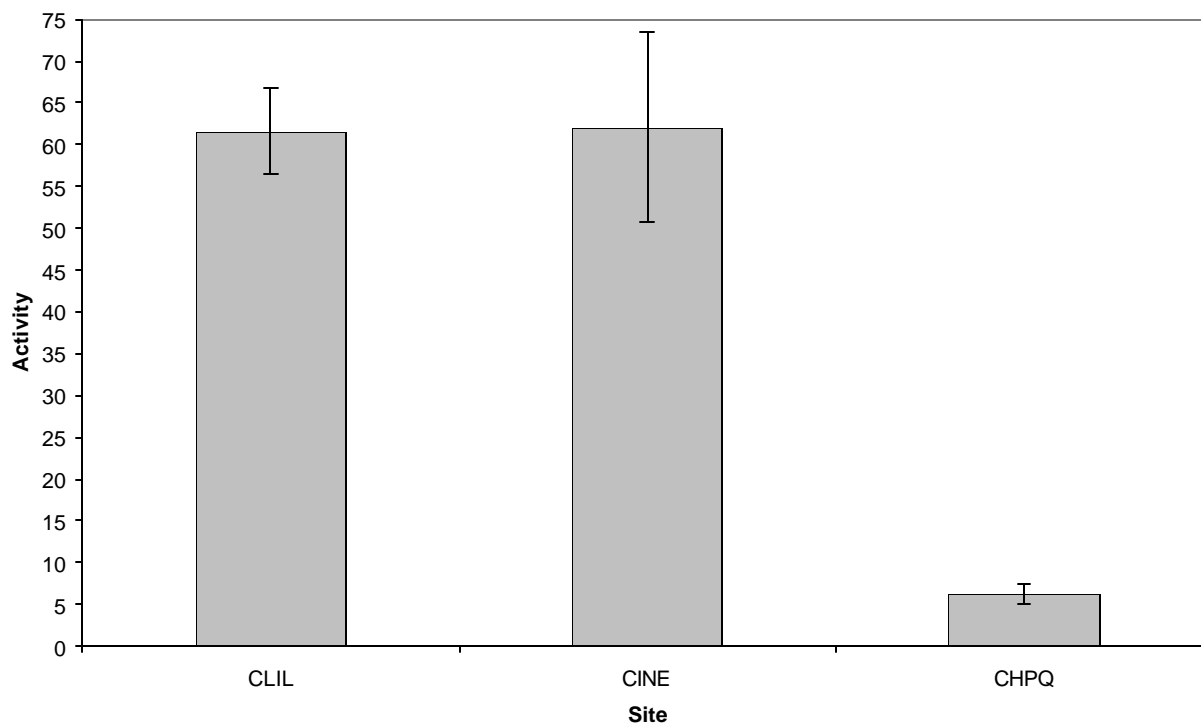
Figure 31. EROD activity (pmole/min*mg) for southern flounder by site.

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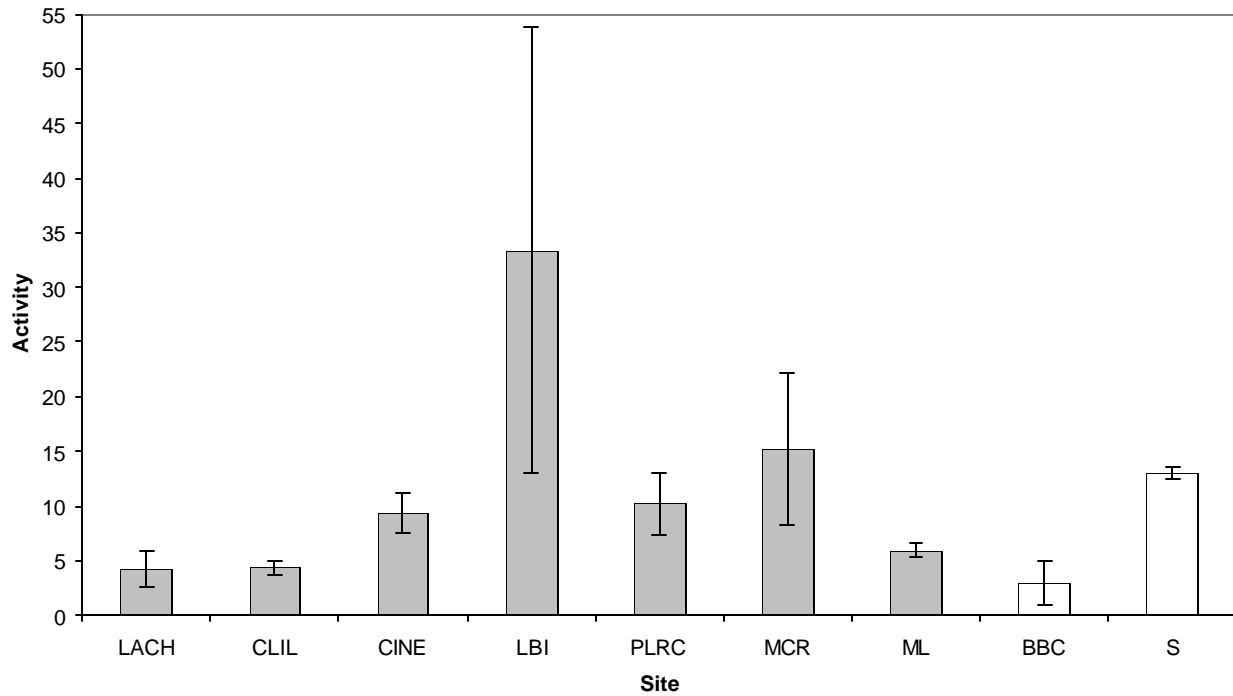
Figure 32. EROD activity (pmole/min*mg) for spotted trout by site.

Condition Factor

Least squares regression analysis of the length-weight relationships indicated a seasonal (fall or spring) difference for red drum, seasonal and sex interaction difference for black drum and spotted trout, and no difference between season, sex, or their interaction for southern flounder. Based on these results, separate equations were developed for the significantly different subpopulations. The following equations are the results, where separate equations were developed for significantly different subpopulations influenced by season and/or sex based on statistical analyses of the length-weight relationships.

Red Drum
Fall: $\log W = -4.817+(2.944)(\log L)$
Spring: $\log W = -5.276+(3.110)(\log L)$

Black Drum
Fall/male: $\log W = -4.861+(3.001)(\log L)$
Spring/male: $\log W = -5.235+(3.149)(\log L)$
Fall/female: $\log W = -5.191+(3.124)(\log L)$
Spring/female: $\log W = -5.084+(3.093)(\log L)$

Spotted Seatrout
Fall/male: $\log W = -5.272+(3.093)(\log L)$
Spring/male: $\log W = -4.938+(2.969)(\log L)$
Fall/female: $\log W = -5.204+(3.066)(\log L)$
Spring/female: $\log W = -5.106+(3.035)(\log L)$

Southern Flounder
Overall: logW = -5.632+(3.267)(logL)

After applying regression equations to Calcasieu data to determine Kn, there were no significant differences in Kn among the four species (p=0.7573) and there was not a site and species interaction (p=0.0538). There were significant site type differences in Kn between reference and contaminated sites (p=0.0008), where Kn from contaminated sites was lower (p=0.0032) (Fig. 33). For black drum, red drum, and spotted trout, there were no significant correlations between Kn and age or these biomarkers: organosomatic indices (liver, spleen, and gonads), splenic macrophage aggregate (MA) area and frequency, blood DNA integrity, or EROD.
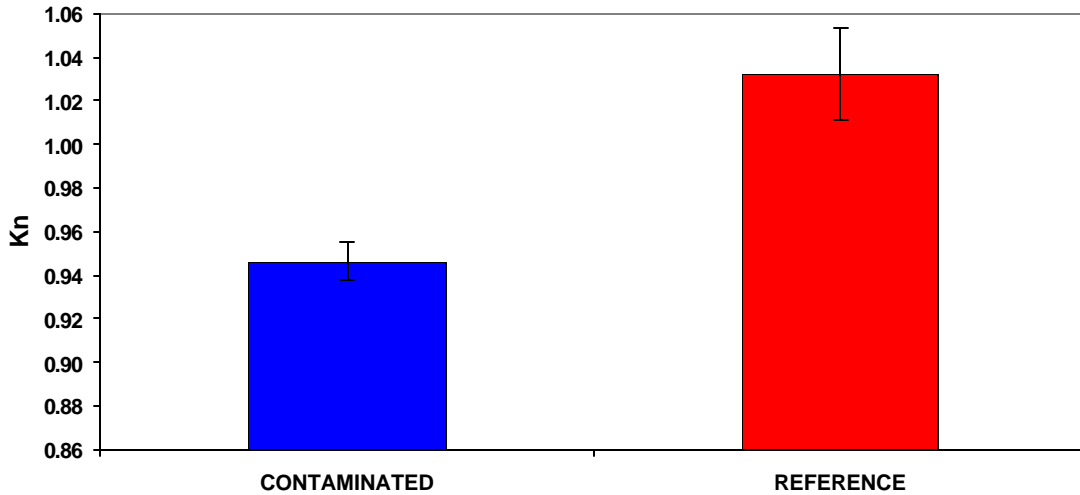
Figure 33. Relative condition factor (Kn) for four primary species of fish collected during Phase I and Phase II samplings periods at the Calcasieu Estuary. Kn was significantly lower for contaminated sites than for the reference sites (p=0.0032). Kn was 0.95±0.01 (n=122) at the contaminanted sites and 1.03±0.02 (n=23) at the reference sites.

		Species		
		Black Drum	Red Drum	Speckled Trout
Biomarkers				
	MA freq		*	*
	MA area			
	DNA	*	*	*
	EROD	*	*	*
	SSI	*		
	HSI			*
	GSI	*		

Table 7. Significant differences in biomarkers for individual species between contaminated and reference sites (asterisks).

Table 8.

SITES AT WHICH SIGNIFICANT DIFFERENCES IN PARTICULAR BIOMARKERS* WERE

NOTED BETWEEN SITES FOR THE FOUR MAIN FISH SPECIES**

SITES	LACH	CLIL	CINE (C)	UBI	MBI	LBI	D	PLRC	MCR	ML (L)	CHPQ	BBC	GB	S
LACH														
CLIL														
CINE (C)	ST-SSI ST-EROD	SF-FREQ SF-AREA ST-EROD												
UBI			RD-KN											
MBI			RD-KN											
LBI	ST-SSI ST-EROD	ST-EROD												
D														
PLRC	ST-SSI													
MCR	ST-EROD	ST-EROD												
ML (L)														
CHPQ		SF-FREQ SF-AREA SF-EROD	SF-FREQ RD-KN SF-EROD	RD-HSI	RD-HSI									
BBC	ST-SSI		BD-GSI BD-SSI ST-EROD	RD-KN	RD-GSI RD-KN	ST-EROD	BD-GSI BD-SSI	ST-EROD	ST-EROD	BD-SSI	RD-HSI RD-KN			
GB							BD-SSI			BD-SSI				
S	ST-HSI ST-SSI ST-EROD	ST-HSI ST-EROD	ST-HSI			ST-HSI		ST-HSI	ST-HSI	ST-HSI	ST-HSI BD-SSI ST-EROD	BD-SSI		

*SSI = SPLENOSOMATIC INDEX

 HSI = HEPATOSOMATIC INDEX

 GSI = GONADOSOMATIC INDEX

 FREQ = MACROPHAGE AGGREGATE FREQUENCY (SPLEEN)

 AREA = MACROPHAGE AGGREGATE AREA (SPLEEN)

 EROD = EROD ACTIVITY (LIVER)

 KN = RELATIVE CONDITION FACTOR

SITES IN BLUE ARE CONTAMINATED

SITES IN RED ARE REFERENCE

NOTE SAMPLES SIZES BY SITE ARE VERY LOW.

Note: See additional information for determining low and high values.

**BD = BLACK DRUM

 RD = RED DRUM

 ST = SPECKLED TROUT (SPOTTED SEATROUT)

 SF = SOUTHERN FLOUNDER

Table 9. Species numbers per site collected within the Calcasieu Estuary.

	Black Drum	Red Drum	Spotted Trout	Southern Flounder
Lake Charles (LACH)	-	-	7	-
Clooney Island Loop (CLIL)	1	-	8	2
Clooney Island NE (CINE)	8	1	8	2
Upper Bayou D'Inde (UBI)	-	6	-	-
Middle Bayou D'Inde (MBI)	-	6	1	-
Lower Bayou D'Inde (LBD)	-	-	11	-
Mouth of D'Inde (D)	5	1	1	1
Prien Lake Old River Channel (PLRC)	-	-	8	1
Middle Calcasieu River (MCR)	-	-	7	1
Moss Lake (ML)	6	-	7	-
Choupique (CHPQ)	-	4	-	4
Bayou Bois Connine (BBC)	2	3	2	-
Grand Bayou (GB)	6	-	-	-
Sabine Johnson Bayou (S)	2	-	3	-

Discussion

The recreational fishery in the Calcasieu Estuary targets sea trout, red drum, black drum, and flounder, and commercial fisheries for crab and shrimp also operate in the southern part of the Calcasieu Estuary. The estuary has been designated by the Louisiana Department of Environmental Quality as having surface waters for both recreation and propagation of diverse fish and wildlife. Because anthropogenic impacts necessitated the evaluation of natural resources, a sound approach that employs methods to demonstrate measurable biological responses (Adams, 2002) for assessing biological damage or injury to fish and wildlife was used. An important reason for working with fish in the field is that fish populations might respond to chronic pollution either by physiological acclimation or by genetic adaptation (Forrester and others, 2003). Similarly, more than one sympatric species is useful in accurately assessing the presence of environmental contaminants (de Sousa-Bueno and others, 2000). Taken together, bioindicator indices account for possible confounding factors such as animal age, health, nutritional status, presence of pathogens, and intra- and interpopulational genetic variability. In this study, grouping data from the contaminated or reference sites served to lessen possible confounding factors.

Fish life history, such as spawning and foraging habits, and age influences its exposure to environmental media. In this study, spotted trout and black drum were the predominant species, where spotted trout spawn in the spring through summer, and black drum spawn in the fall through winter timeframe. The study design allowed for the study of larval representatives exposed to environmental media over the full course of estuarine species spawning. Larvae and embryos are generally more vulnerable to lower levels of contaminants than are adults, juveniles are in intimate contact with sediments, and adults migrate and over-winter in close contact with sediments (Sindermann, 1996). In coastal and estuarine systems, potential toxicants in sediments are the primary form of contamination. Fish have mechanisms of resistance and tolerance to contaminants stressors, and these take various forms, such as behavior modification, increase in surface, gill, and intestinal mucus, increase in immune response, synthesis of heavy metal sequestering proteins (metallothioneins), increased mixed function oxidase enzymes for metabolizing organics, and sequestering fat-soluble material in fat (Sindermann, 1996). Population responses can be seen by the selection of the more resistant animals. These coping responses are measurable and should be considered in interpreting biomarker data.

Fisheries biologists routinely employ the body condition as a straightforward measure of fish condition because it is an inexpensive, non-lethal alternative to proximate analysis of tissues (Sutton and others, 2000), though there are assumptions that may be violated and limitations to its application. Condition factors, when low or having declined, may be interpreted as a depletion of energy reserves, such as stored liver glycogen and body fat. Prudent interpretation of fish condition factor data considers that condition factors can vary seasonally because of changes in feeding activity and nutrient availability, sexual maturational stages when energy is shifted from somatic to gonadal processes, geographically or spatially because of subpopulation differences, and sex. Consequently, the use of the relative condition factor, Kn, takes into account such variables. In this study, significant site differences were found in Kn for both red drum and spotted trout, but not for black drum and southern flounder. The average Kn (pooled over all sex, season, and species data) for the contaminated sites was lower than that for the reference sites. In this study of fish with varying life histories, careful consideration was given to species-specific data in order to generate appropriate statistical models for Kn.

PCBs produce detrimental effects on endocrine function and reproduction in a variety of species (Zhang and others, 2002). PCBs in adult chickens reduced testicular weight, the number of spermatogeic cells, and serum testosterone, and damaged seminiferous tubules. In just hatched animals, PCBs retarded the differentiation of germ cells and decreased the size of testicular

tubules. Several sites within the Calcasieu Estuary include PCBs as a chemical of primary concern including Bayou D'Inde (MacDonald and others, 2001a; 2001b) which has been previously shown to be very impacted (Redmond and others, 1996), and where sediments and biota were sinks for halogenated organics (Demas and Demcheck, 1989).

Interspecies differences in MA can be apparent at the same site. Larger (older) fish may have more and/or bigger aggregates, in keeping with results with age as a noted covariable for MA frequency in spotted trout.

The presence of parasites in marine fish species in the southeastern U.S. might not cause disease in a particular host unless they are present in high density or in a stressed environment (Overstreet 1988). In this study, splenic parasites were investigated, and only 2 fish from the contaminated sites showed high numbers of metacercariae. Neoplasia can result from chemical toxicants, and some can result from infectious agents, from a combination of toxicants and infectious organisms, or from a hereditary condition (Overstreet, 1988). Parasitic infections have served as indicators of water quality.

Body condition can be related to DNA damage, which may indicate a direct relationship or an indirect effect that is ultimately caused by toxicants affecting health (Sugg and others, 1995). Genotoxicant exposure can also produce ecological impacts by affecting the reproductive success of populations (Theodorakis, 2001; Wirgin and Waldman, 1998). Not only do tissues show differential responses to toxicants, but species do as well. That is where life histories can point toward more prevalent exposure pathways for contaminants. In this study, histological identification as female fish versus the morphological identification as male was more predominant for black drum (5 of 5 from contaminated sites) than for spotted trout (1 of 14 from contaminated sites). This may be reflective of higher overall age of black drum with possible increased exposure to endocrine disrupting compounds than the younger spotted trout, and it may be reflective of the foraging behavior differences and consequent endocrinological impact, where black drum get more overall direct and indirect exposure from sediments.

If a realistic understanding of the implications of environmental pollution is to be gained, the effects of compounds must be assessed in terms of the environment in which they occur (McBee and others, 1987). Exposure to genotoxic chemicals can damage DNA in a variety of ways, possibly leading to heritable mutations, carcinogenesis, or cell death (Hook and Lee, 2004). Thus, there is the potential for environmental pollution to impact populations as well as individuals (Gielazyn and others, 2003). The high incidence of tumors on the heads of the Gulf menhaden collected in a single sampling event may be indicative of an epizootic event. Neural tumors have not been generally linked to contaminants, although they have been associated with viruses (J. Fournie, personal communication). These fish are seasonal residents of estuaries along the Gulf coast, where juveniles and adults often are surface feeders (Lassuy, 1983), and where PAHs (El Nemr and Abd-Allah 2003) and other contaminants (Saint-Louis and Pelletier, 2004) may concentrate. A cause-and-effect relationship has been established between the exposure to genotoxins in sediment and water and neoplasm epizootics in wild fish populations (Baumann 1998). Dermal exposure to contaminants or contaminant mixtures may induce tumorigenic risk, whereby tumors may be initiated or promoted. Mutagenic events can result from complex mixtures of genotoxic compounds, and reduced immune responses may be involved in tumorogenic events. Methylmercury has been shown to induce mutagenic effects in killifish *Fundulus heteroclitus* embryos, and to retard embryonic development (Perry and others, 1988). Chromosomal aberrations in humans have been correlated with age and with mercury concentration in blood (Franchi and others, 1994).

Environmental effects of genotoxic contaminants can be determined by assessment of damage to DNA. DNA adducts in marine fish are effective molecular dosimeters of genotoxic

contaminant exposure (Reichert and others, 1998). Genotoxicants are substances that can modify both the structure and integrity of DNA, whereby damage is exerted by several mechanisms. Flow cytometry, a method of choice for sensitively measuring DNA breakage, was used in this study and showed a high percentage of fish with abnormal DNA, overall. Flow cytometry is used commonly because it is an accurate and rapid method of DNA measurement that allows analysis of large numbers of cells (approximately 10K cells per sample).

Metals readily bind to phosphate groups and to the heterocyclic bases of the DNA and can thus change the stability of DNA as well as hinder the normal functioning of DNA. Thus metal-induced DNA damage can include mutagenesis, carcinogenesis, and/or teratogenesis (see Sorensen and others, 2003). Because red blood cells are not actively producing proteins, they may have minimal DNA repair capabilities, which may be consistent with the general observation that DNA damage is greater in blood than in liver (Sugg and others, 1995). Chromosomal aberrations have been noted in rainbow trout from chemically polluted water (Al-Sabti, 1985).

Genotoxicants can also exert metabolically toxic mechanisms such as the cytochrome P450s and Phase II liver enzymes. The CYP1A family of enzymes are inducible by PAHs, holgenated aromatic hydrocarbons such as PCBs, dioxins, and other organochlorines. While the most significant role of CYP1A is the detoxification of xenobiotics, induction can also elicit toxic effects. In this study, abnormal DNA and high EROD activity were significantly more prevalent at contaminated sites than at reference sites. A variety of biological endpoints used in combinations can be effective measures in assessing the effects of environmental stressors on aquatic organisms.

Summary

The biotic integrity of an ecosystem is often reflected by the health of the organisms that reside in that system. In aquatic ecosystems, fish, especially at the top of the food chain, are generally regarded as representative indicators of overall system health (Adams and others, 1993). The purpose of this study was to assess biomarkers in multiple fish species as indicators of the conditions of the Calcasieu Estuary ecosystem.

Biomarkers are biological indicators that change in measurable ways to the environment, thereby reflecting the conditions of the environment. The sample sizes for three fish species (black drum, red drum, and spotted trout) allowed for the most robust analyses. Because of the species differences in routes of exposure to, metabolism of, and modes of action by toxicants, all analyses were performed by species. Because of the low sample numbers at individual collection sites within the designated contaminated or reference areas, most site data were grouped as either reference (n=3) or contaminated (n=11). The analyzed biomarkers included relative condition factor (Kn), organosomatic indices (liver, spleen, and gonads), splenic MA, hepatic EROD activity, and DNA abnormalities. For black drum, significantly more fish with abnormal DNA were found in the contaminated sites; hepatic EROD activity was significantly higher in fish from contaminated sites; the splenosomatic index was significantly higher in fish from contaminated sites, and; the gonadosomatic index was significantly lower in fish from contaminated sites. For red drum, relative condition factor was significantly lower in fish from contaminated sites than reference sites; significantly more fish with abnormal DNA were found in the contaminated sites, and; hepatic EROD activity was significantly higher in fish from contaminated sites. For spotted trout, relative condition factor was significantly lower in fish from contaminated sites than reference sites; frequency of MA in the spleen was significantly higher in contaminated sites; significantly more fish with abnormal DNA were found in the contaminated sites; hepatic EROD activity was significantly higher in fish from contaminated sites, and; HSI was significanlty lower from contaminated sites.

The overall epidemiological evidence is consistent with the hypothesis that sites designated as contaminated negatively impacted fish. The results show that that the end points used as biomarkers in the early detection of genotoxic agents, liver enzyme activity, and condition factor were sensitive bioindicators. The results underscore the utility of the use of multiple species for bioindicator studies. Based on these results, the sole reliance on one species to evaluate biological responses would not be sufficient to estimate damages to an aquatic ecosystem. Similarly, the use of biomarkers on only one level of biological organization would not accurately represent the impact to natural resources.

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