Abstract

Lehmann, Daniel Wade. Oxidative Stress in the Aquatic Environment: Effects of Hypoxia and Polychlorinated Biphenyls in Fish and Bivalve Molluscs (Under the direction of J. M. Law.)

Oxidative damage is hypothesized to be an underlying cause of many chronic disease states. Acutely, oxidative damage can lead to irreversible cell injury and pathological consequences. Reactive oxygen species, while naturally occurring at low levels in biological systems, can also be brought about by severe environmental conditions such as hypoxia or by chemical contaminants.

This body of work encompasses two different areas of toxicological research into the generation of radical species and the resulting oxidative damage to organisms. The first section deals with hypoxia as a causative factor in epizootic ulcerative syndrome (EUS) in Atlantic menhaden (*Brevoortia tyrannus*) in the estuaries of the Atlantic coast. Menhaden have a fairly large impact on the coastal environment and on tourism for the state, being integral to food webs of the estuary, coastal shelf, and an indicator of environmental degradation. Our hypothesis was that environmental exposure to hypoxic conditions in the estuaries leads to oxidative stress in Menhaden sufficient to cause tissue damage. Specific objectives of the study were two-fold: Determination of LC_{50} levels for both Atlantic Menhaden and the hypoxia tolerant species, Nile Tilapia (*Oreochromis niloticus*), at both acute (2 hour) and medium term (96 hours) exposures to hypoxia. Results indicated that tilapia have a very high tolerance and adaptability to hypoxic conditions. We could not ascertain an LC_{50} for tilapia as at 0.24 mg/L (3% saturation) we saw only 28% mortality at 2 hours. Ca^{++} and K^{+} were the only ionic alterations to blood

chemistry due to exposure. Menhaden had an LC_{50} of 1.2 mg/L (16% saturation) dissolved oxygen (at 28 ppt salinity) over 1 hour and had blood chemistry dysregulation affecting Ca^{++}, K^{+}, Na^{+}, and glucose levels. In the 96 hour exposures, no alterations in lipid peroxidation, glutathione, lipid soluble antioxidants, or strand breakage were detected indicating that hypoxia and reperfusion were not sufficient to cause oxidative damage or EUS in these species.

In the second portion, we evaluated the effects of PCBs in *Corbicula* clams as possible surrogates for endangered bivalve mollusks. The Ward Transformer site, in Wake County, North Carolina was found to have Aroclor 1260 levels as high as 1.7 mg/kg in fish collected miles downstream from the source. Concern over the effects of PCB exposure to the native wildlife, especially at-risk populations of native bivalves, prompted the hypothesis that oxidative damage due to Aroclor exposure would cause changes in biomarkers and pathology associated with decreased health status. Specifically, we conducted a laboratory study to determine the effects of Aroclor 1260 in order to validate common biomarkers of oxidative damage in *Corbicula fluminea* clams, without confounding environmental factors. Secondly, we performed field deployments of *Corbicula* into the polluted Brier Creek system to gauge the oxidative status of animals in the field relative to a downstream gradient of Aroclor 1260 concentrations. Results from the laboratory study at 0, 1, 10, and 100 ppb indicated that exposures were detrimental to the clams. Antioxidant biomarkers responded significantly at 3 weeks exposure and morphologic changes included inflammation, anasarca, Brown cell accumulation, severe gonad atrophy, inflammation, and necrosis. In conjunction with

biomarker changes, a linear relationship was seen to exist between sediment and time-weighted average water concentrations of PCBs and total oxidant scavenging capacity (TOSC) values. These studies indicated that oxidative damage occurs as a result of exposure to Aroclor 1260.

Oxidative Stress in the Aquatic Environment: Effects of Hypoxia and Polychlorinated Biphenyls in Fish and Bivalve Molluscs

By

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BIOGRAPHY

Daniel Wade Lehmann was born in Colorado Springs, CO in 1973. Wade is an Eagle Scout. He graduated Clemson University in 1995 with a Bachelor of Science in Microbiology and a minor in Environmental Science. After graduation, he moved to Texas to attend Texas A&M University, in the Department of Medical Microbiology as a PhD candidate. Prior to finishing the degree requirements, he left the program and picked up a position as a Research Associate in the Department of Oceanography. His research revolved around phytoplankton population dynamics and included many research voyages to different oceans around the world to classify and quantify phytoplankton populations. In 1999, he moved to North Carolina and worked as a technician at CIIT, Centers for Health Research in RTP. While at CIIT, he assisted in a large scale benzene inhalation study in rodents. Shortly after, he began graduate studies in the Department of Environmental and Molecular Toxicology under the guidance of Dr. Mac Law via the Department of Population Health and Pathobiology in the Veterinary College at North Carolina State University.

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LITERATURE REVIEW

I. INTRODUCTION

The research detailed in this dissertation encompasses two major projects involving two distinct species of aquatic organisms, Atlantic Menhaden and Asiatic clams, with an over-arching theme of oxidative stress and the toxic impacts of reactive species. The first portion involves the possible role of hypoxia and resulting oxidative stress in relation to epizootic ulcerative syndrome, commonly reported on the Atlantic coast of the U.S and elsewhere in the world. The latter half of the dissertation involves the toxicity and impact of polychlorinated biphenyl exposure and resulting oxidative stress on bivalves in a National Priorities Listed site in Wake County, North Carolina.

II. OXIDATIVE STRESS AND DAMAGE

Oxidative stress can be defined as the production of radicals in a living organism beyond the scope of its antioxidative capacities, leading to an increase in the metabolism of cells in an attempt to counteract possible damage. Oxidative damage is the end product of oxidation reactions hitting critical macromolecules within the cell. Free radicals are defined as any molecule that has an unpaired electron in its outer orbital. Reactive oxygen species (ROS) implicitly deals with radicals of oxygen in various forms. Oxygen, being necessary for aerobic metabolism, is by its very nature, able to be

transformed into a radical in biological electron transfer systems. It is estimated that 1-3% of all oxygen taken into the body becomes converted to ROS [1]. ROS are also integral to cell signaling in pro-inflammatory and growth-related pathways [2]. Oxygen, by itself or in concert with nitrogen or organic compounds, also has various levels of stringency concerning its ability to oxidize molecules. In order of reactivity, hydroxyl ($OH^{\cdot}$), singlet oxygen ($O^{\cdot}$), peroxynitrite (ONOO-), superoxide radical ($O_2^{\cdot}$-), superoxide (O_2-), organic peroxides ($ROO^{\cdot}$), nitric oxides ($NO^{\cdot}$) and hydrogen peroxide (H_2O_2) each have been defined as radical species in biological systems with a multitude of effects derived from nucleophilic attack against macromolecules. Radical half-lives (t ½) are generally inversely proportional to their reactivity, with highly reactive compounds having a short distance of effect due to a high level of reactivity, such as the estimated 1 ns $t_{½}$ of hydroxyl [3]. Radicals have two modes of decomposing. The first is dimerization with another free electron containing molecule creating a stable compound and the second is disproportionation when one compound is oxidized and another is reduced simultaneously. Radical species are kept in check by a system of enzymes and non-enzymatic low molecular weight antioxidants compounds. These antioxidants are present in plasma, cytoplasm, and lipid bilayers in order to prevent radical chain propagation and oxidation of nearby proteins. An antioxidant can be defined as a chemical species able to create a stable radical by donating an electron to highly electrophilic radical compounds and is found in a quantity within biological environments sufficient to overcome radical presence.

Free radicals originate from both normal biological processes and the interaction of exogenous compounds. Natural processes within enzyme systems are a large source of natively produced oxygen radicals *in vivo*. The electron transport system, during the oxidation of molecular oxygen via mitochondrial respiration, is a naturally occurring source of oxygen free radicals. Electron transport overspill can also be exacerbated by anthropogenic toxicants that interrupt the chain of electron transfer. Enzymes can also be a source of cellular radical production. Enzymes such as xanthine oxidase, cytochromes, and nitric oxide synthase can release radicals in the form of reactive oxygen as well as metabolites that have reactive centers. A third native source of reactive compounds *in vivo* is the immune system. Cellular defenses include the production and release of superoxide as well as nitrogen oxides that are effective antibiotics. Most of these systems are under stringent control in order to prevent release of radicals into the surrounding matrix; however, toxicants can interact with various points in many of these biochemical reactions causing release of enough reactive oxygen to overcome local antioxidant defenses [4, 5].

Two other causes of oxyradicals are hypoxia resulting from ischemia and reperfusion when blood supply is returned to the tissue. Ischemia is the localized loss of blood flow to tissue creating a zone of hypoxia and repressed nutrients (partially mimicked by hypoxia in the environment) which has been shown experimentally to produce superoxide, induce various antioxidant enzyme systems, and leads to an effect termed 'stunning' in which cells cease normal function [6-9]. Stunning is reversible if oxygen is

restored fairly rapidly to the tissue. However, reperfusion can also generate oxyradicals [9]. Reperfusion is the rapid infusion of oxygen into hypoxic (ischemic) tissue that results in a burst of oxygen radicals due to enzyme and electron transport backup and overspill which can in turn overburden the local antioxidant abilities of that tissue and results in oxidative stress or damage. This effect has been experimentally defined in both cell culture and in intestine models of ischemia and reperfusion. Implicated to have a role in reperfusion injury are neutrophils releasing locally high levels of reactive species, activity of xanthine oxidase, and the uncoupling of iron from lactoferrin enzyme [10, 11]. In addition, it has been suggested by Paller and Jacob (1994) that P450 enzymes can function directly as iron donors in reperfusion injury [12, 13].

Most chemically induced reactive species are organic molecules, frequently with a cyclic structure, that contain oxygen moieties or a strong electrophilic side group, although select toxicants can interact with cellular processes and cause secondary radical production. Two generalized reactions occur that generate radicals from anthropogenic compounds. The first is a compound being metabolized to produce a reactive secondary metabolite. This is the case with many of the phase I and II metabolized compounds. The cleavage of bonds within the molecule causes a free or unpaired electron to exist such as in the production of nitrenium and carbonium (carbocation) radicals from 2-acetylamonifluorine metabolism by N-hydroxylation. Paraquat is an example of a compound that is enzymatically metabolized to an oxon allowing for reactivity. The second mechanism is by two-electron quinone reductases catalyzing the redox cycling of

polycyclic aromatic hydrocarbons and compounds such as anthracyclines. The presence of uncoupled or enzyme bound transition metals is also a source of radical production in living cells. Lactoferrin, for example, can be a source of hydroxyl radical production by way of the iron core of the enzyme [10]. This effect is countered by antioxidants such as superoxide dismutase (SOD) and catalase [14].

The macromolecules affected by free radicals are very diverse, although in many instances an alteration in a molecule will have little effect beyond destroying the activity of the molecule. Damage to DNA, select proteins, and lipid membranes, however, is very detrimental if uncontested. Lipid membranes, specifically unsaturated fatty acids (FA) contained in those membranes, are very sensitive to radical attack. Oxidation of the side chains of FA causes a stiffening of the membrane and a propagation of the radical throughout nearby fatty acid chains. Lipid peroxidation (LPO) is a series of steps including initiation of the peroxidation by attack from a free radical, chain propagation, and eventually termination. Initiation occurs due to the nature of FA having double bonded carbons separated by methyl groups. Chain propagation proceeds from the production of the lipid radical by the interaction with molecular oxygen creating a lipid peroxide. This peroxide is active in that it attacks a nearby FA which leaves behind a lipid hydroperoxide (LOOH) and generates a new lipid free radical. A second progression or chain propagation step can occur when hydrogen gets abstracted from LOOH generating an alcohol and a new lipid radical. This process has been termed chain branching. This process continues until terminated by antioxidants or dimerization with a

nearby lipid radical. Membrane oxidation causes stiffening thereby decreasing the fluidity of membranes and allows for leakage of cellular constituents into plasma or surrounding tissue. Severe lipid peroxidation can lead to cell death via ionic dysregluation or deterioration of cell structure. Laboratory and field studies of contaminant exposed fish and clams has shown increases in detectable lipid peroxidation [15-18].

LPO leads to the production of numerous chemical byproducts. Peroxidation of lipids can lead to the generation of reactive aldehydes, which in turn can react with nearby molecules. Malondialdehyde (MDA) and 4-hydroxynonenol (4HNE) are two of these reactive compounds which are detectable in biological systems. They are reactive and bind proteins and other molecules which allows for their detection using various methods. Linoleic and arachidonic acids are especially vulnerable to oxidation and result in the release of 4HNE [19]. Another commonly detected target of oxidation are nucleic acids. Both free nucleoside pools and DNA/RNA molecules are subject to radical attack. The structure of purines is such that oxidation can commonly occur at specific locales on the molecule and as such can be used as an indicator of oxidative damage. The effects of the oxidation of DNA are numerous. Repair can occur, whereby the damaged nucleotide is enzymatically cut from the DNA backbone and eliminated. If repair does not occur, misincorporation of nucleotides can occur during synthesis and replication of DNA leading to cancer or reproductive abnormalities. Alteration of the cytoplasmic pool of certain nucleosides, specifically guanine and adenine, can also have negative effects on

cellular function such as signaling systems. Repair of the damaged molecules is also energetically expensive which can cause physiological stress for cells.

Protein oxidation, while negatively impacting the cell, is of minor concern in most instances. Non-functional enzymes are degraded and removed from the cell and are replaced under normal physiologic processes. The replacement is energy expensive which can be detrimental if enough occurs [20]. In some cases, the buildup of oxidized proteins and the reduction in effectiveness can lead to disease states. Alzheimer's Disease is thought to have a link to the buildup of oxidized compounds in cells. Cancer can be accelerated when antioxidant systems are reduced and allow more oxidation of proteins responsible for repair and maintenance. Proteins, and specifically enzymes, that contain valent metals are of concern in that they are also considered producers of free radicals. Due to the rapid reactivity of some oxygen radicals, these enzymes are more vulnerable to oxidation. Iron- and copper-containing enzymes are of concern as the valent states allow for Fenton reactions to occur if the enzyme becomes uncoupled as in experiments with lactoferrin and P450 enzymes [12, 21, 22].

The primary means of protection that cells employ against oxidative damage is an antioxidant system, comprised of two major absorptive compound components: enzymatic defenses and low molecular weight antioxidant compounds. Enzymatic defenses include dismutases, catalases, and peroxidases among others. Enzymatic systems function by coupling two radical containing constituents into a stable byproduct

or by donating, via NADP(H), an electron to the compound, thereby stabilizing the outer orbital of the initial reactive molecule. Non-enzymatic defenses are typically grouped into two categories: lipid-soluble or cytoplasmic. Lipid soluble antioxidants consist mainly of tocopherols, carotenoids, and ubiquinones. These are integrated into lipid bilayers, especially in reticular bodies and mitochondrial membranes in order to protect against LPO and lipophilic oxidants. Cytoplasmic antioxidants consist mainly of ascorbic acid, uric acid, glutathione, bilirubin (extracellular), and dietary sources (typically plant-derived compounds) and are found freely available in the hydrophilic fraction of cells for ready reduction of water soluble compounds. The complex regulation and interaction of the antioxidant system assures that both types of antioxidants work in conjunction with one another for total cellular prevention of oxidation. Various experimental models have been used to test the efficacy of antioxidants to ameliorate the effects of oxidation [23]. Antioxidants that are effective against reperfusion injury include ascorbic acid, trolox (vitamin E homolog that is water soluble), and GSH [24-27]. Increases in antioxidant enzyme systems are also commonly reported in controlled laboratory studies of both chemical and physiological oxidative stress, although they vary between ischemia/hypoxia and reperfusion. For example, during hypoxia, mRNA in rat liver generally decreases in quantity, although reperfusion produces an increase in Cu/Zn-SOD, Mn-SOD, and GPx [28]. The same rise of SOD was evidenced in Spot (*Leiostomus xanthurus*) due to environmentally controlled hypoxia exposure [29].

The oxidation of macromolecules has been implicated in almost all forms of chronic disease including carcinogenesis, atherosclerosis, Alzheimer's disease, neurological declines and aging. At high rates, oxidative damage can cause localized necrosis due to the destruction of cell membranes and proteins. The mechanisms of damage in acute oxidative attack include loss of ionic homeostasis, leading to increased intracellular calcium levels. The increased cytoplasmic calcium concentration causes permeability transition pore opening in mitochondria which effectively uncouples respiration and causes cell death via apoptosis or even necrosis in severe situations [30].

III. MENHADEN LIFE HISTORY AND BIOLOGY

Aquatic species are unique as models in experimentation due to their immersion in the aqueous environment. Dose cannot be used to define ecotoxicological study parameters in the field and in many species, which means that referencing to concentration in the local environment or body burdens at a given times during experimentation is necessary as is the case with hypoxia. In addition to dosing issues, the intimate contact with the environment means that exposure is multifaceted and includes environmental factors and variable, and possibly confounding, inputs such as multiple anthropogenic stressors.

Atlantic Menhaden, *Brevoortia tyrannus*, are a true euryhaline species in the Clupidae family. They begin life in the outer estuaries or on the continental shelf as eggs laid *en masse*. The eggs and hatching larvae are moved by prevailing winds and tidal movement

upriver to regions of low salinity where they begin to develop and migrate downriver back toward the continental shelf [31]. Development accompanies striking physiological change as the larvae mature into juveniles and then further into adults before migrating into open water on the continental shelf [32]. This migratory pattern during development allows menhaden to serve as valuable sentinels of regional conditions and contaminants in a given area at a specific time. As an adult or year 1 fish, menhaden serve as a valuable indicator of whole ecosystem health in regards to bioaccumulative contaminants, having traversed from river freshwater origin to ocean. The fact that these fish have predictable downstream development also lends itself to cohort studies whereby health and population can be monitored throughout their life cycle. These fish are endemic from the Gulf of Maine to Florida where they are replaced by a close relative, the Gulf menhaden (*Brevoortia patronus*) [33].

Juvenile fish, also known as 'peanuts,' (young of the year, 20-100 mm) are most commonly reported to be affected by lesions, and occur in a salinity range of 10-19ppt, as reported during random sampling by the NC Department of Environment and Natural Resources (NCDENR). NOAA estimates fisheries catches for 2001 of 233 thousand metric tons [34]. Estimated population size by age, projected based on Murphy Virtual Population Analysis, indicates that the biomass of *B. tyrannus* in recent years has fallen drastically from that seen in the 1980s, from approximately 14,000 million to 3,160 million in 2000/2001 [34]. Menhaden are one of the most economically important fisheries in the United States [35, 36]. In regards to direct usage, they are harvested by

the hundreds of tons for their oils and protein. As reported by Joseph *et al*. [37] and Dubrow *et al.* [38], menhaden are comprised of 15-20% protein and 8-20% lipid. They are a source of unsaturated fatty acids, especially omega 3 fatty acids, which are touted for their antioxidant properties. Menhaden products can be found in food supplements, cosmetics, and other common use items. Recent research has even created a niche for menhaden oils, as a supplement, in research against the effects of ischemic injury [85]. A second relevance to the economy is their presence and position in the food webs of the estuary and continental shelf. Menhaden are a food source for many of the game fish that inhabit the coastal estuaries and continental shelf offshore which draw tourism dollars. Negative impacts due to massive fish kills and reports of pathogenic organism occurrence in estuarine species can lead to decreases in fishing resulting in reduction in tourism derived dollars. Tourism related to outdoor activities and coastal recreation, a large percentage of which is fishing, according to the North Carolina Department of Commerce 1999 report, is a significant portion of the tourism (81% of 11 million annual visitors, 52% of total state visitation) derived money in the state.

Atlantic menhaden are filter feeding primary consumers that constantly graze on particulate debris and phytoplankton in the river systems where they mature. While in the larval state (14-34 mm) menhaden are particulate omnivores. Distribution of the species within estuaries is defined by phytoplankton gradients and food species concentrations [39]. Due to this adaptation, they selectively feed in areas of high nutrient concentrations that cause plankton to bloom, including eutrophic zones. These zones

typically progress through a cycle of oxygen concentrations, passing through hypoxia and ultimately ending in an anoxic local environment. Concurrently with oxygen cyclicity, perhaps even accentuating it, salinity and temperature wedges form which can physically limit fish movement in the estuaries and rivers leading to increased oxygen demand and ultimately death in the affected zones [32].

Adaptation has provided most fish with a suite of physiological alterations that can be initiated on demand to assist with low oxygen environments. Hemoglobin type shifts, ventilation increases, metabolic change and depression, and oxygen sensing all aid in detection, physiological management, and escape behaviors. Unlike many other estuarine species, obligatorily mobile Atlantic Menhaden do not have the ability to repress motor activity levels during times of stress to compensate for reduction in arterial oxygen. As active swimmers that never cease moving, their physiological behavior is possibly detrimental inside limited and shifting estuary environments.

IV. ASIATIC CLAM LIFE HISTORY AND BIOLOGY

The Asiatic clam, *Corbicula fluminea*, is a species native to Southeastern Asia. Due to their adaptability, however, Asiatic clams can now be found in freshwater environments across Europe and the North America in loose sand and rock aquatic environments that have moderate flow and high oxygenation. *C. fluminea* was first documented in the 1920s in coastal British Columbia and has since progressed to occupy most warm,

freshwater environments. Accidental introduction of the species was likely due to release of clams brought over by immigrants from China where they are utilized as a food source. In North Carolina, documentation of clam presence first appeared in the mid 1970s [40, 41]. The high rate of spread, high growth rate, low generation time, high reproductive capacity, and the ability to reach staggering densities has determined their classification as a pest or nuisance species that likely out compete native unionid and sphaeriid species [42].

Corbicula are simultaneous hermaphrodites, with a capacity for self-fertilization. Fertilized eggs are incubated in inner demibranchs in the mantle until the pediveliger stage (18-25um) which is approximately 30 days. Unlike native unionid mussels, these clams do not have intermediate hosts and the juveniles, when released, can immediately use their muscular foot and byssal threads to attach to the substrate. Water movement can also readily carry these small, unattached larvae downstream [41, 43]. Their hermaphoriditic nature and exceedingly high reproductive output, of approximately 8000 juveniles per year per individual, creates a highly available species for scientific study [44]. Corbicula also utilize two reproductive periods per year, in the spring and fall, most likely dictated by temperature and environment. The intense growth rate, estimated at 16-30mm shell length per year, also provides for a good model for toxicological testing as the high metabolic rates and two reproductive periods per year increase *Corbicula* viability as a model [42]. The localized presence of these organisms in the environments

where native, endangered species exist is also of benefit, since *Corbicula* can be used as a surrogate for toxicological studies so as not to impact wild populations.

Corbicula have been used as a model for toxicity testing and a highly useful sentinel species for field testing for years. Responses to environmental factors, biomarkers of exposure for many compounds, and histological evaluations have been performed using *Corbicula* as a model organism in many locations around the world [45-47]. In a 1988 review, Doherty and Cherry summarized findings on the tolerance of *Corbicula* clams to lethal levels of stressors and environmental factors such as substrate in both adults and juvenile stages [48]. The discussion centers on anti-fouling treatments, but is also relevant to the use of these clams as a model organism in that it defined the parameters necessary for successful culture and testing of the clams: substrate presence, life stage, recovery periods after exposures, median lethal (LC_{50}) concentrations for many toxicants, and temperature effects. Clams are also widely used as uptake models for anthropogenic compounds in the environment, so information exists about the kinetics of many chemical species as well as their metabolism and biomarker responses [48].

V. LESIONS

It has been found, through histological monitoring of thousand of samples, that there is a background level of infection and disease common to Atlantic Menhaden populations in estuaries [49]. A variety of pathogens have been loosely associated with ulcerative

lesions including anchor worms, myxosporidians, viral, and fungal agents. Lesions associated with epizootic ulcerative syndrome (EUS) have been identified in locations worldwide including Japan [50], Southeast Asia [51], and Australia [52]. In the United States, lesioned estuarine species have been documented for years from Maryland to Florida, but are most prominent in Atlantic Menhaden [53-58].

Outbreaks of lesions in Menhaden have been described since 1984 in various estuarine locations along the U.S. Atlantic coast, with episodic outbreaks occurring that frequently correspond to large scale fish kills [55, 59, 60]. In multiple histological sections, it is readily apparent that these lesions are typified by deep muscle necrosis. (fig 1) This is consistent with the deep, necrotic lesions that have resulted from hypoxic conditions in catfish [61]. Transmission electron microscopy of the Menhaden lesions shows this necrosis penetrating into and destroying muscle fiber bundles. (fig 2) However, there is often no evidence of a primary biological pathogen associated with this disease state. We have also noted a significant number of pre-ulcerative lesions upon sectioning of random fish from these sites. The thin epidermis of Menhaden may contribute to their susceptibility to lesion development [62]. The ulcerative lesions were initially described as focal ulcers that were usually singular and found most often on the ventrum of the fish near the anal pore. Occasionally, lesions occur along the dorsal ridge or through the peritoneal cavity wall [63]. Ulcerative lesions are typically classified into early (type 1 or type 2), advanced, end-stage, or healed depending on their characteristics [59]. Early lesions are uncommon as they are difficult to identify, with type 1 being red and flattened

and type 2 being raised and slightly more obvious. Advanced stage lesions penetrate into the peritoneal cavity and adjacent organs and this severe localized damage leads to sloughing of necrotic portions leaving a crater-shaped lesion classified as end-stage. We have even netted fish that had end-stage lesions penetrating through the entire body. Healed lesions were typically small (less than 5 mm) and appeared as smooth, non-unclerative areas of tissue loss [59]. Lesions can result from environmental stressors including hypoxia, temperature, or salinity shifts [57, 61, 62, 64]. Frequently, the lesions are laden with fungal hyphae and other opportunistic parasites, although the timing of lesion development is difficult to determine relative to infection status [55]. Lesions may develop from an 'inside-out' pattern, evidenced by pre-ulcerative lesions seen beneath the epithelium in fish histological sections [65]. (fig 3)

Earlier studies in NC using trawls and pound nets showed that lesioned fish were mostly present in the inland regions of the Pamlico River, but also indicated there was no relationship to water quality, including temperature, DO, or pH [55]. Later studies indicated correlations with regions of lower salinity with the development of lesions, but again showed no relationship to sampling time, DO, or pH [63]. Lesion prevalence and occurrence was monitored by random sampling of river sites by the NC Department of Environment and Resources (NC DENR) in the late 1990s and early 2000s. This sampling has shown lesion prevalence ranging from 0-95% of net caught menhaden in the New, Neuse and Tar-Pamlico river basins. Massive fish kills have also been monitored. Lesion prevalence within those kills ranges from 5-95% and consists

typically of ‘peanut’ sized juvenile fish (young of the year). Adult fish have been found with lesions in the near shore, but first and second year sampling has been unreliable due to the natural habits of the fish and their locations offshore.

Lesion etiology remains a topic of strong debate. Various laboratories have attributed lesion development to a multitude of biological factors including *Aphanomycete* fungus, K*udoa* protozoans and *Pfiesteria* dinoflagelates [58, 63, 65, 66, 66, 67]. Based on histological evaluation of over 2000 samples of Atlantic Menhaden in our laboratory, each of these agents may play primary or secondary roles in EUS at various times and under various environmental conditions.

Lesion development was originally attributed to *Pfiesteria piscicida* in the early 1990s [59, 63]. *Pfiesteria* is a dinoflagellate alga that has many life cycle stages and has been demonstrated to physically attack other algae and fish epithelium [68-70]. There is a putative neurotoxin generated by *P. piscicida*, although it has not yet been isolated and fully characterized [70]. Studies of Pamlico River and Chesapeake Bay fish kills attributed much of the observed mortality with the presence of *Pfiesteria* in the water as determined by polymerase chain reaction. Mixed results have been shown that via cell free exposure to *Pfiesteria sp.*, exposure can cause the acute death of fish, but in other cases no mortality has been reported. No lesion presence was ever observed in these studies [71]. Doubt regarding the viability of *Pfiesteria sp*. toxins as causative agents in lesion development led to the consideration that another agent may be present [71].

Fungal spores were frequently seen in open lesions so work led to the determination that *Aphanomyces* could indeed cause lesion development in closed environments [57, 58]. Koch's postulates have been verified for *Aphanomyces*; however, our own histological evaluations have shown fungal spores are present in only a portion of lesions [55, 72].

In conjunction with biological agents, environmental factors may play a role in lesion development due to stresses placed on estuarine animals and resulting immunocompromise [73, 74]. Pesticides, nutrient loading and resultant eutrophication, and physical environmental changes may play a role in large scale fish kills and lesion development [62]. Paerl *et al.* has shown that correlations with hypoxia exist for fish kills generated through use of the ModMon system in North Carolina rivers, although no such trend has been reported for lesion events[75]. Lesion events do occur mainly in the spring and fall, during times of temperature shift and increased pesticide usage. Temperature changes could be responsible for algal population shifts, depressed immune function, or a host of other physiological alterations possibly leading to susceptibility to or development of lesions. Physical stresses need to be considered in that Menhaden school in tight units in shallow areas of the estuary where they can become entrapped. Physical contact with other fish, contact with substrate, or possibly even predation attempts, are all possible mechanisms of epithelial barrier breakdown in such localized zones. Another effect of being clustered in a small area is the depression of oxygen levels. Rapid depletion of oxygen is possible due to the large numbers of active fish entrapped in creeks or shallow areas.

Lesion events most commonly occur in brackish water regions of estuarine rivers. The so called 'hot spots' have higher lesion incidence and over time have proven to be zones where recurrences are common. These same zones are shown to have periods of hypoxia and anoxia that are cyclic and dependant on prevailing environmental factors such as salt wedges, decreased rainfall, and increased eutrophication [75]. While earlier studies failed to correlate factors such as pH, temperature, and DO levels, it is of value to note that sub-lethal effects in a mobile population are difficult, if not impossible, to ascribe to the occurrence of a specific event, such as anoxia. The mobility of Menhaden is especially high and effects derived due to a chemical or environmental factor may not be visible for days, which could place the sampled cohort miles from the source of the stress [76]. A possible result of isolation in a low oxygen environment is ischemic tissue injury as seen in various health conditions in mammals such as stroke or thrombosis. In this instance, it is hypothesized that animals caught within a region of hypoxia might experience reperfusion injury upon movement into normoxic waters. The resulting oxidative stresses might play a direct role in cell damage as well as depression of the immune system leading to lesion formation [9].

VI. HYPOXIA

The capacity of water to hold oxygen is substantially lower than that of air. Oxygen enters the aquatic environment via diffusion with the surface (or aeration by physical agitation) and photosynthetic creation [77]. The diffusion of oxygen into water is slow

compared to the diffusion of oxygen in a gaseous environment. Oxygen is consumed by respiration of organisms within the environment. Regions containing high levels of nutrients allow for higher respiration rates and, due to overgrowth of bacterial mass when phytoplankton populations crash, will result in hypoxic or anoxic conditions as the surrounding biota consume the dissolved oxygen at rates that exceed replacement. Salinity, atmospheric pressure, and temperature also play defining roles in the holding capacity of water for oxygen, although temperature is the dominant factor. As temperature increases, ambient DO levels fall according to the ideal gas law. Aquatic organisms, being primarily poikilothermic, also have a higher consumption rate of oxygen at higher temperatures, exacerbating the effects of temperature stress. As an example, a trout at 5^{o}C requires approximately 5 times less oxygen than at 25^{o}C due to both increased metabolic demand and the ambient dissolved oxygen concentration.

In the aqueous environment hypoxia is defined as oxygen tension that is too low for maintenance of homeostasis. The exact level at which hypoxia becomes stressful is dependant on differing species tolerances, but levels below 2.8 mg/l of dissolved oxygen are considered to be hypoxic [78]. In the review by Miller (2002), levels of DO were compared in various species at various life stages. Most fish species have an LC_{50} in the range of 0.8 to 3.5 mg/l, with earlier life stages being more susceptible to lethality than later stages [79]. DO levels below 4.0 mg/l also have a drastic effect on the sustainable diversity of fish populations [80, 81]. Anoxia is the lack of oxygen remaining in solution, a common occurrence where biological oxygen demand outstrips input rates. Broad

changes in oxygen concentrations are characteristic of aquatic ecosystems impacted by eutrophication. Eutrophication has been indicated as the most prominent water pollution issue in modern times as the rate and severity of events increases [78]. Daily cycles of oxygen levels in estuaries can range from daytime supersaturation reaching as high as 300% to pre-dawn anoxia (0%) [75, 81, 82]. As noted by Paerl and coworkers, shallow, high organic impacted estuaries have a very high rate of hypoxic episodes brought on by eutrophication that can entrap or endanger animal populations [75]. In addition to temperature, other physical factors such as haloclines, thermoclines, tidal events, and rainfall amounts can all have significant effects on dissolved oxygen concentrations in shallow environments [83, 84].

Periods of hypoxia or anoxia can have profound effects on aquatic organisms. Anoxia has been associated with the loss of benthic invertebrates, commonly evidenced in eutrophied ecosystems [84], as well as fish kills[86]. Also, hypoxic events have been proposed to play a role in more subtle disease conditions such as reproductive or developmental abnormalities [78]. The spectrum of health effects observed in wildlife populations, although implied, is still only partially understood mechanistically. Arrays of changes occur at both the genetic level and the physical level in organisms exposed to hypoxic conditions. Physically, aquatic animals exposed to hypoxia tend to alter behavior to increase avoidance and down regulate energetic activities [85, 87, 88]. Immediate responses also include alterations in blood flow patterns to maximize oxygen exchange with the environment and minimize peripheral blood flow by shunting flow to

necessary tissues [89]. In humans, this shift and dilation is controlled by potassium ATP channels and polarization status that changes with redox status [90]. Penumbra, or the reduced functionality of cells during ischemic stress, is recoverable if the stress does not exceed recovery mechanisms.

Gene modulation by hypoxic exposure is a multifaceted series of events, primarily dictated by the controlled generation of oxygen radicals as signaling molecules intracellularly. One mechanism is the NADPH oxidase control by radicals [81, 91]. A second, and more pronounced, mechanism is by the reduced oxidation of hypoxia-inducible factor (HIF-1) mRNA. Under normal circumstances, the mRNA is degraded rapidly and has little effect on cell signaling. When hypoxia occurs, degradation of the RNA is reduced, allowing for accumulation of the protein product which induces a wide variety of downstream events. HIF-1 dependant changes include increased production of glycolytic enzymes, enolase, VEGF levels, GLUT1 transporter proteins, and erythropoietin [81, 90, 92]. In response to the cell signaling alterations, hypoxia induces changes on a broader scale including repressed immune function[93], repression of lipid mobilization in fish [94], and decreased reproductive capacity as evidenced by decreases in estradiol and testosterone levels [80, 95].

The ability of aquatic species to resist hypoxia (and resulting oxidative stress) has been examined in many species, generally, species native to estuarine environments appear to have sufficient physiological adaptations to be unaffected. It has been shown in multiple

species that increases in antioxidative enzyme systems such as catalase and superoxide dismutase are sufficient to improve survival [96]. Gracey and coworkers noted 120 differentially expressed genes in response to hypoxia in fish [97] which indicates an organism-wide response to the stresses derived from hypoxic exposure.

VII. POLYCHLORINATED BIPHENYLS

Polychlorinated biphenyls (PCBs) are ubiquitous contaminants in the environment due to their early use as high boiling point, stable compounds in electrical transformers, heat transfer applications, vacuum oils and other industrial applications. Due to widespread production and use, and more recently, lack of containment in reclamation operations, PCBs have been introduced into the environment at toxic levels. This widespread use and release, the inherent stability, and toxicity of many of the congeners have resulted in PCBs being a persistent environmental problem [98]. These highly lipophilic compounds partition into soil and sediment readily, with log octanol-water coefficients ranging from 3.76-8.26. They are present in the sediment and water column in aquatic environments, making them available to bioaccumulate and produce effects in native fauna [3]. A review by the U.S. National Biological Service detailed the occurrence, quantities, toxic effects, and risk to wildlife pf PCBs [99].

There are 209 possible congeners created by differing amounts of chlorination and the varying location of the chlorine atoms on the two phenyl rings. Of those, the dioxin-like or coplanar, PCB congeners (12 of 209) have been studied most extensively. Coplanar

PCBs function primarily by interaction with the aryl hydrocarbon receptor (AhR), although non-AhR dependant effects have also been observed. Non-dioxin-like PCBs also have effects within cells, although the mechanism(s) of interaction are not well defined and have less of an effect on cellular status in complex mixtures containing dioxin-like PCBs.

Commercially produced PCBs are labeled according to a four digit system, with the first two digits indicating the category of the mixture and the second two, the percent chlorine content of the congeners in that mixture. In site sampling and analysis, the US Environmental Protection Agency currently uses this designation for reporting environmental concentrations of PCBs. The National Priorities List, maintained by the EPA, currently shows more than 500 sites across the country polluted with PCBs, dibenzofurans, and dioxins (http://www.epa.gov/superfund/sites/npl). The study described herein was prompted by a recent rediscovery of Aroclor 1260 concentrations in aquatic species in a recreational reservoir and the knowledge that endangered species inhabit downstream waterways. Concentrations in fish tissue are as high as 1.7 mg/kg Aroclor 1260 in fillet. The state has posted no fishing signs in portions of the system and restricted intake warnings on downstream sections (http://www.epi.state.nc.us/epi/fish/current.html). Little attention has been paid to the species inhabiting the system in regards to health indices.

The ubiquitous distribution of PCBs means that they are detectable in living organisms from every environment [100]. Their effects, while congener dependant, include both cancer and non-cancer endpoints. Mechanistically, PCBs affect lipid metabolism, endocrine function, and are implicated in contaminant-stimulated reactive oxygen species production in aquatic organisms at many trophic levels [3, 101, 102]. The resulting excessive oxidation leads to non-cancer maladies including reproductive dysregulation, immune system damage, nervous system disorders, skin disorders such as chloracne, and an array of sensory defects [103, 104]. Acutely, high levels of PCBs can also cause localized necrosis and inflammatory responses that cause overt tissue damage. Polychlorinated biphenyls appear to exert their effects by two main mechanisms. The first is receptor interactivity with the aryl hydrocarbon (AhR) receptor. The second mechanism has been shown to be AhR-independent and likely related to hormonal interaction [105]. The oxidative stress due to activation of the monooxygenase system or by activated metabolites performing quinone cycling is related to increases, typically driven by the AhR, which directly oxidizing nearby macromolecules [106]. Although mollusks have not been found to have an AhR, they are still susceptible to oxidative damage and respond by induction of cytochrome P450s (as evidenced by 7-ethoxyresorufin-O-deethylase induction) to exposure to halogenated hydrocarbons [107, 108]. Oxidative damage derived from PCB exposure is associated with carcinogenesis, diabetes, reproductive, and other ill health effects in mammalian systems as evidenced by environmental disasters as in Yusho, Japan [104, 109]. Oxidative stress comes about when the balance of antioxidative systems is overcome by free radical presence [110].

This imbalance leads to physiological stress and the oxidation of critical elements of the cell including nucleic acids, lipid bilayers, and proteins.

VIII. HYPOTHESIS

The hypoxia research is based on the following hypothesis: Oxidative damage, due to hypoxia and reperfusion, cause significant reduction in the health status of Atlantic Menhaden. Specific aims of the hypothesis were to define lethal concentrations of oxygen for Atlantic Menhaden, define biochemical responses to hypoxic exposure in both acute (2 hr) and medium duration (96 hr) time frames, and quantification of oxidative damage based on commonly employed biomarkers.

The research into the effects of PCBs in Asiatic clams was driven by the hypothesis that exposure to Aroclor 1260 causes an increase in oxidative damage in tissues sufficient to create marked changes in biomarkers and pathological indices. The specific aims of the study were to determine the effects of Aroclor 1260 in a laboratory-based study where extraneous inputs were minimized, quantify biomarker changes due to environmental exposure to the PCB polluted system in relation to possible detrimental health effects, and determine if a relationship exists between biomarker responses and concentration of PCBs in passive sampling devices and sediment.

Figure 1. Demonstration of internal, deep, penetrating lesion development. Lesion originates from within the trunk skeletal muscle and appears to be progressing outward toward the epithelium. Sections are in series across the peritoneal cavity.

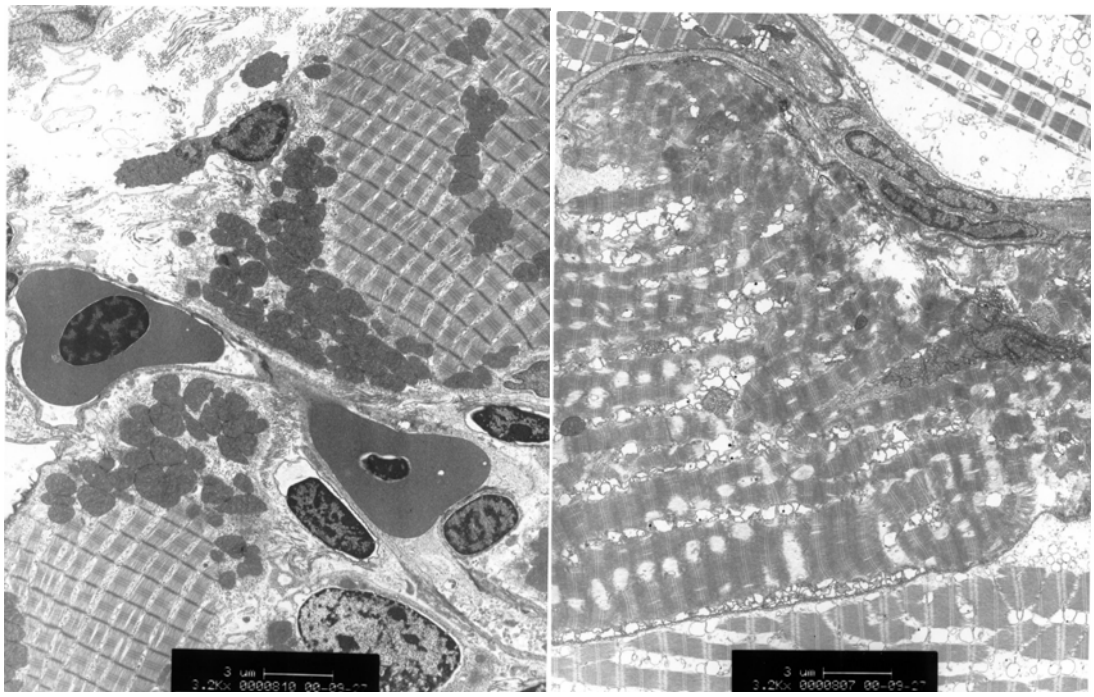

Figure 2. (A) Normal tissue viewed under transmission electron microscopy at 3.2k X magnification. (B) Deranged muscle fiber bundles without evidence of nearby pathogens magnified at 3.2k X. Sample from a lesioned Menhaden from NC DENR sampling of a fish kill on the Neuse River (2001).

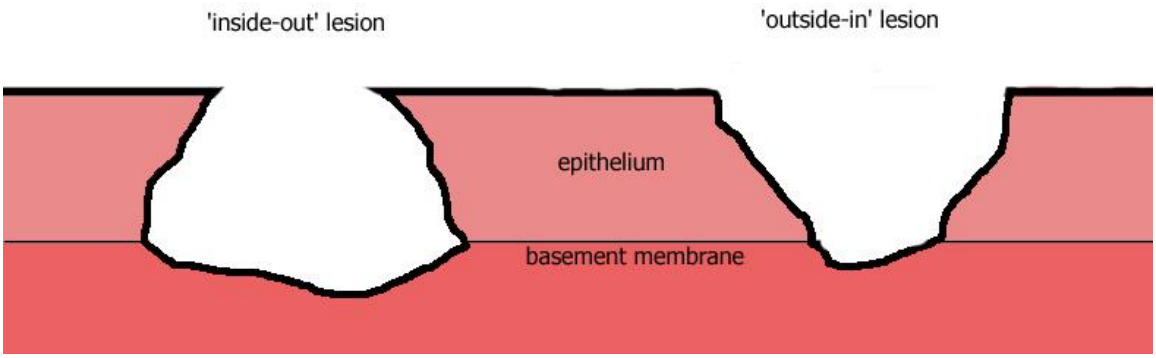

Figure 3. Diagramatic representation of lesion origin and progression. Atlantic Menhaden commonly found in early lesion stages indicated that the lesions were of the 'inside-out' variety.

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DESIGN AND USE OF A HIGHLY RESPONSIVE AND RIGIDLY CONTROLLABLE HYPOXIA EXPOSURE SYSTEM

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INTRODUCTION

Broad changes in oxygen concentrations are characteristic of aquatic ecosystems impacted by eutrophication. Daily cycles of oxygen levels in estuaries can range from daytime supersaturation reaching as high as 300% to pre-dawn anoxia (0%) [1-3]. To test the effects of hypoxia in aquatic animal models we have designed an exposure system capable of rapidly changing dissolved oxygen (DO) conditions in experimental tanks. The system was assembled from readily available components and allows precise, programmable control of DO concentrations in the laboratory setting.

When dealing with sublethal stressors, complicating factors such as time to effect and spatial relevance make field studies impractical. The mobility of free-ranging species, and the dynamic variability of aquatic systems makes clearly characterizing the exposure history of an individual challenging. Exposure to stressors may occur days prior to and miles apart from a fish kill or sampling site[4-6]. Controlled laboratory studies, however, can be used to document the contribution of individual environmental factors to specific health problems.

Periods of hypoxia or anoxia can have profound effects on aquatic organisms. Anoxia has been associated with the loss of benthic invertebrates in eutrophied ecosystems [7], as well as fish kills[8]. In addition, hypoxic events have been proposed to play a role in more subtle disease conditions such as reproductive or developmental abnormalities[9,

10]. The spectrum of health effects observed in fish populations, although implied, is poorly understood mechanistically. An example is the association of hypoxic events with epizootic ulcerative syndrome (EUS), a disease which has affected countless Atlantic menhaden (*Brevoortia tyrannus*) along the east coast of the United States from Chesapeake Bay to the estuaries of the Carolinas[11-13]. Originally ascribed to the *Aphanomyces* fungus[14] and more recently to dinoflagellate *Pfiesteria* spp.,[5] the root cause(s) of EUS remain largely unknown[15-17]. Histopathological studies performed in our laboratory on hundreds of specimens frequently showed no evidence of a specific pathogen that could be associated with the disease[18]. Moreover, EUS occurrence in the estuaries of North Carolina follows a seasonal trend with temperature and hypoxic events [19].

Fish have gained popularity as animal models in aquatic toxicology as recent advances have increased our knowledge of normal physiologic conditions and responses to various stressors[20]. Fish models are also increasingly being used in research leading to information regarding human diseases and genetic and reproductive responses[21]. In this chapter, we describe a laboratory system for examining the response of aquatic species to hypoxia. Experiments with wild caught Atlantic menhaden and laboratory-reared Nile tilapia (*Oreochromis niloticus*) are used to demonstrate the functionality and limits of the system. The tilapia served as a resistant species and the menhaden, based on the association of estuarine hypoxia with ulcerative skin lesions in these fish, as a susceptible species.

The system described provides a means to investigate responses to hypoxia as a single variable or in combination with other stressors. The use of nitrogen gas to reduce the partial pressure of dissolved gases is not novel in aquatic research. The reduction in oxygen tension can be isolated and studied if the proper mixture of gases is used to emulate atmospheric carbon dioxide, argon, and nitrogen ratios. This method works because fish, unlike other vertebrates, sense O_2 in their environment in place of CO_2[22-24]. The system can readily be used for aquatic organisms other than fish or for complex mesocosm studies. Using O_2 in place of N_2 or component air can also rapidly create hyperoxic experimental conditions. Other advantages this system has over traditional methods include its controllability, monitoring systems, rapid oxygen partial pressure changes, and automated data logging and graphing. Herein, we describe the system and provide some example data from two experiments to demonstrate its use. Dissolved oxygen levels for a longer term hypoxia exposure were based on acute LC_{50} values for the two fish species. We chose a series of endpoints to test the hypothesis that hypoxia and subsequent reperfusion create oxidative cellular damage as a factor in the development of ulcerative skin lesions in fish[25-28].

MATERIAL REQUIREMENTS AND SETUP

A wide range of tank sizes can be used with this system depending on the size and flow rating of the protein skimmer, or foam fractionator, employed and species specific

requirements. Our system includes 260 L fiberglass circular tanks for the 1- and 2-hour acute exposures and 855 L dark blue, circular polyethylene tanks for the long term exposures. Each tank has a bulkhead located on the bottom (center) and on the sidewall at the bottom. The center bulkhead was for drainage and tank cleaning purposes. The sidewall bulkhead is plumbed into an external Iwaki-Walchem 55RLXT pump (Walchem Corp, Holliston, MA). Two or more tanks should be employed, each as an independent unit, to house control and exposed animals.

The system functions by use of a Neptune Systems (San Jose, CA) Aquacontroller Pro unit connected to laboratory grade pH, DO, temperature and conductivity probes (Figure 1). Data from the probes are monitored by the controller and logged both by the controller and by Aquanotes (Neptune Systems) software on a computer connected directly to the controller via a serial port. The controller can be operated via the computer (local or internet) or directly on the controller via its simple programming language. User defined settings toggle system components on and off remotely. For example:

Equation 1 $DO > 1.0$ mg/L = ON

$DO < 0.9$ mg/L = OFF

When toggled on, the system functions by opening a solenoid and turning on the Iwaki-Walchem feed pump. The skimmer (AE Tech, ETSS Professional 800) is a passive

device that forces air (or compressed gases) and water to mix at high rates via a downdraft mechanism. The skimmer must be placed at or above the level of the exposure tank's resting water position to allow for gravity feed back to the tank. This allows efficient and rapid mixing of the exposure tank water with contained gases, in this case pre-purified grade nitrogen.

Standard 300 cubic foot nitrogen tanks with nitrogen-rated regulators are used as a source of nitrogen. [Note: Compressed gas tanks should be properly secured to a wall or other stationary structure according to current institutional safety regulations.] The nitrogen gas passes from the tank and regulator through a stainless steel and glass flow meter (Dwyer, model SS-DR12442). Flow meters bracket ranges of gas volume per time so the choice of flow meter model necessary depends on the volume of nitrogen released per hour into the skimmer. The flow meter has a needle valve for controlling 5 to 20 standard cubic feet per minute (scfm) input into the system so that flow rates can be finely adjusted while the regulator is static. The solenoid valve, placed between the regulator on the N_2 source and the skimmer, is turned on and off based on signals from the controller to allow gas to flow to the skimmer.

Signals are relayed from the controller via an x10 control module (www.x10.com). This module codes the signal and passes it along the electrical lines of the building allowing for remote control of x10 appliance modules. The controller specifies the channel and each module set to that channel will respond with an ON/OFF switch. For example,

when the controller reads DO at 1.1mg/L, it then sends a signal to the unit(s) connected to the solenoid and the feed pump for the skimmer thereby initiating the scrubbing of the exposure tank water which circulates from the tank, through the skimmer, and returns to the tank via PVC plumbing with a reduced oxygen concentration.

Water for the menhaden exposures was made with synthetic sea salt (Instant Ocean, Mentor, OH) to 12.5 ppt (19 mS/cm) and allowed to mix for at least 24 hours prior to use. Water was mixed between tanks before introducing the randomized fish to eliminate slight differences between water parameters. Each tank was equipped with a 2 mil clear plastic cover secured by elastic lines and clamps to prevent fish from gulping air at the surface during exposure. The clear plastic allowed easy observation of the fish during the study. (table 1)

Atlantic menhaden were collected from a reference site, the White Oak River, NC. The fish were cast netted, placed into filtered, flow-through tanks and held for a minimum of 2 weeks. Menhaden had a mean fork length of 17.5 cm and mean weight of 71.8 g. Feeding commenced on the second day of holding and continued twice daily with salmon starter crumble (Zeigler Bros Inc, Gardners, PA) ground to a fine consistency. After acclimation, fish were transported to the laboratory in a large, round, enclosed transport tank with heavy aeration and circulation to minimize stress. Temperature was maintained during transport by frozen blocks of water in sealed containers or by aquarium heaters. At the laboratory, fish were acclimated for a further 2 weeks prior to the experiment and

remained apparently healthy. In parallel experiments, healthy tilapia with a mean tail length of 19 cm and 156 g weight were randomized into the exposure tanks using aged and dechlorinated tap water. Tilapia were a gracious donation from NC State University Fish Barn.

Validation of DO concentrations was performed daily using a handheld YSI-85 portable meter (Yellow Springs, OH). All DO probes had new membranes at the start of each experiment and were calibrated daily. All other probes were calibrated at the start of each experiment.

PROCEDURES

All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee (IACUC), NC State University. Acute exposures were performed in 260 L round fiberglass custom tanks with either menhaden or tilapia. Each tank was prepared as above and contained 5 and 7 randomly selected fish, respectively. Fish were acclimated to the exposure tanks for a minimum of 2 days prior to initiating the experiment. Feeding was halted and tanks were cleaned 24 hours prior to initiation. Water quality parameters (ammonia, nitrite, nitrate and hardness (tilapia only)) were monitored daily. A separate tank was used for each oxygen saturation level. Menhaden were exposed to 84/6.7 (control), 20/1.59, 15/1.19, 10/0.79, 5/0.39 (% oxygen saturation/mgL^{-1}) for 1 hour in independent tanks. Tilapia were exposed to 82/6.9

(control), 20/1.68,10/0.83,7/0.58, 3/0.24 (% oxygen saturation/mgL^{-1}) in independent tanks for 2 hours.

Exposures were initiated and a log was kept of DO (% saturation and mg/L), pH, temperature and mortality at 5 minute intervals. Moribund fish, as indicated by uncontrolled swimming behavior or lack of response to physical stimuli, were removed for sampling. At the end of 1 or 2 hours exposure, remaining fish were euthanized and sampled.

Euthanasia was performed by overdose of MS222 (Argent Chemical Laboratories, Redmond, WA) in water from the tank in which the fish was exposed to maintain the oxygen saturation level. Sampling consisted of taking length and weight measurements, drawing blood for clinical pathology, and taking tissue samples for histopathology, oxidative stress, and immune function measurements. Fish were examined for gross abnormalities upon dissection. Blood and spleen samples were taken from all fish for immune function analysis (not covered herein). Samples of heart, liver, anterior (head) kidney, intestine, gonads, gills and spleen were fixed in 10% neutral buffered formalin for 24-48 hours and then held in 70% ethanol for histopathology. Samples of muscle, liver, and blood were placed in 2 ml cryovials and snap-frozen in liquid nitrogen for later analysis of oxidative stress endpoints.

Clinical Pathology

To determine blood parameters, we used a Portable Clinical Analyzer (i-Stat Corp., East Windsor, NJ) with expendable cartridges which self-calibrate upon insertion into the unit. The EG7+ cartridge employed displays results from analysis of a few drops of fresh blood for the following parameters: sodium, potassium, ionized calcium, hematocrit, pO_2, pCO_2. Blood was drawn from the caudal vein of the fish in a syringe free of anti-coagulant. Analyses for lactate dehydrogenase (LDH), creatinine kinase (CK), aspartate aminotransferase (AST), glucose and total protein were performed by the Clinical Pathology Laboratory at the NCSU College of Veterinary Medicine. Blood samples for clinical pathology were taken in heparin coated syringes, kept on ice, and sent directly for plasma chemistry analysis.

Histopathology

For histopathological examination, tissues were fixed in 10% neutral buffered formalin for 24 to 48 hours, routinely processed by paraffin embedment, sectioning at 5 μm, and staining with hematoxylin and eosin (H&E). Tissue sections were evaluated by a single pathologist and assigned a grade from 0 (no remarkable abnormalities) to 5 (severely lesioned)[29].

Data Analysis

Results of clinical pathology and histopathology were analyzed using JMP (SAS, Cary, NC). ANOVA and Dunnett's tests were used for all comparisons using 84 and 80% oxygen saturation levels as controls for menhaden and tilapia respectively.

RESULTS AND DISCUSSION

The controlled DO exposure system described here is very efficient and less demanding of personnel for operation than traditional systems. It responds rapidly to computer or controller commands. Monitoring can be performed remotely and data logging is automated, allowing for better control and replication of experiments. Dissolved oxygen was reduced to 20% saturation (1.6mg/L) in one hour in an 855 L tank and levels remained steady over a period of 96 hours with a standard deviation of +/- 0.091 mg/L during that time (Figure 2). Effective exposure of animals to stressors in toxicologic research hinges on controlled applications and this system increases the control by reducing variability. Isolation of factors from extraneous inputs is also critical for reproducibility and a realistic determination of the biological effects of each factor.

LC_{50} determination with Atlantic menhaden in these experiments mirrored previously published data indicating a DO level of 16 % saturation (1.2 mg/L) for one hour as the approximate lethal concentration. Tilapia were highly resistant to challenge by hypoxia

evidenced by a moderate response at 3% saturation (0.24 mg/L). Tilapia mortality was low, only 28% over the 2 hour exposure period, such that the data generated were insufficient to determine an accurate LC_{50} (Fig 3)[6].

Fish showed behavioral changes as a result of the hypoxic stress. Menhaden are a filter feeding, continually active fish. The inability to rest and preserve energy stores is obvious in comparison with tilapia regarding responses to treatment. At 10% saturation, menhaden were obviously stressed and several began to search for the surface to gasp at the air-water interface. At 5% saturation, they were visibly agitated and all tried to reach the surface of the tank. Tilapia responded to 7% saturation by disengaging territorial behaviors and resting on the bottom and occasionally an individual fish would attempt to gasp from the surface. At 3% many of the tilapia would intermittently gasp at the surface, but then return to the bottom[30]. This response indicates that they have mechanisms for reducing metabolic oxygen demand and are a valid choice as a hypoxia resistant species.

Results of the acute exposures suggest that the exposure system works very efficiently and mimics environmental hypoxia with minimal additional stress on test subjects. Blood electrolyte changes validated the adverse affect of hypoxia on the test fish, and changed sharply as the fishes passed from a mild to a severe stress state with reduced oxygen tensions. Partial pressure of CO_2 in the blood fell as oxygen saturation decreased in the exposure tank, indicating that O_2 availability is coordinately falling. Blood ion

concentrations likely shift in response to a depletion of ATP stores and the lack of ability to regenerate those energy stores during a failure in oxidative phosphorylation[31]. A concomitant drop in pH suggests that anaerobic metabolism occurs as a salvage effort. Reduced ATP concentrations would also lead to a failure of the ATPases that maintain homeostasis in the blood. Failure of the sodium-potassium ATPase in many cells of the body and by the ATPases that drive chloride cell function allow for increases in sodium, calcium, and potassium in the blood. Menhaden showed a significant increase in K and Na at 10% saturation. Menhaden also responded with significant increases in ionized Ca (iCa), potassium (K), sodium (Na), and glucose at 5% saturation. Tilapia showed increases in K and iCa at 3% saturation. (Figures 5 & 6)

Histopathology showed only mild parasitism in both treated and control menhaden, a reflection of being wild caught specimens. No significant difference in lesion prevalence was seen between treated and control menhaden. Likewise, no remarkable microscopic lesions were found in the tilapia specimens to suggest that hypoxia alone causes ulcerative skin lesions in fish. This is consistent with our findings from other biomarkers of oxidative stress (not discussed here) using this system. While both menhaden and tilapia showed strong physiologic responses to extremely low oxygen saturation levels, there appeared to be no overt oxidative damage to cells generated from these exposures. This may suggest an indirect or perhaps supplemental role for hypoxia in EUS, and is an area in need of further study.

This precisely controlled hypoxia system has proven to be useful for the experimental induction of hypoxic responses in fish in our laboratory. With ever increasing influences of anthropogenic inputs into the nation's watersheds, particularly those resulting in eutrophication, this system is likely to serve broader applications that will answer questions in aquatic toxicology where hypoxia may play a role.

ACKNOWLEDGMENTS

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Table 1. Required Materials for Exposure System Setup

ITEM	**MODEL**	**MANUFACTURER**
Skimmer	ETSS Pro 800	A E Tech.
Pump	55 RLXT	Iwaki-Walchem
Solenoid	115V a/c static off/powered on type	National Welders
Tanks	260 L circular fiberglass	Custom
	855 L circular polyethylene	PolyTank Inc, Mn
Regulator	Nitrogen rated - M1-960-PG	National Welders
Controller	Aquacontroller Pro and lab grade probes	Neptune Systems, Ca
Computer	Any Pentium model	Any
PVC pipe	1.5” rigid piping	Any
PVC tubing	1” flexible tubing	Any

Figures:

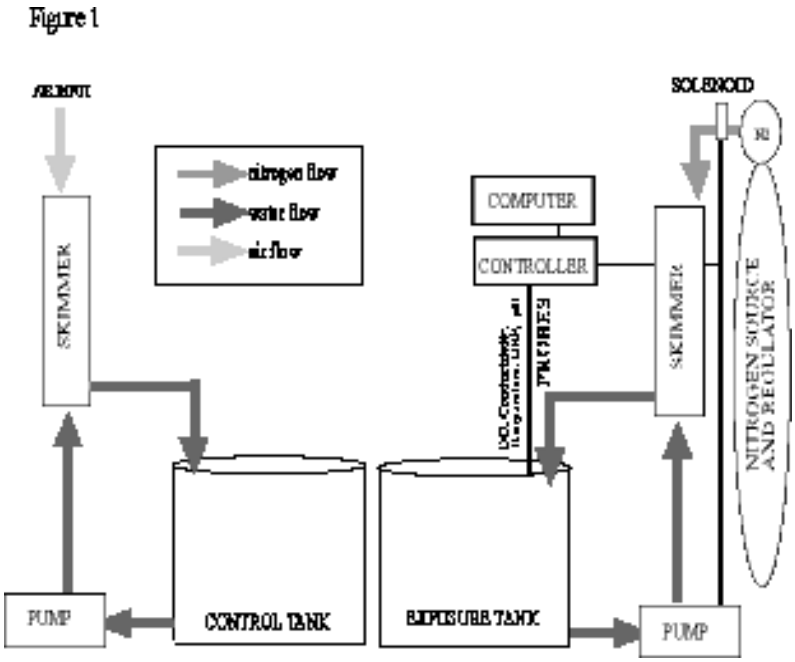

Figure 1. Dissolved oxygen exposure system schematic showing sequence of controller and direction of laminar fluid and gas flow. Tanks of any size from 150 L to 1500 L can be used on this system depending on the capacity and volume rating of the skimmer used.

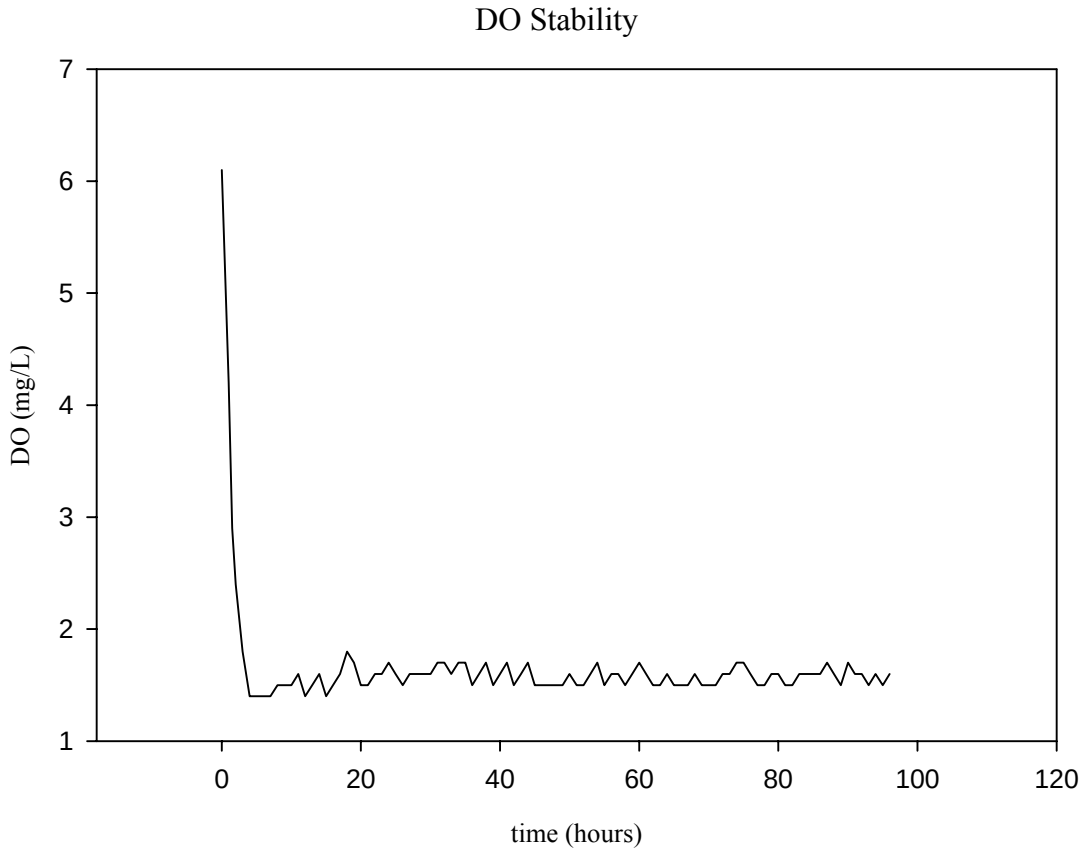

Figure 2. Dissolved oxygen stability over time in laboratory trials. Trials proceeded for 96 hours from initiation time. DO level of 1.6mg/L (20% oxygen saturation) was held within 0.091 mg/L over the time course after initial oxygen reduction.

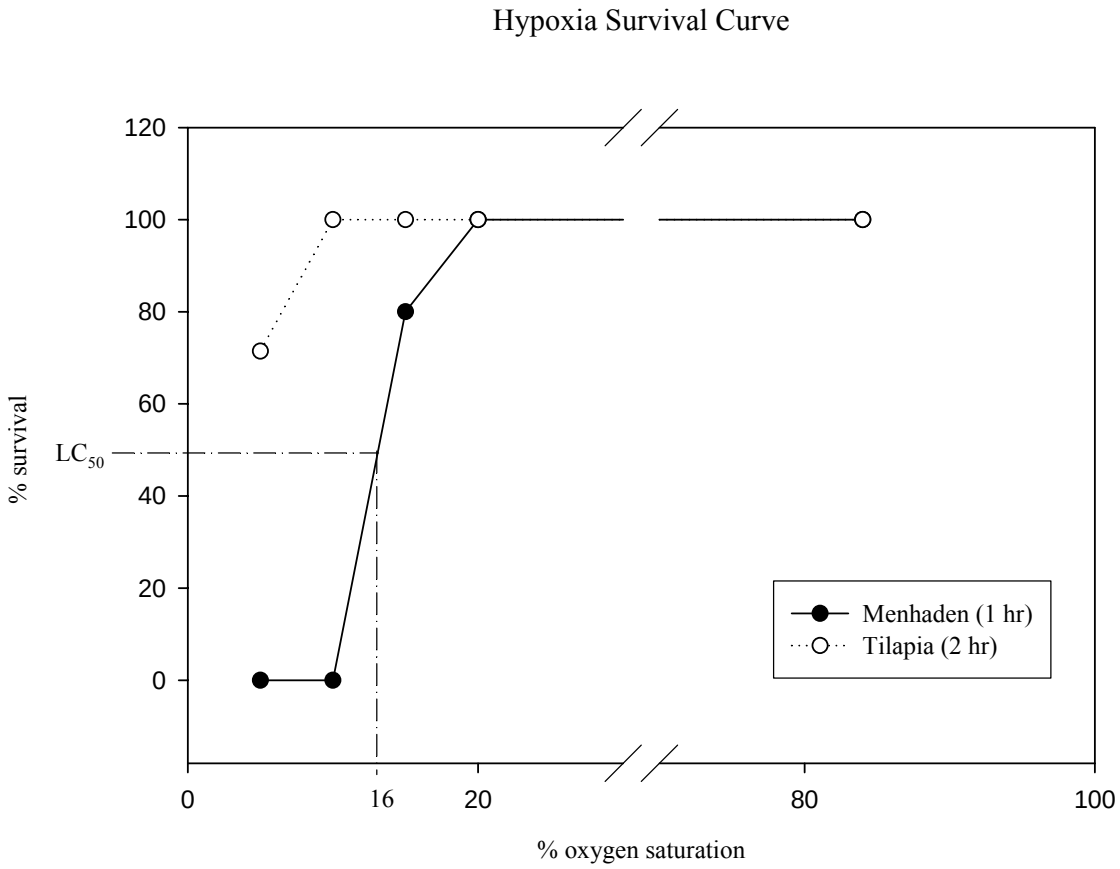

Figure 3. Percent survival in acute hypoxia exposures for both menhaden and tilapia (1 and 2 hr respectively). Menhaden displayed an approximate LC_{50} of 16% oxygen saturation (1.2mg/L) while tilapia proved extremely hardy down to 3% oxygen saturation (0.24 mg/L). N = 5 menhaden and 7 tilapia per saturation level.

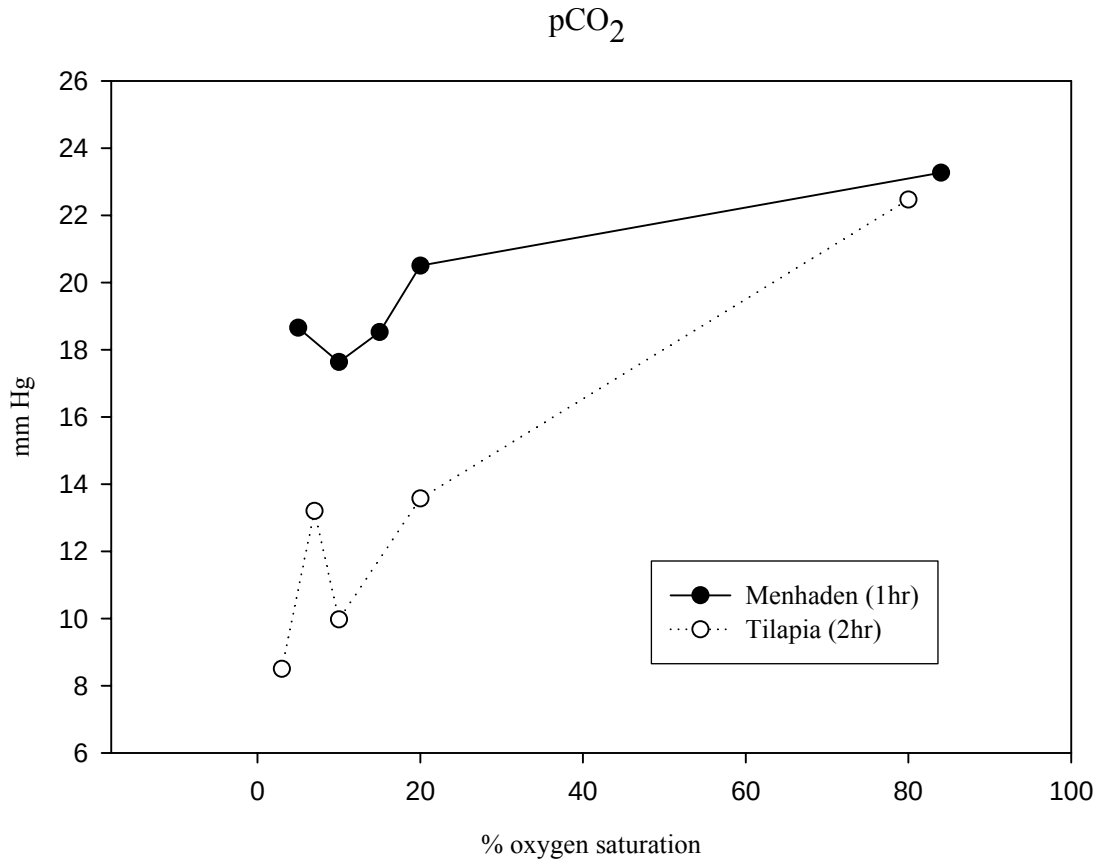

Figure 4. Partial pressure of carbon dioxide (pCO_2) in venous blood of exposed menhaden and tilapia. CO_2 concentrations fell as dissolved oxygen levels in the exposure tank decreased. A sharp drop was evident at approximately 10% saturation in both species. N = 3.

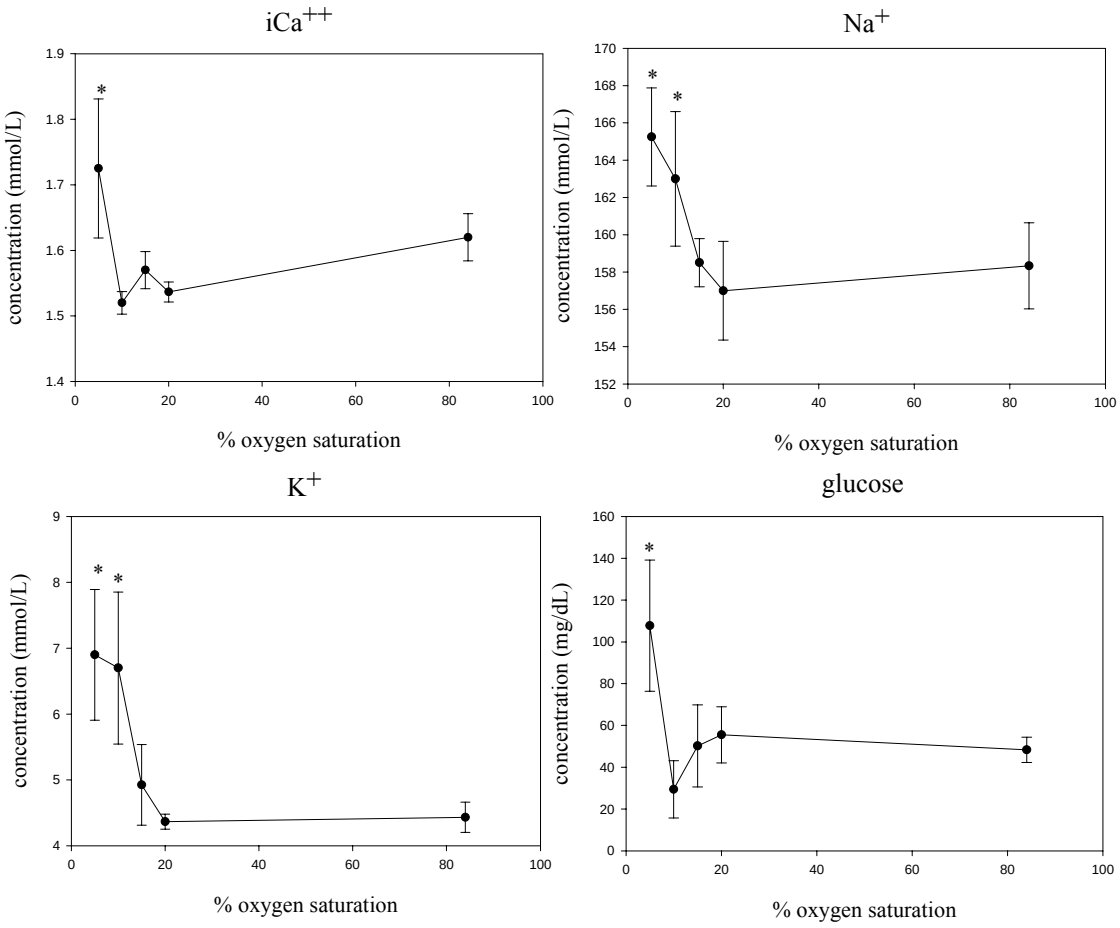

Figure 5. Menhaden blood chemistry parameters. Significant changes (*) were noted at critically stressful levels of hypoxia, indicating physiological failure of oxidative phosphorylation or blood ion homeostasis. Na ($p<0.004$), K ($p<0.0013$), iCa ($p<0.003$), glucose ($p<0.0016$). Error bars are standard deviation.

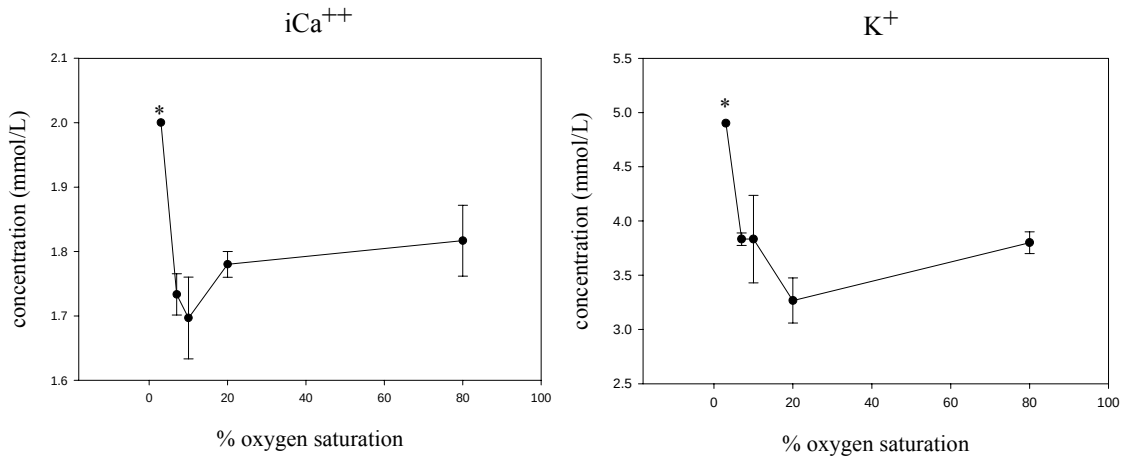

Figure 6. Tilapia blood chemistry parameters. Significant changes were seen in fewer ion types as compared to menhaden, indicating less loss of homeostasis and better energy management in tilapia. K ($p<0.0041$), iCa ($p<0.0041$). Error bars are standard deviation.

POLYCHLORINATED BIPHENYL EXPOSURE CAUSES GONADAL ATROPHY AND OXIDATIVE STRESS IN *CORBICULA FLUMINEA* CLAMS

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ABSTRACT

Polychlorinated biphenyls (PCBs) are widespread environmental contaminants that have been linked to oxidative and other toxic effects in both humans and wildlife. Brier Creek, in central North Carolina, USA, was recently reevaluated by the Environmental Protection Agency and found to contain Aroclor 1260 concentrations that are sufficient to cause closure of the system to fishing. This finding has generated concern that local, endangered species of bivalves are at long term risk within this system. To determine and isolate the possible effects of PCBs on bivalves, we evaluated a suite of biomarkers of oxidative stress and histopathology on laboratory-exposed Asiatic clams, *Corbicula fluminea,* as a surrogate species for endangered bivalves in the region. *Corbicula* have been used extensively as model organisms due to their presence in many varied environments around the world. Clams were exposed to 0, 1, 10, or 100 ppb Aroclor 1260 in the water for 21 days. Measured biomarkers spanned a range of effective levels of organization including low molecular weight antioxidants, lipid-soluble antioxidants, enzymatic antioxidants, and whole tissue radical absorption capacity. These data were augmented by use of histological evaluation of whole samples. Aroclor 1260 significantly increased protein and reduced glutathione (GSH) concentrations at all treatments levels. Significant decreases were also measured in % gonad coverage and γ-tocopherol (γT) in all treatments and total oxidant scavenging capacity (TOSC) and α-tocopherol (αT) values at the 100 ppb exposure. Histologically, Aroclor 1260 caused significant gonadal atrophy, measured morphometrically as decreased gonadal cross-

sectional area in all treatments, inflammation and necrosis in digestive tubules and foot processes, and effacement of gonad architecture with accumulations of Brown cells. Our results indicate that oxidative mechanisms play a significant role in the decrease in the health of these clams due to exposure to Aroclor 1260. The change in the gonads of exposed clams indicates that a serious threat to bivalve reproduction exists due to PCB exposure.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are ubiquitous contaminants in the environment due to their early use as high boiling point, stable compounds in electrical transformers, heat transfer applications, vacuum oils and other industrial applications. Due to widespread lack of containment from operations such as reclamation of electrical components, PCBs were still being introduced into the environment at many sites until the late 1990s. The inherent stability and toxicity of many of the congeners have resulted in PCBs being a persistent environmental problem [1]. These highly lipophilic compounds partition into soil and sediment readily, with log octanol-water coefficients ranging from 3.76-8.26. They are present in the sediment and water column in aquatic environments, making them available to bioaccumulate and produce negative effects in native fauna [2].

Commercially produced PCBs are labeled according to a four-digit system, with the first two indicating the category of the mixture and the second two the percent chlorine content of the congeners in that mixture. The US Environmental Protection Agency currently uses this designation for reporting environmental concentrations of PCBs. The National Priorities List, maintained by the EPA, currently shows more than 500 sites across the country polluted with Aroclor, dibenzofurans, and dioxins. Polychlorinated biphenyls have been detected in organisms at these sites [3]. Their effects, while congener dependant, include both cancer and non-cancer endpoints. Mechanistically, PCBs affect lipid metabolism, endocrine function, and are implicated in contaminant

stimulated reactive oxygen species production in aquatic organisms at many trophic levels [2, 4, 5]. The lengthy list of PCB-related maladies includes reproductive dysregulation, immune system damage, nervous system disorders, skin disorders such as chloracne, and an array of sensory defects [6, 7].

Suspension feeding invertebrates living at tainted sites serve as useful indicators of PCB contamination [8-11]. The Asiatic clam, *Corbicula flumenia*, an invasive species initially introduced into the Northwestern U.S. in the early 20th century, is now wide-spread in freshwater ecosystems of the United States [12]. In North Carolina, they are present in most river and lake systems at relatively high densities. The wide distribution of the Asiatic clam and its nuisance designation has facilitated its use as an environmental sentinel [8, 11, 13, 14]. Typically, bivalves with higher PCB burdens demonstrate increases in both primary and secondary antioxidant systems. Animals from chronically polluted sites typically have higher resistance to episodic exposure compared to animals from control sites [15]. Uptake of PCBs through contaminated or spiked algal diets has been well documented for bivalves [16, 17]. The recent rediscovery of Aroclor 1260 concentrations in aquatic species in a recreational reservoir in Wake County, NC, and the knowledge that at-risk species of freshwater mussels inhabit downstream waterways prompted field studies with sentinel *C. flumenia* to determine the potential biological effects of contaminants in the reservoir on freshwater bivalves. Marked gonadal atrophy and other tissue damage was apparent after the clams were held for three weeks in the reservoir (field study section). However, because the clams were deployed in the

environment, the actual effects of Aroclor exposure could not be differentiated from that of other stressors present in the system. In this study we describe the effects of Aroclor 1260 on Asian clams exposed to known concentrations under controlled laboratory conditions. The responses of a suite of primary and ancillary biomarkers of oxidative stress and histopathology at different levels of biological organization were used to measure the effect of this PCB mixture on the clams.

MATERIALS AND METHODS

Corbicula fluminea clams, 14-20mm in length, were collected from a local, clean watershed (Lake Wheeler, Wake County, NC) and held in the laboratory for a minimum of 2 weeks in a closed recirculating system (~1100 liters) with substrate to assure their health prior to the experiments. While in holding, clams were fed a continuous slow drip of unicellular green algae. *Corbicula* (5 or 6 per container) were then randomized into 4L beakers, 3 beakers per treatment. Two centimeters of sterile sand was placed in the bottom as substrate as clams without substrate show alterations in biomarkers of oxidative stress [18]. Exposure beakers were placed into a single water bath at 20^{o}C for the duration of the exposure, in a chemical fume hood. Sand was sterilized by baking at 300^{o}C for 12 hours and then thoroughly rinsing and rehydrating with reverse osmosis purified water. Air was provided by a slow bubble from a standard aquarium type air pump and sterilized air stone. Exposure water (3L per container) was reverse osmosis purified (18+ MΩ resistance) and reconstituted to 50 uS/s conductivity with Instant

Ocean synthetic sea-salt (Marineland, Moorpark, CA). Clams were treated at 0 (vehicle control), 1, 10, or 100 ppb Aroclor 1260 in a static replacement configuration. Aroclor 1260 was purchased from Chem Service (West Chester, PA). Water changes (50%) were performed twice weekly and included feeding 100ul of commercially available concentrated algal culture (Reef Mariculture, Campbell, CA) as described by Chu and co-workers (2003) [19]. Aroclor was dosed into the water changes in 100ul ethanol carrier and mixed well for 30 minutes to assure dissolution (1.5L water changed twice per week). Control clams were dosed with 100ul ethanol containing no Aroclor 1260. The exposure ran for 21 days as literature suggests that this is sufficient time for uptake and effects in bivalves [20]. Preliminary results from our laboratory indicated that oxidative effects and histological changes were also present at 21 days exposure. Water quality testing was performed weekly and no accumulation of ammonia was detected. Total dissolved organic carbon (DOC) was tested for at the NC State University Department of Soil Chemistry Analytical Services Laboratory in weekly water samples from each treatment vessel (3.3 ± 1.4 mg C/L). This exposure was repeated.

Clams were placed on ice at the conclusion of the experiment. Three clams were randomly removed for histopathology from each treatment level. The remaining clams were euthanized by an overdose of MS-222 (Argent Labs, Redmond, WA), shucked, blotted dry, weighed and then used to create a composite sample from each beaker. The weight of each composite sample was recorded and then homogenized in 4 volumes (w:v) of PBS buffer containing 1mM EDTA using a BioHomogenizer (ESGE,

Switzerland). Aliquots were then taken and processed per the requirements for each assay. Final aliquots were immediately diluted or processed and stored at -80oC until analysis. All chemicals without a specified source were purchased from Sigma-Aldrich (St. Louis, MO).

Specimens were prepared for histopathology by gently prying open the shells and inserting a toothpick between the valves to hold them open. *Corbicula* were euthanized and immediately fixed in cold, 10% neutral buffered formalin for 24 hours. The visceral mass of each specimen was then carefully removed by transecting the adductor muscle close to the shell with a scalpel and peeling up the mantle. Clams were then bisected and placed into 10% formic acid for 24 hours to remove any residual shell or mineral debris. After that time, they were placed in 70% ethanol until histological processing. The tissues were routinely processed, embedded in paraffin, sectioned at 5 microns, stained with hematoxylin and eosin (HE), and examined by light microscopy. Lesions were scored by a single pathologist according to the scale outlined in Hurty, *et al.*, 2002 [21]. In brief, lesions were scored from 0 (no remarkable microscopic abnormalities) to 5 (severe lesions) for each lesion type.

Morphometrics were performed on HE stained slides using Image-Pro Plus (MediaCybernetics, Silver Springs, MD) software. Three random fields from each slide at 4x magnification were photographed and each image was analyzed for total gonad surface area including both ovarian and testicular tissues. Regions of lumen or those

containing no tissue were subtracted from the total tissue area of each field. Fields containing 25% of more of intestinal tissue were discarded and not photographed for purposes of gonadal coverage measurements.

Reduced glutathione (GSH) levels were measured with commercially available kits according to manufacturer's instructions (Cayman Chemical (Ann Arbor, MI)). The GSH kit uses glutathione reductase and DTNB as a basis for colorimetric detection of glutathione. Protein was measured by use of a BCA kit from Pierce (Rockford, IL) using manufacturer's instructions at a dilution of 1:20. This kit uses bicinchoninic acid as a universal reagent measuring the reduction of copper in solution.

Antioxidants were extracted in amber glass vials using sequential extractions with dichloromethane:hexane (9:1). In brief, samples were combined with the DCM:hexane at 1ml volume per 0.1ml sample homogenate. δ-tocopherol was added as a recovery standard. After an overnight shake, the supernatant was removed, and the process repeated for 30 minutes. A third and final extraction was performed after briefly vortexing. The supernatant was then exchanged to ethanol by reduction under nitrogen and brought up to a final volume of 250ul. Fifty ul of each sample was injected onto the column and run at 0.8ml/min. Antioxidants were measured via high pressure liquid chromatography (HPLC) with electrochemical detection (ECD) on an ESA, Inc. Coularray (Boston, MA). Retinol acetate was used as an internal standard. [22]α-, δ-, and γ-tocopherol, β-carotene, retinol, and coenzyme's Q9 and Q10 were measured with

the following voltages: +200, +400, +500, +700, -800, -900, +200, +500 mV using an isocratic method and a C18 nucleosil column (Supelco). Running buffer was 78:20:2 methanol:2-propanol:ammonium acetate. Each sample run was followed by a 1 minute flushing with running buffer that included 10% hexane.

Total oxidant scavenging capacity was assessed according to Winston and Regoli, 1998 [22]. Whole homogenate samples were cleaned up using a 10,000g centrifugation at 4^{o}C for 10 minutes to remove large cellular debris. Due to experimentally increased protein concentrations in our samples, we did not normalize TOSC values to total protein concentration. Instead, samples are normalized to wet tissue weight. We also used whole homogenate in place of S9, cytosolic, or lipid soluble fractions alone, to better observe alterations in the entire animal. Samples were diluted 1:5 in 100mM potassium phosphate buffer with 1mM EDTA and a total of 10ul was used in each 1ml reaction vessel fitted with Mininert® valves (Supelco, Bellefonte, PA). Samples were reacted with 20mM 2,2'-azobis(2-methylpropionamidine)dihydrochloride (ABAP) and 2mM α-keto-γ-methiolbutyric acid (KMBA) over a period of 120 minutes for the measurement of ethylene gas production as an indicator of susceptibility to peroxy radicals. Samples were run on a Hewlett-Packard Series 5890 gas chromatograph (GC) with flame ionization detection (FID). A 30m Rt-QPlot capillary column (ResTek, Bellefonte, PA) was used for separation of ethylene from other gases. Running conditions were injector 165^{o}C, oven 40^{o}C, detector 250^{o}C. 0.5 ml of headspace was injected per sample every 12 minutes over a course of the reaction.

Statistical analyses were performed with JMP or SAS (SAS, Inc., Cary, NC). One way analysis of variance (ANOVA) was performed on all biomarker responses. Effects were further analyzed by use of Dunnett's t-test to compare individual treatments to controls. A P value ≤ 0.05 was considered significant.

RESULTS

Low mortality was observed in the initial experiment (2/18 individuals at 10 ppb, 1/19 at 100 ppb). Clams were immediately removed and discarded when it was determined that they were dead. No mortalities occurred in the repeat experiment. Dissolved organic carbon levels did not vary significantly between treatments or experiments. Ammonia levels were never above detection limits (0.25mg/L).

During dissection, in preparation for homogenization, we noted that the visceral mass of some of the 10 and 100 ppb treated clams was bulging and more turgid than normal, had rounding of the edges, and exuded clear fluid on cut surface. This was interpreted as generalized edema.

Lipid-soluble antioxidant analysis indicated that γ-tocopherol showed a decrease in all treatments relative to controls (fig 2). Alpha-tocopherol levels were highly variable among samples, with the 100ppb dose being reduced in concentration, but not

significantly. Ubiquinol (CoQ9 and CoQ10), β-carotene, and retinol levels were typically below detection in the dilutions used for analysis. Total reduced glutathione levels in whole body were elevated in all treatments compared to control. No apparent dose response was discerned in increasing treatment concentrations between 1 and 100 ppb over the 21 days exposure time. (fig 5)

Protein concentrations were significantly increased in all treatments compared to controls (fig 3). Due to this fact, we chose to use TOSC values that were not normalized to protein concentrations for the assay to prevent data misinterpretation derived from covariance of the assay with experimentally derived differences in total protein concentrations. The highest concentration of Aroclor 1260 caused a sharp reduction in overall antioxidant capabilities. (fig 4) The TOSC assay was only performed on one repetition of the experiment.

Lesions, evaluated on the basis of mean lesion rating, displayed an apparent dose-response, with an increase in lesion severity with increasing treatment levels in both exposures (table 1). *Corbicula* from the control group showed few to no remarkable microscopic lesions. A few controls had scattered hemocytes infiltrating around the digestive glands, but this was considered to be within normal limits. *Corbicula* in the PCB-exposed groups had three major lesion types: gonad atrophy (and increased Brown cell presence), generalized edema, and marked inflammation and necrosis. Total gonad cross-sectional area decreased significantly in all exposures compared to control clams

(fig 6). The gonads in the exposed specimens were often shrunken, necrotic/fragmented, and infiltrated with one to several relatively large, pigmented phagocytes (melanomacrophage-like hemocytes or Brown cells). While control clams had the normal, clustered arrangement of ovarian follicles and ovotestes, exposed clams had marked necrosis and loss of oocytes and ovotestes with both a decrease in the overall gonadal cross-sectional area and a decrease in the size of individual clusters. Morphometric analysis of whole sections verified that changes in gonadal abundance and density occurred in the exposed groups (table 1).

Compared to controls, many exposed clams also had diffuse, moderate to severe edema characterized by increased clear space within connective tissues and paler staining to the viscera. Digestive glands were expanded by heavy infiltrates of activated hemocytes. Epithelial cells in some tubules were swollen, pale staining, and had indistinct to frayed cell borders. In some specimens, aggregates of hemocytes were also noted in the connective tissue away from the digestive glands (fig 7).

DISCUSSION

Corbicula exposed to Aroclor 1260 showed increasing signs of cell injury with increasing concentrations of PCBs. Histologic changes observed were similar to those seen in sentinel clams held in an aquatic system heavily contaminated with PCBs (laboratory studey section). The marked morphologic changes may reflect oxidative alterations

previously associated with PCB exposure. Two mechanisms appear to exist in which PCBs exert their oxidative effects. The first is direct oxidation of macromolecules due to phase I metabolism by the mixed function oxidase system and quinone cycling of the metabolites [23], and the second is indirect release of reactive oxygen species by metabolizing enzymes induced by exposure [24]. Polychlorinated biphenyls interact at the hormonal level by interaction with the aryl hydrocarbon and other cellular receptors and by interfering with lipid metabolism [25]. Dioxin induced reproductive alterations have been shown in some cases to be AhR-independent [26]. Although the AhR is not phylogenetically conserved in mollusks, there may be comparable receptors in bivalves and other invertebrates as evidenced by 7-ethoxyresorufin-O-deethylase (EROD) induction due to halogenated hydrocarbon exposure [27, 28]. Bivalves fed a contaminated diet or exposed via environmental field studies show transfer of PCBs to reproductive tissues with the highest residual concentration of these lipophilic compounds being found in the gonads [19]. Reductions in gonadal size and function are also associated with PCB exposure in rodent studies, even at levels approaching those found in as background in human populations [29, 30]. We observed similar reductions in gonad cross-sectional area in the *Corbicula* we exposed to Aroclor 1260.

Freshwater bivalves serve as highly useful biological sentinels in polluted environments. *Corbicula* sp. clams have been used for this purpose for many years [8, 11]. As an invasive species, these clams are now relatively ubiquitous in warm water environments worldwide. Their broad distribution and nonnative status makes them ideal sentinels and

biomarkers, which provide a consistent model for environmental field studies [8, 13, 31, 32]. *Corbicula* are well adapted to shifting environments and oxidative challenge. Their benthic habit places them into contact with sediment-associated contaminants. These laboratory studies provide a baseline for comparison with field studies, where multiple factors can confound data interpretation, using sentinel clams and for evaluation of the risk of PCB related health effects in native, endangered wildlife.

No single biomarker reflects the complex interactions of variables that define the health of ecosystems. Common biomarkers of oxidative challenge include antioxidative enzymes (such as superoxide dismutase and catalase), ancillary antioxidative enzyme systems and those involved in phase I metabolism (such as glucose-6-P-dehydrogenase and glutathione S transferase), lipid oxidation state, non-enzymatic antioxidants (such as total reduced glutathione levels, vitamins, urea), and total ability of cellular components to reduce oxidation *in vitro* (Total Oxidant Scavenging Capacity) [22, 33-35]. Generally, in single compound exposures, there is a rise in enzymatic activity or concentration as well as increases in non-enzymatic defenses. High levels of oxidative compounds cause a sharp decrease in many of these biomarkers indicating that the antioxidative systems employed have been overburdened or compromised. Highly specific biomarkers, such as reduced glutathione concentrations (GSH) are less relevant at the population level, while more general population level biomarkers give no indication of the mechanism(s) of action of a toxicant. Therefore, it is of benefit to use an array of biomarkers for determination of toxic effects, preferably at different levels of organization, from

molecular markers to genetic alternations to whole animal health indices [36-38]. Included in the analysis of biomarker changes, however, is the need for demonstrable biological or physiological effects of a toxicant that can be compared across phylogenetic levels. This need is well met by the use of histopathology. This idea is in-line with the need to relate the responses of individuals to the responses of populations and communities [39]. Measuring the relative changes of biomarkers in response to environmental and contaminant-related variables will help define the breadth of physiologic changes that can be anticipated in response to specific contaminants. Further definition of these biomarkers in resident aquatic fauna would help refine our understanding of their value in assessing ecosystem health.

The duration of exposure, in this case 21 days, allowed for a whole animal response to the oxidative stress which the PCBs initiate, but not necessarily the time necessary to achieve steady state concentrations due to the quantity of highly (penta-, hepta-, hexa-) chlorinated PCB congeners in the Aroclor 1260 mixture. However, according to Rodriguez *et al.* 2003, approximately 50% maximal uptake is achieved by 21 days in sediment associated uptake monitoring, including proportionately more of the lower (mono-, di-, tri-)chlorinated congeners which are more readily metabolized by monooxygenases [20]. Nevertheless, the rate of uptake and resulting tissue concentrations are sufficient to generate a strong response in oxidant scavenging function. In our study, increases in total glutathione, as well as the decreases in α- and

γ-tocopherol and the decrease in TOSC levels indicated that the course of exposure was sufficient to allow for accelerated cellular metabolism and to indicate a reduction or possible collapse of overall antioxidant capacity at tested concentrations. The oxidative burden placed on the tissues in the clams indicated that oxidative stress resulted from PCB exposure.

Lipid soluble antioxidants appear to be a fairly stable component of the complete antioxidant system in organisms[40]. Many of the lipid soluble antioxidants have critical function in homeostasis of cells and are regulated strongly to maintain those functions [41, 42]. Any measurable change in these lipid soluble antioxidant levels is an indication of a potent oxidative challenge. Gamma-tocopherol is the stronger lipid soluble antioxidant, being more responsive and excreted when consumed in comparison to α-tocopherol. Alpha-tocopherol is typically recycled in conjunction with ascorbic acid, unlike γ-tocopherol, so that levels should be comparatively more stable [40]. In the short term, shifts in relative proportions of tocopherols indicate that the cumulative total of the oxidant side of the oxidant:antioxidant balance is increasing and allow for more oxidation of lipids. Lipofuscin, the "wear and tear" pigment, builds up in cells due to oxidative degradation of lipid and is visible as yellow or brown pigment in cells during histological evaluation [43]. It follows that reduction in lipid protective molecules may increase lipofuscin accumulation as seen in our study as accumulation of Brown cells. In this experiment, it appears that the 100 ppb Aroclor 1260 concentration may take defense mechanisms beyond the compensation point of the antioxidant system. The more

responsive results demonstrated by γ-tocopherol are likely due to perturbance of lipid bilayers by the highly lipophilic PCBs and resulting quinone cycling oxidative metabolites. Tocopherols have been shown experimentally to reduce thiobarbituric acid reactive substances (TBARS) values during oxidative challenge and are known to directly affect reproduction [44]. Alterations in lipid metabolism due to PCB exposure have been demonstrated by Ferreira and Vale, 1998 [45]. Coincident with the lipid metabolism alterations, PCBs are known to interact with signal transduction as evidenced by the release of insulin in cell culture and while many invertebrates are thought to have no aryl hydrocarbon receptor, functional homologs have been described in the literature [26, 46-48]. Taken together with the fact that reproductive tissues accumulate high quantities of PCBs, these possible mechanisms may have contributed to the severe changes identified in the gonads of the *Corbicula* studied.

Reduced glutathione concentrations are used frequently as markers of effects in both field situations and in the laboratory. Experimental supplementation with GSH has been shown to reduce oxidative damage directly induced by some PCB congeners [49]. However, the variability seen in the literature somewhat offsets the usefulness of GSH as a sole marker of oxidative stress. Therefore, in combination with other markers, GSH can remain a useful indicator of early responses to oxidative challenge to cells. In this study, GSH rose in response to increasing concentrations of PCBs, corroborating the results seen in previous studies [50]. An increase in the glutathione system components tested indicates that oxidative stress was a major consequence of exposure. Rodriguez et

al. (2003) noticed a decline in GST levels over a longer term PCB exposure, where the activity continued to fall until 110 days exposure suggesting a slow overburdening of the system due to PCB accumulation while GSH levels rose and fell with time [20].

The TOSC assay has been used as a generalized indicator of overall antioxidant system status by measuring whole sample *in vitro* capacity to absorb peroxyl, hydroxyl, or other radicals depending on the radical generating system employed [22, 36, 51]. Since protein production increased by treatment in this experiment, we did not normalize our values to protein concentration to avoid covariance. As noted in the study by Regoli, ***et al.*** in 1998 [52]of *Mytilus* bivalves, various fractions (cytoplasmic, lipid, s9) have differing degrees of relevance for each contaminant stressor. Due to the nature of the oxidative stress derived from the Aroclor mixture used in this study, we opted to examine whole homogenized sample in lieu of various fractions to evaluate *Corbicula*'s whole sample antioxidant capacity. We found that high concentrations of Aroclor 1260 in the water column caused a significant decrease in TOSC consistent with our hypothesis that PCBs cause significant oxidative stress in clams. Although these levels are three fold higher than EPA water quality criteria levels for protection of aquatic life, the results do match those evidenced in our prior field studies in an Aroclor polluted system. The resistance of clams to lower concentrations of Aroclor is likely related to a reduced ability to metabolically activate the contaminants. Assay values fell in a dose dependant manner with increasing dose of PCB mixture.

Microscopically, gonadal atrophy seems to be the most significant population relevant effect of PCB exposure in these clams. This gonadal alteration may be sufficient to reduce fecundity or cause population level alterations in the local environment as noted by a lack of mature eggs in *C. virginica* oysters after PCB exposure by Chu et al. (2003) [19]. Evidence of inflammation and discrete regions of necrosis were found, indicating that some overt damage was derived from exposure to Aroclor 1260, possibly due to oxidative cellular damage as noted by Koponen in fish from polluted lake systems [53]. Holding the clams under constant exposure conditions beyond the three week period used in this experiment would likely have produced even more negative effects at the cellular and organ levels and would probably have resulted in higher mortalities as noted in the extended study of oxidative effects in *C. gallina* by Rodriguez-Ariza [20]. Many of our higher dose specimens also had severe generalized edema (anasarca) which may be caused by changes in osmotic balance, hydrostatic pressure, increased tissue permeability due to inflammation, or direct injury by the PCBs. In our study clams, the edema was most likely caused by oxidation of lipid membranes, creating increased permeability as well as profound osmotic disturbance. The scattered inflammatory infiltrates seen in various tissues were most likely reactive or secondary to direct tissue injury. This injury is an explicit result of oxidative damage and the resulting peroxidation of lipid chains. Patchy necrosis was also visible in histological sections from some of the treated clams. Necrosis is the end result of too much overt cellular damage that cannot be repaired. This also fits into the model of oxidative damage being primarily responsible for dysregulation of ionic balance and breakdown in cellular structure. In addition to the extensive necrosis

and loss of gonadal tissue, the accumulation of relatively large, pigmented macrophage-like hemocytes or Brown cells amongst the residual necrotic debris of the gonads was interesting. While the origin and specific functions of these cells are still being investigated, recent studies have associated the accumulation of Brown cells in various tissues of bivalves with exposure to organic pollutants [54, 55]. Zaroogian *et al.* have perhaps most thoroughly characterized the brown cells of the red gland or pericardial gland of clams as fixed tissue cells involved in detoxication of metals [43, 56]. This would suggest a liver-like function for the red gland. However, the Brown cells observed in our study are more likely a sub-type of the mobile, phagocytic hemocytes involved in innate immunity. The accumulation of Brown cells in areas of oocyte injury, apparently due to oxidation of lipid membranes, would suggest that these cells serve a somewhat similar function to the melanomacrophages of teleost fish. That is, they probably serve as the "clean-up crew" and accumulate ceroid and other materials that cannot be further broken down. Further research correlating morphology and function of Brown cells is needed.

The specific biomarker responses, as well as the results of whole organism pathology, in this study are consistent with our hypothesis that oxidative damage is a result of exposure to Aroclor 1260, with accumulated damage affecting many levels of organization. The combination of oxidative stress biomarkers and the decline in gonad health visible at the microscopic level indicates that oxidative stress is a direct consequence of exposure to the PCB mixture. While we are unable to define oxidation as a direct cause of the

gonadal atrophy, evidence derived from this study and others indicates that oxidative damage is a direct acting factor in such declines. Relevant changes, whether directly or indirectly due to oxidative stress, occur at the organ and organism levels and will likely result in population wide effects, including reduced fecundity and chronic maladies.

Freshwater mussels native to North America are among the most endangered groups of animals on the planet. Polychlorinated biphenyl contamination is extensive and may be contributing to declines in freshwater mussel fecundity and populations. In some areas, little or no recruitment of juvenile bivalves is taking place. Similar declines have been noted in snail populations. Endangered bivalves and other aquatic macroinvertebrates will remain at risk due to the extensive number of PCB, dioxin, and dibenzofuran polluted sites in this country. Biologists considering the augmentation or reintroduction of extirpated or extinct macro-invertebrate populations should measure and carefully consider PCB levels at potential restoration sites.

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Table 1. Mean Lesion Rating per Treatment. Mean lesion ratings per each lesion type per treatment level. Significant increases in all lesion types are visible microscopically in an apparent dose-dependant manner. Mean values are derived from 3 clams per treatment level and evaluated on a scale of 0 (normal) to 5 (severe lesion).

Treatment	**PMA**	**Atrophy**	**Inflammation**	**Necrosis**	**Other**	**Other Description**
0	0.00	0.00	0.00	0.00	0.20	Pale staining tubules
1	1.33	1.67	0.50	0.17	0.83	Pale staining tubules and matrix
10	0.83	2.50	2.00	0.17	1.33	Generalized edema
100	2.50	3.00	1.50	0.33	0.67	Generalized edema

N=3 per treatment

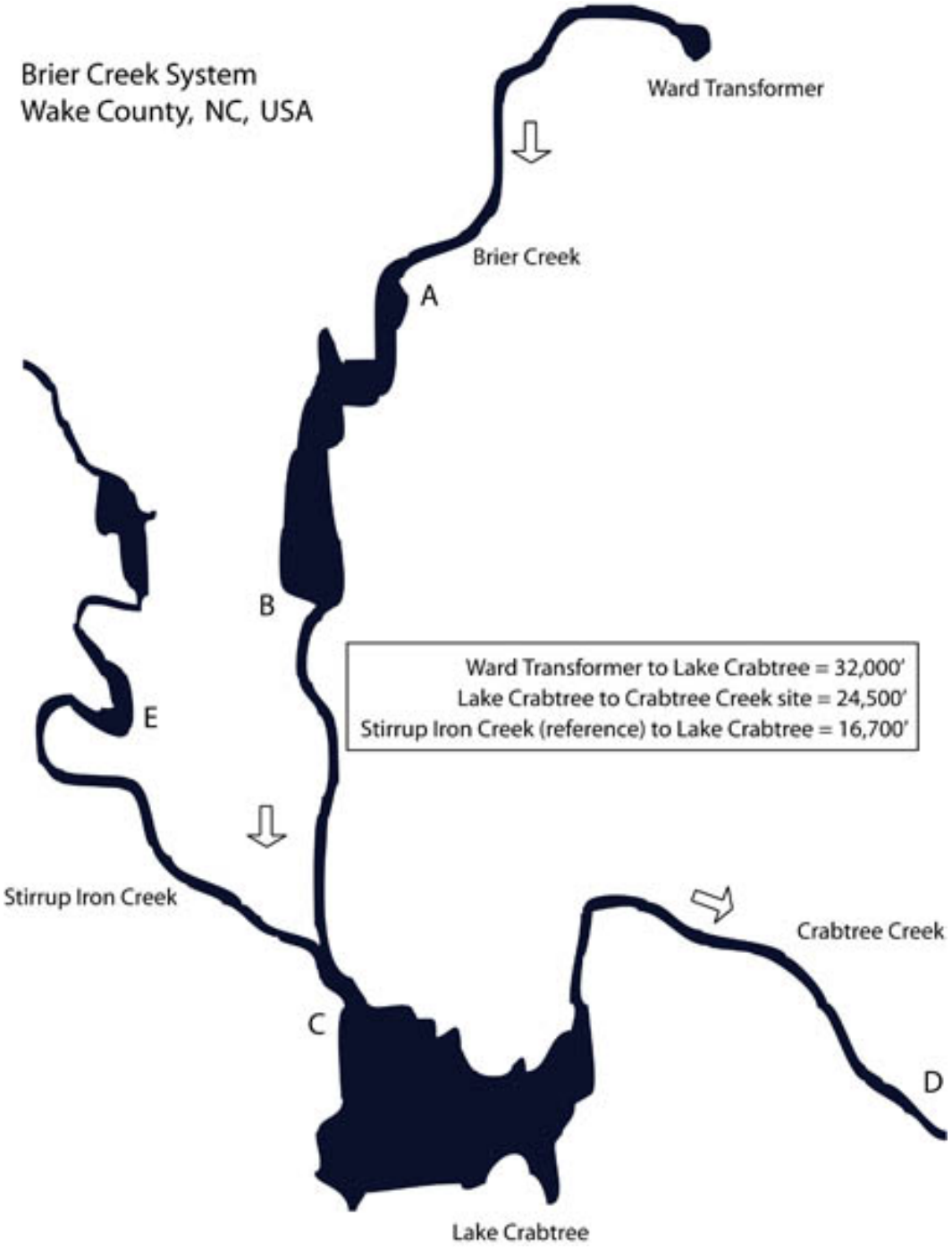

Figure 1: Map detailing the location of sampling sites and relative distances, Brier Creek, NC. [A] Upper Brier Creek Reservoir [B] Lower Brier Creek Reservoir [C] Lake Crabtree (recreational reservoir) [D] Crabtree Creek [E] Stirrup Iron Creek (control). Arrows indicate the direction of water flow.

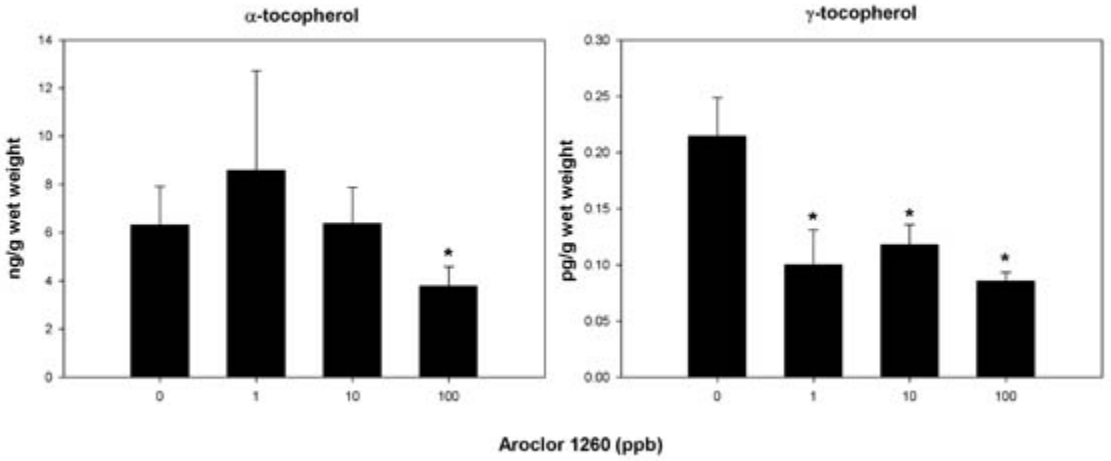

Figure 2: [A] Alpha-tocopherol levels show a reduction only at the highest dose. [B] Gamma-tocopherol levels per treatment concentration in pg/g wet weight, show significant reduction at all exposure levels compared to control in γ-tocopherol. N=6 per treatment level. ($p>0.0226$)

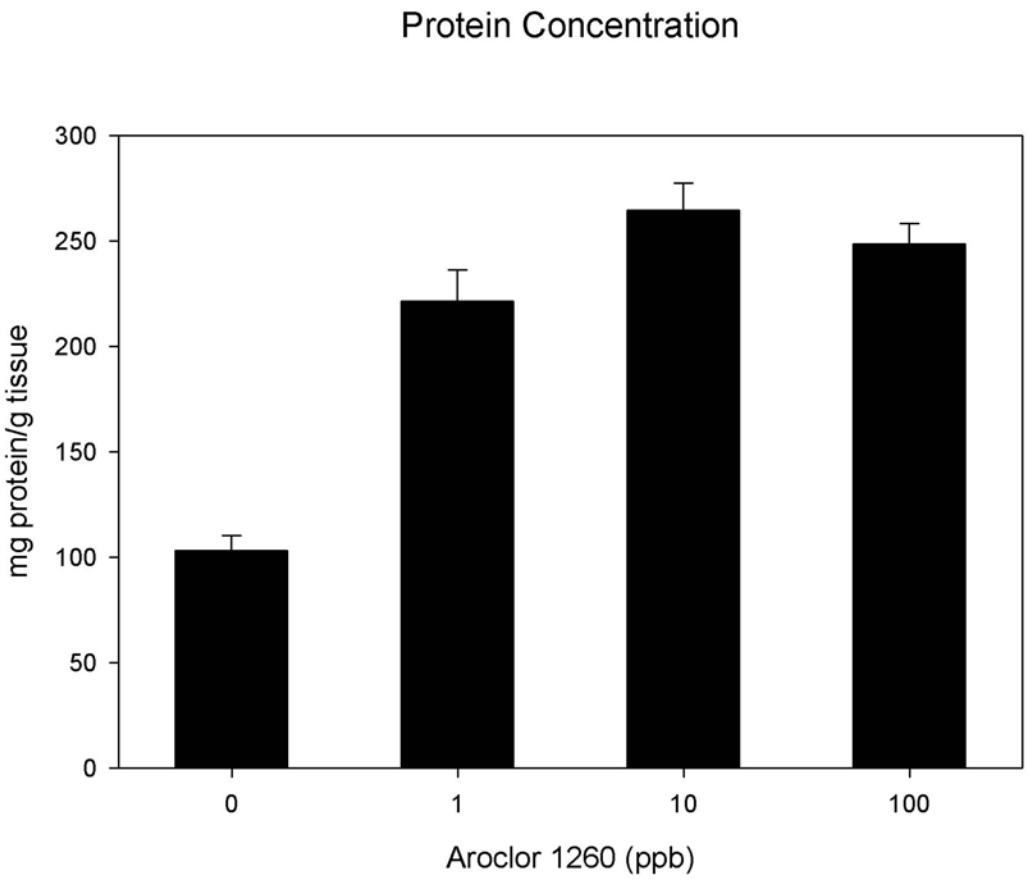

Figure 3: All treatment doses show increased total protein concentrations in comparison to vehicle control. ($p>0.0001$)

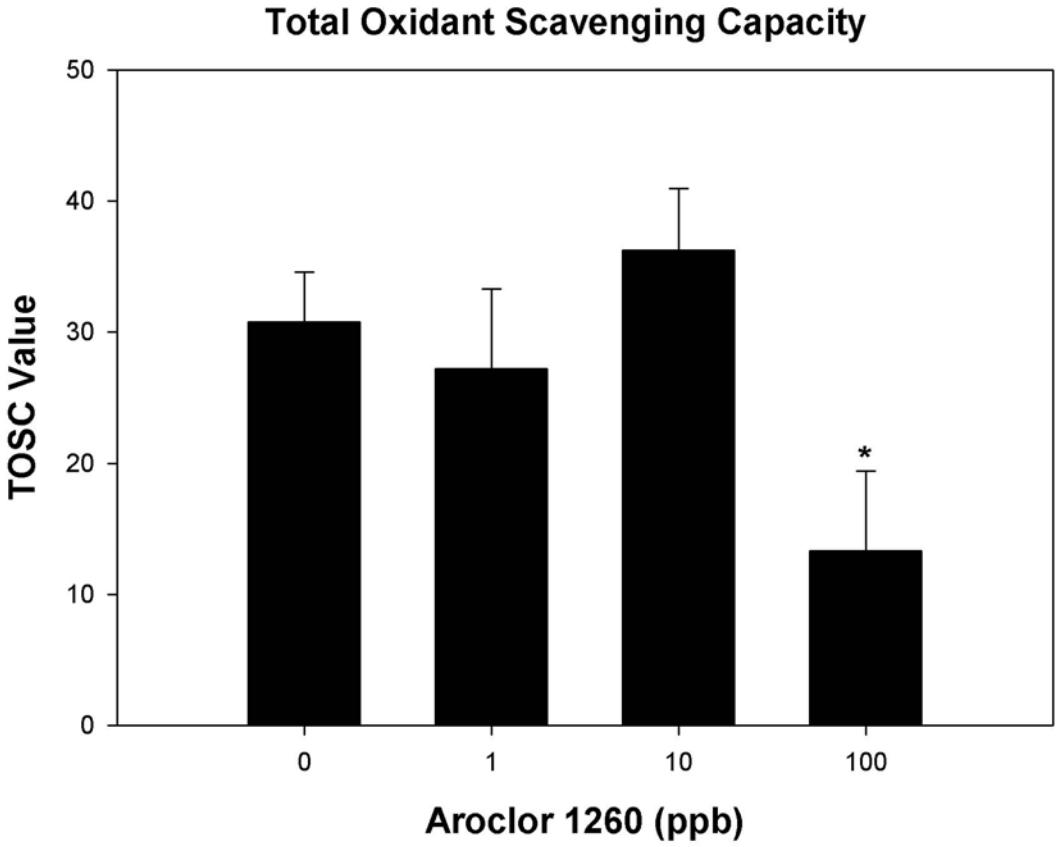

Figure 4: Total Oxidant Scavenging Capacity values are significantly reduced at the 100 ppb dose level. Only one experiment was performed with the TOSC assay. N=3 per treatment level. ($p>0.0379$)

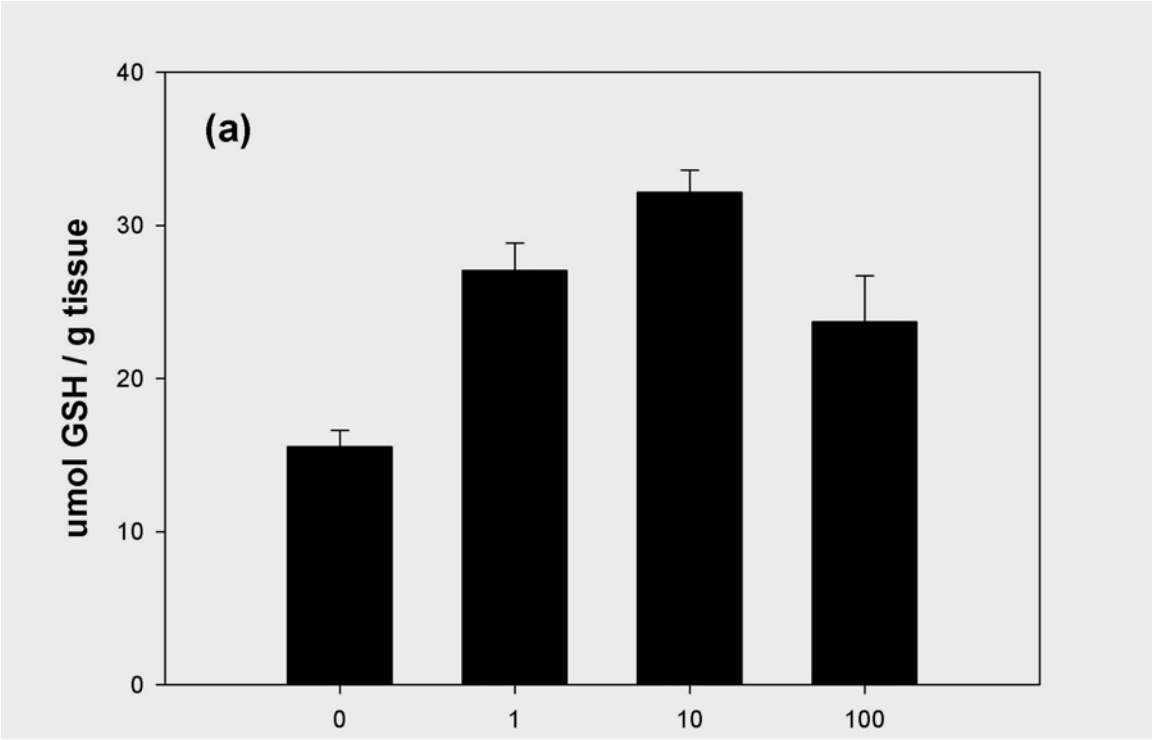

Figure 5: GSH levels show a significant increase at all treatment levels in comparison to control. ($p>0.0001$)

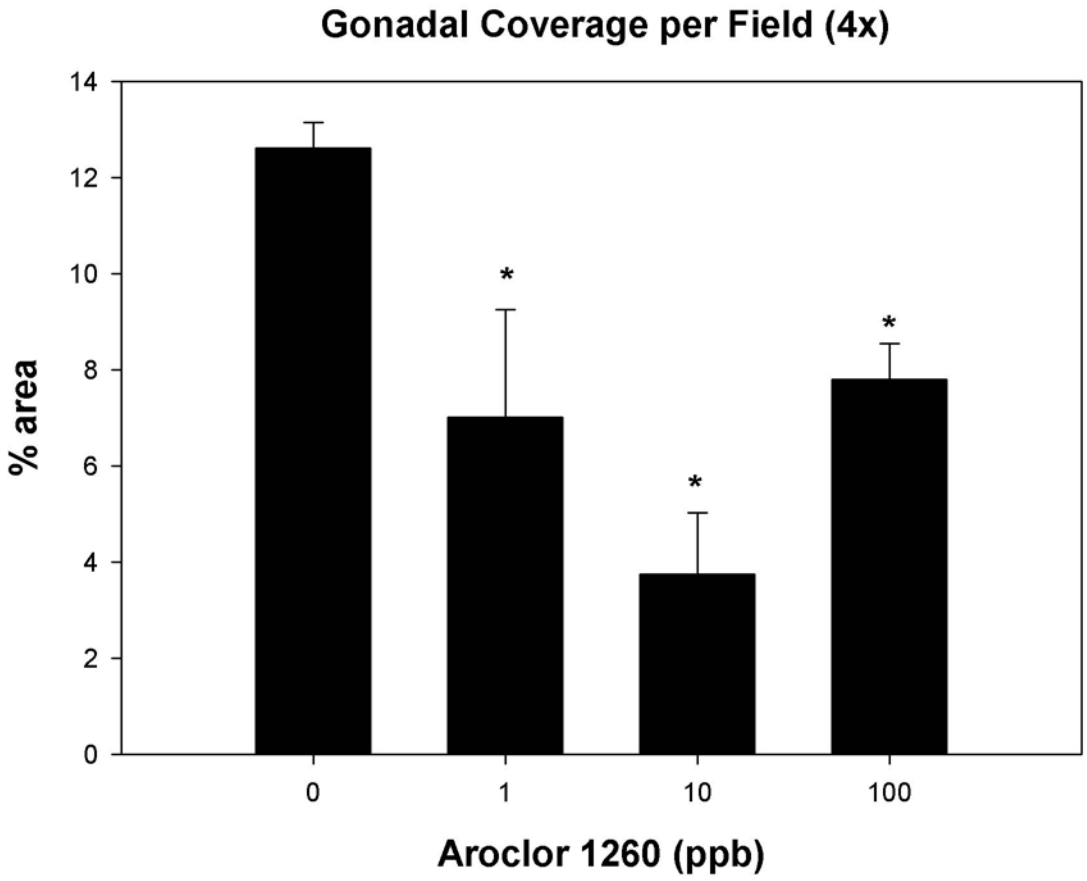

Figure 6: Morphometric analysis of gonadal area per field. Three random fields were digitally photographed through a 4x objective for each sample and analyzed for percent coverage as a function of total surface area. Three animals per treatment level were evaluated. Decreases appear to be somewhat dose responsive and are significantly reduced at all dose levels. N = 6 per treatment level. ($p>0.0020$)

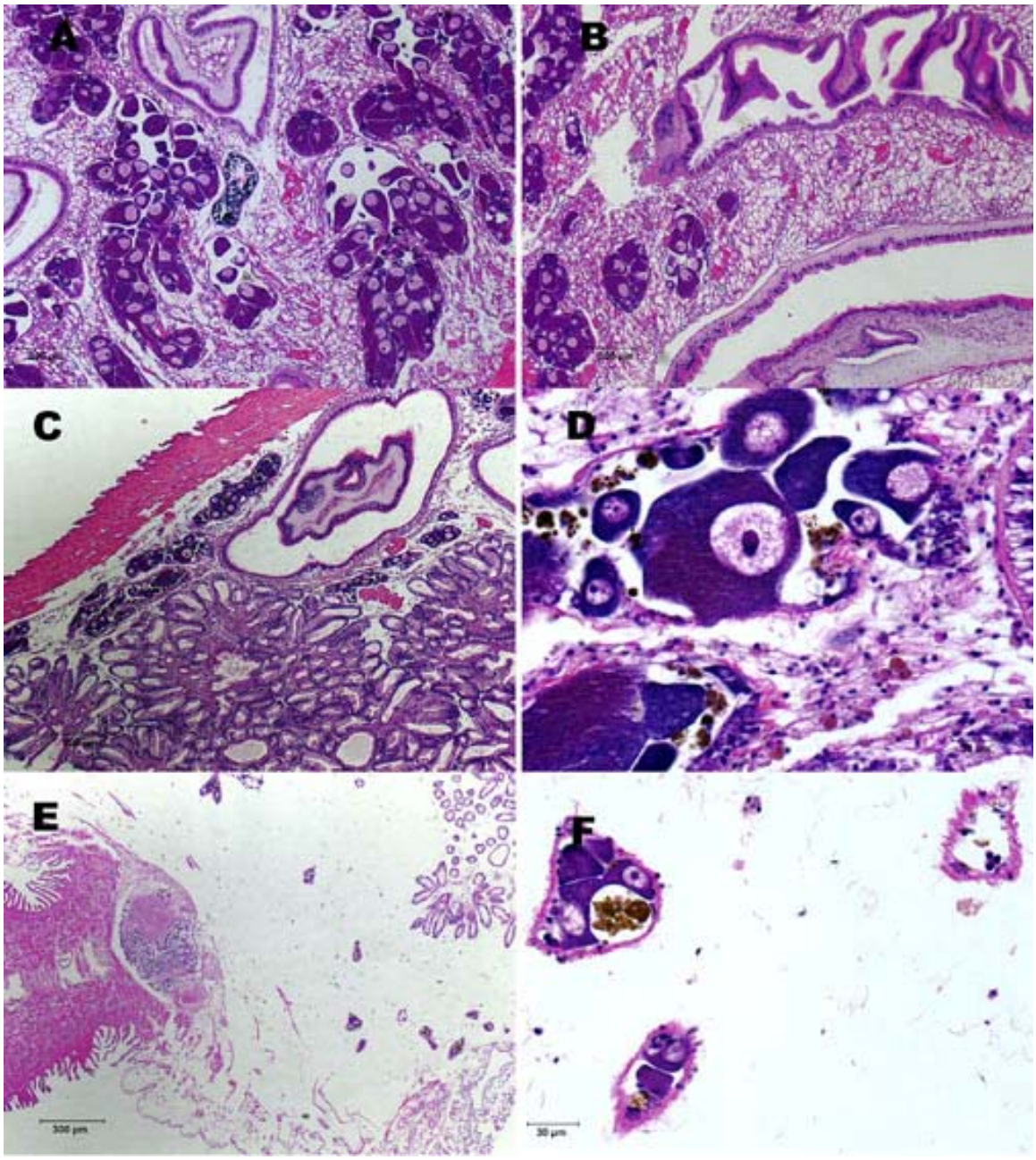

Figure 7: Images of control and Aroclor 1260 exposed clams. (A) 5x objective field, control sample showing normal gonad tissue and surrounding matrix (B) 5x objective field, showing localized necrosis (C) 5x objective gonadal atrophy, note the relative area of each follicle compared to control (D) 40x objective field showing view of single follicle with obvious Brown cell presence and degradation of oocytes (E) 5x objective

field showing generalized edema, note the shrunken gastric tissues(symb1), follicles(symb2), and lack of staining in the interstitial matrix (F) 40x objective field of shrunken oocytes with generalized edema of surrounding tissues.

FIELD EXPOSURE OF *CORBICULA FLUMINEA* CLAMS TO A SITE POLLUTED WITH AROCLOR 1260 RESULTS IN OXIDATIVE DAMAGE AND GONADAL ATROPHY

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ABSTRACT

Polychlorinated biphenyls are persistent environmental pollutants that have been banned from use in the US for the last 30 years. While the ban has reduced the presence of these compounds, concentrations in polluted areas are a concern and environmental release is still occurring in many locations due to improper storage, disposal, and poor reclamation of industrial goods that contain these compounds. Brier Creek, in central North Carolina, USA, was recently reevaluated by the Environmental Protection Agency and fish were found to contain Aroclor 1260 concentrations sufficient to cause closure of the system to fishing. This finding has generated concern that local, at-risk species are at long term risk within this system. To determine the effects of environmental PCB contamination on bivalves, we evaluated a suite of biomarkers of oxidative stress and histopathology on Asiatic clams (*Corbicula fluminea*) that were deployed into the field for 21 days, downstream of the Ward Transformer site (NPL listed). We also deployed polydimethylsiloxane (PDMS) and low density polyethylene (LDPE) passive sampling devices in each cage to correlate PCB concentrations to biomarker responses. Evaluation of sediment and PDMS concentration data indicated that concentrations decreased downstream of the source. Biomarker responses varied by type. Lipid concentrations fell as PCB levels fell while total protein did not vary with location. Reduced glutathione levels were high at most sites compared to concentrations in reference samples. Alpha-tocopherol and total oxidant scavenging capacity were significantly reduced at all sites compared to reference. Histological and morphological analysis revealed that clams at

the high concentration sites had increased inflammation, necrosis and decreased gonad cross-sectional area as determined by morphometry. Results indicate that oxidative damage is a consequence of exposure to this environment and that population level effects such as fecundity and recruitment may be a byproduct of the decrease in size and quality of the gonads of these clams.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are ubiquitous contaminants in the environment due to their use as high boiling point, stable compounds in electrical transformers, heat transfer applications, vacuum oils and other industrial applications prior to 1976. Due to widespread lack of containment from operations such as reclamation of electrical components, PCBs were still being introduced to the environment until recently at many sites. This widespread use and release, the inherent stability, and toxicity of many of the congeners have resulted in PCBs being a persistent environmental problem {{126 Safe,S.H. 1994; }}. These highly lipophilic compounds partition into soil and sediment readily, with log octanol-water coefficients ranging from 3.76-8.26. They are present in the sediment and water column in aquatic environments, making them available to bioaccumulate and produce effects in native fauna {{143 Livingstone,D.R. 2001; }}. Commercially produced PCB mixtures are labeled according to a four digit system, with the first two indicating the category of the mixture and the second two, the percent chlorine content of the congeners in that mixture. In site sampling and analysis, the US Environmental Protection Agency currently uses this designation for reporting environmental concentrations of PCBs and has set action limits of 25 and 1 ppm in soil (or sediment) at commercial and non-commercial sites respectively. The National Priorities List, maintained by the EPA, currently shows more than 500 sites across the country polluted with PCBs, dibenzofurans, and dioxins. The study described herein was prompted by a recent rediscovery of high Aroclor 1260 concentrations in aquatic species

in a recreational reservoir and the resulting concern for at-risk species that inhabit downstream waterways.

The ubiquitous distribution of PCBs means that they are detectable in living organisms from every environment {{136 Tanabe,S. 1987; }}. Their effects, while congener dependant, include both cancer and non-cancer endpoints. Mechanistically, PCBs affect lipid metabolism, endocrine function, and are implicated in contaminant stimulated reactive oxygen species production in aquatic organisms at many trophic levels {{143 Livingstone,D.R. 2001;130 Livingstone,D.R. 1990; 144 Kelly,K.A. 1998; }} leading to non-cancer maladies including reproductive dysregulation, immune system damage, nervous system disorders, skin disorders such as chloracne, and an array of sensory defects {{160 Shimizu,K. 2003;192 Kuratsune, M. 1996; }}. Polychlorinated biphenyls appear to exert their effects by two main mechanisms. The first is interacton at the hormone level, which is mostly responsible for reproductive and developmental effects. The second is interactivity with the aryl hydrocarbon receptor (AhR) which can induce cytochrome P450 expression and increase reactive species and activated metabolite production. These metabolites may cycle in a quinone redox pathway causing the oxidation of nearby macromolecules {{145 McLean,M.R. 2000; }}. Some dioxin induced reproductive alterations have been shown to be AhR-independent {{142 Butler,R.A. 2004; }}. Mollusks have been found to not have an AhR, although induction of cytochrome P450 (as evidenced by 7-ethoxyresorufin-O-deethylase induction) occurs due to exposure to halogenated hydrocarbons {{163 Brown,S.B. 2002;14 Gardinali,P.R.

1998; }}. Oxidative damage at a level that is physiologically detrimental is associated with carcinogenesis, diabetes, reproductive, and other ill health effects in mammalian systems as evidenced by environmental disasters as in Yusho, Japan {{192 Kuratsune, M. 1996;153 Ross,G. 2004; }}. Oxidative stress comes about when the balance of antioxidative systems is overcome by free radical presence {{203 Regoli,F. 1998; }}. This imbalance leads to physiological stress and the oxidation of critical elements of the cell including nucleic acids, lipid bilayers, and proteins. Oxidation of these elements can overcome the antioxidant potential of cells leading to oxidative damage.

After dispersal into soil and surface waters, PCBs in the environment generally undergo some degradation with time. It is not known just how strong an oxidative response can be generated from the remaining larger, less readily metabolized compounds that are unique to every contaminated environment. To answer the question of potency, it is necessary to use field placement of an sentinel species to gauge the alteration of relevant biomarkers. Biomarker responses appear to differ in the scientific literature by toxicant, season, and location. Therefore, it is critical to understand the response of the biomarkers of interest within a specific animal model. Common biomarkers of oxidative challenge include antioxidative enzymes (such as superoxide dismutase and catalase), ancillary antioxidative enzyme systems and those involved in metabolism (such as glucose-6-P-dehydrogenase and glutathione-s-transferase), lipid oxidation state, non-enzymatic antioxidants (such as total reduced glutathione levels, vitamins, urea), and total ability of cellular components to reduce oxidation *in vitro* (Total Oxidant Scavenging

Capacity){{157 Lee,D.W. 2004;52 Sheehan,D. 1999; 171 Winston,G.W. 1998; 165 Koremura,N. 1990; }}. Generally, in single compound exposures, there is a rise in enzymatic activity or concentration as well as increases in non-enzymatic defenses. High levels of oxidative compounds will cause a sharp decrease in many of these biomarkers indicating that the antioxidative systems employed have been overburdened or compromised. Highly specific biomarkers, such as reduced glutathione concentrations (GSH) are less relevant at the population level, while more general population level biomarkers give no indication of the mechanism(s) of action of a toxicant. Therefore, it is of benefit to use an array of biomarkers for determination of toxic effects, preferably at different levels of organization, from molecular markers to genetic alternations to whole animal health indices {{203 Regoli,F. 1998;166 Ham,K.D. 1997; 173 Burton,G.A.,Jr 2005; }}. Included in the analysis of biomarker changes, however, is the need for demonstrable biological or physiological effects of a toxicant that can be compared across phylogenetic levels. This need is well met by the use of histopathology. This concept of transferability is in-line with the need to relate the responses of individuals to the responses of populations and communities to better define end-case scenarios caused by pollution {{218 Hinton, D. E. 2005; }}.

The Asiatic clam, *Corbicula* sp., was initially introduced into the Northwestern U.S. in the early 20th century, and is now spread worldwide as an invasive species that inhabits fresh to brackish water systems {{191 McMahon, R.F. 1983; }}. In North Carolina, they are present in most river and lake systems at relatively high densities. The wide

distribution of the Asiatic clam and its nuisance designation have facilitated its use as an environmental sentinel organism {{176 Barfield,M.L. 2001;22 Colombo,J.C. 1995; 175 Fournier,E. 2005; }}. Typically, clams with higher PCB burdens demonstrate increases in both primary and secondary antioxidant systems which are time and dose dependant. Animals from chronically polluted sites typically have higher resistance to episodic exposure compared to animals from clean sites {{47 Rodriguez-Ortega,M.J. 2002; }}. Uptake of PCBs through contaminated diets has been well documented for bivalves from many environments {{186 Chu,F.L. 2000;196 Thompson,S. 1999; }}. The recent rediscovery of Aroclor 1260 concentrations in aquatic species in a recreational reservoir and the knowledge that at-risk species inhabit downstream waterways prompted these field studies. We have also examined Aroclor 1260 exposed clams in which we recorded marked oxidative stress and gonadal and other tissue injury in the laboratory. (laboratory study section) In this study we describe the responses of a suite of primary and ancillary biomarkers of oxidative stress at different levels of biological organization, including lipid soluble antioxidants, overall ability to resist oxidative challenge (TOSC), glutathione components, and whole animal histopathology.

MATERIALS AND METHODS

Corbicula fluminea clams, collected from a nearby clean site, were held in the laboratory and fed a diet of mixed, live, unicellular algae for a period of 10-14 days prior to deployment in cages. Clams ranged from 12-20 mm in shell length. This size range is

also reproductively viable. Cages were constructed of vinyl coated wire mesh and anchored with nylon string for easy recovery. Fifty clams and suspended strips of PDMS and LDPE were deployed into a single, subdivided cage at each location and water parameters (temperature, pH, conductivity, dissolved oxygen) were measured at each site weekly. Deployed clams and passive sampling devices were collected after 21 days and sorted randomly into 4 groups of 10-12 individuals at each site. A sub-sample of 3 clams from each site were preserved for histological analysis. Brier Creek deployment sites started as near to the source [A] as we could arrange and followed a downstream gradient through flood control reservoirs [B], a recreational use reservoir (Lake Crabtree [C]), and further into a state park [D]. The reference site (Stirrup Iron Creek Reservoir [E]) was less than 3 linear miles from the polluted sites, but in a disconnected system that had no obvious anthropogenic inputs. (fig 1) Clams were placed in mild to low flow environments with sand and rock substrates and moderate silt settling of 0.25-1" depth. During the exposure period, 4.47 inches of rainfall occurred causing a noticeable increase in turbidity and suspended clay at all sites. Three randomly selected clams were removed upon harvest from each site for histological preservation and analysis.

Clams were placed on ice after re-collection for transport to the laboratory where they were immediately shucked, blotted, and weighed. Each composite sample weighed 8-12 grams (wet weight) and was homogenized in 1:2 w:v (20mM Tris-HCl (pH 7.6), 0.5M sucrose, 0.15M KCl, 1mM EDTA, 1mM DTT) homogenization buffer using a BioHomogenizer (ESGE, Switzerland). Aliquots were removed for total antioxidant

scavenging capacity (TOSC) assay, protein quantification, reduced glutathione (GSH) concentration, and lipid soluble antioxidant quantification. All methods were completed on the day of clam recovery except for TOSC and lipid soluble antioxidant samples which were frozen at -80^{o}C in (20mM TRIS-HCl, 1mM EDTA, 1mM DTT, 5ug/ml leupeptin) preservation buffer for later analysis.

Reduced glutathione (GSH) levels were measured with commercially available kits according to manufacturer's instructions (Cayman Chemical (Ann Arbor, MI)). The GSH kit uses glutathione reductase and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) substrate as a basis for colorimetric detection of glutathione. Protein was measured by use of a BCA kit from Pierce (Rockford, IL) using manufacturer's instructions at a dilution of 1:20. This kit uses bicinchoninic acid as a universal reagent measuring the reduction of copper in solution.

Extraction of PCBs from sediment and tissue was performed by sequential extraction using a methylene chloride (DCM) based shaker method. Fully homogenized samples were placed into Teflon tubes. DCM was added at a ratio of approximately 10 ml:1 g sediment or tissue and anhydrous sodium sulfate (approximately 50g) was added to absorb excess water. Surrogate internal standards included PCB 112 and 197 and were added prior to shaker extraction. Supernatants were poured from the tubes and collected in 50ml glass tubes. A matching volume of methylene chloride was added to the sample tubes and the shaking was repeated for 3 hours. Following supernatant removal, the

procedure was repeated once more after 30 minutes of shaking. Samples were reduced under a slow flow of nitrogen and then brought up to 1ml in volume. Gel permeation chromatography (GPC) was run for cleanup purposes and the lipid phase collected and allowed to dry fully and subsequently weighed for lipid content calculation. Separated samples were then exchanged to hexane, chloroxylenol (TCMX) was added as a recovery standard, and PCB congeners were quantified on a Agilent 6890 gas chromatograph (GC) with electron capture detection (ECD) and congeners were verified by mass spectrometry (MS) on a Restek (Bellefonte, PA) RTX-5MS column. Total PCBs are calculated as the sum total of the 20 congeners detected by this ECD method. (table 1)

Specimens were prepared for histopathology by gently prying open the shells and inserting a toothpick between the valves to hold them open. *Corbicula* were euthanized by an overdose of MS-222 (Argent Labs, Redmond, WA) and immediately fixed in cold, 10% neutral buffered formalin for 24 hours. The visceral mass of each specimen was then carefully removed by transecting the adductor muscle close to the shell with a scalpel and peeling up the mantle. Clams were then bisected and placed into 10% formic acid for 24 hours to remove any residual shell or mineral debris. After that time, they were placed in 70% ethanol until histological processing. The tissues were routinely processed, embedded in paraffin, sectioned at 5 microns, stained with hematoxylin and eosin (HE), and examined by light microscopy. Lesions were scored by a single pathologist according to the scale outlined in Hurty, *et al.*, 2002 {{195 Hurty,C.A. 2002;

}}. In brief, lesions were scored from 0 (no remarkable microscopic abnormalities) to 5 (severe lesions) for each lesion type.

Morphometrics were performed on HE stained slides using Image-Pro Plus (MediaCybernetics, Silver Springs, MD) software. Three random fields from each slide at 4x magnification were photographed and each image was analyzed for total gonadal area including both ovarian and testicular tissues. Regions of lumen or those containing no tissue were subtracted from the total tissue area of each field. Fields containing 25% of more of gastric tissue were discarded and not photographed for purposes of gonadal area measurements.

Antioxidants were extracted in amber glass vials using sequential extractions with dichloromethane:hexane (9:1). Samples were combined with the DCM:hexane at 1ml volume per 0.1ml sample homogenate. δ-tocopherol was added as a recovery standard. After an overnight shake, the supernatant was removed, and the process repeated for 30 minutes. A third and final extraction was performed after briefly vortexing. The supernatant was then exchanged to ethanol by drying under nitrogen and brought up to a final volume of 250ul. Retinol acetate was used as an internal standard. 50 ul of each sample was injected onto the column and run at 0.8ml/min. Antioxidants were measured via HPLC with electrochemical detection (ECD) on a ESA, Inc. Coularray (Boston, MA). Alpha-, delta-, and gamma-tocopherol, beta-carotene, retinol, and coenzyme's Q9 and Q10 were measured with the following voltages: +200, +400, +500, +700, -800, -900,

+200, +500 mV using an isocratic method and a C18 nucleosil column (Supelco). Running buffer was 78:20:2 methanol:2-propanol:ammonium acetate. Each sample run was followed by a 1 minute flushing with running buffer that included 10% hexane.

Total oxidant scavenging capacity was assessed according to Winston and Regoli, 1998. Whole homogenate samples were cleaned up using a 10,000g centrifugation at 4^{o}C for 10 minutes to remove large cellular debris. Due to experimentally increased protein concentrations in our samples, we did not normalize TOSC values to total protein concentration. Instead, samples are normalized to wet tissue weight. We also used whole homogenate in place of S9, cytosolic, or lipid soluble fractions alone, to better observe alterations in the entire animal. Samples were diluted 1:5 in 100mM potassium phosphate buffer with 1mM EDTA and a total of 10ul was used in each 1ml reaction vessel fitted with Mininert® valves (Supelco, Bellefonte, PA). Samples were reacted with 20mM 2,2'-azobis(2-methylpropionamidine)dihydrochloride (ABAP) and 2mM α-keto-γ-methiolbutyric acid (KMBA) over a period of 120 minutes for the measurement of ethylene gas production as an indicator of susceptibility to peroxy radicals. Samples were run on a Hewlett-Packard Series 5890 GC with flame ionization detection (FID). A 30m Rt-QPlot capillary column (ResTek, Bellefonte, PA) was used for separation of ethylene from other gases. Running conditions were injector 165^{o}C, oven 40^{o}C, detector 250^{o}C. 0.5 ml of headspace was injected per sample every 12 minutes over a course of the reaction. Evaluation of results is displayed as relative response due to amount of

ethylene produced in the reaction. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Statistical analysis was performed with JMP or SAS (SAS, Inc., Cary, NC). Data was examined for normal distribution and variance using one way ANOVA. Effects versus control were analyzed by use of Dunnett's t-test to check for significance (α= 0.05). Linear regression analysis was performed using JMP to compare PCB concentrations against each biomarker response. Values shown are means +/- standard error. Time weighted average concentrations derived from the quantification of PCBs from the PDMS passive devices was calculated using the formula:

(eq 1) $R_s=N_{cmpnd}/(C_w*t)$

Where R_s is the PDMS sampling rate acquired from Heltsley (2005) {{556 Heltsley, R.M. 2005; }}. N_{cmpnd} is the measured amount of PCB within the PDMS in grams, C_w is the calculated water concentration of PCBs in ng/L. t is time of deployment in days.

RESULTS

Total polychlorinated biphenyl concentrations generally decreased with distance from source with the site closest to the source being by far the most concentrated. (fig2) Sediment analysis indicated a decrease at all sites compared to all upstream sites with

concentrations at the reference site [E] being more than an order of magnitude less than the reference [A] site. Time weighted average concentrations derived from the PDMS placed in the clam cages showed a higher concentration in the higher flow Crabtree Creek [D], the furthest site downstream from the source. The increase is likely due to increased flow rates carrying proportionately more dissolved PCBs across the surface of the passive device and the added inputs from a sewage outfall at the base of Lake Crabtree. PCB concentration data available in Appendix Table A1, A2, A3.

Clam mortality at all field sites did not exceed 6% at the conclusion of the 21 day exposure period.

Total protein concentrations were not significantly altered at any sites. Lipid content of the samples also decreased in a concentration-responsive manner in sites downstream from the source. (fig 3) The lack of changes in total protein and increases in lipid at the high concentration locations indicate that there is no food limitation or starvation affecting antioxidant parameters at these sites.

Lipid-soluble antioxidant analysis indicated a very severe reduction in α-tocopherol levels at all sites in comparison to the reference site, although no differences were detected among exposure sites. (fig 4a) Based on the viability and apparent health of the clams in the reference site, the difference appears to be unrelated to any factors that might increase the background level of α-tocopherol in the clams deployed at the reference site.

γ-tocopherol was undetectable in most samples so statistical analysis was not possible, as it appears that environmental exposure may have reduced levels to below detection limits or be diet limited.

Total reduced glutathione levels in whole body homogenates were significantly increased between reference and exposure sites. (fig 4b) In regards to comparisons with the reference site, sites [A], [B], and [D] were higher in GSH, while the [C] site did not differ significantly. Although the trend in increased GSH amounts is still visible.

The TOSC assay indicated that there was a strong reduction in whole body antioxidant potential in all sites compared to reference. (fig 4c) Exposure sites did not significantly differ from one another in regards to the ability of whole homogenates to absorb peroxyl radicals in the *in vitro* reaction.

Morphometric analysis of whole sections indicated that changes in gonad cross-sectional area occurred due to field exposure. Total ovotestis area displayed an apparent dose-response at downstream sites as compared to reference values, although the only significant decrease was at the upstream site [A] where contaminated sediments contain the most PCB contamination. (fig 5)

Microscopically, *Corbicula* from the reference site showed few to no remarkable lesions. There was evidence of mild bacterial presence in the mantles of some control clams.

Exposed *Corbicula* had three major lesion types: necrosis, inflammation, and gonadal atrophy. Compared to reference animals, a couple exposed clams had diffuse, moderate to severe edema characterized by increased clear space in connective tissue and paler staining of the viscera. (fig 6) In exposed clams at the [A] and [B] sites, there were amorphous regions of basophilic debris typical of necrosis in the foot processes and/or mantle of all examined. The clams from these sites also had inflammation in muscle or mantle tissues. Clams from the [C] and [D] sites downstream showed only mild inflammation in their mantles. Many follicles in the clams from [A] and [B] sites were shrunken, fragmented, and were infiltrated with Brown cells (multiple phagocytes containing golden brown pigment granules) Gonad tissue was characterized by reduction in the size of or loss of ovarian clusters, with decreased overall oocyte area and a decrease in the size of individual clusters. In some animals, there appeared to be senescent decreases in total ovarian size, so this was not considered to be a lesion.

DISCUSSION

Freshwater bivalves serve as highly useful biological sentinels in polluted environments. *Corbicula* sp. clams have been used for this purpose for many years {{22 Colombo,J.C. 1995;172 Labrot,F. 1999; }}. As an invasive species, these clams have managed to settle in most warm freshwater environments in the US and Europe, allowing them to be used as sentinels and biomarkers without risk of further corruption to local watersheds. The fact that this species is distributed worldwide also presents researchers with a

consistent model sentinel organism for environmental field studies {{176 Barfield,M.L. 2001;22 Colombo,J.C. 1995; 43 Vidal,M.L. 2002; 46 Vidal,M.L. 2002; }}. Due to the high population densities present, we chose to use *C. fluminea* collected from a local clean site as they are well adapted to local conditions. In addition, *Corbicula* clams are well adapted to shifting environments being benthic dwelling organisms. Their substrate association places them into contact with sediment-associated contaminants and exposes them to periodic hypoxia/anoxia. Behaviorally, bivalves are adapted to low oxygen situations due to their ability to seal their shells against environmental input and modify their metabolism accordingly {{178 Pampanin,D.M. 2002; }}. Our objective was to determine relevant changes in biomarkers and histological sections due to the environmental Aroclor 1260 exposure. In combination with concentration data from PDMS and sediment, we also evaluated relatedness of biomarkers to concentration data in an attempt to validate these various biomarkers as independent measures of biological effect due to exposure to specific concentrations.

EPA action levels, for non-industrial sites, are set at 1 ppm total Aroclor in sediment and soil. Our detected levels, based on the cumulative total of the 20 congeners in the analysis, ranged from 2 – 28 ng/g (ppb) dry weight in sediment. However, estimated water concentrations at our sites indicated concentrations from 0.15-0.20 ng/L and EPA the water quality criteria for protection of aquatic species in freshwater in 0.014 ng/L which is ten times lower. *Corbicula*, when exposed to this environment, showed signs of cell injury even though PCB concentrations are below action levels at these downstream

sites. We feel that the marked histological changes that were noted in these exposures are a good indicator of the oxidative alterations that occur in PCB polluted environments. Recent studies have shown that bivalves exposed via environmental field studies show transfer of PCBs to reproductive tissues and have indicated that the highest residual concentration of these lipophilic compounds is found in the gonads {{185 Chu,F.L. 2003; }}. Reduction in gonad size and function are also associated with PCB exposure in rodent studies, even at levels approaching those found at background levels in human populations {{193 Wade, T.L 1992;206 Johnson,L. 1994; }}. This is corroborated by our demonstration of severe alterations in gonad cross-sectional area due to exposure to Aroclor.

The duration of exposure, in this case 21 days, allowed for a whole animal response to the oxidative stress initiated by the PCBs, but most likely not the time necessary to achieve steady state concentration. However, according to Rodriguez *et al.* 2003, approximately 40-50% maximal uptake is achieved by 21 days at 10ppb in aqueous exposures, including proportionately more of the lower chlorinated congeners which are more readily metabolized by monooxygenases {{9 Rodriguez-Ariza,A. 2003; }}. In our study, increases in total reduced glutathione and lipid, as well as the decreases in α-tocopherol and in TOSC levels indicate that the course of exposure was sufficient to allow for a reduction or possible collapse of overall antioxidant capacity.

Lipid soluble antioxidants are a fairly stable component of the complete antioxidant system in organisms {{181 Kohar,I. 1995; }}. Many of the lipid soluble antioxidants have critical functions in the homeostasis of cells and are regulated strongly to maintain those functions {{182 Jiang,Q. 2000;327 Jiang Q,A.B. 2003; 183 Saldeen,T. 1999; }}. Any measurable change in these lipid soluble antioxidant levels is an indication of a potent oxidative challenge. Alpha-tocopherol is typically recycled in conjunction with ascorbic acid, unlike γ-tocopherol which is excreted, so that levels should be more stable {{181 Kohar,I. 1995;182 Jiang,Q. 2000; }}. Shifts in the relative proportion of α-tocopherol indicate that the cumulative total of the oxidant side of the oxidant:antioxidant balance is increasing and can be detrimental over the long term, even when cellular function is not overtly perturbed. The lack of measurable γ-tocopherol and the overt depression of α-tocopherol concentrations, at all exposure sites, indicate that oxidation of macromolecules is a factor in the overall decline in histological status of these clams.

The reduction of α-tocopherol is likely due to perturbance of lipid bilayers by the highly lipophilic PCBs and resulting quinone cycling oxidative metabolites. Tocopherols have been shown experimentally to reduce lipid peroxidation (measured as TBARS) during oxidative challenge and are known to directly affect reproduction {{188 Kawai-Kobayashi,K. 1986; }}. Lipofuscin, the "wear and tear" pigment, builds up in cells due to oxidative degradation of lipid and is visible as yellow or brown pigment in cells during histological evaluation {{184 Zaroogian,G. 2000; }}. It follows that reduction in lipid protective molecules may increase lipofuscin accumulation as seen in our study as an

accumulation of Brown cells. In this experiment, it appears that the site with the highest PCB concentrations [A] may take defense mechanisms beyond the compensation point of the antioxidant system as indicated by increased Brown cell presence and degredation of gonadal architecture. Alterations in lipid metabolism due to PCB exposure have been demonstrated by Ferreira and Vale, 1998 {{15 Ferreira,A.M. 1998; }}. Coincident with the lipid metabolism alterations, PCBs are known to interact with signal transduction as evidenced by the release of insulin in cell culture and while many invertebrates are thought to have no aryl hydrocarbon receptor, oxidative stress has been demonstrated due to exposure {{142 Butler,R.A. 2004;187 Hahn,M.E. 2002; 163 Brown,S.B. 2002; }}.

Reduced glutathione concentrations are used frequently as markers of effects in both field situations and in the laboratory. However, the variability seen in the literature somewhat offsets the usefulness of GSH as a sole marker of oxidative stress. Experimental supplementation with GSH has been shown to reduce oxidative damage directly caused by exposure to PCB congeners {{189 Slim,R. 2000; }}. Therefore, in combination with other markers, GSH can remain a useful indicator of early responses to stress. In this study, GSH rose in response to increasing concentrations of PCBs, corroborating the results seen in previous studies {{161 Sheehan,D. 1995; }}. Rodriguez et al. (2003) noticed a decline in GST levels over a longer term PCB exposure, where the activity continued to fall until 110 days exposure suggesting a slow overburdening of the system due to PCB accumulation, but GSH levels rose and fell on a much shorter time scale {{9 Rodriguez-Ariza,A. 2003; }}.

The TOSC assay is suggested as a generalized indicator of overall antioxidant system status by measuring whole sample *in vitro* capacity to absorb peroxyl, hydroxyl, or other radicals depending on the radical generating system employed {{203 Regoli,F. 1998;171 Winston,G.W. 1998; }}. TOSC values were normalized to total protein concentration. As noted in the study by Regoli, ***et al.*** in 1998 of *Mytilus* bivalves, various fractions (cytoplasmic, lipid, s9) have differing proportions of the total antioxidant activity for each contaminant stressor {{203 Regoli,F. 1998; }}. Due to the nature of the oxidative stress derived from the Aroclor mixture used in this study, we opted to examine whole homogenized sample *in lieu* of various fractions to evaluate *Corbicula*'s total antioxidant capacity. We found a significant decrease in TOSC values at all contaminant polluted sites. The decrease in TOSC pushes the burden of evidence toward oxidative damage being a high risk factor in these exposures.

Microscopically, gonadal atrophy seems to be the most significant population relevant effect of PCB exposure in these clams. This gonadal alteration may be sufficient to reduce fecundity or cause population level alterations in the local environment as noted by a lack of mature eggs in *C. virginica* oysters after PCB exposure by Chu et al. (2003) {{185 Chu,F.L. 2003; }}. We found evidence of inflammation and discrete regions of necrosis were found, indicating that some overt damage was derived from exposure to Aroclor 1260, possibly due to oxidative cellular damage as noted by Koponen in fish from polluted lake systems {{190 Koponen,K. 2001; }}. Holding the clams under

constant exposure conditions beyond the three week period used in this experiment would likely have produced even more negative effects at the cellular and organ levels and would probably have resulted in higher mortalities as noted in the extended study of oxidative effects in *C. gallina* by Rodriguez-Ariza 2003 {{9 Rodriguez-Ariza,A. 2003; 219 Morado, J.F. 1997; }}. Many of our higher dose specimens also had severe generalized edema (anasarca) which may be caused by changes in osmotic balance, hydrostatic pressure, increased tissue permeability due to inflammation, or direct injury by the PCBs. In our study clams, the edema was most likely caused by oxidation of lipid membranes, creating increased permeability as well as profound osmotic disturbance. The scattered inflammatory infiltrates seen in various tissues were most likely reactive or secondary to direct tissue injury. Gamma-tocopherol has been noted to curtail inflammatory reactions, and the lack of detectable γ-tocopherol in these clams shows that the high level of oxidation present had already overcome antioxidant defenses. Patchy necrosis was also visible in histological sections from some of the treated clams. Necrosis is the end result of too much overt cellular damage that cannot be repaired. This also fits into the model of oxidative damage being primarily responsible for dysregulation of ionic balance and breakdown in cellular structure.

In addition to the extensive necrosis and loss of gonadal tissue, the accumulation of relatively large, pigmented macrophage-like hemocytes or Brown cells amongst the residual necrotic debris of the gonads was interesting. While the origin and specific functions of these cells are still being investigated, recent studies have associated the

accumulation of Brown cells in various tissues of bivalves with exposure to organic pollutants {{220 Smolowitz, R. 1996;221 Zaroogian, G. 1995; }}. Zaroogian *et al.* have perhaps most thoroughly characterized the brown cells of the red gland or pericardial gland of clams as fixed tissue cells involved in detoxication of metals {{184 Zaroogian,G. 2000;221 Zaroogian, G. 1995; 20 Gilek,M. 1996; }}. This would suggest a liver-like function for the red gland. However, the Brown cells observed in our study are more likely a sub-type of the mobile, phagocytic hemocytes involved in innate immunity. The accumulation of Brown cells in areas of oocyte injury, apparently due to oxidation of lipid membranes, would suggest that these cells serve a similar function to the melanomacrophages of teleost fish. That is, they probably serve as the "clean-up crew" and accumulate ceroid and other materials that cannot be further broken down. Further research correlating morphology and function is needed.

The specific biomarker responses, as well as the results of whole organism pathology, in this study are consistent with our hypothesis that oxidative damage is a result of exposure to environmental Aroclor 1260, with accumulated damage affecting many levels of organization even over only 21 days of exposure. The combination of oxidative stress biomarkers and the decline in gonad health visible at the microscopic level indicates that oxidative stress is a direct consequence of exposure to the PCB mixture. While we are unable to define oxidation as a direct cause of the gonadal atrophy, evidence derived from this study and others indicates that oxidative damage is a direct acting factor in such declines. Relevant changes, whether directly or indirectly due to oxidative stress, occur

at the organ and organism levels and will likely result in population wide effects, including reduced fecundity and chronic maladies. Freshwater mussels native to North America are among the most endangered groups of animals on the planet. Polychlorinated biphenyl contamination is extensive and may be contributing to declines in mussel fecundity and populations {{110 Williams,J.R. 2004;53 Cossu,C. 1997; }}. In some areas, little or no recruitment of juvenile bivalves is taking place. Similar declines have been noted in snail populations. Endangered bivalves and other aquatic macroinvertebrates will remain at risk due to the extensive number of PCB, dioxin, and dibenzofuran polluted sites in this country. Biologists considering the augmentation or reintroduction of extirpated or extinct macroinvertebrate populations should measure and carefully consider PCB levels at potential restoration sites.

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Table 1

Listing of the 20 PCB congeners analyzed in environmental samples.

Figure 1.
Scale map displaying deployment sites [A] – [E] used in this study. Ward transformer is the source of PCBs in this system and direction of flow is indicated with arrows. [E] is the reference site that has low anthropogenic inputs. Concentrations are indicated at each site from sediment (ng/g dry weight) and calculated time weight averages in the water from PDMS samplers (ng/L).

Figure 2.
Polychlorinated biphenyl concentrations in sediment and estimated from PDMS sampling devices at each site of deployment. Note the background level of PCBs at the reference site compared to all treated sites. Values derived from composite samples.

Figure 3.
Lipid content at each deployment site rises in a dose dependant manner as you approach the source. Lipid data is from composite samples and not replicated.

Figure 4.
Antioxidant responses to field deployment of Corbicula clams. (A) Alpha-tocopherol concentrations mirror the response of whole sample oxidation resistance (TOSC) and fall at all sites relative to reference values. ($p>0.0001$) (B) Reduced glutathione concentrations rise significantly at all sites compared to reference. ($p>0.0021$) (C) Total Oxidant Scavenging Capacity values are all repressed significantly in comparison to reference sites. ($p>0.0094$) n = 4 per site

Figure 5.
Cross-sectional area of gonad tissues in individual clams at each deployment site at the end of exposure. Gonad size appears to follow a concentration dependant decrease as you approach the source with those clams at the highest concentration site [A] showing significant repression of gonadal area. ($p>0.00x$) n = 3 per site

Figure 6.
Histological sections, HE stained. (A) Normal visceral mass from Corbicula clams. Note the densely staining connective tissues and the fullness of the gonads. 4x (B) Inflammation in the muscle/mantle of clams from the [B] site. 4x (C) Anasarca with shrunken gonad tissues and pale staining connective tissues from [A] site. 4x (D) Higher

magnification image of a gonad from the highest concentration [A] site showing Brown cells. 40x.

Table 1. Listing of the 20 PCB congeners analyzed in environmental samples by the MS-ECD method.

Polychlorinated Biphenyl Congeners			
Cl2 (08)	Cl4 (66)	Cl5 (105)	Cl7 (180)
Cl3 (18)	Cl5 (101)	Cl6 (138)	Cl7 (170)
Cl3 (28)	Cl4 (77)	Cl7 (187)	Cl8 (195)
Cl4 (44)	Cl5 (118)	Cl5 (126)	Cl9 (206)
Cl4 (52)	Cl6 (153)	Cl6 (128)	C10 (209)

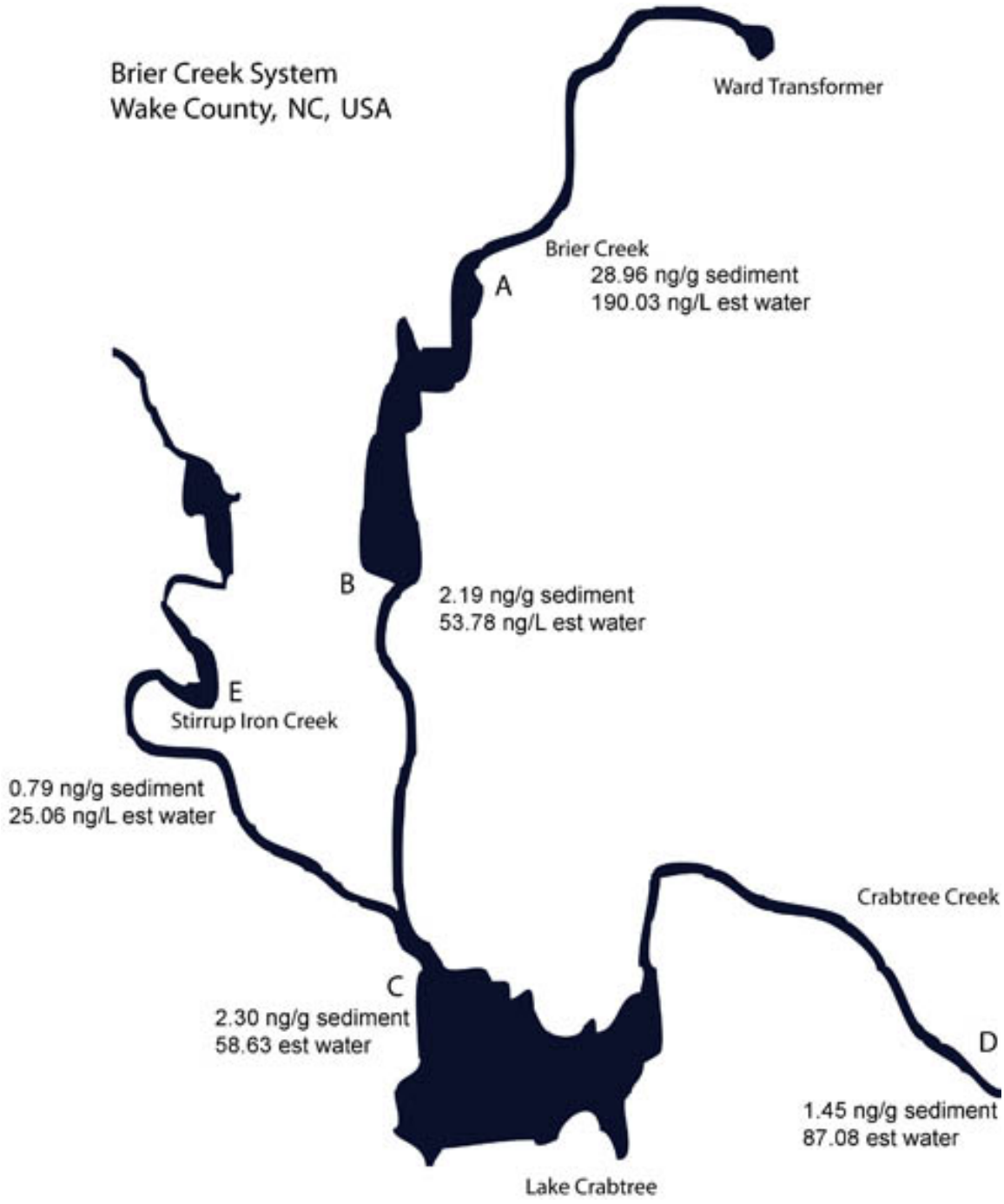


Figure 1. Scale map displaying deployment sites [A] – [E] used in this study. Ward transformer is the source of PCBs in this system. [E] is the reference. Concentration data is displayed as sediment ng/g OC of PCBs and ng/L time-weighted average concentrations derived from PDMS on top and bottom respectively.

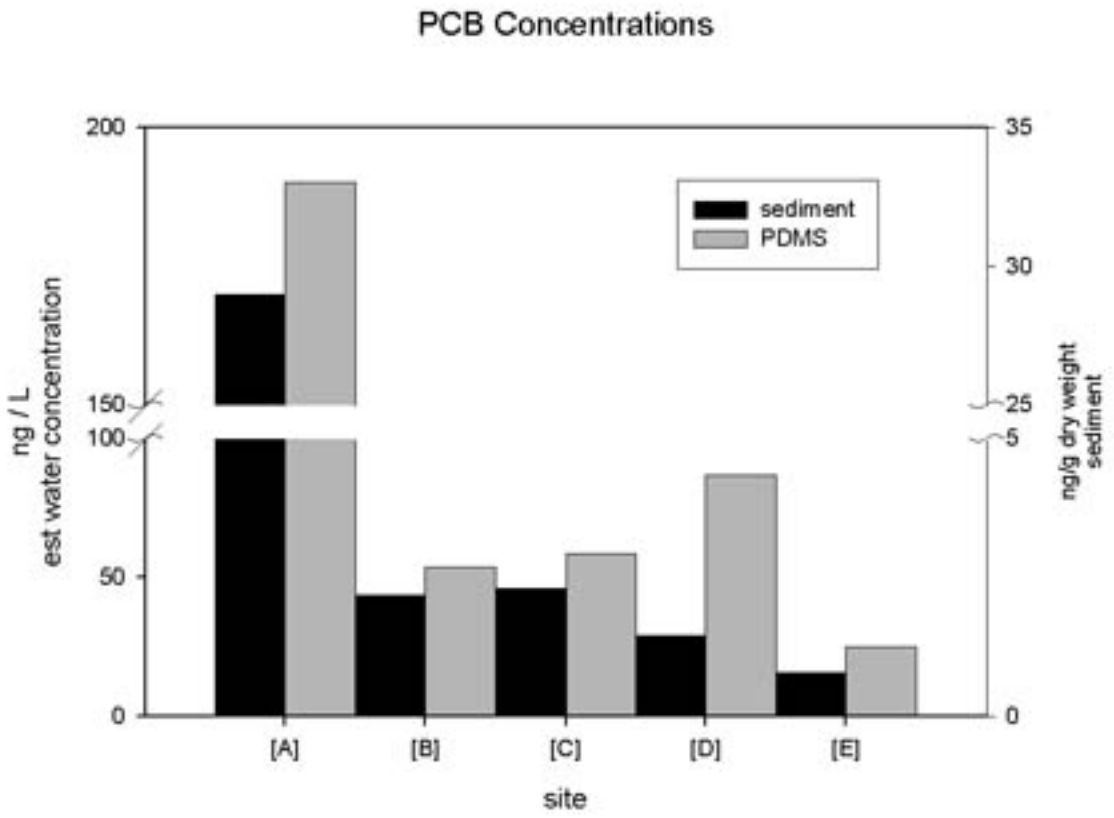


Figure 2. Polychlorinated biphenyl concentrations in sediment and estimated from PDMS sampling devices at each site of deployment. Values derived from composite samples.

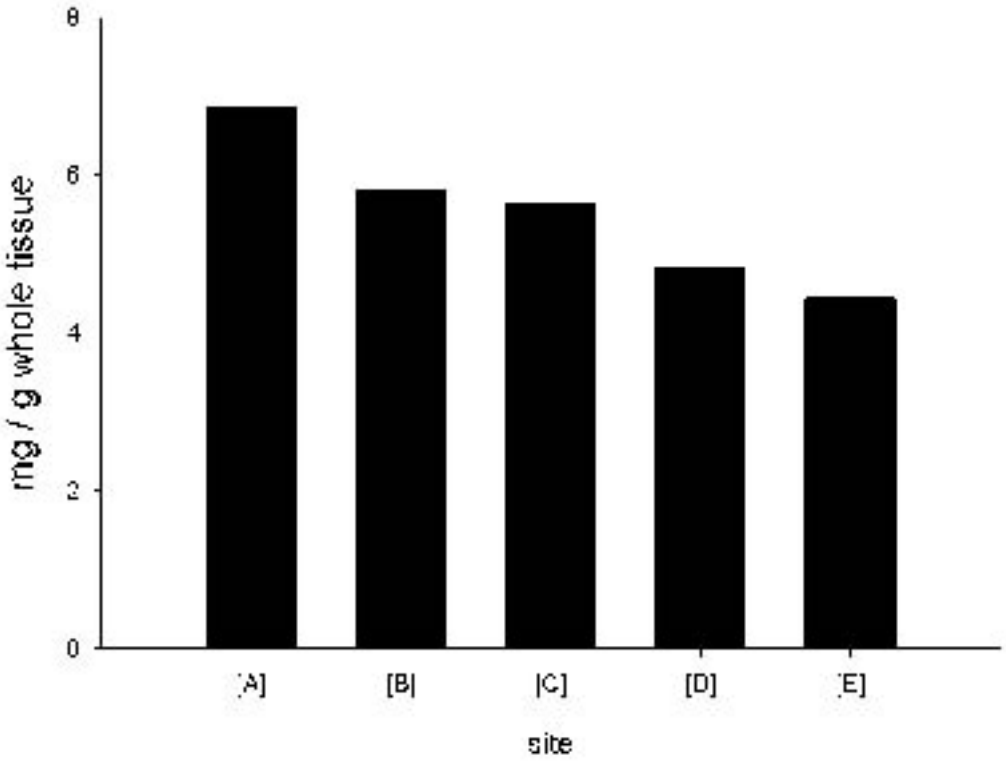


Figure 3. Lipid content at each deployment site. Lipid data is from composite samples and not replicated.

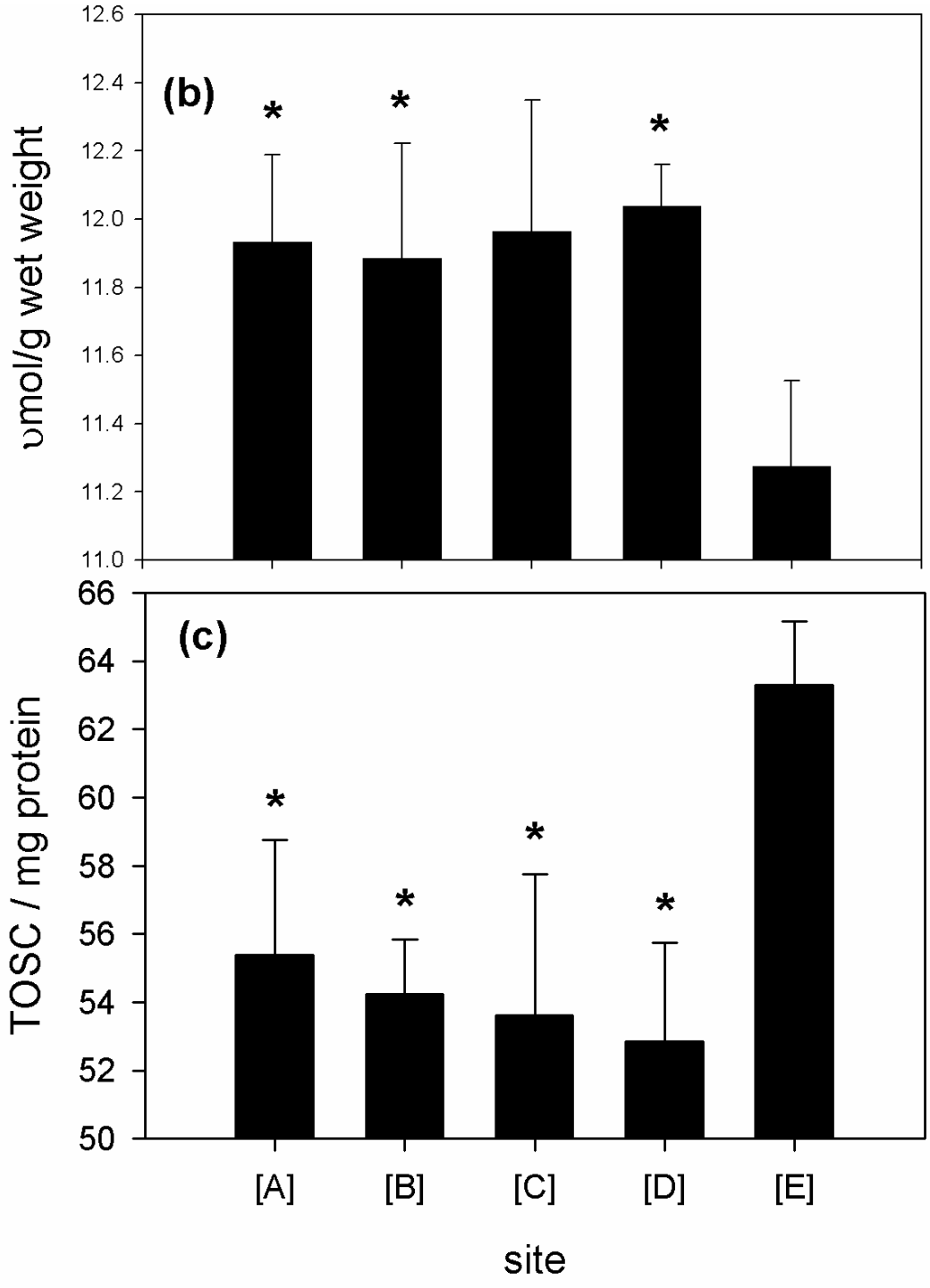


Figure 4. Antioxidant responses to field deployment of *Corbicula* clams. (A) Alpha-tocopherol concentrations ($p>0.0001$) and (B) Total Oxidant Scavenging Capacity values in comparison to reference site ($p>0.0094$). n = 4 per site.

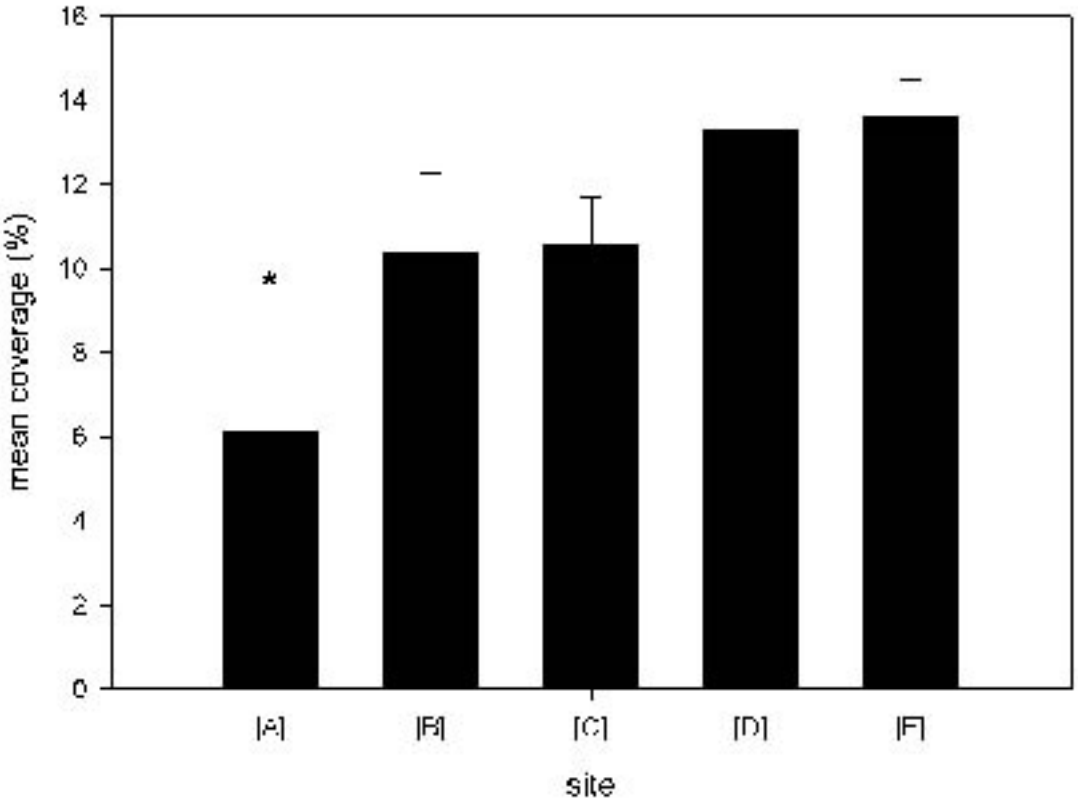


Figure 5. Cross-sectional area of gonad tissues in individual clams at each deployment site at the end of exposure. Gonad size appears to follow a concentration dependant decrease as you approach the source with those clams at the highest concentration site ($p>0.002$). n = 3 per site.

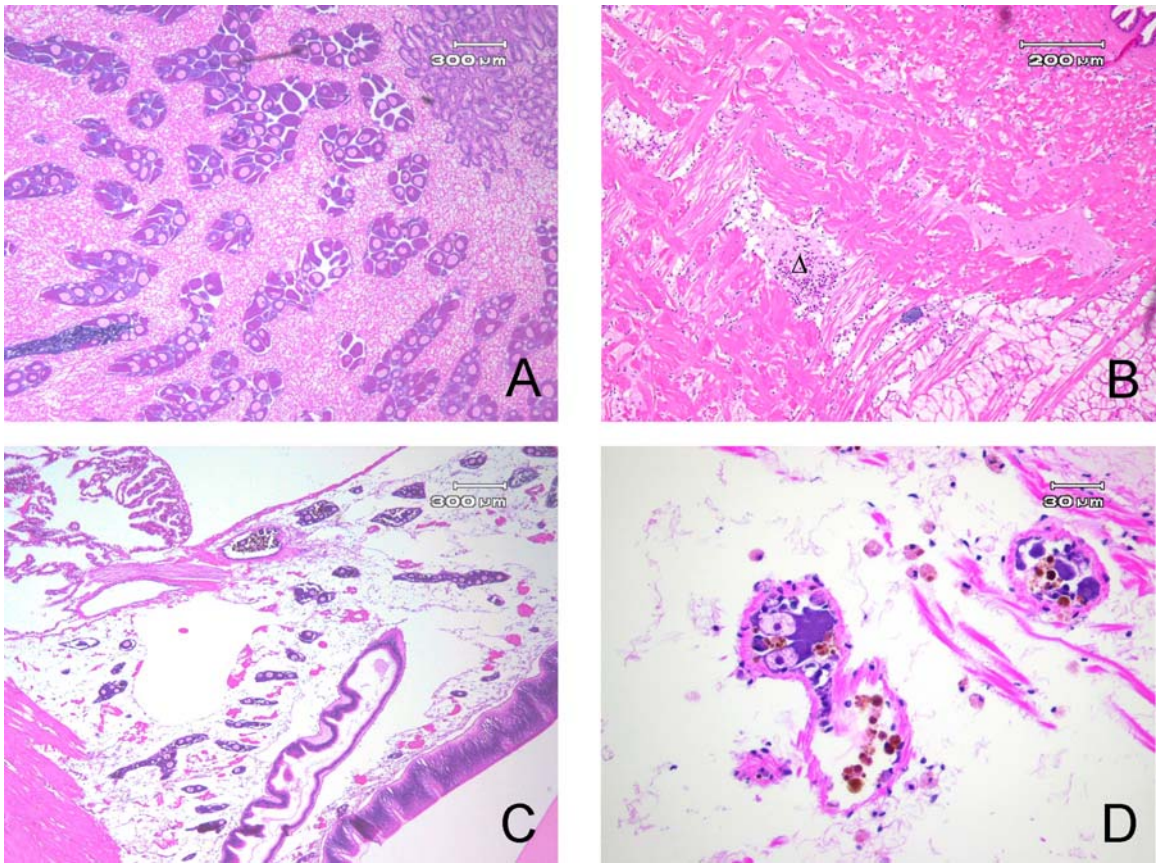


Figure 6. Histological sections, HE stained. (A) Normal visceral mass from Corbicula clams. Note the densely staining connective tissues and the fullness of the gonads. 4x (B) Inflammation in the muscle/mantle of clams from the [B] site. 4x (C) Anasarca with shrunken gonad tissues and pale staining connective tissues from [A] site. 4x (D) Higher magnification image of a gonad from the highest concentration [A] site showing Brown cells. 40x.

CONCLUSIONS AND SUMMARY

Oxidative stress is difficult to attribute to a specific source in biological samples. Stress, by its very nature, is an oblique response to a multitude of etiologies. The oxidation of compounds within cells is a natural process and is under the control of various mechanisms including the antioxidant system. Oxidative stress can lead to oxidative damage, or the oxidation of high amounts of critical macromolecules, when those compensatory systems are overcome by the generation of oxidants due to environmental changes or toxification by anthropogenic chemicals. Detection of oxidative stress is fairly routine, although linking changes in biomarkers to a source is only done through a weight of evidence approach consisting of biomarkers that are relevant at differing levels of biological organization (or population structure).

In the studies we performed, oxidative damage did not stand as a causative factor in lesion development in Atlantic menhaden. Aside from changes in the ionic content of the blood, no evidence of oxidative stress was found. This could be caused by a lack of sensitivity in the biomarkers selected, a highly variable natural fluctuation in the parameters measured in the fish population as a whole, or a sufficiently robust antioxidant system to compensate for the increase in radical production evidenced in other studies during reperfusion. Cyclicity in oxygen concentrations has been occurring in estuaries for a long time and adaptation has likely accounted for sufficient resistance to the changes induced by hyperoxic and hypoxic conditions in the native environment.

Exposure to PCBs has been documented to cause increases in oxidative stress biomarkers in many sentinel and native species where contamination occurs. Laboratory studies back up the claims of oxidative damage due to PCB mixture exposures. In our studies, we attempted to determine the effects of PCB contamination on a surrogate species in order to assess possible negative outcomes, by extrapolation, to native at-risk species of bivalves present in this polluted system. Follow-up laboratory studies validated the alterations in biomarkers noted from the field study in *Corbicula* clams. Sharp declines in antioxidative defenses, increases in tissue damage, and greatly affected reproductive tissues were in evidence in both studies. The laboratory and field deployments indicated agreement in effects observed due to Aroclor 1260 exposure via the water column and water column plus sediment, respectively. The end result of the reduction in antioxidant potential and decreased gonadal indices were not addressed in these studies. However, I would hypothesize that in the short-term, direct damage to tissues is the most prominent acute outcome. In the longer term, population level effects are likely to occur due to decreases in fecundity and survivability in the affected system.

The knowledge that reproductive changes occur in these clams due to PCB exposure begs the question: Is there a similar effect in native bivalve species that inhabit this system? Assumptions on the transferability of this body of exposure data to other species would need to be verified by future research. Knowing if this pollutant exposure would cause similar effects in endangered, threatened, or of special concern species could allow for

cleanup of this polluted environment with the objective of protecting and retaining at-risk species such as the Neuse river waterdog or the rainbow lipped mussel. Additional future directions for continuing this research could include laboratory studies detailing oxidative stress and histological alterations that occur due to exposure at concentrations bordering that found in the water column within the polluted system in *Corbicula* clams and performing population studies in which subsets of bivalves are sampled and analyzed histologically for negative tissue level effects within and downstream of the Brier Creek system.

Eutrophication and hypoxic events are negative impacts of human population expansion in our local waterways. While we could find no evidence of these events causing oxidative stress in local species, it would be prudent to regulate nutrient inputs to these nursery systems in order to protect the assets that large estuaries provide. Due to the persistent nature of PCBs in the environment and the findings of this research, I would recommend the immediate cleanup of the PCB source and enforcement of containment methods for any ongoing operations. With sufficient removal, the current PCB burden in this system would slowly begin to decline due to dilution downstream and deposition of the contaminants into sediment layers. System wide cleanups are not possible due to the sheer volume of polluted sediments and the fact that moving contaminated sediments does not remove the problem, but rather it transfers the contamination to another site. In the short term, an initial thorough cleanup of highly contaminated soils at Ward Transformer would alleviate the input and initiate a phase of contamination decline in the

Brier Creek system. Monitoring of at-risk species at the site should be performed, and perhaps in time, populations will adapt or repopulate the creek systems. In a broader context, PCB contaminated sites need to be monitored and any active inputs need to cease in order for the dispersal of PCBs to be contained. Little can be done to remove the level of PCBs now present in the environment, but monitoring of chronic effects in at-risk species and the elimination of new inputs is vital to ecosystem health.

APPENDIX

Table A1. Sediment concentrations per PCB congener. PCBs labeled with chlorine content and (congener number) in ng/L calculated for time-weighted average concentration and ng/g organic carbon.

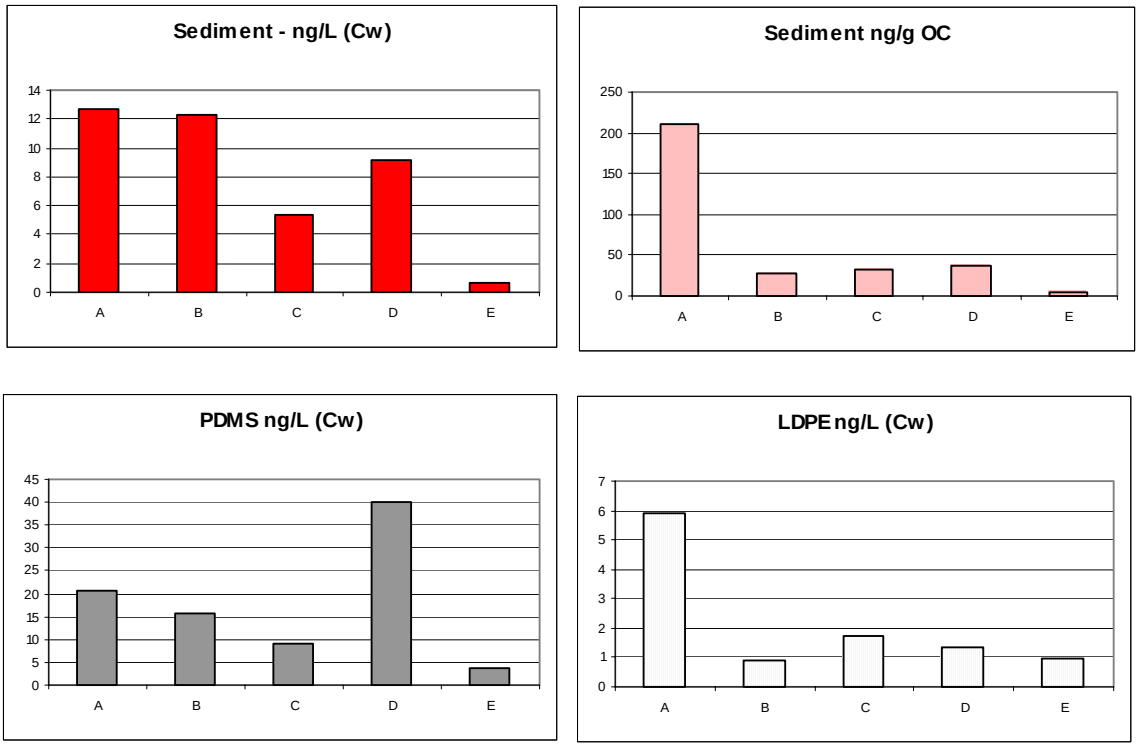
	[A] Cw sed (ng/L)	[B] Cw sed (ng/L)	[C] Cw sed (ng/L)	[D] Cw sed (ng/L)	[E] Cw sed (ng/L)	[A] Csed (ng-g OC)	[B] Csed (ng-g OC)	[C] Csed (ng-g OC)	[D] Csed (ng-g OC)	[E] Csed (ng-g OC)
Cl2 (08)	14.398	6.014	2.909	4.149	0.475	7.818	3.266	1.110	1.685	0.295
Cl3 (18)	4.778	2.068	0.707	2.168	0.115	14.257	6.170	1.483	4.838	0.391
Cl3 (28)	6.775	3.124	0.193	0.247	0.010	54.413	25.091	1.089	1.481	0.096
Cl4 (44)	0.549	0.248	0.155	0.249	0.027	5.301	2.391	1.052	1.797	0.304
Cl4 (52)	0.000	0.000	1.148	2.302	0.000	0.000	0.000	9.575	20.456	0.000
Cl4 (66)	0.116	0.061	0.015	0.004	0.002	3.163	1.649	0.278	0.072	0.075
Cl4 (77)	0.421	0.000	0.017	0.012	0.001	17.327	0.000	0.495	0.375	0.049
Cl5 (101)	0.617	0.190	0.019	0.025	0.016	24.273	7.482	0.512	0.744	0.703
Cl5 (118)	0.043	0.019	0.036	0.004	0.004	4.022	1.775	2.372	0.296	0.385
Cl5 (105)	0.149	0.058	0.000	0.009	0.000	11.407	4.439	0.000	0.527	0.009
Cl5 (126)	0.000	0.000	0.002	0.001	0.000	0.033	0.025	0.170	0.079	0.034
Cl6 (138)	0.405	0.163	0.024	0.002	0.000	57.802	23.221	2.419	0.204	0.011
Cl6 (128)	0.167	0.071	0.012	0.005	0.000	42.519	18.085	2.053	0.902	0.000
Cl6 (153)	0.698	0.295	0.034	0.013	0.000	93.023	39.257	3.156	1.277	0.009
Cl7 (187)	0.496	0.212	0.027	0.007	0.010	46.761	20.050	1.812	0.513	1.041
Cl7 (180)	0.197	0.081	0.007	0.002	0.000	77.633	31.781	2.019	0.652	0.015
Cl7 (170)	0.138	0.057	0.005	0.002	0.000	44.091	18.129	1.171	0.500	0.019
Cl8 (195)	0.078	0.025	0.012	0.003	0.001	19.864	6.341	2.175	0.625	0.374
Cl9 (206)	0.007	0.003	0.001	0.001	0.000	4.382	1.959	0.238	0.487	0.024
C10 (209)	0.000	0.000	0.000	0.000	0.000	1.152	0.577	0.034	0.000	0.000

Table A2. PCB congener concentrations in ng/L/day time weighted average concentration in PDMS passive device.

Cw (ng/L)	[A]	[B]	[C]	[D]	[E]
Cl2 (08)	0.170	0.213	0.208	0.557	0.246
Cl3 (18)	0.728	0.878	0.736	0.689	0.638
Cl3 (28)	12.036	4.191	2.804	9.905	1.208
Cl4 (44)	0.251	0.039	0.056	0.000	0.000
Cl4 (52)	0.000	0.265	0.000	15.916	0.000
Cl4 (66)	0.000	0.026	0.077	0.191	0.281
Cl4 (77)	0.491	0.207	0.161	3.193	0.161
Cl5 (101)	0.491	0.164	0.088	0.075	0.082
Cl5 (118)	0.233	0.000	0.268	0.000	0.000
Cl5 (105)	0.390	0.141	0.187	0.359	0.000
Cl5 (126)	0.279	0.143	0.143	0.271	0.111
Cl6 (138)	0.531	1.528	1.619	4.624	1.114
Cl6 (128)	0.903	0.195	0.266	0.195	0.000
Cl6 (153)	0.708	1.631	0.865	0.000	0.000
Cl7 (187)	0.767	0.269	0.249	0.110	0.000
Cl7 (180)	1.759	4.932	0.762	3.735	0.000
Cl7 (170)	0.367	0.048	0.072	0.000	0.000
Cl8 (195)	0.192	0.598	0.235	0.107	0.000
Cl9 (206)	0.207	0.038	0.150	0.000	0.000
C10 (209)	0.000	0.000	0.000	0.000	0.000

Table A3. Time weighted average concentrations in water derived from LDPE passive devices in ng/L/day.

Cw (pg/L)	[A]	[B]	[C]	[D]	[E]
Cl2 (08)	0.221	0.208	0.141	0.144	0.218
Cl3 (18)	0.321	0.273	0.354	0.285	0.286
Cl3 (28)	1.814	0.060	0.229	0.511	0.063
Cl4 (44)	0.249	0.009	0.066	0.020	0.010
Cl4 (52)	0.030	0.000	0.050	0.016	0.000
Cl4 (66)	0.033	0.000	0.004	0.005	0.000
Cl4 (77)	0.000	0.045	0.000	0.000	0.047
Cl5 (101)	0.371	0.023	0.077	0.061	0.024
Cl5 (118)	0.086	0.033	0.045	0.000	0.035
Cl5 (105)	0.148	0.000	0.000	0.003	0.000
Cl5 (126)	0.004	0.013	0.009	0.012	0.014
Cl6 (138)	0.671	0.000	0.105	0.016	0.000
Cl6 (128)	0.415	0.114	0.092	0.122	0.119
Cl6 (153)	0.635	0.058	0.217	0.062	0.061
Cl7 (187)	0.317	0.053	0.125	0.037	0.056
Cl7 (180)	0.333	0.000	0.174	0.016	0.000
Cl7 (170)	0.203	0.007	0.052	0.019	0.007
Cl8 (195)	0.047	0.000	0.000	0.000	0.000
Cl9 (206)	0.024	0.014	0.017	0.021	0.014
C10 (209)	0.000	0.000	0.000	0.000	0.000



Appendix Graph B1. Sediment, PDMS, LDPE graphs detailing concentrations on a per site basis.