BIOMARKERS OF OXIDATIVE STRESS IN FRESHWATER CLAMS (*CORBICULA FLUMINEA*) AS MECHANISTIC TOOLS TO EVALUATE THE IMPAIRMENT OF STREAM ECOSYSTEM HEALTH BY LAWN CARE PESTICIDES

by

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(Under the Direction of Marsha C. Black)

ABSTRACT

Many chemicals including fertilizers, herbicides, insecticides and fungicides are routinely applied to lawns, and have the potential to leach into nearby aquatic ecosystems and adversely affect biota. The purpose of this dissertation was to develop sensitive biological markers of stress in freshwater clams (*Corbicula fluminea*) for use as mechanistic tools to evaluate the degradation of streams by turf contaminants. Many xenobiotics cause damage in aquatic organisms via oxidative stress mechanisms, therefore measurements of stress used in this study included antioxidants (superoxide dismutase, catalase, glutathione) and indicators of cellular (lipid peroxidation, DNA strand breaks) and physiological (condition index) oxidative damage. Clams exposed *in situ* to pesticide runoff from residential lawns and a golf course accumulated a variety of persistent metals and organic pesticides in their tissues. Concentrations of turf contaminants were typically highest in tissues of clams deployed in streams that drain residential properties of high socioeconomic status during times of heavy rain and in a stream draining a golf course. Clams exposed to turf contaminants exhibited transient signs of oxidative stress (i.e., elevated levels of superoxide dismutase, catalase, glutathione and lipid peroxidation), whereas condition indices were reduced only at high exposures. These results suggest that clams may be able to compensate for adverse cellular effects of pesticides but that energy required for amelioration can eventually affect physiological processes. A laboratory experiment was conducted to investigate if the observed effects on clam health were caused by turf chemicals accumulated by clams, and not by other

contaminants that may co-occur in the streams but were not measured. Realistic concentrations of turf contaminants induced oxidative stress in laboratory-exposed clams, but not to the extent as observed in the field where exposures may have been more severe due to the presence of other contaminants. Together these data suggest that turf contaminants may be important contributors to the etiology of stress in freshwater clams from urbanized streams. Furthermore, these data when combined with other indicators of stream ecosystem health (e.g., community indices and ecosystem processes) highlight the utility of oxidative stress biomarkers in freshwater clams for use as mechanistic tools in biomonitoring.

INDEX WORDS: Oxidative stress, Superoxide dismutase, Catalase, Glutathione, Lipid peroxidation, DNA damage, Condition index, Bivalves, *Corbicula fluminea*, Biomonitoring, Pesticides, Turf, Headwater streams, Urbanization

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DEDICATION

This dissertation is dedicated to my late grandmother, Catherine Strauch, who was a wonderful naturalist and inspired me to pursue a career in environmental science.

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CHAPTER 1

INTRODUCTION

Lawns represent a small but important portion of greenspace in many developed countries. Lawns consist of a monoculture or oligoculture of grasses and have been created in many residential areas, parks and sports fields for aesthetic and recreational purposes. Lowen (1991) also attributes the prevalence of lawns in suburban and urban societies to an ingrained genetic preference for areas where predators such as snakes can be easily detected, and as a way for people to display social status and alleviate agrarian energy. Nowadays, many residential communities have legal mandates regarding the care and maintenance of lawns. Turf grass in the culturally defined, ideal green lawn often requires adequate sunlight, water and nutrients, frequent trimming and the use of chemicals.

A variety of chemicals are routinely applied to turf for the care and maintenance of lawns. Fertilizers include nutrients and essential metals, and these chemicals are used on turf to promote grass growth. Herbicides are applied to turf to eradicate opportunistic weeds and to control nuisance vegetation such as poison ivy and briars. Insecticides are applied to turf to prevent excessive insect herbivory and to control arthropod pests such as ticks and fire ants. Fungicides are applied to turf to prevent fungal infestation and disease. In the United States, pesticide use on homes, lawns and gardens increased between 13 and 20 % from 1995 to 1999, and during these years an average of 60 million kilograms of active ingredient were applied annually (Donaldson et al. 2002). The benefits of chemical use on turf may include the protection of human health in situations where they are used to control hazardous pests, but often chemicals are used for purely aesthetic reasons. Risks may be associated with pesticide use on turf if chemicals travel offsite at concentrations that are detrimental to human and ecosystem health.

Pesticides applied to turf that do not reach their intended target will either persist or degrade onsite via photolysis, hydrolysis and microbial activity or move offsite via volatilization and leaching. Pesticides often leach from application sites during rainfall, and travel as dissolved forms or bound to particles into nearby aquatic systems via surface water runoff or groundwater recharge (Richards and Baker 1993). Surface water runoff of pesticides may occur overland or by movement through direct conduits such as stormwater drains. The loading of turf care pesticides to streams is largely influenced by: (1) user attributes (e.g., amount and timing of applications, educational training), (2) turf condition (e.g., grass height, shading, watering frequency), (3) chemical properties (e.g., water solubility, sorption to soils, volatility, degradation rates), (4) watershed characteristics (e.g., topographic grade, drainage area, width and type of riparian vegetation, impervious surface cover, soil type) and (5) local climatic conditions (e.g., temperature, rainfall).

Turf care pesticides have been detected frequently in streams draining urbanized watersheds throughout the United States (Larson et al. 1999). Pesticides in streams may partition into water, sediment or biota, degrade via physicochemical and biological processes or dissipate via downstream transport. Nutrients move through streams in a spiral pattern whereby their downstream transport is offset by retention in pools of water, sorption to sediments and consumption by biota (Newbold et al. 1981, Munn and Meyer 1990). The movement of pesticides through streams has not been widely studied, but may also be conceptualized as occurring in a dampened spiral similar to that of nitrogen and phosphorus with a component added for degradation. Pesticides in streams may become problematic and degrade the health of local aquatic habitats if they are sufficiently retained, bioavailable and bioaccumulated by biota to toxic levels. Uptake of pesticides by stream biota may occur via diffusion through surface membranes in contact with water or sediments, or by the ingestion of contaminated food or particles (Nowell et al. 1999). While pesticides in current use are often designed for their low environmental persistence and reduced ability to bioaccumulate, many are still detected in aquatic biota (Nowell et al. 1999). The risks associated with the exposure of freshwater flora and fauna to realistic mixtures of turf care pesticides and their degradation products are largely unknown.

Streams in the southeastern United States are unique in that they were not altered by Pleistocene glaciation and contain a large percentage of global freshwater biological diversity (Lydeard and Mayden 1995). Many species of fish, crayfish, mollusks and amphibians indigenous to this region are endangered due to water quality degradation, habitat loss and the introduction of non-native species (Williams et al. 1993, Richter et al. 1997). Turf care pesticides have been detected more frequently and at higher concentrations in southeastern watersheds that drain urbanized areas than in watersheds that drain agricultural areas (Hippe and Garrett 1997). This dissertation is part of a larger collaborative project that was designed to study the impact of lawn care practices on aquatic ecosystems in urban and suburban watersheds of the southeastern United States (US Environmental Protection Agency STAR Grant R828007). Objectives of the larger project were to examine the cultural beliefs and values that influence the use of turf care chemicals on lawns, to assess the loading of pesticides and nutrients to streams and to determine if such chemicals may impair stream ecosystem health. The biological indicators of stream ecosystem health used in this project ranged across many levels of the ecological spectrum of organization and included measurements of molecular responses (biomarkers of oxidative stress in bivalves), community indices (algal and benthic invertebrate community structure) and ecosystem processes (leaf decomposition). The use of multihierarchical approaches in biomonitoring is becoming more common and is advantageous because such an approach reduces the need for a making a trade-off between sensitivity and ecological relevance when choosing endpoints (Clements 2000, Adams et al. 2001, Hyne and Maher 2003, Sherry 2003).

Bivalves are widely regarded as good bioindicator species because of their widespread distribution and abundance in many aquatic habitats, sedentary traits, hardiness and ability to bioaccumulate xenobiotics from water and sediments (Elder and Collins 1991). The purpose of this dissertation was to develop pragmatic and sensitive biomarkers in freshwater clams (*Corbicula fluminea*) that may be used as mechanistic tools to evaluate the adverse effects of chemicals applied to lawns on non-target aquatic invertebrates during field exposures. Biomarkers of oxidative stress were selected for analyses because a variety of different chemicals can cause damage in aquatic organisms by this

mechanism of toxicity (Winston and Di Giulio 1991, Livingstone 2001). The freshwater clam, *C. fluminea*, was chosen as a bioindicator species because it is abundant in many aquatic habitats throughout the southeastern United States (McMahon 1982), and this species shares some physiological and ecological attributes with imperiled freshwater mussels (Hull et al. 2002).

The use of biomarkers in biomonitoring requires well-established relationships between xenobiotic exposures and molecular responses, and the relevance of such responses to biological and ecological damage (De Zwart et al. 1999). Chapter two of this dissertation further outlines the rationale for the use of biomarkers of oxidative stress in bivalves for biomonitoring, and critically evaluates their status of validation. Environmental variables such as temperature, dissolved oxygen and food availability via their influence on metabolism and reproduction are known to affect oxidative stress responses in marine bivalves (Sheehan and Power 1999). Chapter three investigates how these confounding environmental variables may affect biomarkers of oxidative stress in freshwater clams from lotic and lentic habitats. A field assessment of oxidative stress in clams exposed to pesticide runoff from residential lawns and a golf course is presented in chapter four. Chapter five describes a laboratory study of oxidative stress during the chronic exposure and recovery of clams to suites of turf care chemicals that were identified as contaminants of concern in the field.

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CHAPTER 2

VALIDATING THE ROLE OF BIOMARKERS OF OXIDATIVE STRESS IN BIVALVES AS MECHANISTIC TOOLS FOR ASSESSING THE DEGRADATION OF AQUATIC HABITATS BY XENOBIOTICS [1]

[1] Conners DE, Ringwood AH, Black MC. To be submitted to *Ecotoxicology and Environmental Safety*

Abstract. Biomarkers in freshwater and marine bivalves may be valuable as mechanistic tools for assessing the degradation of aquatic habitats by xenobiotics. The use of biomarkers in biomonitoring necessitates well-established relationships between xenobiotic exposures and molecular responses, and the relevance of such responses to biological and ecological damage. Diverse xenobiotics can cause oxidative stress in aquatic organisms, and it has been proposed that this mechanism of toxicity would be useful for examining stress in biota exposed to mixtures of chemicals in the field. This paper reviews the status and challenges associated with validating the use of biomarkers of oxidative stress in bivalves for biomonitoring. Present evidence supports the concept that a wide variety of xenobiotics may enhance oxyradical production in bivalves, that bivalves are capable of mounting an adaptive response to prevent the indiscriminate attack of oxyradicals on biomolecules, and that oxidative damage may ensue when defenses are overwhelmed. However, more oxyradical research on bivalves is needed to (1) deduce the shape of dose-response curves for biomarkers over a realistic range of xenobiotic exposures, (2) develop methods to control for confounding environmental variables and identify what variables are most important for a particular bioindicator species and (3) identify links between biomarker responses and relevant biological and ecological damage. Addressing such issues would further validate the use of biomarkers of oxidative stress in bivalves for biomonitoring.

Introduction

Biological monitoring or biomonitoring has been defined as the systematic use of living organisms or their responses to assess the quality of the environment (Rosenberg and Resh 1996). The advantages of applying field-based biomonitoring data to ecological risk assessments are many and include: (1) organisms provide an integrated sample of the environment versus direct chemical measurements of media (i.e., sediment, water), in which xenobiotics often fluctuate over time, (2) the use of indigenous organisms negates the need for extrapolating data from standard test species to local species of concern and (3) the characterization of exposures and effects in the field accounts for environmental

complexities that can modify deleterious responses, and are difficult to replicate in laboratory settings. Such environmental complexities include factors that can alter bioavailability, the presence of multiple chemical and environmental stressors and compensatory responses of organisms that may mitigate the detrimental effects of xenobiotics. Munkittrick and McCarty (1995) importantly note that xenobiotics may exert indirect effects on aquatic populations that are mediated by bottom-up factors that can affect the availability of food and habitat and top-down factors on higher trophic-level consumers. In essence, biomonitoring provides a realistic and integrated description of how xenobiotics can alter the structural and functional attributes of aquatic ecosystems. Responses measured in biomonitoring can range across all levels of the ecological spectrum of organization (i.e., cellular biomolecules, physiology, individual health, populations, communities and ecosystem processes), but population and community level indices are predominately used as endpoints because of their direct relevance to water quality legislation. A recent trend in biomonitoring is to incorporate multihierarchical approaches whereby different levels of the ecosystem are evaluated concomitantly to reduce the need for a trade-off between sensitivity and ecological relevance when choosing endpoints (Clements 2000, Adams et al. 2001, Hyne and Maher 2003, Sherry 2003).

Biomarkers are biochemical, physiological or histological indicators of either exposure to, susceptibility to or effects of xenobiotic chemicals measured at the suborganismal or organismal level (Huggett et al. 1992). The use of biomarkers in biomonitoring is based on the concept that aquatic communities adversely affected by xenobiotics progress through a series of stress responses that begin at the molecular level and advance to the community level where ecological damage occurs (Bayne 1985). Therefore, biomarkers that are predictive of advanced toxicity at higher biological levels have historically been heralded as potentially powerful tools for use in biomonitoring because of their sensitivity and specificity to xenobiotic exposures and also for pragmatic reasons such as the cost and time associated with measuring a stress response. Much recent research in aquatic toxicology has been devoted to testing the sensitivity and specificity of biomarkers used for biomonitoring. Emerging from this research are important examples that biomarkers are not always sensitive and are not always specific to xenobiotic

exposures. Also, with the advent of rapid bioassessment protocols for measuring community level responses to xenobiotics, biomarkers may not always represent the most cost-effective endpoints. Biomarkers are, however, the mechanistic link necessary to address the cause of xenobiotic-induced alterations in aquatic communities; hence their use in biomonitoring will likely continue and expand in the future.

The intent of this review is to summarize and evaluate the progress made toward validating the use of biomarkers of oxidative stress in bivalves for biomonitoring. Three monumental reviews were published in the late 1980s and early 1990s that outlined how diverse xenobiotics can cause oxidative stress in aquatic organisms, and proposed that this mechanism of toxicity would be useful for examining stress in organisms exposed to complex mixtures of chemicals in the field (Di Guilio et al. 1989, Livingstone et al. 1990, Winston and Di Guilio 1991). Since that time, there has been a substantial increase in ecotoxicological research of oxidative stress in many aquatic organisms including fish, bivalves, gastropods, crustaceans and annelids (reviewed by Livingstone 2001). Lackner (1998) provides a concise conceptual overview of oxidative stress in fish. Besides fish, bivalves are a second widely studied taxon in ecotoxicological oxygen radical research. Bivalves are widely regarded as good bioindicator species for use in biomonitoring because of their widespread distribution and abundance in many aquatic habitats, sedentary traits, hardiness and ability to bioaccumulate xenobiotics (Elder and Collins 1991). Therefore, this review focuses on some of the idiosyncrasies of oxyradical research in bivalves and critically addresses issues regarding their validation. Although Sheehan and Power (1999) have reviewed how seasonal factors may influence biomarkers of oxidative stress in bivalves, this review is intended to be more comprehensive. In a mammalian review, De Zwart et al. (1999) eloquently state that the use of biomarkers of oxidative stress necessitates a (1) a well-established relationship between xenobiotic exposures and biomarker responses and (2) a well-established relationship between biomarker responses and relevant damage, and this review is organized around these primary validation concerns. Other important validation concerns to consider include issues of cost, stability of biomarkers upon storage and standardization of assay methods, but these are not addressed specifically in this review.

Overview of biomarkers for measuring oxidative stress

Minute quantities of oxygen were present in the oceans when life first evolved on earth, arising from photochemical reactions between ultraviolet radiation and water (Dole 1965). The evolution of cyanobacteria that produce oxygen during photosynthesis approximately three billion years ago greatly increased oxygen concentrations and correspondingly all other forms of life were profoundly altered (Mukhopadhyay and Das 1994). As oxygen is an excellent electron acceptor, this molecule was quickly recruited by eukaryotes for use in aerobic respiration. Aerobic respiration can produce 38 energy rich adenosine triphosphate (ATP) molecules, whereas anaerobic respiration yields only 2. Clearly an advantage exists in the use of oxygen as the ultimate electron acceptor in respiration, but such use also has a cost. During the reduction of oxygen to water, which can proceed via four steps of electron transfer (1), various oxygen intermediates are produced that are highly reactive and hence toxic i.e., they can bind quickly and indiscriminately to biomolecules such as lipids, proteins, nucleic acids and carbohydrates.

$$O_2 \xrightarrow{e^-} O_2^{\bullet -} \xrightarrow[2H^+]{e^-} H_2O_2 \xrightarrow[(+\,OH^-)]{e^-} {}^{\bullet}OH \xrightarrow[H^+]{e^-} H_2O \qquad (1)$$

Reactive oxygen species (ROS) produced during the tetravalent reduction of oxygen to water include the superoxide anion ($O2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$). Furthermore, these oxygen species by reaction with other molecules can yield alkoxy radicals ($RO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$) and organic hydroperoxides (ROOH). Those species containing unpaired electrons (i.e., radicals) are typically the most toxic because of their short half-lives and indiscriminant reactivity. The hydroxyl radical has the shortest half-life (one nanosecond) and is likely the most potent contributor to oxidative damage (Yu 1994).

Mitochondrial generation of ROS is a major source of oxyradical production in cells. Of oxygen consumed by mitochondria, approximately 1-3 % is converted to ROS (Chance et al. 1979, Davies 1995).

Similar to Davies' (1995) estimate for humans, we calculated that a bivalve with a rather high oxygen consumption rate like *Corbicula fluminea* may produce anywhere between 37 to 461 μmol of superoxide anion per day depending on the time of season (oxygen consumption rates obtained from McMahon 1991). Basal production of ROS may also occur via the actions of non-respiratory enzymes that catalyze oxidation reactions (e.g., oxidases and oxygenases), by other electron transport systems (e.g., nuclear membrane transporters and mixed function oxidases located in the endoplasmic reticulum) and by nonbiologically mediated chemical reactions (e.g., Haber-Weiss and metal catalyzed Fenton-like reactions).

As in mammals, aquatic organisms have evolved a suite of enzymatic and non-enzymatic defenses to cope with the production of ROS. Antioxidant enzymes include superoxide dismutase, catalase and glutathione peroxidase. Non-enzymatic antioxidants or scavengers include fat-soluble compounds like tocopherol (vitamin E) and β-carotene (provitamin A) and water-soluble compounds like glutathione and ascorbic acid (vitamin C). Many of these antioxidants interact in a concerted manner to eliminate ROS and prevent damage to cellular components (Figure 2.1). Organisms also possess secondary antioxidant defenses that repair oxidative damage to biomolecules such as phospholipases, proteases and DNA repair enzymes (Yu 1994, Davies 1995).

Oxidative stress has been defined as a disturbance in the prooxidant-antioxidant balance in favor of the former (Seis 1985). Measurements of oxidative stress in bivalves have included increases in oxyradical production, elevations in antioxidant defenses and the occurrence of oxidative cellular damage (Winston and Di Guilio 1991). Oxyradical production is very difficult to measure directly because ROS have such short half-lives and expensive electron spin resonance technology is needed. Alternatively, ROS production can be assessed by the use of redox sensitive dyes that change in response to being oxidized by a particular oxygen species or by many oxygen species, in which case specific enzymes are added to the reaction mixture to investigate the production of individual radicals. Measurements of ROS production are often used in mechanistic *in vitro* laboratory studies with bivalves. *In vivo* studies of oxidative stress in bivalves from the laboratory or field rely heavily on surrogate measurements of

elevated antioxidant defenses and indicators of oxidative damage to demonstrate that organisms are experiencing stress from ROS.

Antioxidants commonly measured in biomonitoring with bivalves include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione (GSH). Superoxide dismutase catalyzes the dismutation of two superoxide anions to molecular oxygen and hydrogen peroxide. Bivalves as with other eukaryotes, contain two major forms of superoxide dismutase: a copper and zinc homodimer located in the cytoplasm and nucleus, and a manganese tetramer located in the mitochondria (Fridovich 1998). An iron-containing superoxide dismutase is thought to exist exclusively in prokaryotes and plants (Bordo et al. 1994). Catalase is a heme-containing enzyme that decomposes hydrogen peroxide into water and molecular oxygen, and is located primarily in peroxisomes (Jones et al. 1981), but minimal activities may also be present in the cytoplasm and mitochondria. Hydrogen peroxide is also detoxified in the cytosol by glutathione peroxidase. Glutathione peroxidase is a selenium-containing enzyme that uses glutathione as a cosubstrate to detoxify many types of inorganic and organic peroxides (Mannervik 1985). Glutathione is a ubiquitous tripeptide (L-γ-glutamyl-L-cysteinylglycine) that functions to detoxify ROS either enzymatically as a cosubstrate or nonenzymatically by direct conjugation. Glutathione also functions in many other important processes including protein maintenance, synthesis of precursors to DNA and detoxification of metals and organic xenobiotics (Meister and Anderson 1983, Ketterer 1986, Stohs and Bagchi 1995). Levels of antioxidants in bivalves are often highest in metabolically active tissue like the digestive gland (Livingstone et al. 1990, Prakash and Rao 1995, Viarengo et al. 1995, Irato et al. 2003). Despite the importance and existence of other antioxidants besides those listed above, the concomitant measurement of a diverse array of defenses in biomonitoring is often prohibitively costly. Winston et al. (1998) have developed a novel and useful technique, the total oxyradical scavenging capacity (TOSC) assay that measures the complete repertoire of cellular ROS detoxification potential; yet it is not in widespread use.

Indicators of oxidative damage commonly measured in biomonitoring with bivalves include lipid peroxidation and DNA strand breaks. Lipid peroxidation is a self-propagating sequence of chemical

reactions that occur in the bulk phase of lipid bilayers. Lipid peroxidation consists of four phases (initiation, propagation, decomposition and termination) whereby oxygen radicals react with unsaturated fatty acids in membranes to produce lipid radicals (Gutteridge and Halliwell 1990). These lipid radicals in turn react with molecular oxygen to produce lipid peroxyl radicals that can continue to react with other lipids to produce more lipid radicals and lipid hydroperoxides. Eventually the lipid hydroperoxides are decomposed into lipid aldehydes and malondialdehyde. Termination of lipid peroxidation is difficult and completed only when both ROS and lipid radicals have been eliminated. Direct interactions of ROS with DNA can cause damage to bases and the sugar phosphate backbone. Strand breaks are expressed in DNA during sugar fragmentation events and when excision repair enzymes remove damaged bases (Imlay and Linn 1988). Furthermore, ROS may indirectly damage DNA via the production of oxidized lipid and protein byproducts that form adducts (Møller and Wallin 1998). Proteins represent a third major biomolecule in cells susceptible to ROS attack, but measurements of oxidative damage to proteins (i.e., protein carbonyl groups) have only been investigated in a few bivalves (Kirchin et al. 1992, Walker et al. 2000)

Relationships between xenobiotic exposures and biomarker responses

Xenobiotic mediated oxyradical production

Xenobiotics may enhance the production of oxyradicals in organisms by a variety of different mechanisms that may be classified by the molecular source, product and cellular loci of single electron transfer reactions. Radionucleotides are potent inducers of oxidative stress (Holloman et al. 2000, Walker et al. 2000). ROS production from irradiation has been detected immediately in mice after exposures (Hardmeir et al. 1997), and occurs presumably through the action of excited electrons that indiscriminately abstract other electrons from water in the cytosol or from biomolecules. Chemicals that disrupt mitochondrial electron transport are also potent inducers of oxidative stress (e.g., antimycin A, strobilurin fungicides). Damage to mitochondrial membranes or individual components of the electron

transport chain can cause a buildup of reduced electron carriers and increase their propensity to undergo autoxidation with molecular oxygen to yield superoxide anions (Di Giulio et al. 1989). Redox cycling chemicals are potent inducers of oxidative stress. Many organic xenobiotics or their metabolites (e.g., bipyridyl herbicides, polycyclic aromatic hydrocarbon (PAH) quinone metabolites, nitroaromatics) are converted into radicals in the endoplasmic reticulum or cytosol by the action of NAD(P)H-dependent reductases, oxidases and oxioreductase enzymes (Di Giulio et al. 1989, Burczynski and Penning 2000). These xenobiotic radicals may then be further detoxified, but often will easily undergo autoxidation reactions with molecular oxygen, producing superoxide anions and regenerating the original xenobiotic that can repeatedly undergo the cycle and produce large quantities of ROS. Some metals (e.g., iron and copper) are well-known for their ability to redox cycle via interacting with hydrogen peroxide and catalyzing Haber-Weiss and Fenton-like reactions that produce highly toxic hydroxyl radicals. Liu et al. (2001) suggest that arsenic may also cause cytotoxicity by reacting with hydrogen peroxide to produce hydroxyl radicals. The first metal observed to react with hydrogen peroxide was titanium, and others besides copper and iron exist (e.g., chromium, cobalt-EDTA) (Eberhardt 2001). Many other metals can produce superoxide anions via simple autoxidation reactions with molecular oxygen (e.g., silver, cadmium, zinc) (Eberhardt 2001). Still other xenobiotics may cause oxidative stress by inducing enzymes that participate in oxidation reactions like cytochrome P450s. Notably, many organic xenobiotics (e.g., PAHs, polychlorinated biphenyls (PCBs), organophosphate insecticides) induce P450s to facilitate phase I detoxification. Finally, xenobiotics may cause oxidative stress by depleting antioxidants. The pesticide endrin inhibits glutathione peroxidase (Bagchi et al. 1995). Glutathione may be depleted by a variety of xenobiotics, including many metals with a high affinity for sulfhydryl groups and organic xenobiotics that are metabolized by glutathione-S-transferase catalyzed conjugation reactions and exported from cells.

While there appears to be a well-established relationship between xenobiotic exposures and enhanced production of ROS, much of this research has been done on mammalian systems or on aquatic organisms other than bivalves (e.g., fish). In biomonitoring studies with bivalves, it may suffice to extrapolate on the ability of xenobiotics to produce ROS from research on other species or from research

on different chemicals within the same class, but this can lead to erroneous assumptions. For instance, many chemicals within a class may have very different ROS producing mechanisms and potencies. Viarengo et al. (1990a) exposed mussels to three metals of the borderline class (copper, cadmium and zinc) but only copper produced lipid peroxidation during the six days of exposure. Likewise, others have observed that copper is more potent than cadmium in causing lipid peroxidation in scallops (Chelomin and Belcheva 1992) and clams (Roméo and Gnassia-Barelli 1997). Caution is also warranted in extrapolating across species, as a xenobiotic that mainly produces ROS via induction of cytochrome P450s in fish may not be as potent of a producer in bivalves because of their low ability to induce this biotransformation enzyme (Livingstone 1998). Unfortunately, measurements of xenobiotic mediated ROS production in bivalves have only been achieved for a few model pro-oxidants like paraquat (Wenning et al. 1988), various nitroaromatics (Garcia-Martinez et al. 1995, Hetherington et al. 1996), PAH quinone compounds (Garcia-Martinez and Livingstone 1995, Sjölin and Livingstone 1997), and iron-EDTA (Winston et al. 1996). However, much indirect evidence on xenobiotic mediated ROS production in bivalves can be derived from controlled *in vitro* and *in vivo* laboratory studies that document alterations in antioxidants and oxidative damage during exposures (Table 2.1). These studies do support the concept that a wide variety of xenobiotics may produce oxidative stress in bivalves, but often it is difficult to distinguish whether ROS production is the primary mechanism driving responses or merely a byproduct of nonspecific cell and tissue injury.

A second major concern for validating the relationship between xenobiotic exposures and ROS production in bivalves during biomonitoring is that factors other than xenobiotics may also enhance oxyradical production. Enhanced ROS production may occur naturally (i.e., not xenobiotic mediated) during respiratory bursts when aquatic organisms move from hypoxic to hyperoxic conditions (Pannunzio and Storey 1998, Hermes-Lima and Zenteno-Savín 2002), in older organisms with depleted antioxidant defenses (Viarengo et al. 1990b, Canesi and Viarengo 1997) and during phagocytosis of foreign pathogens (Adema et al. 1991, Anderson et al. 1992). Also, many studies have implied that ROS production may vary seasonally in bivalves paralleling fluctuations in oxygen consumption rates that are

affected by temperature, reproduction and food availability (Viarengo et al. 1991, Power and Sheehan 1996, Niyogi et al. 2001, Wilhelm Filho et al. 2001, Orbea et al. 2002). Hence, during biomonitoring with biomarkers of oxidative stress in bivalves it is absolutely necessary to attempt to control for factors that may confound xenobiotic mediated ROS production i.e., by concurrent measurements of environmental variables like temperature, dissolved oxygen and food, and in use of animals of similar sizes.

Interactions of oxyradicals with oxidative stress biomarkers

Organisms challenged by enhanced oxyradical production undergo an adaptive response to increase the number of nondestructive binding sites for ROS, thereby preventing their indiscriminate attack on biomolecules. These binding sites represent the active sites of antioxidant enzymes and conjugation sites on antioxidant scavengers. Antioxidant enzyme activity may increase immediately after ROS challenge via post-translational control mechanisms (Hardmeir et al. 1997). Post-translational control of enzymes is usually achieved via phosphorylation events mediated by protein kinases, by the formation of redox sensitive disulfide bonds on allosteric regions of proteins that alter their overall conformation and activity, and by the addition of any necessary cofactors. A longer duration of enhanced ROS production will likely necessitate an increase in the amount of enzyme produced via transcription and translation. In prokaryotes, antioxidant enzyme production is regulated by the binding of redox sensitive transcription factors to an upstream region of the regulatory gene known as the antioxidant response element (ARE) (Zheng and Storz 2000, Johnson 2002). These transcription factors, which only bind to DNA when oxidized, include the *oxyR* protein that controls production of mRNA for catalases and peroxidases and *soxR* and *soxS* proteins that control transcription of mRNA for superoxide dismutase and glucose-6-phosphate dehydrogenase. Less is known about the control of antioxidant enzyme production in eukaryotes. Eukaryotes do not appear to have ARE sequences upstream of the gene for catalase (Johnson 2002), although ARE-like sequences exist for glutathione peroxidase (Ho and Howard 1992) and Mn-superoxide dismutase (Zhu et al. 2001). The identification of transcriptional factors used by

eukaryotes to mount an adaptive oxidative stress response is presently a very active area of research, and Dalton et al. (1999) and Nguyen et al. (2003) offer excellent reviews. The antioxidant response element in mammals may be regulated by a number of transcription factors and co-transcription factor activator/repressors of the AP-1, AP-2, NF-κB, MAF and CNC-bZIP protein families, and controls the production of mRNA for a variety of Phase I and Phase II detoxification enzymes (e.g., NAD(P)H quinone oxidoreductase and glutathione-S-transferase), heme oxygenase and an enzyme responsible for glutathione synthesis (γ-glutamylcysteine synthetase). The antioxidant response element (ARE) in mammals is also known as the electrophile response element (EPRE) because of its regulatory control over of those enzymes used for the detoxification of electrophilic xenobiotic metabolites.

In aquatic organisms, there is a paucity of research addressing the regulatory mechanisms of antioxidant defenses. Carvan III et al. (2000) transfected zebrafish with reporter constructs for mammalian EPREs and observed dose-dependent gene induction during exposures to a wide variety of organic and metallic xenobiotics, suggesting that fish do contain a complement of transcription factors for regulation of EPREs. Translational assembly of antioxidant enzymes likely occurs in ribosomes of the endoplasmic reticulum for superoxide dismutase and glutathione peroxidase, but catalase may be assembled by isolated polyribosomes in the cytosol similar to other peroxisomal proteins (Cancio and Cajaraville 2000). Gamble et al. (1995) observed that glutathione peroxidase activity may be regulated at the level of translation in marine mussels. Additional research on the transcriptional and translational mechanisms involved with regulating the expression and activity of antioxidant defenses in bivalves would be fruitful for many areas of biology and toxicology because bivalves are facultative aerobes that represent an intermediate taxon between prokaryotes and vertebrates.

Enhanced antioxidant enzyme activity is often observed in bivalves exposed to xenobiotics in the laboratory or field, but the responses tend to be transitory (reviewed by Livingstone 2001). A similar transitory dose-response pattern has been suggested to occur in fish (Lackner 1998). The increase of antioxidant enzyme activity in bivalves exposed to xenobiotics presumably represents the result of ROS mediated transcriptional, translational and post-translational control similar to what has been observed in

other species. Alternatively, elevations in antioxidant enzymes may occur concomitantly with the condition of hypertrophy and hyperplasia in bivalves. Decreases of antioxidant enzymes in xenobiotic-exposed bivalves after a period of elevation are more difficult to interpret. Possible reasons for such decreases in antioxidant enzyme activity may include (1) negative feedback mechanisms (i.e., SOD is inhibited by hydrogen peroxide, the endproduct of its enzymatic reaction), (2) lower internal xenobiotic concentrations at loci of ROS generation either because of reduced xenobiotic uptake or increased metabolism of the xenobiotic, (3) lower metabolic generation of ROS caused by reduced oxygen consumption and (4) enzyme damage or destruction from apoptotic or narcotic cell death and tissue atrophy.

Glutathione concentrations in bivalves exposed to xenobiotics in the laboratory or field are highly variable, with decreases (Viarengo et al. 1990a, Regoli and Principato 1995, Doyette et al. 1997, Regoli et al. 1998, Ringwood et al. 1999, Cossu et al. 2000, Torres et al. 2002), increases (Wenning et al. 1988, Michel et al. 1993, Yan et al 1997, Cheung et al. 2002) or transient responses (Canesi et al. 1999, Zaroogian and Norwood 2002, Romero-Ruiz et al. 2003) being observed for a variety of species, chemicals and exposure conditions. Where transient responses in glutathione concentrations were observed during sublethal exposures of bivalves to xenobiotics, the scavenger often first decreased, and then increased over time. A similar transitory dose-response pattern has been suggested to occur in fish (Lackner 1998). Early decreases of glutathione concentrations in xenobiotic-exposed bivalves may result from the conjugation of the chemical or its metabolite to glutathione and ultimate export from cells. Alternatively, decreases in glutathione concentrations may occur because of reduced food intake. Decreases in glutathione concentrations during starvation are often rapid and have been observed for many species including rodents (Strubelt et al. 1981, Shimizu and Morita 1990), crayfish (Almar et al. 1987) and oysters (Conners 1998). Starvation causes glutathione depletion because the synthesis of the tripeptide requires sulfur containing amino acids (cysteine and methionine) in the diet. In the laboratory, reduced food uptake by xenobiotic-exposed bivalves may occur during shell valve closure initiated in response to noxious stimuli. In the field, reduced food uptake by xenobiotic-exposed bivalves may

represent an indirect effect of contaminants on food availability, as algal and microbial cells are likely very sensitive to xenobiotics due to their small size. Elevations of glutathione concentrations in xenobiotic-exposed bivalves after a period of depletion are likely due to increases of γ-glutamylcysteine synthetase, the rate-limiting enzyme responsible for glutathione synthesis, as this enzyme is regulated via feedback inhibition by cellular glutathione status (Meister 1983). During exposures to lethal levels of organophosphate pesticides, glutathione concentrations were severely depleted in bivalves (Peña-Llopis et al. 2002), likely because of ATP depletion, synthetic enzyme destruction and the conversion of severely injured cells from a reduced to an oxidized state.

Increases in oxidative damage are often observed in bivalves exposed to xenobiotics in the laboratory or field, but again the responses tend to be transitory through time. Transitory increases in lipid peroxidation have been observed in bivalves exposed to metals (Prakash and Rao 1995, Ringwood et al. 1998, Geret et al. 2002c, Romero-Ruiz et al. 2003) and pesticides (Wenning et al. 1988). Transitory increases in DNA strand breaks have been observed in bivalves exposed to benzo[a]pyrene (Ching et al. 2001). Early increases of oxidative damage in xenobiotic-exposed bivalves are likely due to the attack of oxyradicals on biomolecules. Decreases of oxidative damage in xenobiotic-exposed bivalves after a period of elevation presumably result from the induction of primary antioxidant defenses that reduce ROS concentrations and the induction of secondary antioxidant defenses that repair oxidative damage. Also, reductions in oxidative damage may occur from the induction of detoxification enzymes that lower internal xenobiotic concentrations at loci of ROS generation. In addition to responses being transitory through time, measures of DNA strand breaks in bivalves have been observed to be greater at lower concentrations of lead (Black et al. 1996), benzo[a]pyrene (Bihari et al. 1990) and various pesticides (Conners and Black 2004) than at higher concentrations, suggesting that there may be a threshold for induction of defenses. Near the point of lethality, oxidative damage in bivalves will likely return to high levels as severely injured cells change from a reduced to oxidized state.

Together, these studies of antioxidant enzymes, scavengers and oxidative damage in bivalves illustrate that the relationship between xenobiotic-mediated oxyradical production and biomarker

responses is not well-established. Presently, there is insufficient information on the mechanisms by which oxyradicals regulate antioxidant expression in bivalves, and such relationships must be assumed from limited research on other species. More importantly for biomonitoring, there is insufficient data to deduce accurately the shape of the dose-response curves for biomarkers of oxidative stress over a wide range of exposure conditions. However, current data on bivalves and fish are suggestive of transient dose-response patterns whereby antioxidant enzymes increase then decrease during exposures whereas scavengers behave in an opposite manner. A hypothetical graph of dose-response patterns for biomarkers of oxidative stress in xenobiotic-exposed bivalves is presented in Figure 2.2. The transient nature of the dose-response curves exemplifies why it would be difficult to observe a correlation between contaminants and any one biomarker of oxidative stress during field exposures. Instead, suites of biomarkers are likely necessary to identify what stage of stress an organism is experiencing. Multiple biomarker responses can be modeled with multivariate statistics. Multivariate statistics have been used successfully to discriminate among contaminated sites when applied to biomarkers of oxidative stress in bivalves (Vidal et al. 2001, Blaise et al. 2002), with other biomarkers in fish (Adams et al. 1996, Machala et al. 1997, Van der Oost et al. 1997, Adams et al. 1999) and in community level aquatic assessments (Reynoldson et al. 1997, Anderson and Clements 2000).

Factors other than xenobiotics may affect measures of antioxidants and oxidative damage in bivalves, and confound their use in biomonitoring. Temperature may affect antioxidants in bivalves by altering the catalytic rate of enzymes. Antioxidant enzyme activities are often low in cold winter months and measures of oxidative damage are often high (reviewed by Sheehan and Power 1999). Dissolved oxygen may affect biomarkers of oxidative stress in bivalves. Past studies have demonstrated that invertebrates adapted to environments with high dissolved oxygen (e.g., polar habitats and organisms with photosynthetic symbionts) often have high levels of antioxidants to protect them from associated increases in oxyradical production (Shick and Dykens 1985, Regoli et al. 2000). Conversely, antioxidant suppression may occur in molluscs during anoxic or hypoxic conditions (Viarengo et al. 1989, Pannunzio and Storey 1998). Food availability could potentially affect oxidative stress responses in bivalves by a

variety of mechanisms, including altering the amount of amino acids available for antioxidant production and changing the composition of polyunsaturated fatty acids in membranes thereby affecting lipid peroxidation rates. Again, during biomonitoring with biomarkers of oxidative stress in bivalves it is absolutely necessary to attempt to control for factors that may confound antioxidant and oxidative damage responses by making concurrent measurements of environmental variables like temperature, dissolved oxygen and food.

Summary of status and challenges for sensitivity and specificity issues

The use of biomarkers in biomonitoring necessitates an established causal relationship between xenobiotic exposures and molecular responses. In bivalves there is adequate evidence that a wide variety of xenobiotics may enhance ROS production, that bivalves are capable of mounting an adaptive response to prevent the indiscriminate attack of oxyradicals on biomolecules, and that oxidative damage may ensue prior to the induction of defenses or when defenses are overwhelmed. What is not readily apparent are the specific mechanisms that mediate such relationships, and whether or not xenobiotics may cause oxidative stress at environmentally realistic concentrations. Future research on mechanisms by which xenobiotics can cause oxidative stress in bivalves will be essential for correct elucidation and interpretation of dose-response curves. Also, controlled studies that expose bivalves to complex mixtures such as those by Cajaraville et al. (1992) and Camus et al. (2003) will be important for documenting that realistic concentrations of xenobiotics can cause oxidative stress, as descriptive field studies are often confounded by environmental stressors and laboratory exposures to single chemicals remain highly unrealistic. Despite these limitations, present data suggest that biomarkers of oxidative stress in bivalves are valuable for biomonitoring because of their sensitivity (i.e., responses can be detected well before lethality) and their ability to be perturbed by a wide variety of chemical contaminants. However, biomarkers of oxidative stress in bivalves are not specific to xenobiotics and will respond to fluctuating environmental conditions; hence, there is a need in biomonitoring to control for confounding variables and to determine which confounding variables are most important for a particular bioindicator species.

Relationships between biomarker responses and relevant biological and ecological damage

Individual Health

Xenobiotics in aquatic habitats are often present at sublethal concentrations but may affect the health of bivalves by impairing growth, reproduction and immunological competency. Perhaps the most evoked relationship between xenobiotic exposures, molecular stress and organismal health is that of reduced energy allocation whereby the detoxification of xenobiotics and oxyradicals may shift energy towards maintenance tasks and away from tissue and shell production (Widdows et al. 1995, Smolders et al. 2004). Ringwood and others observed that juvenile oysters deployed at a superfund site had low glutathione concentrations (Ringwood et al. 1999) and reduced rates of shell growth (Ringwood et al. 1996). Likewise, Couillard et al. (1995) observed that increases in lipid peroxidation coincided with reduced shell growth and tissue biomass in freshwater mussels transplanted to metal contaminated lakes. Pellerin-Massicotte (1994) observed that perturbations in antioxidants coupled with reduced tissue biomass only occurred in marine mussels indigenous to but not transplanted at sites receiving pulp and paper effluents. Other biomonitoring studies on transplanted (Amiard-Triquet et al. 1998) and indigenous (Veinott et al. 2003) bivalves that have measured tissue biomass in conjunction with biomarkers of oxidative stress, did not observe any pronounced affects on organismal health despite perturbations in oxidative stress. These latter studies possibly indicate that bivalves may be able to assimilate some degree of contamination without harm to growth and maintenance processes.

Reproduction may be impaired in xenobiotic-exposed bivalves experiencing oxidative stress. Reproduction is an energy demanding process and any energy diverted to detoxification and repair of oxidative damage could potentially reduce fecundity in bivalves. Couillard et al. (1995) observed a low number of gravid females in populations of freshwater mussels experiencing oxidative stress from metal contaminants. Oxyradicals may also directly interfere with bivalve fertilization and development. Many studies have observed elevated superoxide dismutase activity in bivalves during periods of reproduction (Viarengo et al. 1991, Solé et al. 1995, Niyogi et al. 2001, Wilhelm Filho et al. 2001). Superoxide

dismutase catalyzes the dismutation of superoxide anions to hydrogen peroxide, and bursts of hydrogen peroxide are used to cross-link fertilization envelopes in sea urchin eggs (Turner et al. 1988). Furthermore, oxyradicals are important mediators of cell differentiation in rapidly dividing embryonic cells (Allen 1991). Any interference in the timing or concentration of oxyradical production could potentially impair reproductive processes. Ringwood and Conners (2000) selectively depleted glutathione in gonads of adult oysters and observed that gamete fertilization success was enhanced but the development of larvae during metal challenge was severely impaired. Native oysters from a superfund site had 28 percent less glutathione in gonadal tissues than oysters from a reference site (Ringwood and Conners unpublished data), and spat settlement at the superfund site was low (Geoff Scott, National Marine Fisheries, Charleston SC, personal communication).

The immunological defense system of bivalves may be altered by a variety of xenobiotics including metals, PAHs, and nitroamines (reviewed by Fisher et al. 2000). An important mechanism used by bivalves to kill foreign pathogens is the release of superoxide anions by the enzyme NAD(P)H-oxidase located in the plasma membrane of circulating hemocytes (Adema et al. 1991). Copper has been shown to reduce superoxide anion production in hemocytes of bivalves presumably via the attack of excess ROS on functional sulfhydrl groups of NAD(P)H oxidase (Pipe et al. 1999, Matozzo et al. 2001). In fish, the depletion of glutathione potentiated the ability of chlorothalonil to suppress superoxide anion production in macrophages, again suggesting that toxicity results from ROS inhibition of NAD(P)H oxidase (Baier-Anderson and Anderson 2000). Fisher et al. (2000) observed that superoxide anion production was significantly higher in oysters from contaminated sites and suggested that hemocyte activities may be stimulated at low contaminant concentrations, but depressed at higher concentrations. Suppression of hemocyte immune functions by xenobiotics may be associated with an increased susceptibility to infections (Fisher et al. 1999). Freshwater mussels transplanted downstream of a municipal effluent had depressed phagocytic activity and higher numbers of bacteria in their hemolymph than mussels transplanted upstream (Gagné et al. 2002).

Population, Community and Ecosystem Impairment

The use of biomarkers of oxidative stress in bivalves to assess the quality of chemically degraded aquatic habitats has not progressed to a level where molecular responses can be linked definitively to impairment of populations, communities and ecosystems. Natural resource managers urgently need such ecological links. Protecting populations of bivalves from xenobiotics is important because many species are commercially valuable to the seafood industry, function as keystone species or are in danger of extinction. Commercially important bivalves include many species of oysters, scallops and clams and population declines would result in large economic losses. Commercial landings of *Crassostrea virgincia* in the Chesapeake Bay, USA have declined from a peak of 615,000 tons in 1884 to 12,000 tons in 1992 due to problems associated with habitat loss, water quality degradation, disease and overharvesting (Rothschild et al. 1994). Bivalves can function as keystone species in environments where their shells provide stable habitat structure and protection for other aquatic organisms. Oyster beds in coastal estuaries may support up to 300 infaunal and epifaunal species (Burrell 1986). Likewise, freshwater bivalves provide habitat for many epizoic invertebrates and periphyton that colonize shells in running waters (Vaughn and Hakenkamp 2001). Benthic invertebrate abundance was higher in areas adjacent to beds of freshwater mussels (Sephton et al. 1980). Freshwater mussels (Bivalvia: Unionidae) are among the world's most imperiled freshwater fauna. North America has the greatest diversity of Unionids (297 recognized taxa), of which 72 percent have been listed as threatened, endangered or in decline due to factors such as habitat loss, water quality degradation and commercial harvesting (Williams et al. 1993). Extinction rates for North American freshwater mussels are comparable to those of terrestrial species from tropical rainforests (Ricciardi and Rasmussen 1999).

Bivalves provide many ecosystem services that are critical to the integrity of aquatic habitats and human health (reviewed by Vaughn and Hakenkamp 2001). Dense populations of bivalves filter large quantities of water and can reduce the abundance of phytoplankton and improve water clarity. Bivalves may act as nutrient pumps and retain and release nutrients in aquatic habitats at times when food may be scarce, thereby supporting the production of other invertebrates species and associated vertebrate

consumers. Furthermore, bivalves may alter the composition of aquatic sediments by aerating substrates via bioturbation or by excreting organically rich fecal matter.

Summary of status and challenges for ecological relevance issues

The use of biomarkers of oxidative stress in bivalves for biomonitoring has largely been relegated to a sentinel approach whereby molecular responses are solely used to demonstrate that xenobiotics are bioavailable and may disrupt cellular processes in aquatic organisms. While such an approach is valuable, information much more relevant to water quality protection could be gained by linking molecular responses to higher levels of biological organization. Plausible links exist between xenobiotic-induced oxidative stress in bivalves and impaired health. Hence, endpoints that measure aspects of growth, reproduction and immunocompetency need to be incorporated more frequently in biomonitoring programs with bivalves. Emerging research suggests that while oxyradicals may be harmful, they may also be beneficial during circumstances when the timing and concentration of ROS production is tightly controlled (Nordberg and Arnér 2001). More research on the benefits of oxyradicals to bivalves would assist in targeting physiological processes that may be sensitive to perturbations by prooxidant xenobiotics. Links between oxidative stress in bivalves and impairment of populations, communities, and ecosystem processes have not been determined. As bivalves often play important roles in aquatic ecosystem health, such links may be vital to the conservation or restoration of many aquatic habitats. Hence, ecologically relevant endpoints of bivalve functions (e.g., filtration rates, ammonium excretion, biodeposition, biomass production) should be incorporated into biomonitoring programs when appropriate.

Conclusions

Biomarkers of oxidative stress in bivalves are increasingly being incorporated into biomonitoring programs to assess water quality in aquatic habitats. Present research on bivalves supports the concept

that a wide variety of xenobiotics may enhance ROS production, that bivalves are capable of mounting an adaptive response to prevent the indiscriminate attack of oxyradicals on biomolecules, and that oxidative damage may ensue when defenses are overwhelmed. However, validation of the relationship between xenobiotic exposures and oxidative stress responses remains incomplete. The mechanisms by which many xenobiotics may enhance oxyradical production and affect measures of antioxidants and oxidative damage are not well studied in bivalves and must be assumed from data on other species. Given that such mechanisms are often widely conserved in biology, this area may not be as critical as other issues of validation for biomonitoring. A major concern for biomonitoring is that there are insufficient data to deduce accurately the shape of the dose-response curves for biomarkers of oxidative stress in bivalves over a wide range of exposure conditions. Future research must resolve this issue if biomarkers of oxidative stress in bivalves are to be used appropriately in biomonitoring. Furthermore, bivalves are thermoconformers and faculative aerobes that adapt to fluctuating environmental conditions. Not surprisingly, biomarkers of oxidative stress in bivalves will often respond to variables such as temperature, dissolved oxygen and food availability. Hence, there is a need in biomonitoring to develop methods that can control for confounding effects and determine which confounding variables are most important for a particular bioindicator species. Lastly, links between oxidative stress responses and damage at higher levels of biological organization would be valuable for water quality protection efforts, as legislation is aimed at protecting populations, communities and ecosystems, not individual health. The development of such links across ecosystems would be facilitated by more collaboration between biochemical toxicologists and ecologists. Future research aimed at the above validation concerns may advance the use of biomarkers of oxidative stress in bivalves as mechanistic tools for assessing the degradation of aquatic habitats by xenobiotics.

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Table 2.1. Laboratory studies on bivalves that documented responses in one or more biomarkers of oxidative stress during xenobiotic exposures.

Chemical Type	Xenobiotic	Species[1]	Endpoint(s)[2]	Citation
Metals	Aluminum	*Perna viridis*	SOD, CAT, LPX	Prakash and Rao 1995
	Cadmium	*Crassostrea gigas & Mytilus edulis*	LPX	Geret et al. 2002a
		Corbicula fluminea	CAT, LPX	Vidal et al. 2001
		Perna viridis	SOD, CAT, LPX	Prakash and Rao 1995
		Ruditapes decussatus	SOD, CAT, GPX, LPX	Geret et al. 2002b
	Copper	*Adamussium colbecki*	SOD, CAT, GPX, GSH	Regoli et al. 1998
		Crassostrea gigas & Mytilus edulis	LPX	Geret et al. 2002a
		Crassostrea virginica	GSH, LPX	Conners and Ringwood 2000
		Crassostrea virginica	GSH, LPX	Ringwood et al. 1998
		Mercenaria mercenaria	GSH	Zaroogian and Norwood 2002
		Mizuhopecten yessoensis	LPX	Chelomin and Belcheva 1992
		Mytilus edulis	SOD	Manduzio et al. 2003
		Mytilus edulis	LPX	Kirchin et al. 1992
		Mytilus galloprovincialis	GSH	Canesi et al. 1999
		Mytilus galloprovincialis	GSH, LPX	Viarengo et al. 1990a
		Mytilus galloprovincialis	SOD, CAT, GSH	Regoli and Principato 1995
		Ruditapes decussatus	SOD, CAT, GPX, LPX	Geret et al. 2002c
		Ruditapes decussatus	LPX	Roméo and Gnassia-Barelli 1997
		Tapes phillippinarum	SOD	Matozzo et al. 2001
		Unio tumidus	SOD, CAT, GPX, GSH, LPX	Doyette et al. 1997
	Lead	*Perna viridis*	SOD, CAT, LPX	Prakash and Rao 1995
		Perna viridis	GSH	Yan et al. 1997
	Mercury	*Adamussium colbecki*	SOD, CAT, GPX, GSH	Regoli et al. 1998
		Mytilus edulis & Crassostrea gigas	LPX	Geret et al. 2002a
		Perna viridis	GSH	Yan et al. 1997
		Ruditapes decussatus	LPX	Roméo and Gnassia-Barelli 1997
	Methyl Mercury	*Mytilus galloprovincialis*	GSH	Canesi et al. 1999
	Silver	*Mytilus edulis & Crassostrea gigas*	LPX	Geret et al. 2002a
	Uranium	*Corbicula fluminea*	CAT, GPX, LPX	Labrot et al. 1996

Table 2.1 continued

Chemical Type	Xenobiotic	Species[1]	Endpoint(s)[2]	Citation
PAHs	Benzo[a]pyrene	*Mytilus edulis*	DNA SB[3]	Michelmore et al. 1998
		Mytilus galloprovincialis	ROS, GSH	Michel et al. 1993
		Mytilus galloprovincialis	DNA SB	Bihari et al. 1990
	Methyl Napthoquinone	*Mytilus edulis*	GSH, LPX	Ribera et al. 1991
	Mixture	*Mytilus edulis*	Lipofuchin	Krishnakumar et al. 1997
	Coal Tar	*Corbicula fluminea*	CAT, LPX	Vidal et al. 2001
	Oil Pollution	*Mytilus galloprovincialis*	CAT	Cajaraville et al. 1992
	Oil Pollution	*Mytilus galloprovincialis*	Carotenoids	Karnaukhov et al. 1977
Pesticides & PCBs	Aroclor 1254	*Mytilus edulis*	CAT	Krishnakumar et al. 1997
	Fenitrothion	*Mytilus galloprovincialis & Flexopecten flexuosus*	GSH	Peña-Llopis et al. 2002
	Hexachlorobiphenyl	*Mytilus galloprovincialis*	ROS, GSH	Michel et al. 1993
	Paraquat	*Geukensia demissa*	SOD, CAT, GSH, LPX	Wenning et al. 1988
	Tetrachlorobiphenyl	*Mytilus galloprovincialis*	ROS, GSH	Michel et al. 1993
	Thiram	*Unio tumidus*	SOD, CAT, GPX, GSH, LPX	Doyette et al. 1997
Nitroaromatics	1-nitropyrene	*Mytilus edulis*	DNA SB[3]	Michelmore et al. 1998
	Nitrofurantonin	*Mytilus edulis*	DNA SB[3]	Michelmore et al. 1998
Others	Carbon Tetrachloride	*Mytilus edulis*	GSH, LPX	Ribera et al. 1991
	Cobalt γ Radiation	*Mytilus edulis & Dosinia lupinus*	Protein Carbonyls	Walker et al. 2000
	Hydrogen Peroxide	*Crassostrea virginica & Mercenaria mercenaria*	DNA SB[3]	Gielazyn et al. 2003
	t-Butyl Hydroperoxide	*Rangia cuneata*	GPX, GSH, LPX	Darby et al. 1993
	Trichloroethylene	*Corbicula fluminea*	CAT, LPX	Vidal et al. 2001
	Toluene	*Corbicula fluminea*	CAT, LPX	Vidal et al. 2001

[1] The majority of species were marine or brackish bivalves, the only freshwater species were *Corbicula fluminea* and *Unio tumidus*

[2] Endpoints listed are those that were measured and relevant to this review, they do not necessarily represent those that responded to exposures or all of the endpoints measured by a particular study (ROS = reactive oxygen species production, SOD = superoxide dismutase, CAT = catalase, GPX = glutathione peroxidase, GSH = glutathione, LPX = lipid peroxidation, DNA SB = DNA strand breaks)

[3] Studies that used DNA strand breaks were included only if methods were used to ensure damage was specifically caused by ROS

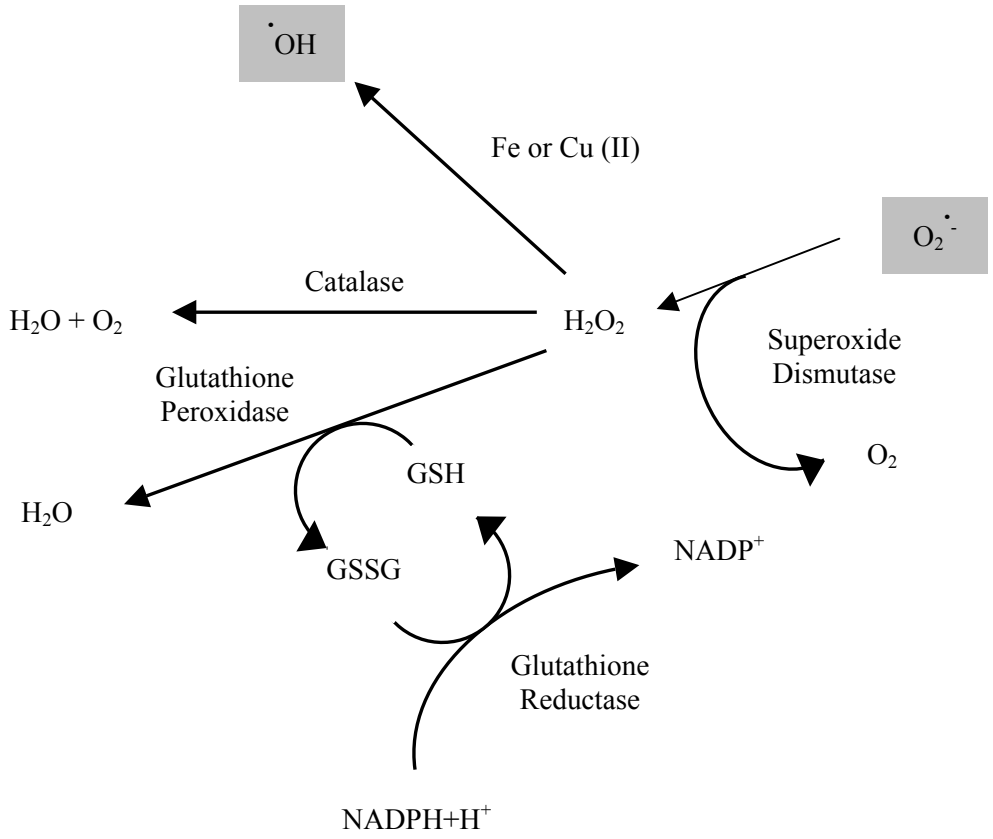

Figure 2.1. Nonstoichiometric overview of the detoxification of reactive oxygen species by antioxidants (adapted from Kappus 1985). Toxic oxyradicals are highlighted in grey, GSH = reduced glutathione, GSSG = oxidized glutathione, $NADPH+H^+$ = reduced nicotinamide adenine dinucleotide phosphate, NADP = oxidized nicotinamide adenine dinucleotide phosphate.

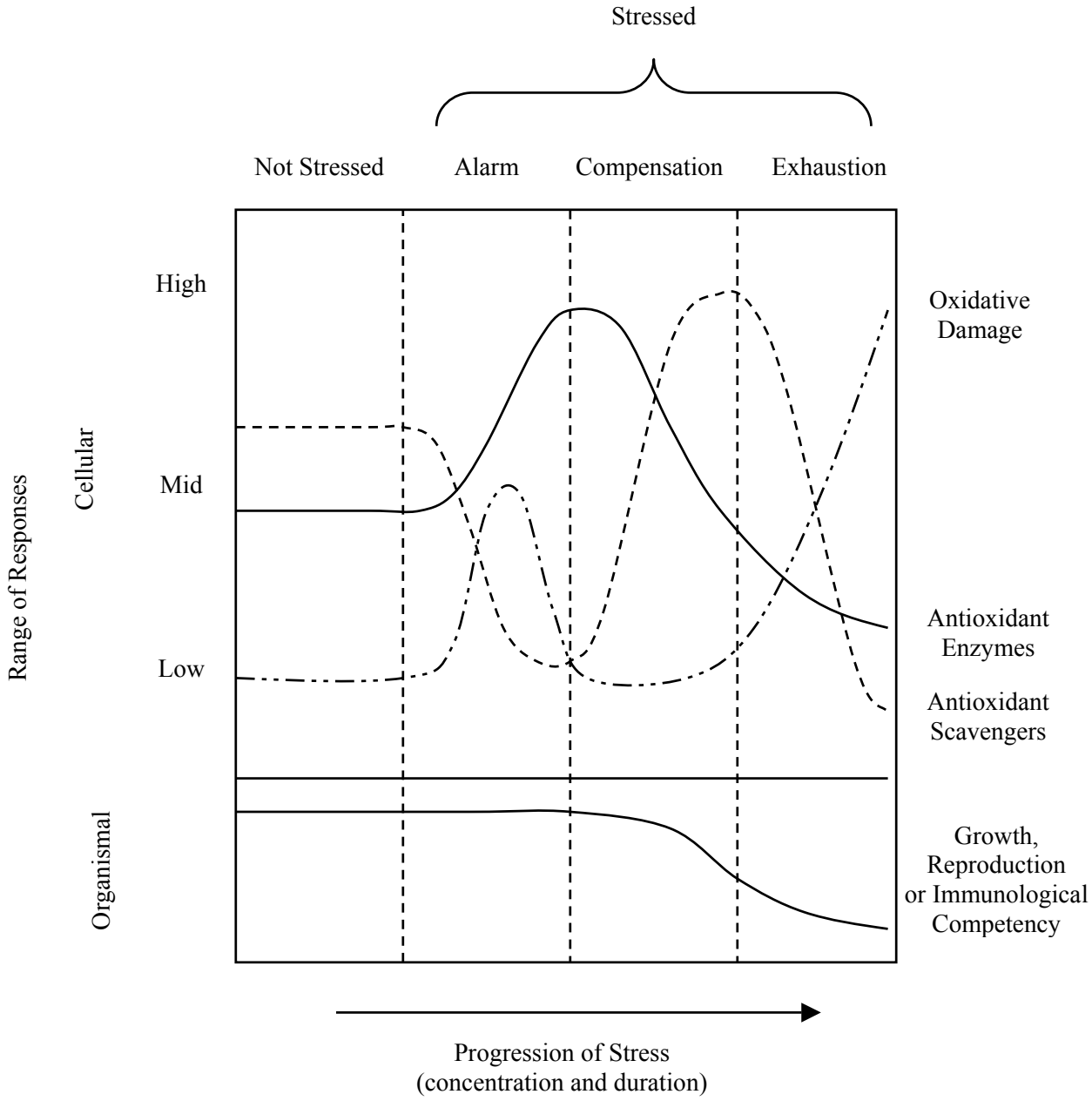

Figure 2.2. Hypothetical model of the responses of antioxidant enzymes (—), scavengers (---) and oxidative damage (-···-) in bivalves during the progression of xenobiotic-induced stress and relationship of these biomarkers to overall health (adapted from Depledge 1994).

CHAPTER 3

SEASONAL TRENDS OF OXIDATIVE STRESS IN FRESHWATER CLAMS (*CORBICULA FLUMINEA*) FROM LOTIC AND LENTIC HABITATS [1]

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[1] Conners DE and Black MC. To be submitted to *Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology*

Abstract. Measures of oxidative stress in bivalves have been used as indicators of adverse effects in contaminated aquatic habitats because they are sensitive and respond to a variety of xenobiotics. Natural environmental factors may also affect oxidative processes in bivalves, and this can confound attempts to use such biomarkers in biological monitoring. This study evaluated how seasonal and habitat related fluctuations in temperature, dissolved oxygen and food affect measures of oxidative stress (superoxide dismutase, catalase, glutathione, lipid peroxidation and DNA single-strand breaks) in the digestive glands of freshwater clams (*Corbicula fluminea*). Clams and water quality measurements were collected monthly for one year from relatively pristine lotic and lentic habitats. Seasonally, antioxidant enzyme activities (superoxide dismutase and catalase) were low during winter months and indicators of oxidative damage (lipid peroxidation and DNA single-strand breaks) were high. Superoxide dismutase activities were also low in the summer and peaked during times of reproduction (spring and fall). Glutathione concentrations were highest in the winter. Lentic clams had lower antioxidants (catalase and glutathione) and higher levels of lipid peroxidation than lotic clams. Many of the observed trends in oxidative stress among clams from different seasons and habitats were related to changes in temperature and dissolved oxygen, and are likely caused by their influence on metabolism and reproduction. Overall, these data suggest that freshwater clams may be more susceptible to oxidative damage in the winter or when in hypoxic environments, and that temperature and dissolved oxygen should be measured in biological monitoring programs to assist in determining the etiology of any adverse effects.

Introduction

Many xenobiotics cause damage in aquatic organisms by stimulating the production of harmful oxyradicals via processes of redox cycling, interfering with electron transport, inducing enzyme systems such as cytochrome P450s that mediate oxidation reactions or by depleting protective antioxidants (Livingstone et al. 1990, Winston and Di Giulio 1991, Lackner 1998). As such, measures of oxidative stress have been used as sensitive, early-warning indicators of adverse effects in contaminated aquatic

habitats. However, natural environmental variables (e.g., temperature, dissolved oxygen and food) via their influence on metabolism and reproduction, are known to affect oxidative stress responses in a wide variety of aquatic organisms including bivalves (reviewed by Sheehan and Power 1999), gastropods (Abele et al. 1998, Pannunzio and Storey 1998), crustaceans (Niyogi et al. 2001a), echinoderms (Lukyanova and Khotimchenko 1995) and fish (Lopes et al. 2001, Wilhelm Filho et al. 2001a). The influence of factors other than xenobiotics on oxidative stress responses in aquatic organisms, if not characterized, can confound attempts to use such biomarkers in biological monitoring.

The purpose of this study was to evaluate how seasonal and habitat related fluctuations in temperature, dissolved oxygen and food affect oxidative stress responses in the digestive glands of freshwater clams (*Corbicula fluminea*). We measured a variety of oxidative stress responses in clams including protective antioxidants (superoxide dismutase, catalase and glutathione) and indicators of oxyradical mediated damage to cell membranes (lipid peroxidation) and DNA (DNA single-strand breaks). Digestive glands were used for analyses because this tissue in bivalves is metabolically active (i.e., high O_2 consumption) and contains high levels of antioxidants (Livingstone et al. 1990, Prakash and Rao 1995, Viarengo et al. 1995). We hypothesize that clams from lentic habitats experiencing hypoxia due to summer stratification events, will have lower antioxidants than clams from well-mixed lotic habitats. Past studies have demonstrated that marine invertebrates adapted to environments with high dissolved oxygen (e.g., polar habitats and organisms with photosynthetic symbionts) often have high levels of antioxidants to protect them from associated increases in basal oxyradical production (Shick and Dykens 1985, Regoli et al. 1997, Regoli et al. 2000). Conversely, antioxidant suppression has been demonstrated in mollusks exposed acutely to anaerobic conditions (Viarengo et al. 1989, Pannunzio and Storey 1998). By season, we hypothesize that clams in winter months will have lower levels of antioxidants than clams from warmer months, as this pattern has been observed in a variety of marine bivalve species and attributed to the effects of temperature on enzymes and overall metabolic activity (Viarengo et al. 1991, Power and Sheehan 1996, Niyogi et al 2001b, Wilhelm Filho 2001b, Orbea et al. 2002). Vidal et al. (2002a) were the first to report on seasonal changes of the antioxidant enzyme catalase

in a freshwater bivalve (*C. fluminea*), and observed activities to be lower in the winter than in warmer months. Likewise, catalase activities were positively correlated with temperature in the freshwater mussel *Anodonta cygnea* (Robillard et al. 2003). This present work extends observations on baseline oxidative stress in freshwater bivalves to other important antioxidants and indicators of oxidative damage.

The Asiatic clam (*C. fluminea*) is a hermaphroditic bivalve that has invaded and is now a common inhabitant of many freshwater systems in North America and Europe (McMahon 1982, Araujo et al. 1993). North America has the greatest diversity of freshwater mussels (Bivalvia: Unionidae) in the world (297 recognized taxa), of which 72 percent have been listed as threatened, endangered or in decline due to factors such as habitat loss, water quality degradation and commercial harvesting (Williams et al. 1993). The southeastern United States contains much of the continent's freshwater biological diversity because watersheds there were not altered by Pleistocene glaciation (Lydeard and Mayden 1995). Because *C. fluminea* is abundant in aquatic habitats throughout this region, and shares some physiological and ecological attributes with imperiled freshwater mussels, this clam may be valuable as an indicator species for *in situ* ecotoxicological studies on freshwater bivalves that are of critical importance for the protection of biological diversity.

Materials and Methods

Field Sampling

Native clams of a uniform size (17.4 ± 2.7 mm length) were collected from lotic and lentic habitats every month for one year during 2001. The lotic habitat (Broad River, Danielsville, GA, USA) was located in a free-flowing river (145 km) that drains forests and rural lands. The lentic habitat (Lake Chapman, Athens, GA, USA) was located in a 105 ha reservoir constructed in l978, and surrounding lands are a recreational park. Both habitats remain ice-free throughout the year. Three locations approximately 6 m apart were sampled within each habitat (n = 3), and at each location six clams were collected at depths of 0.6 to 1.0 m with a D-frame net. For the lentic site, clams were collected from

organically rich substrates within the littoral zone, while at the lotic site clams were collected from riffle areas of course sand and gravel near the center of the channel. Sampling was typically performed mid-morning and on the same day for both lotic and lentic habitats. Clams were transported to the laboratory (~ 1 h) in aerated site water, and digestive glands were dissected immediately and divided for biochemical analyses (i.e., two or three biomarkers were measured on one clam as tissue size allowed). DNA single-strand breaks were measured on fresh tissues, and other tissues were frozen in liquid nitrogen and stored at -80.0 ^{o}C for later analyses.

Water Quality

Monthly measurements of temperature and dissolved oxygen were collected *in situ* from bottom waters (0.6 to 1.0 m) at each location within lotic and lentic habitats (n = 3) by the use of handheld portable meters. Additional water quality measurements (alkalinity, hardness, pH and conductivity) were taken monthly from surface waters at one location per habitat to further characterize these aquatic systems. Alkalinity and hardness were measured in the laboratory by Hach® titration kits, and pH and conductivity were measured *in situ* with portable meters. Concentrations of organic seston were measured monthly as an indicator of food availability. Water samples (2 L) from each location were collected at 60 % depth, filtered to remove large debris (1 mm) and composited. Samples were then analyzed in the laboratory for organic seston by standard methods (Wallace and Grubaugh 1996). Briefly, three replicate water samples were vacuum filtered through pre-ashed (500 ^{o}C for 1 h) and pre-weighed 47 mm glass fiber filters. Filters containing suspended matter were oven dried (50 ^{o}C for 24 h), dessicated (24 h) and weighed for dry mass. Filters were then ashed (500 ^{o}C for 1 h) in a muffle furnace, rewet with distilled water, and again were oven dried (50 ^{o}C for 24 h) and dessicated (24 h). Filters were then weighed to determine ash mass and organic seston or ash-free dry mass was calculated: (dry mass – ash mass) / volume of water filtered.

Indicators of Oxidative Stress

Total superoxide dismutase (SOD) activities were measured by pyrogallol auto-oxidation as described originally by Marklund and Marklund (1974), but modified for measurements of all SOD forms (Cu-Zn SOD and Mn SOD) (Zidenberg-Cherr et al. 1989). Briefly, tissues were homogenized in 50 mM potassium phosphate buffer (pH 7.0) and combined with three parts SOD buffer (pH 8.2, containing 50 mM N-tris[Hydroxymethyl]methyl-3-aminopropanesulfonic acid and 1 mM diethylenetriamine pentaacetic acid). Samples were then centrifuged (14,000 rpm, 4 ^{o}C, 5 min) and the supernatant (150 μL) was combined with 770 μL SOD buffer and 80 μL 4 mM pyrogallol. Changes in sample absorbency were monitored spectrophotometrically at 320 nm every 30 s for a total of 120 s, and activities were expressed as units SOD per mg protein. One unit of SOD activity was defined as the amount of enzyme that inhibits the auto-oxidation of pyrogallol by 50 %.

Catalase (CAT) activities were measured by the decomposition of H_2O_2 as described by Clairborn (1985). Tissues were homogenized in 50 mM potassium phosphate buffer (pH 7.0), and a subsample of the homogenate (100 μL) was combined with 20 μL 5 % Triton X-100. Samples were then centrifuged (14,000 rpm, 4 ^{o}C, 5 min) and the supernatant (8 μL) was combined with 800 μL 19 mM H_2O_2. Changes in sample absorbency were monitored spectrophotometrically at 240 nm every 30 s for a total of 120 s, and activities were expressed as umol H_2O_2 consumed per min per mg protein.

Total glutathione concentrations were quantified by the glutathione reductase recycling assay (Anderson 1985). Tissues were homogenized in 5 % sulfoslicyclic acid and centrifuged (14,000 rpm, 4 ^{o}C, 5 min). A subsample of the supernatant (25 μL) was added to sodium phosphate buffer (143 mM, pH 7.5) for a final volume of 1 mL, which contained 200 μM β-NADPH and 1 mM 5,5'-dithio-bis(2-nitrobenzoic acid). Samples were then vortexed and warmed to 30 ^{o}C in a water bath for 10 min. Glutathione reductase (15 μL at 50 units/mL) was added to initiate the enzymatic reaction and the rate of 5-thionitrobenzoic acid formation was monitored spectrophotometrically at 412 nm every 30 s for a total

of 120 s. Standards were prepared from reduced glutathione, and concentrations were expressed as nmol per g wet weight.

Lipid peroxidation was quantified by the thiobarbituric acid assay for malondialdehyde (MDA) concentrations (Aust 1985). Tissues were homogenized in 50 mM potassium phosphate buffer (pH 7.0) and centrifuged (14,000 rpm, 4 ^{o}C, 5 min). A subsample of the supernatant (71 μL) was combined with 1 mL 15 % trichloroacetic acid containing thiobarbituric acid (0.375 %) and 10 μL butylated hydroxytoluene (20 mg/mL in absolute alcohol). Samples were then heated (100 ^{o}C, 15 min) in a boiling water bath and centrifuged (14,000 rpm, 20 ^{o}C, 5 min) to remove precipitates. Malondialdehyde concentrations were measured spectrophotometrically at 532 nm. Standards were prepared as described by Csallany et al. (1984), and the results were expressed as nmol per mg protein.

Protein concentrations of supernatants were used for determinations of superoxide dismutase, catalase and lipid peroxidation. Proteins were quantified by the use of a Bio-Rad™ protein assay kit based on the method of Bradford (1976). Samples (25 μL) were combined with 1 mL diluted dye reagent (Coomassie Brilliant Blue G-250 diluted 1:4 with distilled water), vortexed and incubated for 5 min in the dark. Protein concentrations were determined spectrophotometrically at 595 nm with bovine serum albumin used for standards.

DNA single-strand breaks were measured with the Comet assay described by Steinert (1996), but modified to account for reduced osmolality of hemolymph in freshwater bivalves. Pieces of digestive gland were suspended in 490 μL of an osmotically modified Ca^{2+}/Mg^{2+}-free Hanks Balanced Salt Solution (HBSS) containing 4.2 mM $NaHCO_3$, 26.2 mM NaCl, 1.3 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4 and 5.55 mM D-glucose (pH 7.3). Proteinase K was added (10 μL at 10 mg/mL) and tissues were crushed gently with a hand-held, ground-glass tissue homogenizer (i.e., three light pressured turns with a homogenizer having a large clearance between pestle and tube of 0.09 to 0.16 mm). Cell suspensions were filtered to remove large debris (100 μm nylon mesh), centrifuged (2000 rpm, 5 min, 10 ^{o}C) and resuspended in 500 μL 0.65 % low melting agarose (made with HBSS, melted in a microwave

then cooled to a constant temperature of 37 ^{o}C). Samples (50 μL) were transferred to microscope slides pre-coated with 1 % normal melting agarose (made with 40 mM Tris-acetate EDTA) and cells were spread out by the use of cover-slips. Slides were then solidified (5 min at 4 ^{o}C), cover-slips were removed and cells were top-coated with 50 μL 0.65 % low melting agarose. Once solidified, slides were transferred to light-protected coplin jars containing cold lysis buffer (10 mM Tris-HCl, 2.5 M NaCl, 100 mM EDTA, 10 % DMSO and 1 % Triton X-100, pH = 10.0), and incubated at 4 ^{o}C overnight. To prevent confounding DNA damage from ultraviolet-light, samples were light-protected during all subsequent steps. Slides were removed from lysis buffer, rinsed three times in cold water and transferred to a submarine gel electrophoresis chamber containing cold DNA unwinding buffer (300 mM NaOH, 1 mM EDTA, pH = 13.1). DNA was allowed to unwind for 15 min and samples were electrophoresed at 25 V, ~300 mA for 10 min. After electrophoresis, samples were transferred to coplin jars and neutralized by rinsing in 400 mM Tris (pH 7.5), 3 times for 2 min. Samples were then immersed in cold 100 % ethanol for 5 min, air dried and stored in a dessicator until analyzed. For analyses, slides were stained with 50 μL ethidium bromide (20 μg/mL) and viewed under epifluorescent microscopy (200X magnification, 510-560 nm excitation filter, 590 nm barrier filter). DNA damage results in increased DNA migration away from individual cells and produces a characteristic comet shape. DNA damage was quantified by measuring tail moment (product of % DNA in comet tail and length of tail) with a Loats Image Analysis System®. Thirty cells were scored per slide, and geometric means were used to describe the damage because distributions of tail moments among cells on a slide were skewed.

Statistics

All data are means ± standard deviations (SD) of three locations per habitat except for measures of organic seston, which are replicate samples composited from each habitat. Measures of oxidative stress were analyzed on two or three individual clams at each location, and were averaged prior to obtaining monthly means for each habitat. Data were checked for normality and homogeneity of variance

prior to statistical testing, and were transformed if necessary. Effects of season and habitat on measures of oxidative stress were evaluated with a two-way analysis of variance (ANOVA) ($P < 0.05$). If data were auto-correlated, a repeated measures ANOVA was used. Relationships between water quality variables and measures of oxidative stress were analyzed separately for each habitat by the use of Pearson correlation coefficients ($P < 0.05$). All statistical tests were run with SYSTAT® software, version 10.2 (SPSS Inc.).

Results

Water Quality

Water temperature, dissolved oxygen and organic seston varied seasonally in lotic and lentic habitats (Figure 3.1). Temperatures ranged from a high of 28.9 ^{o}C in the summer to a low of 6.4 ^{o}C in the winter. The lentic habitat was typically warmer than the lotic habitat by an average of 3.4 ^{o}C throughout the year. Dissolved oxygen concentrations were highest during cold winter months and lowest during warm months in the summer and early fall. The lotic habitat remained well-oxygenated throughout the year and close to 100 % saturation, while oxygen concentrations in the lentic habitat were depleted below 7 mg/L in May and again during September and October with the minimum reaching 61.8 % saturation. Organic seston, used as an indicator of food, had an annual average of 4.3 ± 4.2 mg/L for the lotic habitat and 3.2 ± 1.5 mg/L for the lentic habitat. At both habitats, two peaks in organic seston were observed annually, the first in the early spring and the second in the summer. Peaks of organic seston in the lotic habitat coincided with heavy rains during the 48 h preceding sampling. Measurements of other water quality parameters were similar between habitats and annual averages were pH = 7.08, conductivity = 53.1 ± 6.6 μs, alkalinity = 27 ± 11 mg/L and hardness = 30 ± 17 mg/L.

Indicators of Oxidative Stress

Significant seasonal trends were observed for all antioxidants measured in clams (Figure 3.2). Antioxidant enzyme activities (superoxide dismutase and catalase) were typically low during winter months. Superoxide dismutase activities were also low in the summer and peaked during the spring and fall. Glutathione concentrations were highest in the winter. For lotic clams, indicators of oxidative damage (lipid peroxidation and DNA single-strand breaks) were highest in the early winter months (Figure 3.3). DNA single-strand breaks decreased dramatically in both lotic and lentic clams after peaking in December, and remained low until the fall. Levels of lipid peroxidation also decreased throughout the spring and summer in lotic clams but not in lentic clams. In lentic clams, lipid peroxidation levels were highly variable throughout the year.

By habitat, lentic clams had significantly lower antioxidants (catalase and glutathione) and a higher level of lipid peroxidation than lotic clams. No differences in superoxide dismutase activities or DNA single-strand breaks were observed between clams from lotic and lentic habitats.

Many of the observed trends in oxidative stress among clams from different seasons and habitats were significantly related to changes in temperature (Figure 3.4) and dissolved oxygen (Figure 3.5). Increasing temperatures were correlated with increasing catalase activities in lentic clams ($r^2 = 0.37$, $P < 0.001$), decreasing levels of lipid peroxidation in lotic clams ($r^2 = 0.52$, $P < 0.001$) and decreasing glutathione concentrations in both lotic ($r^2 = 0.45$, $P < 0.001$) and lentic clams ($r^2 = 0.67$, $P < 0.001$). Increasing dissolved oxygen concentrations were correlated with increasing glutathione concentrations in lotic ($r^2 = 0.36$, $P = 0.001$) and lentic clams ($r^2 = 0.25$, $P = 0.008$), and increasing levels of lipid peroxidation in lotic clams ($r^2 = 0.57$, $P < 0.001$). Organic seston was not significantly correlated with any of the antioxidants measured in clams or with indicators of oxidative damage in clams, although there were trends approaching significance for oxidative damage to be lower at food concentrations above 5 mg/L (Figure 3.6).

Discussion

Biomarkers used in biological monitoring require validation of their biological relevance, sensitivity and specificity (NRC 1987). Many *in vivo* laboratory studies have demonstrated that biomarkers of oxidative stress in bivalves are sensitive, and respond readily during sublethal exposures to metals and other organic contaminants (Wenning et al. 1988, Livingstone et al. 1990, Viarengo et al. 1990, Michel et al. 1993, Prakash and Rao 1995, Regoli and Principato 1995, Doyotte et al. 1997, Yan et al. 1997, Ringwood et al. 1998). Fewer studies have attempted to address the biological relevance of chemically-mediated alterations in antioxidants and oxidative damage to bivalves, probably because of the inherent difficulty involved. However, links between some pro- or anti- oxidant perturbations in bivalves and effects at higher biological levels (e.g., growth, reproduction, disease and survival) have been observed (Couillard et al. 1995, Oliver and Fisher 1999, Ringwood and Conners 2000, Peña-Llopis et al. 2002). Alterations in oxidative stress responses have been associated with reduced fitness in fish (Palace et al. 1998, Meyer et al. 2003, Meyer and Di Giulio 2003), and in humans such responses are highly relevant to many diseases including atherosclerosis, diabetes mellitus and neurodegenerative disorders (e.g., Alzheimer's and Parkinson's) (Yu 1994, Eberhardt 2001). Hence, the biological relevance of oxidative stress in bivalves will likely be shown in the future as this important area of research is given more attention. In regards to specificity, there is little evidence suggesting that biomarkers of oxidative stress in bivalves respond solely to chemical contaminants. Many studies on bivalves indicate that antioxidants and levels of lipid peroxidation are affected by environmental variables such as temperature, dissolved oxygen, and food availability (Viarengo et al. 1991, Solé et al. 1995, Power and Sheehan 1996, Frenzilli et al. 2001, Niyogi et al. 2001b, Wilhelm Filho et al. 2001b, Orbea et al. 2002, Regoli et al. 2002, Vidal et al. 2002a, Vidal et al. 2002b). Likewise, we observed in our study that oxidative stress responses in *C. fluminea* were influenced by temperature and dissolved oxygen.

Temperature may affect oxidative stress responses in bivalves either directly by altering the catalytic rate of enzymes or indirectly via its influence on reproduction or on other physical, chemical and

biological attributes of the aquatic ecosystem. Catalase activities in *C. fluminea* were higher in the summer than in the winter, likely because of the direct affect of temperature on enzymes. Abele et al. (1998) observed Q_{10} coefficients (factor by which a rate changes with a short-term 10 ^{o}C increase in temperature) for catalase in limpets to be approximately 2 between the temperatures of 4 and 20 ^{o}C, which is typical for many rate functions in aquatic ectotherms. Q_{10} values computed for seasonally acclimated animals (Q_{10acc}) are highly variable in freshwater bivalves, and if these values are lower than the acute Q_{10}, acclimation is inferred, whereas values equal to 2.0 – 2.5 or the acute Q_{10} indicate that the species is incapable of acclimating (McMahon 1991). In our study, the Q_{10acc} of catalase was 1.07 for lotic clams and 1.57 for lentic clams indicating that *C. fluminea* in warm southern habitats of North America may be able to partially regulate antioxidant enzyme activity during annual fluctuations in temperature. Such regulation of antioxidant enzymes would have the advantage of allowing organisms to maintain some degree of competency in detoxifying oxyradicals during colder months.

Unlike catalase, superoxide dismutase activities in clams were not affected by temperature extremes (i.e., activities during the summer and winter were comparably low), but were significantly elevated during the spring and fall. Many *C. fluminea* populations reproduce twice each year during the spring and fall in response to temperature cues (McMahon 1991). Hence, elevations in superoxide dismutase during the spring and fall are suggestive of an indirect affect of temperature via its influence on reproduction. While the role of superoxide dismutase in bivalve reproduction is unknown, others have observed an elevation of this enzyme in marine bivalves during reproduction, often attributing this pattern to superoxide dismutase's ability to detoxify oxyradicals produced in accord with increases of aerobic respiration necessary to meet the high metabolic demands of gravid animals (Viarengo et al. 1991, Solé et al. 1995, Niyogi et al. 2001b, Wilhelm Filho et al. 2001b). While this explanation is plausible for *C. fluminea*, Williams (1985) demonstrated that embryo brooding in this species does not alter metabolic rates. Superoxide dismutase catalyzes the dismutation of the superoxide anion radical to hydrogen peroxide (Fridovich 1995), and bursts of hydrogen peroxide production are known to control fertilization and early embryonic development in sea urchins (Turner et al.1988). In oysters, Ringwood and Conners

(2000) observed that sperm penetration of embryonic membranes and fertilization success may be enhanced by free radicals. Hence, elevations of superoxide dismutase during reproduction in *C. fluminea* may result from the enzymes' direct involvement in fertilization and embryonic development, albeit this postulate requires further research.

The effects of temperature on all other oxidative stress responses in clams from this study were likely caused by the indirect influence of temperature on the water solubility of dissolved oxygen. *C. fluminea* is a poor regulator of oxygen consumption rates during oxygen depletion, and therefore is less tolerant of hypoxia than many other freshwater and marine bivalve species (McMahon 1979, Johnson and McMahon 1998). We observed that clams from lentic habitats with less dissolved oxygen had lower catalase activities and glutathione concentrations and higher levels of lipid peroxidation than clams from well-oxygenated lotic habitats. Oxyradicals produced by aerobic respiration, which amount to approximately 1 to 3 % of oxygen consumed (Chance et al. 1979, Davies 1995), were likely low in *C. fluminea* from lentic habitats and this may explain why protective antioxidants were either physiologically suppressed or genetically down-regulated in this population. Antioxidant suppression has been demonstrated in mollusks exposed acutely to anaerobic conditions (Viarengo et al. 1989, Pannunzio and Storey 1998). Interestingly, dissolved oxygen concentrations may also explain why glutathione concentrations were high in clams during the winter, an observation that contrasts other studies of oxidative stress in bivalves (i.e., glutathione concentrations are often lower in the winter) (Viarengo et al. 1991, Power and Sheehan 1996, Wilhelm Filho et al. 2001b). Several aquatic researchers (Winston and Di Giulio 1991, Ringwood et al. 1996, Pannunzio and Storey 1998) have proposed that the reintroduction of oxygen in aquatic systems may pose a greater threat than hypoxia, similar to the process of ischemic reperfusion injury observed in mammals (Oliver et al. 1990, Levine 1993). Pannunzio and Storey (1998) have shown that glutathione concentrations and related enzymes (glutathione peroxidase, glutathione reductase and glutathione-S-transferase) rise more rapidly than other defenses (superoxide dismutase and catalase) during aerobic recovery in marine gastropods, which is suggestive of their role as first-responders to oxyradical detoxification. During the seasonal increases of dissolved oxygen observed in

this study, glutathione may also be functioning as a first-responder to elevations of harmful oxyradicals in clams. This hypothesis may also explain why measures of oxidative damage were high in clams during early winter months, but decreased following the increased production of glutathione.

Food availability could potentially affect oxidative stress responses in bivalves by a variety of mechanisms including altering the amount of amino acids available for antioxidant production, changing the composition of polyunsaturated fatty acids in membranes thereby affecting lipid peroxidation rates, or causing shifts in metabolic rates via affects on filtration. While studies on marine bivalves have noted that food availability may affect antioxidants or measures of oxidative damage (Viarengo et al. 1991, Solé et al. 1995, Power and Sheehan 1996, Conners 1998, Niyogi et al. 2001b, Regoli et al. 2002, Vidal et al. 2002a), we did not observe any relationship between organic seston and measures of oxidative stress in *C. fluminea*. Mattice (1979) observed that filtration rates in *C. fluminea* were not altered over a wide range of seston concentrations (7-25 mg/L). Also, freshwater bivalves, including *C. fluminea*, may supplement filter feeding of organic seston by pedal feeding on sediment detritus (Hornbach et al. 1984, Way et al. 1990, McMahon 1991). Hornbach et al. (1984) estimated that only 35 % of organic carbon assimilated by a stream population of Sphaeriid clams is derived from filter feeding, the remainder coming from detritus. Hence, our data on organic seston may have reflected only a portion of the food available to *C. fluminea*, and was a poor surrogate of food availability for this species.

In conclusion, we have found that responses of oxidative stress in freshwater clams are affected by seasonal and habitat related fluctuations in temperature and dissolved oxygen, and that clams may be more susceptible to oxidative damage in the winter or when in hypoxic environments. We concur with others (Wilhelm Filho et al. 2001b, Orbea et al. 2002, Vidal et al. 2002a, Vidal et al. 2002b) that it is highly unlikely to find biomarkers that respond specifically to chemical contaminants in thermoconforming aquatic organisms, but that a lack of specificity should not negate the use of such biomarkers in biological monitoring. As long as the effects of natural variables such as temperature and dissolved oxygen on biomarkers are characterized, measuring them simultaneously during effect assessments can allow for their control as confounding influences.

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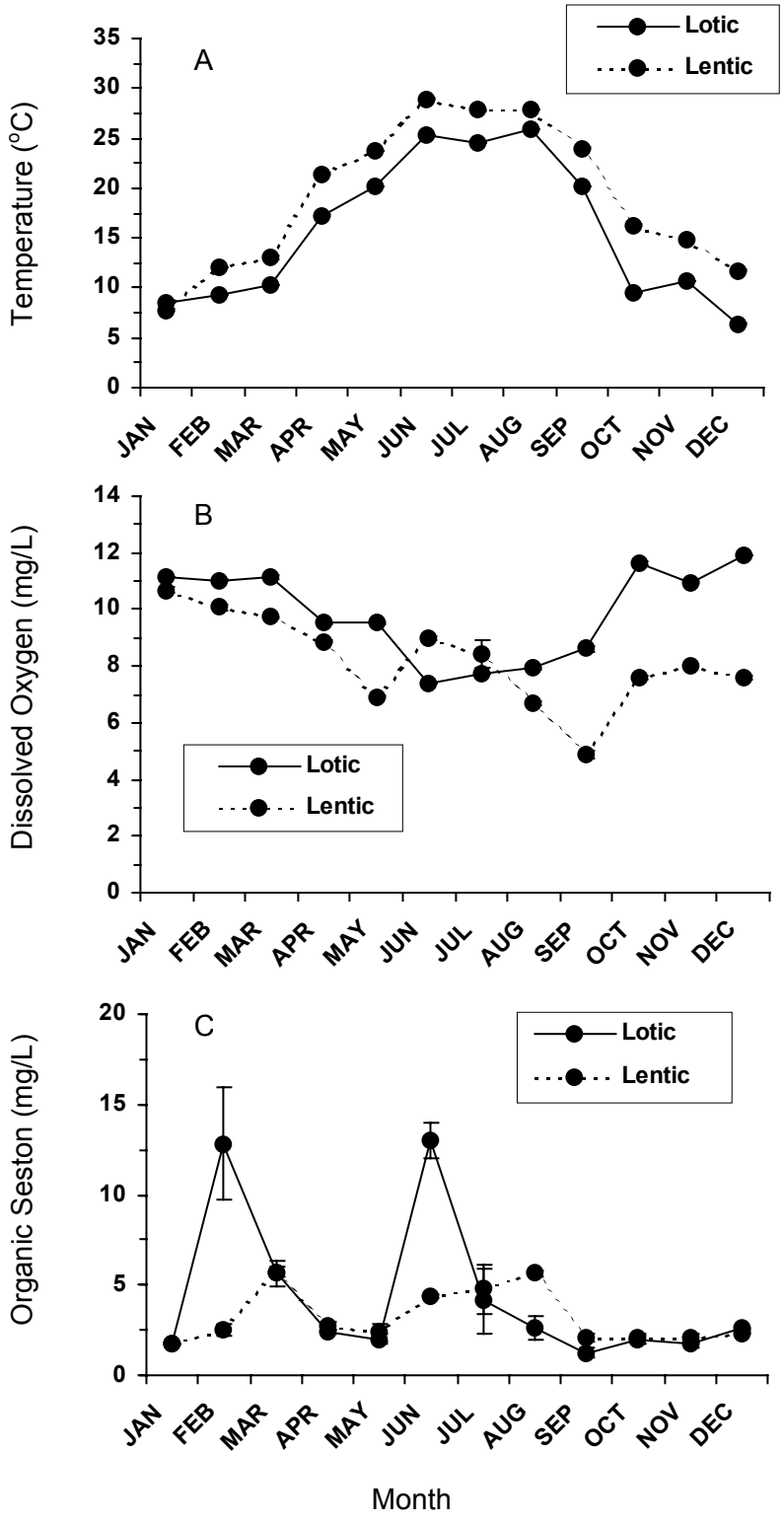

Figure 3.1. Seasonal trends of temperature (A), dissolved oxygen (B) and organic seston (C) at lotic and lentic habitats.

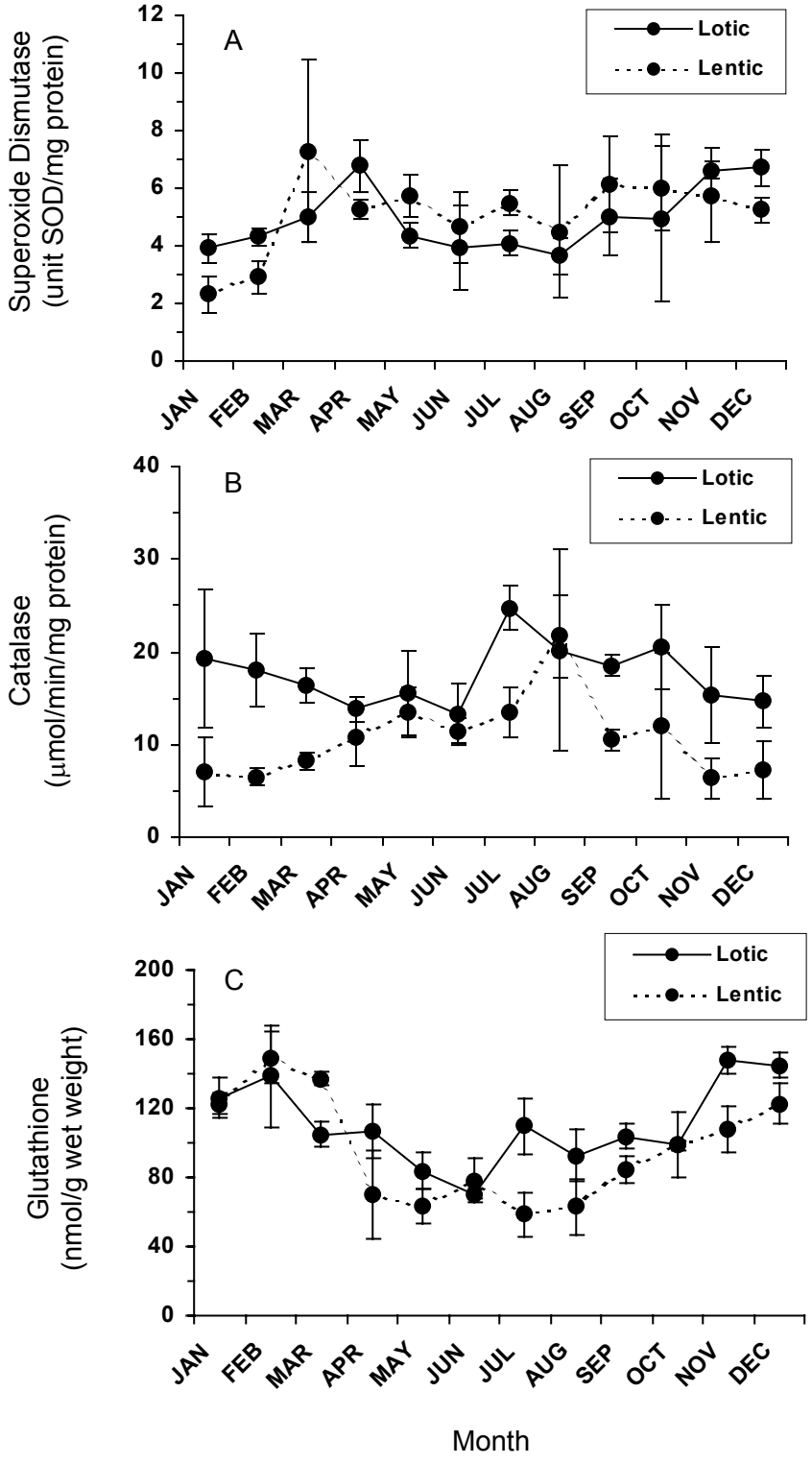

Figure 3.2. Seasonal trends of superoxide dismutase (A), catalase (B) and glutathione (C) in the digestive glands of clams from lotic and lentic habitats.

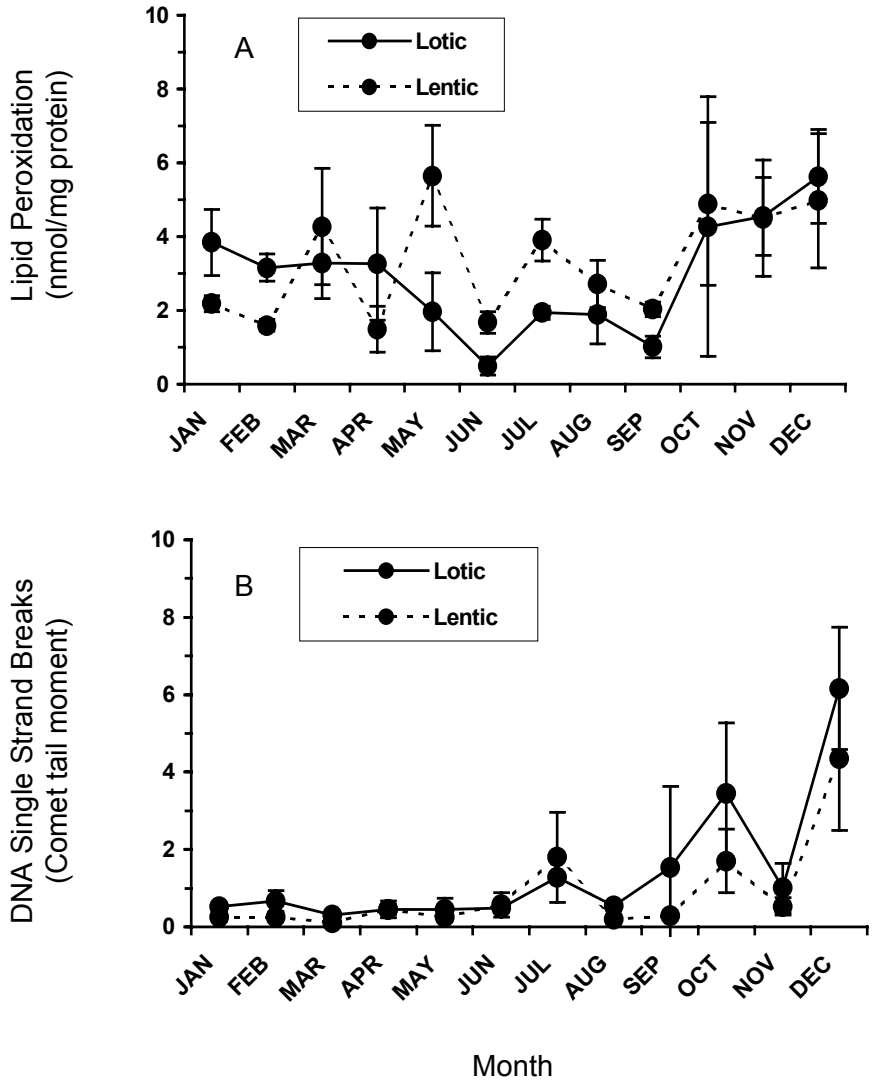

Figure 3.3. Seasonal trends of lipid peroxidation (A) and DNA single-strand breaks (B) in the digestive glands of clams from lotic and lentic habitats.

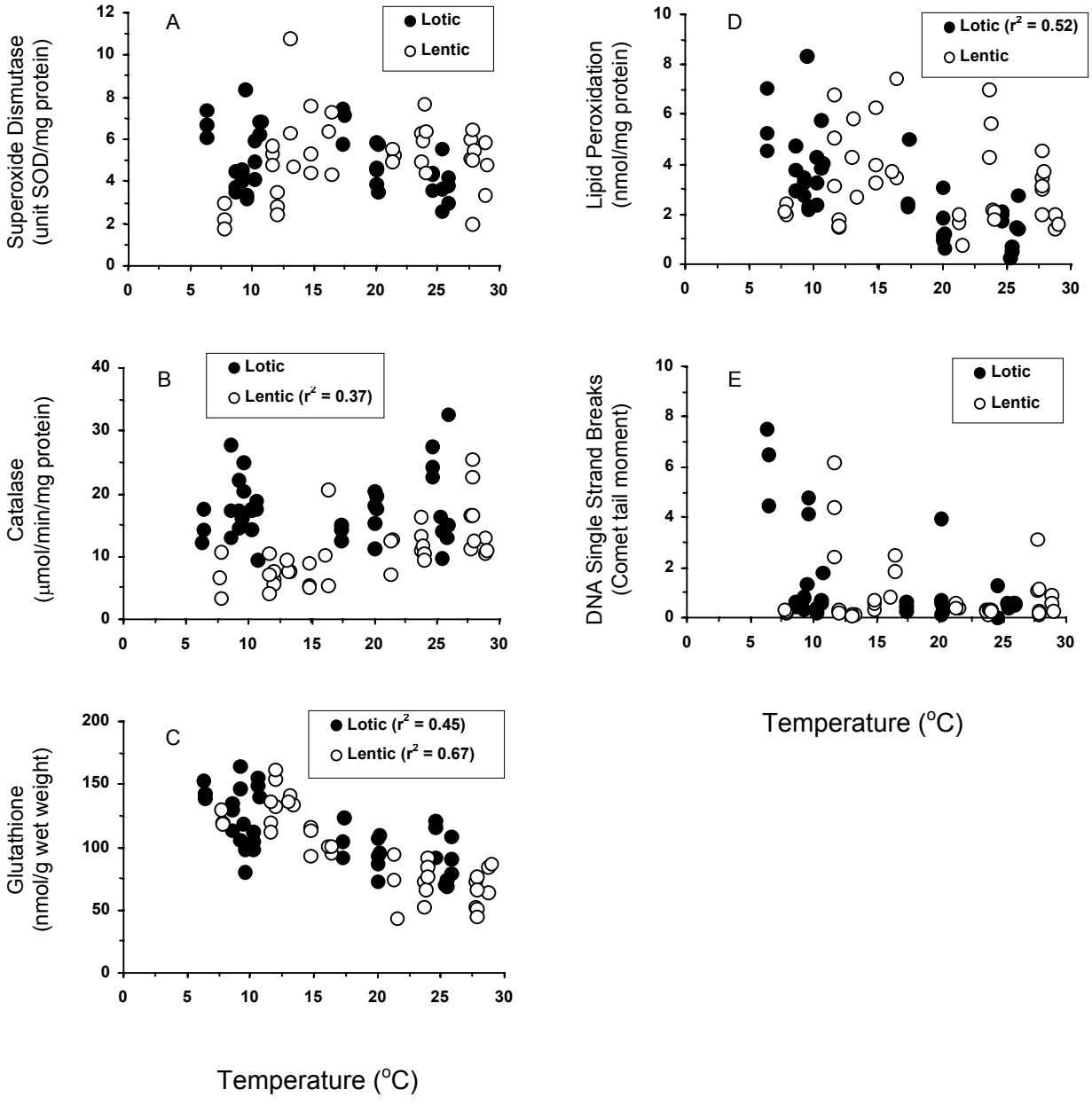

Figure 3.4. Relationships between temperature and measures of oxidative stress in digestive glands of clams from lotic and lentic habitats (A = superoxide dismutase, B = catalase, C = glutathione, D = lipid peroxidation and E = DNA single-strand breaks). The coefficient of determination (r^2) is shown for relationships that were significant ($P < 0.05$).

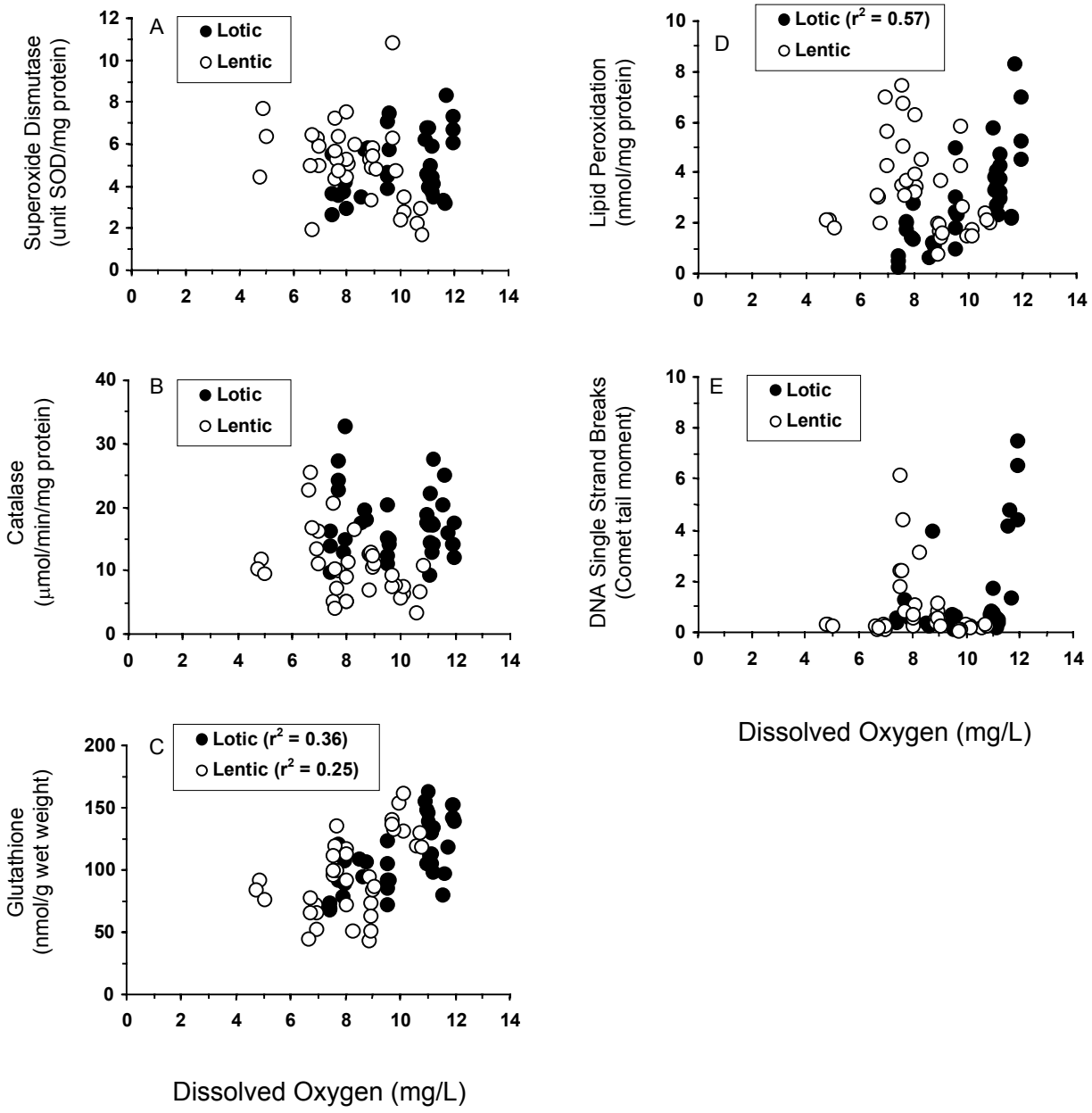

Figure 3.5. Relationships between dissolved oxygen and measures of oxidative stress in digestive glands of clams from lotic and lentic habitats (A = superoxide dismutase, B = catalase, C = glutathione, D = lipid peroxidation and E = DNA single-strand breaks). The coefficient of determination (r^2) is shown for relationships that were significant ($P < 0.05$).

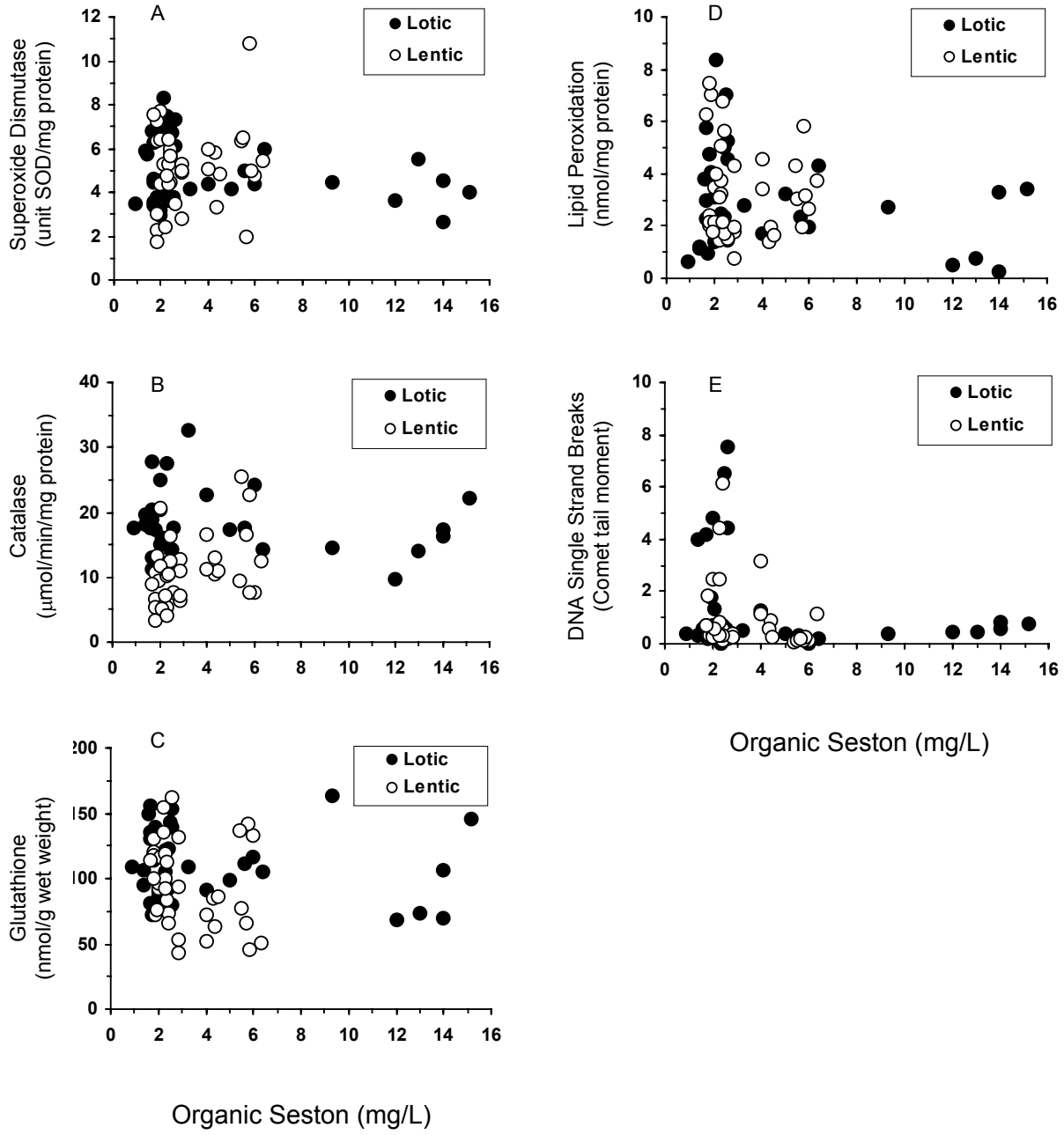

Figure 3.6. Relationships between food availability (organic seston) and measures of oxidative stress in digestive glands of clams from lotic and lentic habitats (A = superoxide dismutase, B = catalase, C = glutathione, D = lipid peroxidation and E = DNA single-strand breaks). No relationships were significant ($P < 0.05$).

CHAPTER 4

OXIDATIVE STRESS IN FRESHWATER CLAMS (*CORBICULA FLUMINEA*) EXPOSED TO PESTICIDE RUNOFF FROM RESIDENTIAL LAWNS AND A GOLF COURSE [1]

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[1] Conners DE, Armbrust KL, Shuman LM, Black MC. To be submitted to *Environmental Toxicology and Chemistry*

Abstract. Many chemicals, including fertilizers, herbicides, insecticides and fungicides, are routinely applied to turf in the care and maintenance of lawns, and have the potential to leach into nearby surface waters and adversely affect aquatic biota. Because oxidative stress is a common mechanism of toxicity by which diverse chemicals exert adverse effects, we evaluated biomarkers of oxidative stress (superoxide dismutase, catalase, glutathione and lipid peroxidation) and condition index in clams (*Corbicula fluminea*) that were exposed *in situ* to suites of turf care chemicals. Clams were deployed in freshwater streams receiving pesticide runoff from residential lawns or a golf course for 4 and 8 weeks during the spring/summer of a dry year and a wet year. We observed that clams from contaminated sites accumulated persistent pesticides (log Kow > 5.00) in their tissues. Concentrations of pesticides in clam tissues were typically highest for clams deployed in (1) residential streams from high property value neighborhoods during the wet year and (2) the stream draining a golf course. Clams exposed to contaminants exhibited transient signs of oxidative stress (i.e., elevated levels of superoxide dismutase, catalase, glutathione and lipid peroxidation), whereas condition indices were reduced only at high exposures. These data suggest that clams may be able to compensate for adverse cellular effects of pesticides, but that energy required for amelioration will eventually affect physiological processes. From these data, we developed a discriminate model that describes how cellular responses can be linked to higher levels of biological organization, and illustrates how multivariate analyses can be useful for evaluating the contribution of contaminants in the etiology of adverse effects in *in situ* bioassays. Future research will involve replicating exposures in a laboratory setting to investigate if the observed adverse effects on health were caused by the turf care chemicals accumulated by clams, and not by other contaminants that may co-occur in the streams but were not measured. In conclusion, this research highlights the sensitivity and utility of the use of suites of oxidative stress biomarkers for detecting adverse effects during environmentally relevant exposure scenarios, and illustrates how such data may be simplified to a health score that would facilitate communication of risks to environmental managers and the general public.

Introduction

Many chemicals, including fertilizers, herbicides, insecticides and fungicides, are routinely applied to turf in the care and maintenance of lawns. In the United States, pesticide use on homes, lawns and gardens increased between 13 and 20 % from 1995 to 1999, and during these years an average of 60 million kilograms of active ingredient were applied annually (Donaldson et al. 2002). Pesticides may leach from application sites during rainfall and travel as dissolved forms or bound to particles into nearby aquatic systems via surface water runoff or groundwater recharge (Richards and Backer 1993). A variety of pesticides have been detected more frequently and at higher concentrations in watersheds of the southeastern United States that drain urbanized areas than in watersheds that drain agricultural areas (Hippe and Garrett 1997). This phenomena has also been observed elsewhere in the United States (Hoffman et al. 2000) and in other developed countries (e.g., France) (Chevreuil et al. 1999).

Streams and rivers in the southeastern United States are unique in that they were not altered by Pleistocene glaciation, and contain a large percentage of global freshwater biological diversity (Lydeard and Mayden 1995). Many species of fish, crayfish, mollusks and amphibians indigenous to this region are endangered due to factors such as water quality degradation, habitat loss and the introduction of non-native species (Williams et al. 1993, Richter et al. 1997). Ricciardi and Rasmussen (1999) project that many North American freshwater mussels (Bivalvia: Unionidae) will become extinct over the next 100 years (6.4 % per decade), at a rate that is comparable to estimates of species loss in tropical rainforests (1 to 8 % per decade). The purpose of this study was to assess the effects of pesticide runoff from residential lawns and a golf course on sensitive molecular markers of stress in freshwater clams.

Bivalves are widely regarded as good bioindicator species because of their widespread distribution and abundance in many aquatic habitats, sedentary traits, hardiness and ability to bioaccumulate xenobiotics from water and sediments (Elder and Collins 1991). The freshwater clam, *Corbicula fluminea*, was chosen as a bioindicator species because it is abundant in many aquatic habitats

throughout the southeastern United States (McMahon 1982), and this species shares some physiological and ecological attributes with imperiled freshwater mussels (Hull et al. 2002).

A variety of different xenobiotics may cause damage in aquatic organisms by increasing levels of harmful oxygen radicals via processes of redox cycling, disrupting mitochondrial electron transport, inducing enzyme systems such as cytochrome P450s that mediate oxidation reactions or by depleting levels of protective antioxidants (Winston and Di Giulio 1991, Livingstone 2001). We hypothesized that many chemicals used in turf care may produce toxicity via oxidative stress mechanisms, and that suites of such biomarkers would be sensitive and useful for characterizing adverse effects in bivalves exposed to complex pesticide mixtures. Antioxidant enzymes (superoxide dismutase, catalase), an antioxidant scavenger (glutathione), an indicator of oxidative membrane damage (lipid peroxidation) and an indicator of physiological health (condition index) were measured in clams that were exposed *in situ* to turf care pesticides for four and eight weeks. Oxidative stress has been observed in bivalves exposed to the pesticides paraquat (Wenning et al. 1988), thiram (Doyette et al. 1997) and fenitrothion (Peña-Llopis et al. 2002). Additionally, copper is often applied to lawns as an essential metal in fertilizers, and is widely known to produce oxidative stress in bivalves (Viarengo et al. 1990, Doyette et al. 1997, Regoli et al. 1998, Conners and Ringwood 2000, Geret et al. 2002).

The streams evaluated in this study were allochthonous headwaters of the Apalachicola-Chattahoochee-Flint River Basin (Georgia, USA). Six of these streams were located in a watershed that drains residential lawns. These streams were chosen based on their location in neighborhoods of differing socioeconomic status with the assumption that in low property value neighborhoods, homeowners likely apply pesticides themselves whereas in high property value neighborhoods, pesticides are likely applied more intensely by professional applicators. Robbins and Sharp (2003) offer a review of studies that have documented increased chemical use on lawns by homeowners with higher incomes and more educational training. To obtain a high exposure scenario, clams were also deployed upstream, adjacent to a tile drain and downstream in a waterway running through a golf course.

Materials and Methods

Clam Deployments

Clams of a uniform size (16.2 ± 1.4 mm length) were collected from a free-flowing river (145 km) that drains forests and rural lands, and supports abundant populations of *Corbicula fluminea* (Broad River, Danielsville, GA, USA). Clams were transported to the laboratory (30 min) in aerated site water, and were held for one to seven days prior to deployments in a large flow-through system (265 L) containing aerated and dechlorinated tap water adjusted to within 2 ^{o}C of the water temperature from the site of collection. During holding, clams were fed a manufactured diet for filter-feeders (30 mg Microfeast® L-10 Larval Diet) supplemented with algae (0.5 mL *Selanastrum capricornutum* per clam) every day. Clams were deployed in six streams that receive residential lawn runoff during the summers of 2000 and 2001 (Peachtree City, GA, USA). Streams were chosen based on their location in reference (R1 and R2), low property value (L), intermediate property value (I) or high property value (H1 and H2) watersheds. Clams were also deployed upstream (US), adjacent to a tile drain (TD) and downstream (DS) in a waterway running through a golf course (Alpharetta, GA, USA) during the spring of 2001. All study steams were headwaters of the Apalachicola-Chattahoochee-Flint River Basin that is located in Georgia's Piedmont physiographic province. Additional characteristics of these streams are presented in Table 4.1. A preliminary survey revealed that small but viable populations of native *C. fluminea* were present in study streams. Clams were deployed in cages (18 x 36 x 18 cm) constructed from rigid polyethylene mesh (1.27 cm x 1.27 cm square openings) and PVC pipe. Three cages were deployed in each stream approximately 3 to 4 m apart (n = 3, 36 clams per cage). Cages were sunk approximately 4 to 6 cm into sediments to allow for both water and sediment exposures. Cages were deployed one week prior to the addition of clams, so that sediments would have time to settle. Clams were sampled at four and eight weeks of exposure, and were transported to the laboratory (2 h) in aerated site water that was adjusted to within 5 ^{o}C of the water temperature from the site of collection. Clams were processed for analyses on

the same day of collection. Visually, it was observed that clams depurated approximately 50 to 70 % of their gut contents prior to processing.

Physicochemical Properties of Streams

Physicochemical properties of streams were determined mid-stream at 60 % depth. Measurements of water temperature, dissolved oxygen, pH and conductivity were collected *in situ* with handheld portable meters every week during clam deployments. Velocity was measured weekly in streams by use of a portable flow meter. Monthly, water samples were collected and analyzed in the laboratory for turbidity, alkalinity, hardness and organic seston. Turbidity was measured on a HF Scientific Micro 100 Laboratory Turbidimeter®. Alkalinity and hardness were measured by Hach® titration kits. Organic seston was measured by methods described in Wallace and Grubaugh (1996). Briefly, water samples (5 L) from each location were collected and filtered to remove large debris (1 mm). Three replicate water samples from each site were vacuum filtered through pre-ashed (500 ^{o}C for 1 h) and pre-weighed 47 mm glass fiber filters. Filters containing suspended matter were oven dried (50 ^{o}C for 24 h), dessicated (24 h) and weighed for dry mass. Filters were then ashed (500 ^{o}C for 1 h) in a muffle furnace, rewet with distilled water, and again were oven dried (50 ^{o}C for 24 h) and dessicated (24 h). Filters were then weighed to determine ash mass and organic seston or ash-free dry mass was calculated: (dry mass – ash mass) / volume of water filtered.

Exposure Assessments

Whole tissues were dissected from clams for metal and organic pesticide analyses. For metals, tissues were dissected from three to five individual clams per cage, dried overnight (100 ^{o}C) and dessicated (24 h). Tissues were then pooled into acid washed plastic vials for storage. Tissues were acid digested (EPA Method 200.3, McDaniel 1992) and analyzed for arsenic, copper and zinc by graphite furnace atomic absorption spectroscopy (EPA Method 200.9, Creed et al. 1992). A special EDL II power supply was used in the analysis of arsenic. Data were expressed as µg/g dry weight and were not

corrected for percent recoveries, which were 126 % for arsenic, 131 % for copper and 43 % for zinc. For organic pesticides, tissues were dissected from three to five individual clams per cage, pooled and stored frozen (-80 ^{o}C) in acetone rinsed glass vials. Samples were extracted with hexane: acetone then cleaned by gel permeation chromatography. Extracts were analyzed for a variety of pesticides by electron capture gas chromatography mass spectrometry with a 60 m DB-5 capillary column (0.25 internal diameter). Pesticides analyzed included herbicides (atrazine, dithiopyr, oxadiazon, pendimethalin, prodiamine, simazine and trifluralin), insecticides (chlorpyrifos, diazinon and malathion) and fungicides (chlorothalonil, the degradation product 4-OH-chlorothalonil and flutolanil). Data were adjusted for percent tissue water, normalized by lipid content and were expressed as ng/g dry weight without correcting for percent recoveries. Percent recoveries for organic pesticides were between 63 and 95 % for all chemicals except chlorothalonil (28.1%) and oxadiazon (44.0 %). Additional characteristics of these metals and organic pesticides are presented in Table 4.2.

Bioeffect Assessments

Digestive glands were dissected from clams for analyses of biomarkers of oxidative stress. Tissues were snap frozen in liquid nitrogen and stored at –80 ^{o}C until assayed. Biomarkers were analyzed on tissues from three individual clams per cage i.e., a total of nine clams were analyzed per stream at four and eight weeks of exposure.

Total superoxide dismutase (SOD) activities were measured by pyrogallol auto-oxidation as described originally by Marklund and Marklund (1974), but modified for measurements of all SOD forms (Cu-Zn SOD and Mn SOD) (Zidenberg-Cherr et al. 1989). Briefly, tissues were homogenized in 50 mM potassium phosphate buffer (pH 7.0) and combined with three parts SOD buffer (pH 8.2, containing 50 mM N-tris[Hydroxymethyl]methyl-3-aminopropanesulfonic acid and 1 mM diethylenetriamine pentaacetic acid). Samples were then centrifuged (14,000 rpm, 4 ^{o}C, 5 min) and the supernatant (150 μL) was combined with 770 μL SOD buffer and 80 μL 4 mM pyrogallol. Changes in sample absorbency

were monitored spectrophotometrically at 320 nm every 30 s for a total of 120 s, and activities were expressed as units SOD per mg protein. One unit of SOD activity was defined as the amount of enzyme that inhibits the auto-oxidation of pyrogallol by 50 %.

Catalase (CAT) activities were measured by the decomposition of H_2O_2 as described by Clairborn (1985). Tissues were homogenized in 50 mM potassium phosphate buffer (pH 7.0), and a subsample of the homogenate (100 μL) was combined with 20 μL 5 % Triton X-100. Samples were then centrifuged (14,000 rpm, 4 ^{o}C, 5 min) and the supernatant (8 μL) was combined with 800 μL 19 mM H_2O_2. Changes in sample absorbency were monitored spectrophotometrically at 240 nm every 30 s for a total of 120 s, and activities were expressed as umol H_2O_2 consumed per min per mg protein.

Total glutathione concentrations (GSH) were quantified by the glutathione reductase recycling assay (Anderson 1985). Tissues were homogenized in 5 % sulfoslicyclic acid and centrifuged (14,000 rpm, 4 ^{o}C, 5 min). A subsample of the supernatant (25 μL) was added to sodium phosphate buffer (143 mM, pH 7.5) for a final volume of 1 mL, which contained 200 μM β-NADPH and 1 mM 5,5'-dithio-bis(2-nitrobenzoic acid). Samples were then vortexed and warmed to 30 ^{o}C in a water bath for 10 min. Glutathione reductase (15 μL at 50 units/mL) was added to initiate the enzymatic reaction and the rate of 5-thionitrobenzoic acid formation was monitored spectrophotometrically at 412 nm every 30 s for a total of 120 s. Standards were prepared from reduced glutathione, and concentrations were expressed as nmol per g wet weight.

Lipid peroxidation (LPX) was quantified by the thiobarbituric acid assay for malondialdehyde concentrations (Aust 1985). Tissues were homogenized in 50 mM potassium phosphate buffer (pH 7.0) and centrifuged (14,000 rpm, 4 ^{o}C, 5 min). A subsample of the supernatant (71 μL) was combined with 1 mL 15 % trichloroacetic acid containing thiobarbituric acid (0.375 %) and 10 μL butylated hydroxytoluene (20 mg/mL in absolute alcohol). Samples were then heated (100 ^{o}C, 15 min) in a boiling water bath and centrifuged (14,000 rpm, 20 ^{o}C, 5 min) to remove precipitates. Malondialdehyde

concentrations were measured spectrophotometrically at 532 nm. Standards were prepared as described by Csallany et al. (1984), and the results were expressed as nmol per mg protein.

Protein concentrations of supernatants were used for determinations of superoxide dismutase, catalase and lipid peroxidation. Proteins were quantified by the use of a Bio-Rad™ protein assay kit based on the method of Bradford (1976). Samples (25 μL) were combined with 1 mL diluted dye reagent (Coomassie Brilliant Blue G-250 diluted 1:4 with distilled water), vortexed and incubated for 5 min in the dark. Protein concentrations were determined spectrophotometrically at 595 nm with bovine serum albumin used for standards.

Condition index (CI) is a ratio of tissue biomass to the capacity of a shell for supporting tissue production, and high values reflect that bivalves are in a good physiological health (Lawrence and Scott 1982). Whole soft tissues from clams were dissected, dried overnight (100 ^{o}C), dessicated (24 h) and weighed. Condition index values were measured on five individual clams per cage, and were expressed as g dry tissue weight per mm shell length.

Statistical Analyses

All statistical analyses were performed at a $P < 0.05$ level of significance. Sample sizes for the majority of data were n = 3 cages per site, but cages were lost at a few locations during severe storms (n = 2 for L at 8 weeks in 2000, R1 and I at 8 weeks in 2001 and the golf course sites US and DS at 8 weeks) or by vandalism (no data were available for L at 8 weeks in 2001). Bioeffects data were checked for normality and homogeneity of variances prior to use in statistical tests, and data were transformed if necessary to meet these assumptions. Data were also checked for temporal correlation via the Durbin-Watson D statistic. Effects data that were not auto-correlated (SOD, GSH, LPX) were analyzed with a one way Analysis of Variance (ANOVA), while data that were auto-correlated (CAT, CI) were analyzed with a repeated measures ANOVA. If significant differences were detected among the deployment sites, multiple comparison tests were used to determine what sites were different from the control site (R1 =

reference stream for the residential watersheds, and US = upstream control reach for the golf course stream). Specifically, Dunnett's multiple comparison procedure was used for data that were not auto-correlated, while Bonferroni's multiple comparison procedure was used for data that were auto-correlated.

Alternatively, discriminate analysis was used to identify differences in bioeffects data among streams. Discriminate analysis is a multivariate statistical technique whereby multiple measures of effects are integrated and used to mathematically define distinct groups; then measures of exposures are evaluated for their ability to explain the processes underlying the grouping (Fairbrother and Bennett 2000). Before bioeffects data were used in the discriminate analysis, data were adjusted by an additive time series function (1) to control for known seasonal variation of these biomarkers between the spring and summer deployment periods i.e., values comparable to baseline monthly averages in source clams (Broad River, Danielsville, GA, USA) were unaffected by the adjustment, whereas values that were higher or lower than monthly averages were adjusted accordingly.

[observed value in deployed clams – baseline monthly value] + observed value in deployed clams] (1)

Data on baseline monthly values for each biomarker are presented in Conners and Black (Chapter 3). In essence, this seasonal adjustment accounted for lower catalase activities and higher levels of lipid peroxidation that are often observed in bivalves during colder months (Sheehan and Power 1999).

The groups identified by linear discrimination functions were given health scores to reflect bioeffect response patterns in healthy clams (= 1), clams that display evidence of cellular stress but no adverse effects at the organismal level (= 2 or 3) and clams whose compensatory responses are overwhelmed and display adverse effects at the organismal level (=4 or 5). Measures of exposures were summarized by use of the Pesticide Toxicity Index (PTI) (Munn and Gilliom 2001), which is a toxic unit based approach for assessing exposures to multiple pesticide contaminants. The PTI used in this study (2) represents the sum of toxicity quotients for each contaminant of concern measured in clam tissues:

$$PTI = \Sigma (E_i/MTC_{x,i}) \quad (2)$$

where E_i = concentration of pesticide $_i$ and $MTC_{x,i}$ = median toxicity concentration of pesticide $_i$ for taxonomic group $_x$. While Munn and Gilliom (2001) provide toxicity data for benthic invertebrates, this data set was incomplete for many of the chemicals analyzed in this study. Therefore, toxicity data for cladocerans (Table 4.2) were used to calculate PTIs. Cladoceran toxicity data for 4-OH-chlorothalonil were not available, hence the value for the parent compound was used as a surrogate. Contaminants of concern were identified as those chemicals detected frequently (> 20 %) and at concentrations exceeding the limit of quantification in deployed clam tissues. The relationship between health scores of deployed clams and their total body burden of relevant pesticides expressed as PTI, was evaluated with a nonparametric Kruskal-Wallis ANOVA. Then, Dunn's multiple comparison procedure was used to identify if health scores for clams in various stages of stress were significantly different from healthy clams. Statistical tests were run with SYSTAT software, version 9.0 (SPSS Inc.) or Microsoft Excel spreadsheets.

Results

Physicochemical Properties of Streams

Physicochemical properties of streams measured during clam deployments are presented in Table 4.3. Qualitative analyses of these data revealed that many of the parameters measured (temperature, dissolved oxygen and pH) were similar among sites during deployments, but differed among the deployment periods. Water temperatures were warmest in residential streams during the summer of 2000, slightly cooler in residential streams during the summer of 2001 and coldest in the golf course stream during the spring of 2001. Trends for dissolved oxygen concentrations were reverse of those for temperatures (i.e., DO of golf course stream, spring 2001 > residential streams, summer 2001 >

residential streams, summer 2000). Measurements of pH were similar among residential streams during the summers of 2000 and 2001, but were higher in the spring at sites located in the golf course stream. Climatic factors (rainfall and air temperature) can control seasonal and yearly trends in stream temperatures, dissolved oxygen and pH. Rainfall was higher in residential watersheds during the 2001 deployments versus the 2000 deployments (Figure 4.1). Hence, surface runoff to streams was likely high during the wet year (average stream velocities of residential streams taken at the same location in 2000 = 3.2 ± 1.5 cm/s and 2001 = 13.4 ± 3.7 cm/s). Conductivity was typically highest at sites located in large streams or those with greater than 7 % impervious surface cover in their watershed (R2, L, I, H1, US, TD and DS). Measurements of turbidity were highly variable, but tended to be high (> 20 NTU) at sites located in large streams (R2, US, TD and DS). Measures of alkalinity and hardness were reflective of soft surface waters common to this granite-based physiographic province. Organic seston concentrations, which reflect food available to filter-feeders, were different among sites during deployments and by season and year. Among residential streams, organic seston was consistently high at R2, the stream with the largest watershed area. By deployment period, organic seston concentrations were lowest in residential streams during the wet year and highest in the golf course stream.

Exposure Assessments

Of 16 chemicals evaluated, only 7 were detected frequently (> 20 %) and at levels above the limit of quantification (loq) in deployed clam tissues. These contaminants of concern (COC) included the herbicides trifluralin (83.3 %) and pendimethalin (79.2 %), the insecticide chlorpyrifos (83.3 %), the fungicide degradation product 4-OH-chlorothalonil (66.7 %) and the metals arsenic (100.0 %), copper (100.0 %) and zinc (100.0 %). High detection frequencies were also observed for the herbicides oxadiazon (54.2 %) and prodiamine (37.5 %), but concentrations of these xenobiotics in clam tissues were not above the loq and did little to differentiate the degree of contamination among streams. While the presence of COCs in clam tissues is suggestive of their use by homeowners and professional applicators in the watersheds (i.e., as pesticides or fertilizers applied to turf), detection frequencies were also related

to chemical persistence (log Kow > 5.00 for all organics except 4-OH-chlorothalonil). Tissue concentrations of each COC are shown in Figure 4.2 (herbicides), Figure 4.3 (insecticides and fungicides) and Figure 4.4 (metals). Clams accumulated all of the COCs except for copper, which was depurated by clams at most sites. Clams deployed at the high property value residential streams (H1 and H2) had more pendimethalin in their tissues during the wet year (551 to 770 ng/g dry weight) versus the dry year (76 to 195 ng/g dry weight). Likewise, chlorpyrifos was higher in clams deployed at all of the residential streams during the wet year (176 to 340 ng/g dry weight) versus the dry year (58 to 142 ng/g dry weight). The degree of contamination among residential streams was variable and depended on the type of contaminant and the deployment time. However, contaminants were often high in clams deployed at the intermediate and high property value sites (I, H1 and H2) and at R2, a proposed reference stream where after this study was performed, it was discovered that pesticides were being used in the watershed for management of surrounding pine plantations. Within the golf course study area, clam body burdens were typically elevated at all of the study reaches (US, TD and DS), but clams from the tile-drain and downstream sites did have higher herbicides, chlorpyrifos and zinc in their tissues than clams from the upstream reach. Overall, organic pesticides were often higher in clams from the golf course stream than clams from residential streams, while metal concentrations were comparable.

Bioeffect Assessments

Indicators of oxidative stress and physiological condition measured in deployed clams are presented in Table 4.4. Antioxidant enzyme activities (SOD and CAT) were elevated in clams deployed for four weeks in many of the residential streams during 2000 (R2, L, H1 and H2). However, by eight weeks these enzyme activities were not significantly different from the reference clams deployed at R1. Lipid peroxidation was also elevated in clams deployed at R2 for four weeks, but not at 8 weeks. Conversely, no effects on condition were observed in clams at four weeks, but values for clams deployed at R2 and H1 were significantly lower than R1 clams at eight weeks of exposure. In residential streams during 2001, the wet year, few effects on antioxidant enzymes were observed. However, glutathione

concentrations were significantly depleted in clams from I, H1 and H2 at four weeks of exposure. Also, low condition indices were observed in clams as early as four weeks of exposure (R2, L, H2). By eight weeks of exposure, only clams from R2 had significantly lower condition indices. Condition indices of clams from H1 and H2 were also low at eight weeks, but values were not significantly different from reference clams because of the low sample size (n = 2 for R1 clams at eight weeks). Few effects were observed on biomarkers of oxidative stress in clams deployed at the golf course stream, probably because the upstream reach was also contaminated. However, condition indices were significantly lower in clams from the tile-drain and downstream sites than clams from the upstream reach at both four and eight weeks of exposure.

A discriminate analysis of bioeffects data identified five groups that were given health scores to reflect bioeffect response patterns in healthy clams (= 1), clams that display evidence of cellular stress but no adverse effects at the organismal level (= 2 or 3) and clams whose compensatory responses are overwhelmed and display adverse effects at the organismal level (=4 or 5). Once discriminated, the model correctly reclassified 60 % of the data. The relationships between individual bioeffect endpoints to health scores of clams are depicted in Figure 4.5. Furthermore, we propose in Figure 4.5 how these biomarker responses may be related to a concept of Seylean stress refined by Newman (1995) for ecotoxicological applications whereby organisms progress through stages of alarm, compensation and exhaustion when exposed to detrimental factors. Tissue contaminant burdens and health scores of clams deployed in streams draining residential lawns or a golf course are shown in Figure 4.6. These data suggest that contaminants were highest in tissues of clams deployed at residential streams draining high property value neighborhoods (H1 and H2) or large watersheds (R2) during the wet year and at all of the golf course sites (US, TD and DS). Likewise, the worst health scores were observed in clams deployed at residential streams draining high property value neighborhoods (H1 and H2) or large watersheds (R2) during the wet year and at all of the golf course sites (US, TD and DS). There was a significant relationship between tissue contaminant burdens and the health scores of clams (Figure 4.7), but only clams with the worst health scores (5) were distinguishable from healthy clams (1) ($P < 0.05$).

Discussion

A number of multivariate statistical techniques exist for examining the effects of anthropogenic stress on aquatic ecosystems (reviewed by Fairbrother and Bennet 2000), and such approaches are becoming more common in ecological risk assessment. Multivariate statistics have been used successfully to discriminate between disturbed and undisturbed aquatic habitats when applied to biomarkers of oxidative stress in bivalves (Vidal et al. 2001, Blaise et al. 2002), with other biomarkers in fish (Adams et al. 1996, Machala et al. 1997, Van der Oost et al. 1997, Adams et al. 1999) and in community level aquatic assessments (Reynoldson et al. 1997, Anderson and Clements 2000). In this study, discriminate analysis was used to evaluate oxidative stress responses in clams exposed *in situ* to pesticide runoff from residential lawns and a golf course.

We observed that clams exposed to turf contaminants exhibited transient signs of oxidative stress (i.e., elevated levels of superoxide dismutase, catalase and lipid peroxidation). Such responses have been observed in other bivalve species (reviewed in Conners et al. Chapter 2) and in fish (reviewed in Lackner 1998). Decreases of antioxidant enzymes in xenobiotic-exposed bivalves after a period of elevation are common and may occur because of (1) negative feedback mechanisms (e.g., SOD is inhibited by hydrogen peroxide, the endproduct of its enzymatic reaction), (2) lower internal xenobiotic concentrations at loci of oxyradical generation due to the induction of detoxification enzymes, (3) lower metabolic generation of oxyradicals caused by reductions in oxygen consumption and (4) enzyme damage or destruction during apoptotic or narcotic cell death. Early increases of lipid peroxidation in xenobiotic-exposed bivalves are likely due to the attack of oxyradicals on polyunsaturated fatty acids in cell membranes. Decreases of lipid peroxidation in xenobiotic-exposed bivalves after a period of elevation presumably result from the induction of primary antioxidant defenses that reduce oxyradical concentrations and the induction of secondary antioxidant defenses that repair oxidative damage (e.g., phospholipases). Also, reductions in lipid peroxidation may occur from the induction of detoxification enzymes that lower internal xenobiotic concentrations at loci of oxyradical generation. Interestingly, the

discriminate model suggests that glutathione concentrations were elevated in clams with high body burdens of turf contaminants. This contrasts other studies that often observe depleted glutathione concentrations in bivalves exposed to xenobiotics (reviewed by Livingstone 2001). While clams with depleted glutathione concentrations were identified in many residential streams during the 2001 deployment by traditional statistical analyses, this was likely because of unusually high levels of glutathione in control clams that coincided with a peak of trifluralin in their tissues (GSH in R1 control clams = 159.5 ± 28.0 nmol/g versus GSH in baseline seasonal clams = 109.6 ± 15.9 nmol/g). Increases in glutathione concentrations have been observed in other bivalves exposed to a variety of organic (Wenning et al. 1988, Michel et al. 1993, Cheung et al. 2002) and inorganic xenobiotics (Yan et al. 1997), and presumably can occur via upregulation of γ-glutamylcysteine synthetase, the rate-limiting enzyme responsible for glutathione synthesis.

While measures of oxidative stress in deployed clams were transitory, condition indices were reduced only during high exposures. These data suggest that clams may be able to compensate for the adverse cellular effects of pesticides, but that energy required for amelioration will eventually affect physiological processes. Reduced energy allocation is a commonly evoked paradigm in bivalve research (Widdows et al. 1995, Smolders et al. 2004), and this concept describes how detoxification and repair processes initiated by xenobiotic exposures may shift energy towards maintenance tasks and away from tissue and shell production. Reduced rates of tissue and shell growth have been observed in freshwater mussels (Couillard et al. 1995) that were experiencing oxidative stress.

Depledge (1994) provides an excellent graphical illustration of how suites of biomarkers with various response patterns can be integrated and linked to adverse effects at higher levels of biological organization. Similarly, we attempted to link the transitory cellular responses observed in clams from this study to physiological condition. The groups identified by discriminate analysis were assigned health scores to reflect bioeffect response patterns in healthy clams (= 1), clams that displayed evidence of cellular stress but no adverse effects at the organismal level (= 2 or 3) and clams whose compensatory responses were overwhelmed and displayed adverse effects at the organismal level (=4 or 5).

Furthermore, we propose that the bioeffect response patterns observed in this model can be related to the concept of stress proposed by Seyle (reviewed by Newman 1995), i.e., elevations of antioxidant enzymes and lipid peroxidation occur during the alarm phase of stress, that these responses often disappear during the compensation phase of stress, and that adverse responses at the physiological level are most likely during exhaustion. The dose-responses observed in our discriminant model were similar to proposed dose-response patterns for these endpoints in aquatic organisms (Conners et al. Chapter 2) with the exception that clams did not appear to reach a late stage of exhaustion, which is often observed in organisms exposed acutely to contaminants in laboratory studies. While the development of such a multivariate model of physiological health is rather complex and requires a good deal of prior knowledge regarding these endpoints, the outputs of the model are simplified health scores. Because these health scores are based on a biomedical metaphor of health, such results may facilitate communication of risks to environmental managers and the general public. Hence, we observed that clams with poor health scores had significantly elevated levels of contaminants in their tissues. Clams with the worst health scores were those deployed at residential streams draining high-property value neighborhoods or large watersheds during the wet year, and clams deployed at all of the golf course sites. These data suggest that intense pesticide use on turf may be adversely effecting biota in nearby streams during rain events.

In addition to the utility of multivariate statistics for integrating bioeffect responses to evaluate the chemical etiology of stress, Reynoldson et al. (1997) review how such techniques are valuable for reducing the need to make prior assumptions regarding the suitability of reference sites. Clearly, the references sites used in this study were problematic. Among the residential streams, R2 was classified as one of the worst sites likely because of pesticide use in the watershed for forestry management. Also, because of the large size of this stream and its watershed, there was probably more sediment organic matter available to trap pesticides as they flowed through the stream. The upstream reach at the golf course stream was also classified as one of the worst sites. This stream flows through and receives nonpoint source runoff from a highly urbanized area located just outside one of the largest cities in North America (Atlanta, GA). Hence, we found the use of discriminate analysis in this study to be extremely

useful because it allowed for simultaneous assessment of clams deployed at reference and contaminated sites. Furthermore, these data illustrate the difficulty of finding suitable reference sites in urbanized watersheds, and highlight the need for preservation of these limited natural resources for scientific research.

Despite the above advantages, the multivariate model of physiological health used in this study has associated uncertainties and limitations that warrant discussion. Importantly, other contaminants may co-occur in the streams but were not measured. Glyphosate is a widely used herbicide in residential settings, but was not measured in this study because of methodological difficulties. Likewise, these streams may have some level of petroleum hydrocarbon contamination from roads and automobiles, but these xenobiotics were not the focus of this research. To investigate if the effects we observed on clam health were caused by turf contaminants accumulated by clams and not by other co-occurring contaminants, future research will involve replicating exposures in a laboratory experiment.

A second uncertainty in this research is that is difficult to identify whether the problematic contaminants identified in clams are actually being applied to turf. Notably, arsenic has been widely used as a fungicide to preserve wood in residential settings, although this use has recently been phased out in the United States (US EPA 2003). Some of this uncertainty associated with pesticide sources should be reduced by on-going collaborations with social scientists on the research grant that are conducting home owner surveys of pesticide use in these watersheds (Ted Gragson, The University of Georgia).

A third uncertainty in this research stems from the use of a Pesticide Toxicity Index based on cladocerans (Munn and Gilliom 2001) to obtain toxicity quotients for turf contaminants. Ideally, toxicity quotients for our multivariate model of physiological health should represent potencies of each chemical for producing oxidative stress in bivalves, but these data do not exist. Also, acute toxicity information is sparse for many current-use pesticides and their degradation products to bivalves and other benthic invertebrates. Hence, we assumed that the rank toxicity of these pesticides to cladocerans would be similar to their rank toxicity in bivalve tissues, and that the toxicity of the degradation product 4-OH-chlorothalonil would be comparable to the toxicity of the parent chemical. These assumptions are

problematic. While toxicity tests with juvenile life stages of freshwater mussels that cannot avoid exposures by shell closure indicate that mussels may be comparable in sensitivity to cladocerans for metals (Keller and Zam 1991, Jacobson et al. 1997) and some current-use pesticides (Conners and Black 2004), they may be less sensitive than cladocerans to organophosphate insecticides requiring bioactivation (Keller and Ruessler 1997, Conners and Black 2004). These studies suggest that we may have overestimated the toxicity of the organophosphate chlorpyrifos, which requires bioactivation by cytochrome P450s. Likewise, these studies were conducted on freshwater mussels (Bivalvia: Unionidae) that may be more sensitive to contaminants than *Corbicula fluminea* (Hull et al. 2002). Additionally, the degradation product 4-OH-chlorothalonil has been observed to be more toxic to stream fungi than the parent chemical, chlorothalonil (Herbert 2003); hence we may have underestimated the toxicity of this contaminant. Preliminary results from our laboratory indicate that 4-OH-chlorothalonil is less toxic to cladocerans then the parent fungicide (Jeremy Peacock, personal communication).

A final limitation of the multivariate model of physiological health used in this study is that it does not reflect impairments of stream ecosystem health at levels of biological organization relevant to water quality legislation (e.g., population, community and ecosystem level responses). Therefore, future efforts will be made to incorporate the results of this study with those of project collaborators that are evaluating study streams for benthic invertebrate community structure (Ray Noblet, The University of Georgia) and leaf degradation rates and algal biomass (Judy Meyer, The University of Georgia).

In conclusion, the results from this study suggest that many chemicals, when applied intensely to turf in the care and maintenance of lawns, have the potential to leach into nearby surface waters and adversely affect aquatic biota during rain events. However, there are important uncertainties and limitations associated with the data so future research will focus on (1) replicating exposures in a laboratory setting to investigate if the observed adverse effects on health were caused by the turf contaminants accumulated by clams, and not by other co-occurring chemicals, (2) improving the toxic unit estimates of turf contaminants used for exposure assessment and (3) integrating these results on bivalves with other measures of stream ecosystem integrity. If turf care chemicals are adversely affecting

stream biota, several management options exist that may reduce their negative impact. Loading of turf chemicals to streams can be reduced through diverting storm water to retention ponds, increasing the width of riparian buffers and educating consumers against applying pesticides before rain. Additionally, reducing pesticide use via integrated pest management strategies or eliminating nonessential pesticide use would be valuable for protecting water quality in streams. In conclusion, this research highlights the sensitivity and utility of the use of suites of oxidative stress biomarkers for detecting adverse effects during environmentally relevant exposure scenarios, and illustrates how such data may simplified to a health score that would facilitate the communication of risks to environmental managers and the general public.

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Table 4.1. Watershed characteristics of study streams located in the Apalachicola-Chattahoochee-Flint River Basin, Georgia, USA.

Land Use [1]	Site Code	Potential Sources of Contamination	Mean Property Value ($)	Stream Order	Watershed Area (km^2)	Impervious Surfaces (%) [2]
Suburban Residential	R1	Reference stream, no obvious sources	---	3	5.75	3.0
	R2	Reference stream, no obvious sources	---	4	21.28	2.4
	L	Turf runoff from low property value neighborhoods	136,900	2	0.75	15.4
	I	Turf runoff from intermediate property value neighborhoods	187,700	2	2.59	10.1
	H1	Turf runoff from high property value neighborhoods	388,900	1	0.39	7.1
	H2	Turf runoff from high property value neighborhoods	326,200	2	1.12	2.4
Urban Golf Course	US	Upstream reference site, nonpoint urban runoff	---	~ 3	---	16.0
	TD	Site adjacent to a golf course tile drain	---	~ 3	---	16.0
	DS	Site immediately downstream of a golf course green	---	~ 3	---	16.0

[1] Residential streams were located in the Upper Flint River Basin, and the golf course stream was located in the Upper Chattahoochee River Basin

[2] Impervious surface data for residential areas were obtained from an individual watershed GIS database (Nathan Piekielek, The University of Georgia, personal communication), while the data for the golf course stream were estimated from Big Creek watershed, to which the study stream is a tributary (Fischenich et al. 2000)

Table 4.2. Properties of turf care chemicals measured in deployed clam tissues. [1]

Type	Common Name	Class	CAS Number	Molecular Wt (g)	Water Solubility (mg/L)	Log Kow	Koc	Henry's Law Constant ($Pa\ m^3/mol$)	Cladoceran PTI ($\mu g/L$)[2]
Herbicides	Atrazine	Triazine	1912-24-9	215.68	33.0	2.68	147	2.48×10^{-4}	41500.00
	Dithiopyr	Pyridine	97886-45-8	401.42	1.4	4.75	1500	1.53×10^{-1}	---
	Oxadiazon	---	19666-30-9	345.23	0.7	4.70	3345	7.20×10^{-3}	---
	Pendimethalin	Dinitroaniline	40487-42-1	281.3	0.275	5.2	13400	1.23	2690.00
	Prodiamine	Dinitroaniline	29091-21-2	350.3	0.013	4.10	13000	8.90×10^{-2}	---
	Simazine	Triazine	122-34-9	201.66	6.2	2.10	140	9.80×10^{-5}	1100.00
	Trifluralin	Dinitroaniline	1582-09-8	335.28	0.32	5.07	7200	1.53	625.00
Insecticides	Chlorpyrifos	Organophosphate	2921-88-2	350.62	1.18	5.0	9930	7.43×10^{-1}	0.40
	Diazinon	Organophosphate	333-41-5	304.35	60.0	3.30	1520	7.20×10^{-2}	1.22
	Malathion	Organophosphate	121-75-5	330.4	130.0	2.7	1200	1.14×10^{-3}	1.80
Fungicides	Chlorothalonil	Organochlorine	1897-45-6	265.92	0.6	2.88	5000	2.20×10^{-2}	97.00
	4-OH-Chlorothalonil	Degradation Product	---	---	---	---	---	---	---
	Flutolanil	Benzanilide	66332-96-5	323.32	6.53	3.70	905	3.22×10^{-4}	---
Metals	Arsenic	Metal	7440-38-2	74.92	---	---	---	---	10730.00
	Copper	Metal	7440-50-8	63.55	---	---	---	---	92.80
	Zinc	Metal	7440-66-6	65.38	---	---	---	---	210.00

[1] Physical properties of chemicals were obtained from the US Department of Agriculture, Agriculture Research Service, Pesticide Properties Database (http://www.arsuda.gov/acsl/services/ppdb/)

[2] Cladoceran toxicity data for organic pesticides are median 48h EC50s obtained from the US Geological Survey's Pesticide Toxicity Index (PTI) (Munn and Gilliom 2001), data for metals were supplementary information obtained by similar methods from the US Environmental Protection Agency's ECOTOX database (http://www.epa.gov/ecotox/)

Table 4.3. Physicochemical properties of streams during clam deployments. Data are means ± SD for parameters measured weekly (temperature, dissolved oxygen, pH and conductivity) (n = 8 or 9) or monthly (turbidity, alkalinity, hardness and organic seston) (n = 2) during the eight weeks of deployment.

Deployed	Site	Temperature (°C)	Dissolved Oxygen (mg/L)	pH [1]	Conductivity (µs)	Turbidity (NTU)	Alkalinity (mg/L)	Hardness (mg/L)	Organic Seston (mg/L)
Residential, Summer 2000	R1	23.0 ± 0.9	6.51 ± 0.49	6.75	58.7 ± 1.5	---	25.0 ± 1.4	23.5 ± 6.4	1.97 ± 0.33
	R2	23.7 ± 0.8	6.29 ± 0.64	6.56	66.9 ± 6.4	---	34.5 ± 5.0	26.5 ± 0.7	2.69 ± 0.69
	L	22.7 ± 0.9	6.05 ± 0.68	6.18	65.1 ± 3.5	---	29.5 ± 0.7	20.8 ± 2.5	2.07 ± 0.90
	I	24.5 ± 1.1	6.73 ± 0.78	6.83	68.1 ± 18.1	---	40.0 ± 1.4	30.5 ± 0.7	1.34 ± 0.19
	H1	21.6 ± 0.6	6.41 ± 0.54	6.60	72.5 ± 5.0	---	34.0 ± 1.4	28.3 ± 0.4	1.22 ± 0.40
	H2	22.6 ± 0.8	6.93 ± 0.78	6.47	47.1 ± 7.7	---	20.5 ± 0.7	19.8 ± 0.4	1.90 ± 0.38
Residential, Summer 2001	R1	22.0 ± 0.5	7.63 ± 0.17	6.49	47.9 ± 1.7	19.4 ± 18.4	25.5 ± 0.7	50.0 ± 19.8	0.82 ± 0.26
	R2	22.3 ± 1.2	7.33 ± 0.36	6.62	55.5 ± 4.1	28.5 ± 15.7	33.3 ± 4.6	46.5 ± 23.3	1.52 ± 0.17
	L	20.5 ± 0.5	7.30 ± 0.28	6.88	54.9 ± 0.8	11.9 ± 11.0	42.0 ± ---	64.0 ± ---	0.93 ± ---
	I	23.6 ± 1.0	7.52 ± 0.32	6.72	71.0 ± 3.2	13.4 ± 7.1	42.0 ± 7.1	81.0 ± 24.0	1.08 ± 0.07
	H1	20.6 ± 0.5	7.75 ± 0.21	6.47	62.7 ± 2.5	10.4 ± 6.3	37.5 ± 5.0	60.0 ± 2.8	1.20 ± 0.57
	H2	21.0 ± 0.6	8.08 ± 0.26	6.50	38.1 ± 7.5	13.0 ± 6.9	23.5 ± 3.5	39.5 ± 27.6	1.00 ± 0.47
Golf Course, Spring 2001	US	12.3 ± 5.8	9.49 ± 1.07	7.26	67.1 ± 6.6	20.4 ± 5.8	54.5 ± 2.1	28.5 ± 0.7	3.45 ± 0.94
	TD	11.7 ± 5.3	9.13 ± 1.15	7.05	66.9 ± 6.5	21.7 ± 9.0	55.0 ± 0.0	31.0 ± 4.2	2.78 ± 0.94
	DS	11.3 ± 4.4	8.92 ± 1.20	7.17	67.7 ± 6.1	22.2 ± 8.2	62.5 ± 12.0	29.0 ± 1.4	3.00 ± 0.79

[1] pH data were averaged from hydrogen ion concentrations

Table 4.4. Indicators of oxidative stress and physiological condition measured in clams deployed in streams draining residential lawns or a golf course for four and eight weeks. Data are means ± SD (n = 2 to 3 cages per stream). Superscripts (a) indicate significant differences from controls ($P < 0.05$), where R1 = reference stream for the residential watersheds and US = upstream control reach for the golf course stream. Values shaded in grey indicate levels were outside of baseline seasonal ranges, and values shaded in black indicate levels were significantly different from controls and were outside baseline seasonal ranges.

		SOD (unit/mg protein)		CAT (µmol/min/mg protein)		GSH (nmol/g wet wt)		LPX (nmol/mg protein)		CI (g dry wt/mm) x10^3	
Deployed	Site	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
Residential											
Summer	R1	2.7±0.6	5.4± 0.9	5.5±0.5	8.7±5.8	81.4±14.7	93.8±7.4	2.7±0.6	3.6±1.8	3.2±0.7	2.8±0.1
2000	R2	**7.7±1.2[a]**	3.9±1.4	23.7±6.3[a]	8.9±1.3	66.0±4.0	97.1±16.3	**8.6±5.9[a]**	2.8±0.3	3.0±0.2	2.2±0.4[a]
	L	4.6±0.5[a]	5.9±2.6	19.4±1.1[a]	19.1±4.2	68.3±16.8	95.2±18.6	4.1±1.3	6.2±0.8	2.9±0.1	2.3±0.2
	I	2.9±0.7	3.6±0.9	5.7±2.6	4.6±1.2	89.3±11.4	95.3±8.8	2.2±0.4	4.4±3.2	3.0±0.3	2.6±0.1
	H1	5.4±1.5[a]	3.6±0.8	19.0±6.4[a]	10.0±2.5	103.9±3.9	78.3±11.0	3.5±1.8	3.4±1.0	3.0±0.1	2.2±0.2[a]
	H2	**5.6±1.5[a]**	5.8±1.2	21.0±3.4[a]	12.0±3.8	74.5±8.3	99.4±10.7	4.7±1.3	4.0±1.4	3.1±0.4	2.5±0.1
	Baseline[b]	(2.6, 5.5)		(6.6, 32.7)		(68.2, 121.3)		(0.2, 3.2)		(2.0, 3.4)	
Residential											
Summer	R1	5.2±1.8	4.1±0.4	14.4±7.9	9.5±0.3	159.5±28	91.0±5.7	2.5±0.2	0.6±0.9	2.9±0.3	2.5±0.2
2001	R2	5.4±1.1	4.2±0.9	13.9±6.4	10.6±2.2	110.7±21.4	107.9±1.8	0.3±0.2	0.3±0.3	2.2±0.4[a]	**1.8±0.1[a]**
	L	6.8±4.7	---	17.2±5.7	---	112.9±9.6	---	4.5±5.7	---	2.0±0.3[a]	2.3±0.1
	I	3.6±0.9	3.0±0.4	17.2±5.9	8.0±0.2	103.2±5.6[a]	108.4±18.4	0.5±0.6	2.4±2.3	2.6±0.3	---
	H1	3.7±0.8	5.5±1.7	18.2±4.8	14.6±4.7	89.4±34.6[a]	108.0±15.5	0.7±1.1	0.3±0.3	2.3±0.3	1.9±0.5
	H2	5.6±2.8	3.5±0.9	20.8±5.6	14.1±4.7	99.9±18.9[a]	131.2±26.4	2.1±2.2	2.6±2.6	2.1±0.2[a]	1.8±0.3
	Baseline[b]	(2.6, 5.5)		(6.6, 32.7)		(68.2, 121.3)		(0.2, 3.2)		(2.0, 3.4)	
Golf											
Course	US	4.7±0.6	3.9±1.0	21.3±9.5	10.9±0.7	173.7±37.5	120.1±46.1	2.9±0.9	1.9±0.1	2.6±0.4	2.6±0.4
Spring	TD	4.7±1.5	5.1±0.9	18.5±8.0	12.6±5.3	186.6±20.7	186.3±96.1	5.2±2.7	2.9±0.4[a]	2.2±0.1[a]	2.3±0.1[a]
2001	DS	7.2±4.2[a]	6.4±0.6[a]	24.4±11.8	14.3±5.9	160.9±28.0	104.0±11.9	3.0±0.3	2.3±0.2	2.1±0.1[a]	2.2±0.1[a]
	Baseline[b]	(3.2, 7.5)		(11.1, 20.4)		(47.6, 123.1)		(1.0, 5.0)		(2.0, 3.4)	

[b] Baseline seasonal ranges are minimum and maximum values observed for each endpoint in unexposed clams during the spring and summer, and values were derived from a combination of time zero data and seasonal data on clams from their native habitat (Conners et al. Chapter 3)

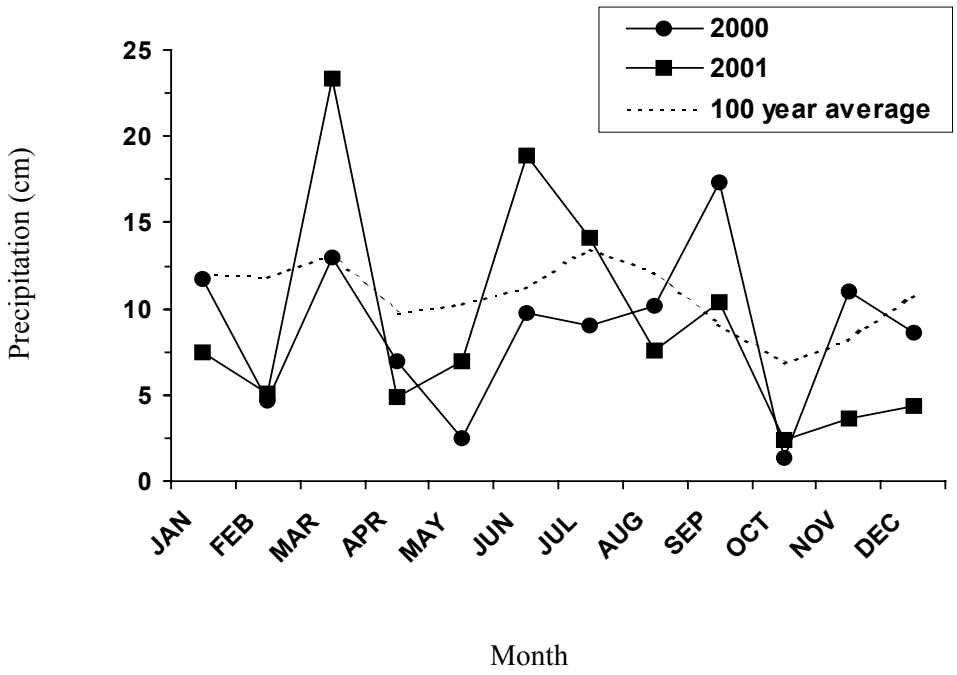

Figure 4.1. Monthly wet precipitation in Peachtree City, GA during the 2000 and 2001 deployment of clams, and statewide averages over the past 100 years. All data are from the Southeast Regional Climate Center (http://www.dnr.state.sc.us/climate.sercc/products/monthly/monthly.html) except for values for February and June in 2001, which were obtained from the National Weather Service (http://www.noaa.gov/ftproot/ffc/html/bubbalast.shtml#sec1).

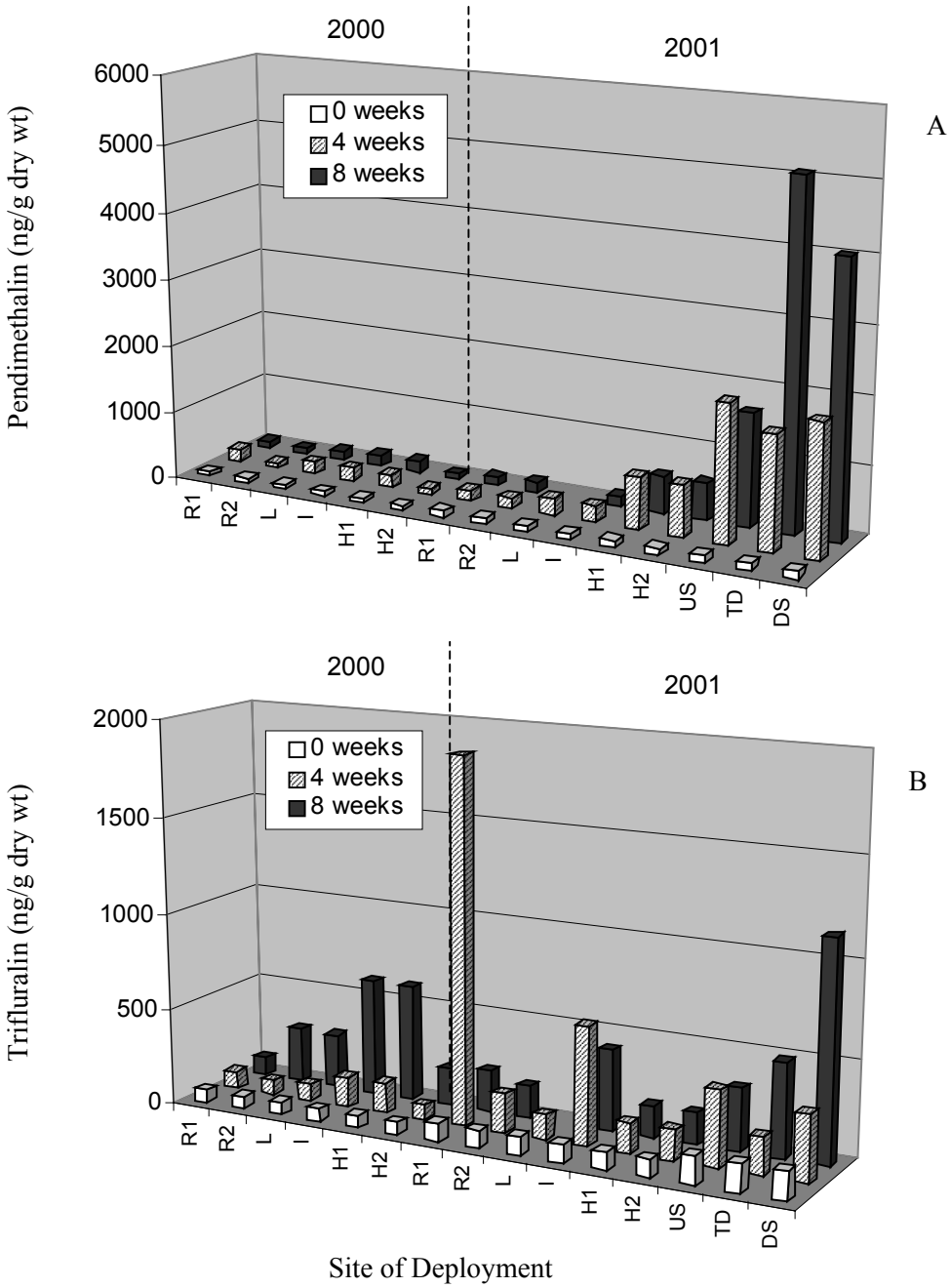

Figure 4.2. Median tissue concentrations of the herbicides pendimethalin (A) and trifluralin (B) in clams deployed in streams draining residential lawns (R1, R2, L, I, H1 and H2) or a golf course (US, TD and DS) during the years 2000 and 2001.

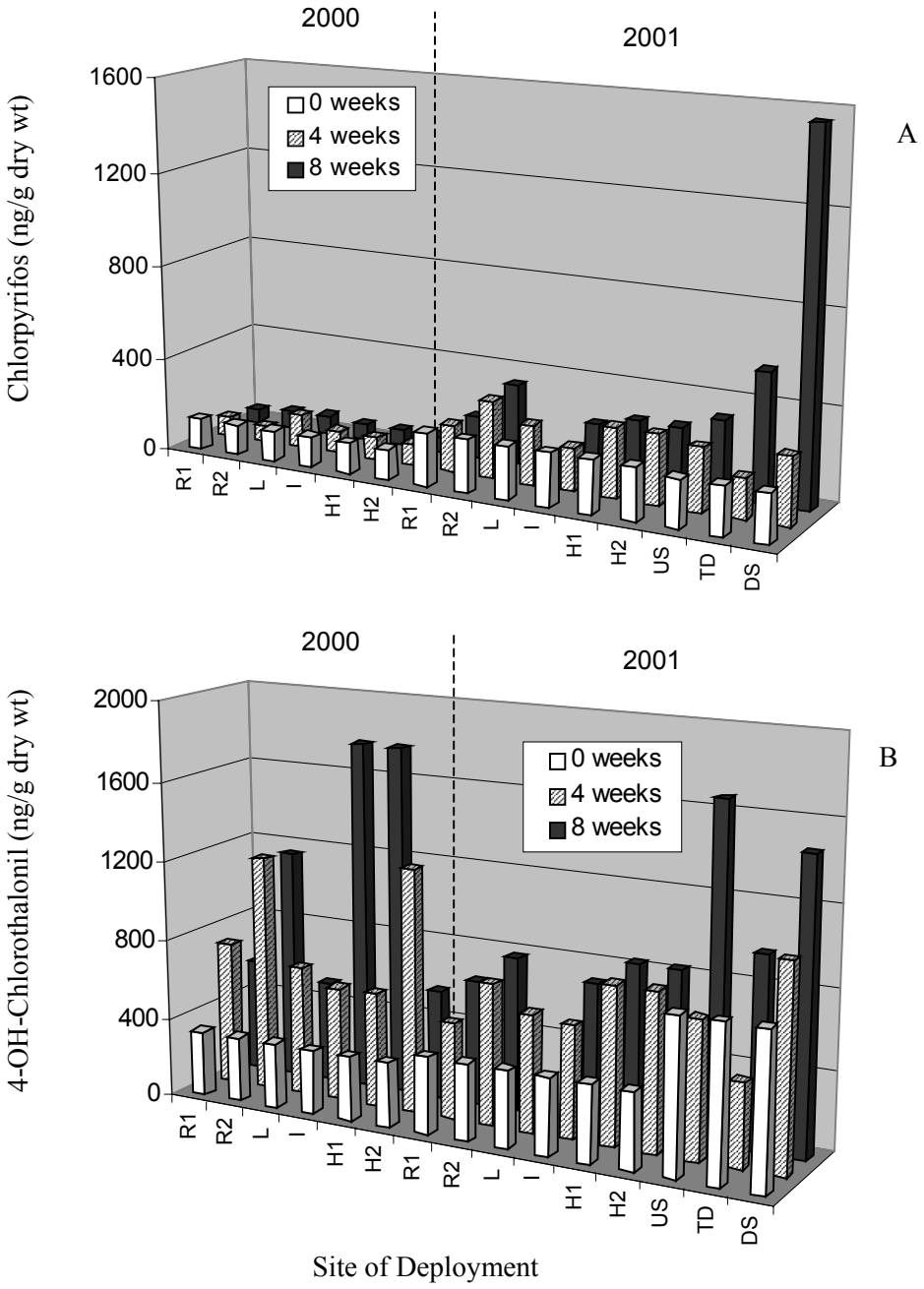

Figure 4.3. Median tissue concentrations of the insecticide chlorpyrifos (A) and the fungicide degradation product 4-OH-chlorothalonil (B) in clams deployed in streams draining residential lawns (R1, R2, L, I, H1 and H2) or a golf course (US, TD and DS) during the years 2000 and 2001.

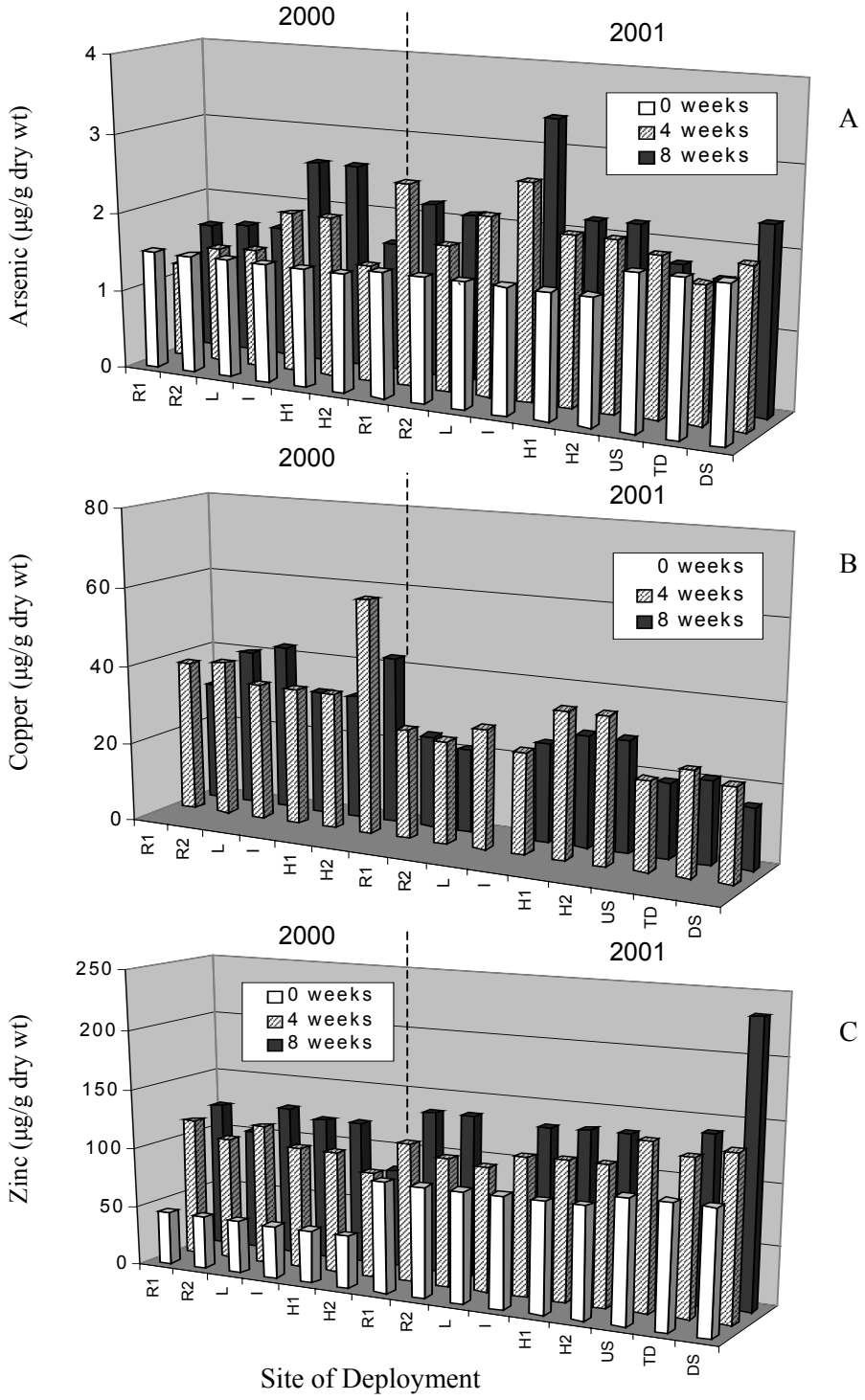

Figure 4.4. Median tissue concentrations of the metals arsenic (A), copper (B) and zinc (C) in clams deployed in streams draining residential lawns (R1, R2, L, I, H1 and H2) or a golf course (US, TD and DS) during the years 2000 and 2001. Data on copper for 0 weeks are not shown for illustrative purposes, but were equal to 67.7 µg/g and 29.1 µg/g for residential streams in 2000 and 2001 respectively, and 45.3 µg/g for the golf course stream.

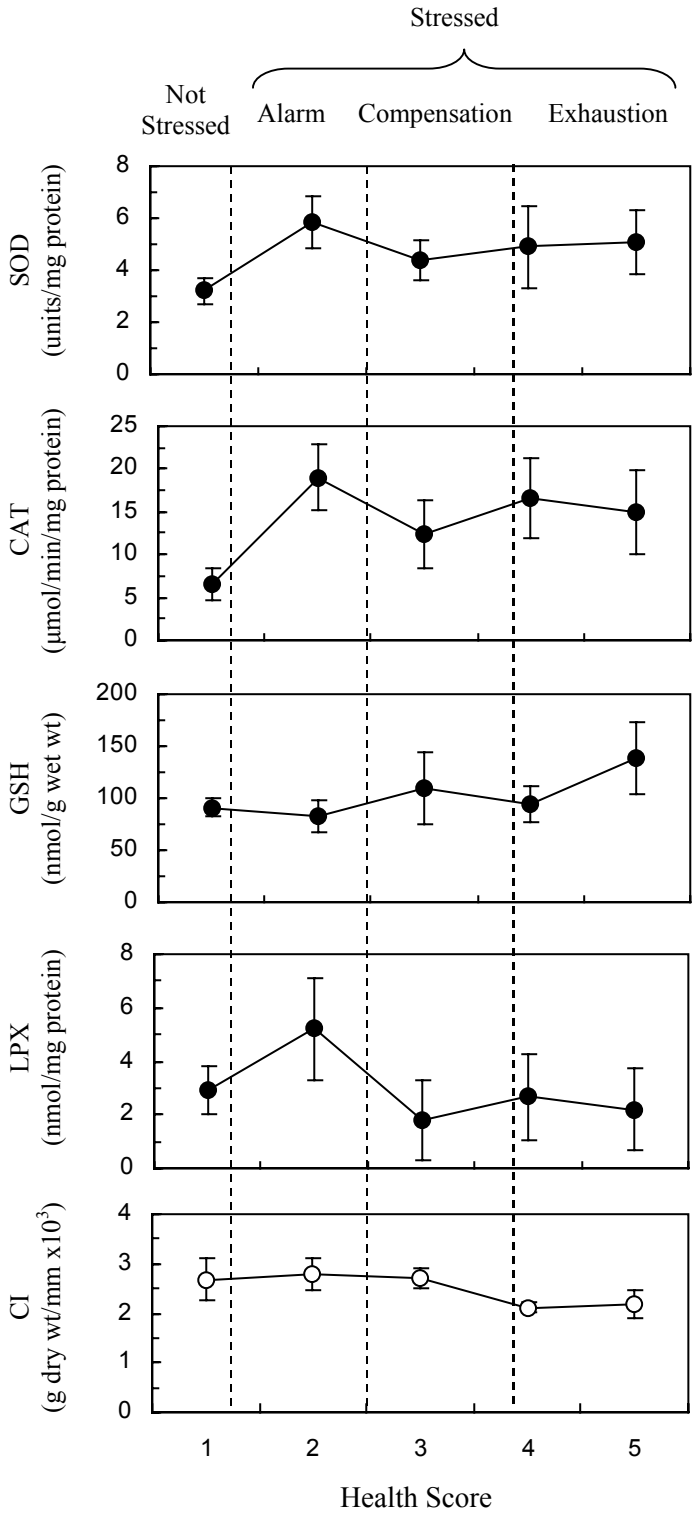

Figure 4.5. Relationships identified by discriminate analysis for cellular (-●-) and physiological (-○-) effects in clams exposed *in situ* to turf contaminants to health scores that reflect the stages of Seylean stress. Effects data for discriminate groups are means ± SD.

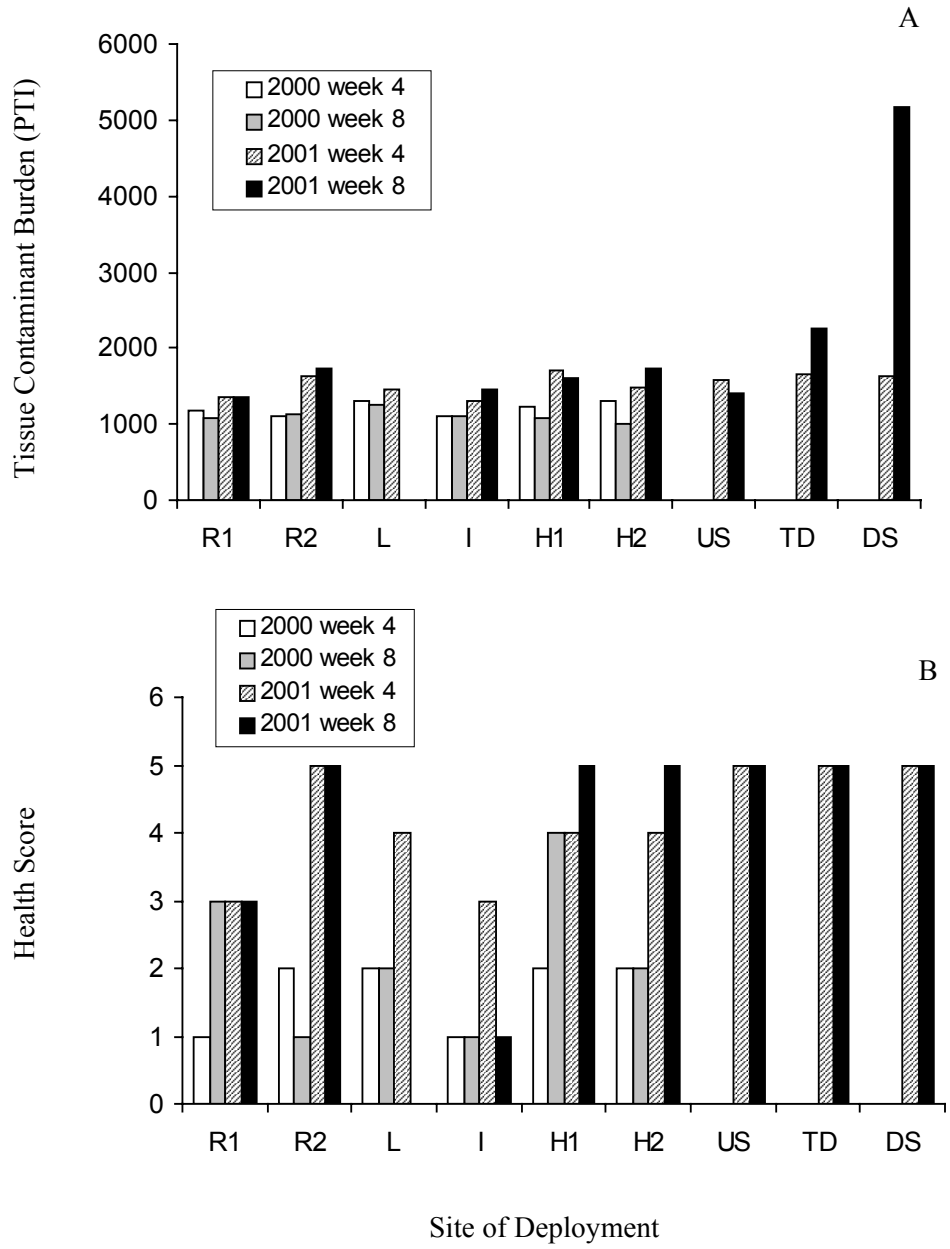

Figure 4.6. Tissue contaminant burdens (A) and health scores (B) of clams deployed in streams draining residential lawns (R1, R2, L, I, H1 and H2) or a golf course (US, TD and DS). Health scores reflect discriminate groups and tissue contaminant burdens are median concentrations of chemicals expressed relative to the Pesticide Toxicity Index (PTI) (Munn and Gilliom 2001).

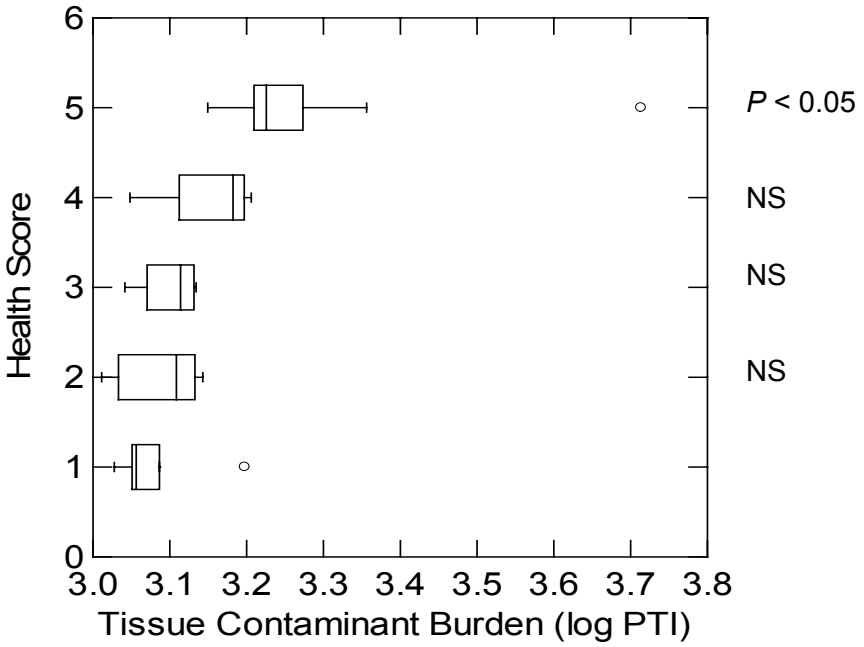

Figure 4.7. Box plot depicting the relationship between tissue contaminant burdens and health scores in clams exposed *in situ* to turf contaminants. For symbols, the center vertical line marks the median of the sample data, the edges of the box mark the first and third quartiles and the error bars mark the range of the data within 1.5 times of the interquartile spread. Values outside of the error bars (> 3 times the interquartile spread) are denoted by empty circles. Differences from health group one are noted by a NS for not significant, or if significant $P < 0.05$.

CHAPTER 5

RESPONSES OF OXIDATIVE STRESS DURING THE CHRONIC EXPOSURE AND RECOVERY OF FRESHWATER CLAMS (*CORBICULA FLUMINEA*) TO A COMPLEX MIXTURE OF TURF CARE CHEMICALS [1]

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Abstract. Many chemicals, including fertilizers, herbicides, insecticides and fungicides, are routinely applied to turf in the care and maintenance of lawns, and have the potential to leach into nearby aquatic systems and adversely affect non-target organisms. This study evaluated oxidative stress in freshwater clams (*Corbicula fluminea*) chronically exposed to a complex mixture of chemicals used in turf care. Components of the mixture included trifluralin and pendimethalin (dinitroaniline herbicides), chlorpyrifos (an organophosphate insecticide), 4-OH-chlorothalonil (a degradation product of the fungicide chlorothalonil) and the metals arsenic, copper and zinc. Clams were exposed to the mixture at low environmentally realistic concentrations and at high concentrations for 21 days in a static-renewal system, then were depurated for an additional 14 days. Antioxidant enzymes (superoxide dismutase and catalase), an antioxidant scavenger (glutathione), measures of oxidative damage (lipid peroxidation and DNA single-strand breaks) and an indicator of physiological health (condition index) were evaluated in clams at days 0, 2, 7, 14, 21 and 35 of the experiment. Glutathione concentrations were significantly elevated in the digestive glands of clams from the high exposure treatment at 14 and 21 days. Glutathione concentrations returned to control levels after depuration. No effects on glutathione were observed in clams from the low exposure treatment. Measures of lipid peroxidation and DNA single-strand breaks were significantly elevated in digestive glands of clams exposed to both the low and high mixture of turf care chemicals, but were not different from controls after depuration. No effects on condition indices were observed. These data indicate that combinations of turf care contaminants may cause reversible cellular damage in freshwater clams at realistic concentrations, and highlight the utility of biomarkers of oxidative stress for evaluating the sublethal toxicity of complex mixtures.

Introduction

Many chemicals, including fertilizers, herbicides, insecticides and fungicides, are routinely applied to turf in the care and maintenance of lawns. In the United States, pesticide use on homes, lawns and gardens increased between 13 and 20 % from 1995 to 1999, and during these years an average of 60

million kilograms of active ingredient were applied annually (Donaldson et al. 2002). Pesticides may leach from application sites during rainfall and travel as dissolved forms or bound to particles into nearby aquatic systems via surface water runoff or groundwater recharge (Richards and Baker 1993). Turf care pesticides have been detected frequently in streams draining urbanized watersheds throughout the United States (Larson et al. 1999). Assessments of the risks associated with the exposure of non-target freshwater organisms to turf care chemicals are complicated by a paucity of information on how realistic combinations of multiple chemicals interact to produce adverse effects.

Chemicals within mixtures may interact to produce additive, antagonistic or synergistic toxic effects. Toxicity assessment of chemical mixtures is a very active area of research. Often, the toxicity of multiple chemical mixtures is described by a bottom-up approach through the use of concentration addition models for chemicals with similar modes of action or response addition models for chemicals with different modes of action (Faust et al. 2000). Recent guidelines for chemical mixture research outline how data on complex mixtures of immediate concern provide the most conclusive evidence for risk assessments (US EPA 2000). Such a top-down approach to mixture toxicity has been successfully applied to study the risks associated with the atmospheric deposition of pesticides on aquatic systems (George et al. 2003).

The purpose of this study was to evaluate oxidative stress responses in freshwater clams (*Corbicula fluminea*) chronically exposed to a complex mixture of turf care chemicals. In previous research we observed that clams deployed in streams draining residential lawns or a golf course had elevated concentrations of persistent pesticides in their tissues (Conners et al. Chapter 4). These contaminants of concern were trifluralin and pendimethalin (dinitroaniline herbicides), chlorpyrifos (an organophosphate insecticide), 4-OH-chlorothalonil (a degradation product of the fungicide chlorothalonil) and the metals arsenic, copper and zinc. Furthermore, concentrations of these chemicals in deployed clam tissues were related to measures of oxidative stress. A variety of xenobiotics may cause damage in aquatic organisms by increasing levels of harmful oxygen radicals, and biomarkers of oxidative stress have been proposed to represent sensitive and useful mechanistic tools for examining stress in biota

during sublethal exposures to complex mixtures of contaminants (Winston and Di Giulio 1991, Livingstone 2001). Antioxidant enzymes (superoxide dismutase and catalase), an antioxidant scavenger (glutathione), measures of oxidative damage (lipid peroxidation and DNA single-strand breaks) and an indicator of physiological health (condition index) were evaluated in clams that were chronically exposed to turf care chemicals for 21 days, then depurated for 14 days. These data were used to determine if oxidative stress may be induced by the turf contaminants of concern at realistic exposure concentrations, and to investigate if oxidative stress is readily reversible when exposures are terminated. Reversibility of biomarker responses during depuration is important for understanding the relationship between molecular perturbations and relevant biological and ecological damage (Depledge 1999). Furthermore, studies of the recovery of aquatic organisms to pesticide exposures are highly relevant to predicting adverse effects in the field because organisms are often exposed to pulses of pesticides that are initiated by leaching of chemicals from application sites during rain (Naddy et al. 2000).

Materials and Methods

Exposures to turf care chemicals

Clams of a uniform size (14.9 ± 4.2 mm length) were collected from a headwater stream that drains forests and rural lands (Hannah Creek, Danielsville, GA, USA). Clams were transported to the laboratory (30 min) in aerated site water, and were held for approximately two weeks prior to exposures in a large flow-through system (265 L) containing aerated and dechlorinated soft tap water. During holding clams were fed a manufactured diet for filter-feeders (30 mg Microfeast® L-10 Larval Diet) supplemented with algae (7.5 mg Algae-feast® *Spirulina* microfine dry powder per clam) every day.

Clams were exposed for 21 days to a complex mixture of turf chemicals in glass aquaria containing 10 L aerated and dechlorinated soft tap water (60 clams per aquarium). Exposure concentrations were equivalent to geometric means of annually observed maximum water concentrations among seven streams draining residential lawns or a golf course (Kevin L. Armbrust, unpublished data).

Treatments included a control (dechlorinated tap water), a vehicle control (0.001 % acetone), a low exposure concentration (observed stream values) and a high exposure concentration (10 times the concentration of observed stream values) (n = 4 aquariums per treatment) (Table 5.1). Stock solutions of organic pesticides were prepared from technical grade chemicals (> 99 % purity). Metal stock solutions were prepared from technical grade arsenic pentoxide, copper sulfate and zinc chloride according to their free ion concentrations (> 98 % purity). Water in aquaria was renewed three times per week. On the same day but prior to water changes, water quality measures of temperature, dissolved oxygen, pH and conductivity were collected with handheld probes, and ammonia-nitrogen was monitored with Nessler's reagent by a Lamotte® titration kit (Model AQ-2). After water changes, clams were fed a mixture of protein powder and green algae (5 mg Microfeast® L-10 Larval Diet and 1.25 mg Algae-feast® *Spirulina* microfine dry powder per clam). The use of reduced food concentrations during exposures was necessary to keep total ammonia levels below detection limits (< 0.2 ppm). After 21 days of exposure, clams were depurated in clean water for an additional 14 days. Clams were sampled at a variety of time points during the experiment (0, 2, 7, 14, 21 and 35 days) for measures of cellular and physiological health.

Measurements of oxidative stress and physiological health

Digestive glands were dissected from clams for analyses of biomarkers of oxidative stress. DNA single-strand breaks were measured on fresh tissues, and other tissues were frozen in liquid nitrogen and stored at –80 ^{o}C until assayed. Biomarkers were analyzed on tissues from two individual clams per aquarium i.e., a total of eight clams were analyzed per treatment at each exposure time.

Total superoxide dismutase (SOD) activities were measured by pyrogallol auto-oxidation as described originally by Marklund and Marklund (1974), but modified for measurements of all SOD forms (Cu-Zn SOD and Mn SOD) (Zidenberg-Cherr et al. 1989). Briefly, tissues were homogenized in 50 mM potassium phosphate buffer (pH 7.0) and combined with three parts SOD buffer (pH 8.2, containing 50 mM N-tris[Hydroxymethyl]methyl-3-aminopropanesulfonic acid and 1 mM diethylenetriamine pentaacetic acid). Samples were then centrifuged (14,000 rpm, 4 ^{o}C, 5 min) and the supernatant (150 µL)

was combined with 770 μL SOD buffer and 80 μL 4 mM pyrogallol. Changes in sample absorbency were monitored spectrophotometrically at 320 nm every 30 s for a total of 120 s, and activities were expressed as units SOD per mg protein. One unit of SOD activity was defined as the amount of enzyme that inhibits the auto-oxidation of pyrogallol by 50 %.

Catalase (CAT) activities were measured by the decomposition of H_2O_2 as described by Clairborn (1985). Tissues were homogenized in 50 mM potassium phosphate buffer (pH 7.0), and a subsample of the homogenate (100 μL) was combined with 20 μL 5 % Triton X-100. Samples were then centrifuged (14,000 rpm, 4 ^oC, 5 min) and the supernatant (8 μL) was combined with 800 μL 19 mM H_2O_2. Changes in sample absorbency were monitored spectrophotometrically at 240 nm every 30 s for a total of 120 s, and activities were expressed as umol H_2O_2 consumed per min per mg protein.

Total glutathione concentrations were quantified by the glutathione reductase recycling assay (Anderson 1985). Tissues were homogenized in 5 % sulfoslicyclic acid and centrifuged (14,000 rpm, 4 ^oC, 5 min). A subsample of the supernatant (25 μL) was added to sodium phosphate buffer (143 mM, pH 7.5) for a final volume of 1 mL, which contained 200 μM β-NADPH and 1 mM 5,5'-dithio-bis(2-nitrobenzoic acid). Samples were then vortexed and warmed to 30 ^oC in a water bath for 10 min. Glutathione reductase (15 μL at 50 units/mL) was added to initiate the enzymatic reaction and the rate of 5-thionitrobenzoic acid formation was monitored spectrophotometrically at 412 nm every 30 s for a total of 120 s. Standards were prepared from reduced glutathione, and concentrations were expressed as nmol per g wet weight.

Lipid peroxidation was quantified by the thiobarbituric acid assay for malondialdehyde (MDA) concentrations (Aust 1985). Tissues were homogenized in 50 mM potassium phosphate buffer (pH 7.0) and centrifuged (14,000 rpm, 4 ^oC, 5 min). A subsample of the supernatant (71 μL) was combined with 1 mL 15 % trichloroacetic acid containing thiobarbituric acid (0.375 %) and 10 μL butylated hydroxytoluene (20 mg/mL in absolute alcohol). Samples were then heated (100 ^oC, 15 min) in a boiling water bath and centrifuged (14,000 rpm, 20 ^oC, 5 min) to remove precipitates. Malondialdehyde

concentrations were measured spectrophotometrically at 532 nm. Standards were prepared as described by Csallany et al. (1984), and the results were expressed as nmol per mg protein.

Protein concentrations of supernatants were used for determinations of superoxide dismutase, catalase and lipid peroxidation. Proteins were quantified by the use of a Bio-Rad™ protein assay kit based on the method of Bradford (1976). Samples (25 μL) were combined with 1 mL diluted dye reagent (Coomassie Brilliant Blue G-250 diluted 1:4 with distilled water), vortexed and incubated for 5 min in the dark. Protein concentrations were determined spectrophotometrically at 595 nm with bovine serum albumin used for standards.

DNA single-strand breaks were measured with the Comet assay described by Steinert (1996), but modified to account for reduced osmolality of hemolymph in freshwater bivalves. Pieces of digestive gland were suspended in 490 μL of an osmotically modified Ca^{2+}/Mg^{2+}-free Hanks Balanced Salt Solution (HBSS) containing 4.2 mM $NaHCO_3$, 26.2 mM NaCl, 1.3 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4 and 5.55 mM D-glucose (pH 7.3). Proteinase K was added (10 μL at 10 mg/mL) and tissues were crushed gently with a hand-held, ground-glass tissue homogenizer (i.e., three light pressured turns with a homogenizer having a large clearance between pestle and tube of 0.09 to 0.16 mm). Cell suspensions were filtered to remove large debris (100 μm nylon mesh), centrifuged (2000 rpm, 5 min, 10 ^{o}C) and resuspended in 500 μL 0.65 % low melting agarose (made with HBSS, melted in a microwave then cooled to a constant temperature of 37 ^{o}C). Samples (50 μL) were transferred to microscope slides pre-coated with 1 % normal melting agarose (made with 40 mM Tris-acetate EDTA) and cells were spread out by the use of cover-slips. Slides were then solidified (5 min at 4 ^{o}C), cover-slips were removed and cells were top-coated with 50 μL 0.65 % low melting agarose. Once solidified, slides were transferred to light-protected coplin jars containing cold lysis buffer (10 mM Tris-HCl, 2.5 M NaCl, 100 mM EDTA, 10 % DMSO and 1 % Triton X-100, pH = 10.0), and incubated at 4 ^{o}C overnight. To prevent confounding DNA damage from ultraviolet-light, samples were light-protected during all subsequent steps. Slides were removed from lysis buffer, rinsed three times in cold water and transferred to a

submarine gel electrophoresis chamber containing cold DNA unwinding buffer (300 mM NaOH, 1 mM EDTA, pH = 13.1). DNA was allowed to unwind for 15 min and samples were electrophoresed at 25 V, ~300 mA for 10 min. After electrophoresis, samples were transferred to coplin jars and neutralized by rinsing in 400 mM Tris (pH 7.5), 3 times for 2 min. Samples were then immersed in cold 100 % ethanol for 5 min, air dried and stored in a dessicator until analyzed. For analyses, slides were stained with 50 μL ethidium bromide (20 μg/mL) and viewed under epifluorescent microscopy (200X magnification, 510-560 nm excitation filter, 590 nm barrier filter). DNA damage results in increased DNA migration away from individual cells and produces a characteristic comet shape. DNA damage was quantified by measuring tail moment (product of % DNA in comet tail and length of tail) with a Loats Image Analysis System®. Thirty cells were scored per slide, and geometric means were used to describe the damage because distributions of tail moments among cells on a slide were skewed.

Condition index is a ratio of tissue biomass to the capacity of a shell for supporting tissue production, and high values reflect that bivalves are in a good physiological health (Lawrence and Scott 1982). Whole soft tissues from clams were dissected, dried overnight (100 ^{o}C), dessicated (24 h) and weighed. Condition index values were measured on three individual clams per aquarium, and were expressed as g dry tissue weight per mm shell length.

Statistical analyses

All data are means ± standard deviations (SD) (n = 4 aquaria per treatment). Data on individual clams from each aquarium were averaged prior to obtaining treatment means. Data were checked for normality and homogeneity of variances prior to statistical testing, and were transformed if necessary. Measures of oxidative stress in clams exposed to the vehicle (0.001 % acetone) were not significantly different from control clams ($P > 0.05$); hence the data were combined for statistical (i.e., evaluation of treatment effects) and illustrative purposes. All data were auto-correlated, and were evaluated with a repeated measures analysis of variance (ANOVA) ($P < 0.05$). If significant differences were detected

among treatments or exposure times, Bonferroni's multiple comparison test was used to determine whether treatments differed significantly from controls and whether values after depuration differed from the preceding exposure time of 21 days (alpha of 0.05 adjusted for the appropriate number of planned comparisons). Statistical tests were run with SYSTAT® software, version 9.0 (SPSS Inc.) or Microsoft Excel spreadsheets.

Results

Water quality in aquaria during the experiment averaged 21.4 ± 0.8 ^{o}C for temperature, 8.54 ± 0.46 mg/L for dissolved oxygen, 7.28 ± 0.20 for pH and 124.2 ± 15.2 μs for conductivity. Total ammonia-nitrogen levels in aquaria water remained below the limit of detection throughout the experiment (< 0.2 ppm). The majority of clams were actively filtering during exposures. Very few clams died during the experiment (< 0.8 % mortality), and dead clams were removed immediately from aquaria.

Among the antioxidants measured, only glutathione concentrations were significantly altered in clams exposed to the mixture of turf care chemicals (Figure 5.1). Glutathione concentrations were significantly elevated in clams from the high exposure treatment at 14 and 21 days. After depuration glutathione concentrations in exposed clams returned to control levels. No effects on glutathione were observed in clams from the low exposure treatment. While not significant, superoxide dismutase activities in clams tended to increase over 7, 14 and 21 days of exposure to the high chemical mixture ($P < 0.1$). Likewise, trends approaching significance were observed for catalase activities in clams; the enzyme was elevated in the low exposure treatment at 21 days, and in the high exposure treatment at 14 and 21 days ($P < 0.1$).

Cellular indices of oxidative damage were significantly elevated in clams exposed to the mixture of turf care chemicals (Figure 5.2). Lipid peroxidation was elevated in clams from the low exposure treatment at 14 and 21 days, whereas values were only elevated in clams from the high exposure treatment at 21 days. After depuration, lipid peroxidation in clams exposed to the mixture of turf

chemicals was not significantly different from controls, largely because of an increase in control values at this time. DNA single-strand breaks were elevated in clams from the low exposure treatment only at 7 days; by 14 and 21 days of exposure values had declined to control levels. Transient increases in DNA single-strand breaks were also observed in clams from the high exposure treatment. At 14 days of exposure, DNA single-strand breaks in clams from the high exposure treatment were 3.8 fold greater than control clams. By 21 days of exposure, values in these exposed clams substantially decreased (0.26 ± 0.17 tail moment), yet were still significantly different from control clams (0.05 ± 0.02 tail moment). Data on DNA single-strand breaks in clams from the high exposure treatment at day 7 were not obtained because of a power surge from a lightning strike that ruined gels during electrophoresis. Condition indices of clams were not significantly different among treatments or through time, although there was a tendency for all values to be lower at day 35 of the experiment.

Discussion

A variety of xenobiotics may cause damage in aquatic organisms by increasing levels of harmful oxygen radicals via processes of redox cycling, disrupting electron transport, inducing enzyme systems such as cytochrome P450s that mediate oxidation reactions or by depleting levels of protective antioxidants (Winston and Di Giulio 1991, Livingstone 2001). Many of the turf care contaminants evaluated in this study for their toxicity to freshwater clams are known inducers of oxidative stress. Copper is often applied to lawns as an essential metal in fertilizers, and is widely known to produce oxidative stress in bivalves (Viarengo et al. 1990, Doyette et al. 1997, Regoli et al. 1998, Conners and Ringwood 2000, Geret et al. 2002). Copper readily interacts with hydrogen peroxide via Harber-Weiss and Fenton-like metal catalyzed reactions to produce highly toxic hydroxyl radicals, which may indiscriminately bind to and damage membranes, proteins and DNA. Liu et al. (2001) suggest that arsenic may also cause cytotoxicity in mammals by reacting with hydrogen peroxide to produce hydroxyl radicals. The widespread use of arsenic as a fungicide to preserve wood in residential settings has

recently been phased out in the United States (US EPA 2003). While zinc is not a potent inducer of oxidative stress in bivalves (Viarengo et al. 1990), this metal could potentially enhance oxyradical production by depleting the antioxidant glutathione. Transient decreases in glutathione were observed in mice injected with zinc chloride (Kawata and Suzuki 1983). Alternatively, zinc may act antagonistically to reduce oxidative stress by displacing redox active metals from site-specific loci of oxyradical production and damage (Stohs and Bagchi 1995). Like copper, zinc is widely applied to lawns as an essential metal in fertilizers.

Few organic pesticides have been evaluated for their ability to produce oxidative stress in bivalves. Oxidative stress has been observed in bivalves exposed to the bipyridyl herbicide paraquat (Wenning et al. 1988), the organophospate insecticide fenitrothion (Peña-Llopis et al. 2002) and the dithiocarbamate fungicide thiram (Doyette et al. 1997). Research on other organisms however supports the concept that a wide variety of pesticides may induce oxidative stress in eukaryotes (reviewed by Rakitsky et al. 2000, Kovacic and Jacintho 2001). Bagchi et al. (1995) observed that various pesticides dissimilar in structure (e.g., chlorinated hydrocarbons and acetamides, organophosphates) could enhance oxyradical production, and induce lipid peroxidation and DNA single-strand breaks in rats. Prakasam et al. (2001) observed that humans working as agricultural pesticide sprayers had altered antioxidants and increased products of lipid peroxidation in their blood.

Of the organic pesticides evaluated in this study, there is substantial evidence implicating chlorpyrifos and chlorothalonil as inducers of oxidative stress. Oxidative stress has been observed in rats exposed to chlorpyrifos (Bachi et al. 1995, Bebe and Panemangalore 2003), and additions of antioxidants (e.g., vitamins E and C) reduced the cytotoxicity of this pesticide (Gultekin et al. 2001). In amphipods, chlorpyrifos inhibited glutathione-S-transferase activity (Steevens and Benson 1999). Chlorpyrifos was recently phased out of use for domestic applications in the United States (US EPA 2001). Chlorothalonil is a widely used fungicide that is metabolized by glutathione-S-transferase to glutathione conjugates that are exported from cells (Rosner et al. 1996). Catfish exposed to sublethal concentrations of chlorothalonil (13 μg/L) for 72 hours had elevated tissue glutathione levels (Gallagher et al. 1992a). Elevated

glutathione concentrations in chlorothalonil exposed organisms presumably occur via upregulation of the rate-limiting enzyme responsible for glutathione synthesis, γ-glutamylcysteine synthetase. Experimental inhibition of γ-glutamylcysteine synthetase by buthionine sulfoximine potentiated the cytotoxicity of chlorothalonil in catfish (Gallagher et al. 1992b) and striped bass (Baier-Anderson and Anderson 2000). No research that we are aware of has investigated the ability of the degradation product 4-OH-chlorothalonil to produce oxidative stress, but its structural similarity to the parent fungicide suggests that it too may form glutathione conjugates. Many xenobiotic metabolites with hydroxyl functional groups are further detoxified by conjugation to glutathione, glucuronides or sulfates.

Direct evidence of the ability of the dinitroaniline herbicides used in this study to produce oxidative stress is lacking, but indirect evidence is suggestive of toxicity via oxyradical production. Trifluralin is metabolized by cytochrome P450s in green sunfish (Reinbold and Metcalf 1976), so the potential for oxyradical production exists; however, bivalves have a low ability to induce this biotransformation enzyme (Livingstone 1998). More importantly, dinitroaniline herbicides degrade into nitrobenzimidazoles (reviewed by Harris et al. 2000), and these compounds may undergo redox cycling to produce toxic superoxide anions prior to their further metabolism by DT-diaphorase (Šarlauskas et al. 1997). The reductive metabolism of similar nitroaromatic chemicals is known to result in the formation of oxyradical production in bivalves (Hetherington et al. 1996).

When present in mixtures, pesticides and metals may interact to produce toxicity by similar or dissimilar mechanisms of action. In this study, we observed that a realistic combination of turf care contaminants produced oxidative stress in bivalves. As the individual components of the mixture have been observed to produce oxidative stress in other organisms, these results are suggestive of toxicity occurring via a common mechanism of action, albeit these data cannot distinguish whether oxyradical production is the primary mechanism driving responses or merely a byproduct of nonspecific cell and tissue injury. Glutathione concentrations were significantly elevated in exposed clams presumably because of an upregulation of the rate-limiting enzyme responsible for glutathione synthesis, γ-glutamylcysteine synthetase. Antioxidant enzyme activities, while not significant, were also elevated in

exposed bivalves. Other research has noted that glutathione is sensitive and often the first responder to stress induced by metals (Singhal et al. 1987, Conners and Ringwood 2000, Zaroogian and Norwood 2002) or by aerobic transitions from hypoxic to oxic states (Pannuzio and Storey 1998, Conners and Black Chapter 3). Alterations in antioxidants were only observed in bivalves from the high exposure treatment, in which concentrations of contaminants are less likely to occur in nature. However, the low exposure treatment was highly realistic of contaminant concentrations in surface waters, and did increase measures of oxidative damage in bivalves. Lipid peroxidation was elevated in bivalves exposed to the low exposure treatment at 14 and 21 days. DNA single-strand breaks were observed transiently in bivalves exposed to the low exposure treatment. Transitory increases in DNA strand breaks have been observed in bivalves exposed to benzo[a]pyrene (Ching et al. 2001). Decreases of oxidative damage in xenobiotic-exposed bivalves after a period of elevation presumably result from the induction of primary antioxidant defenses that reduce oxyradical concentrations and the induction of secondary antioxidant defenses (e.g., DNA repair enzymes) that remove oxidative damage (Conners et al. Chapter 2). Also, reductions in oxidative damage may occur from the induction of detoxification enzymes that lower internal xenobiotic concentrations at the site of oxyradical generation.

Oxidative stress may affect the health of bivalves by impairing growth (Couillard et al. 1995), reproduction (Ringwood and Conners 2000) and immunological competency (Fisher et al. 2000). We did not observe impairments of physiological health in this study, i.e., conditions indices of clams were not altered by exposures to the turf care chemical mixture. In relation to concept of Selyean stress (reviewed by Newman 1995), clams in this study were likely in the compensation phase versus the initial alarm phase or the latter exhaustion phase of stress, as defense mechanisms (glutathione) were increasing and cellular damage (DNA single-strand breaks) was decreasing at the longest time of exposure. Effects on physiological health are probably more likely to occur when organisms enter a stage of exhaustion. The removal of contaminants reversed the occurrence of oxidative stress in clams. Biomarkers that are early warning indicators of reversible xenobiotic-induced stress would represent advantageous tools for use in

proactive environmental management activities that aim to prevent irreversible ecological damage (Ringwood et al. 1999).

In conclusion, these data indicate that combinations of turf care contaminants may cause reversible cellular damage in freshwater clams at realistic surface water concentrations. Furthermore, these data highlight the utility of biomarkers of oxidative stress for evaluating the sublethal toxicity of complex mixtures.

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Table 5.1. Nominal concentrations of chemicals composing the complex mixture of turf care contaminants that clams were exposed to for 21 days.

	Exposure Treatments (µg/L) [1]		
Chemical	Low	High	Water Quality Criteria (µg/L)
Arsenic	1.16	11.6	150[2]
Copper	3.21	32.1	4[2,3]
Zinc	7.19	71.9	57[2,3]
Trifluralin	0.00384	0.0384	0.20[4]
Pendimethalin	0.00384	0.0384	---
Chlorpyrifos	0.00593	0.0593	0.0041[2]
4-OH-Chlorothalonil	0.05804	0.5804	0.18[4,5]

[1] Low exposure concentrations represent geometric means of observed annual maximum water concentrations among seven streams draining residential lawns and a golf course, high exposure concentrations are 10 times the low exposure concentrations

[2] Values are national recommended water quality criteria for the United States (US EPA 1999), and numbers represent the Criteria Continuous Concentration (CCC) for the protection of freshwater aquatic life

[3] Metal criteria were corrected for the hardness of water used in this experiment (45 mg/L) as outlined by the US EPA (1999)

[4] Values are 2002 Canadian Environmental Quality Guidelines for the protection of freshwater aquatic life, and were used because data were not available for the United States (http://www.ccme.ca/publications/can_guidelines.html#110)

[5] Value is a surrogate derived from the water quality criteria for the parent compound, chlorothalonil

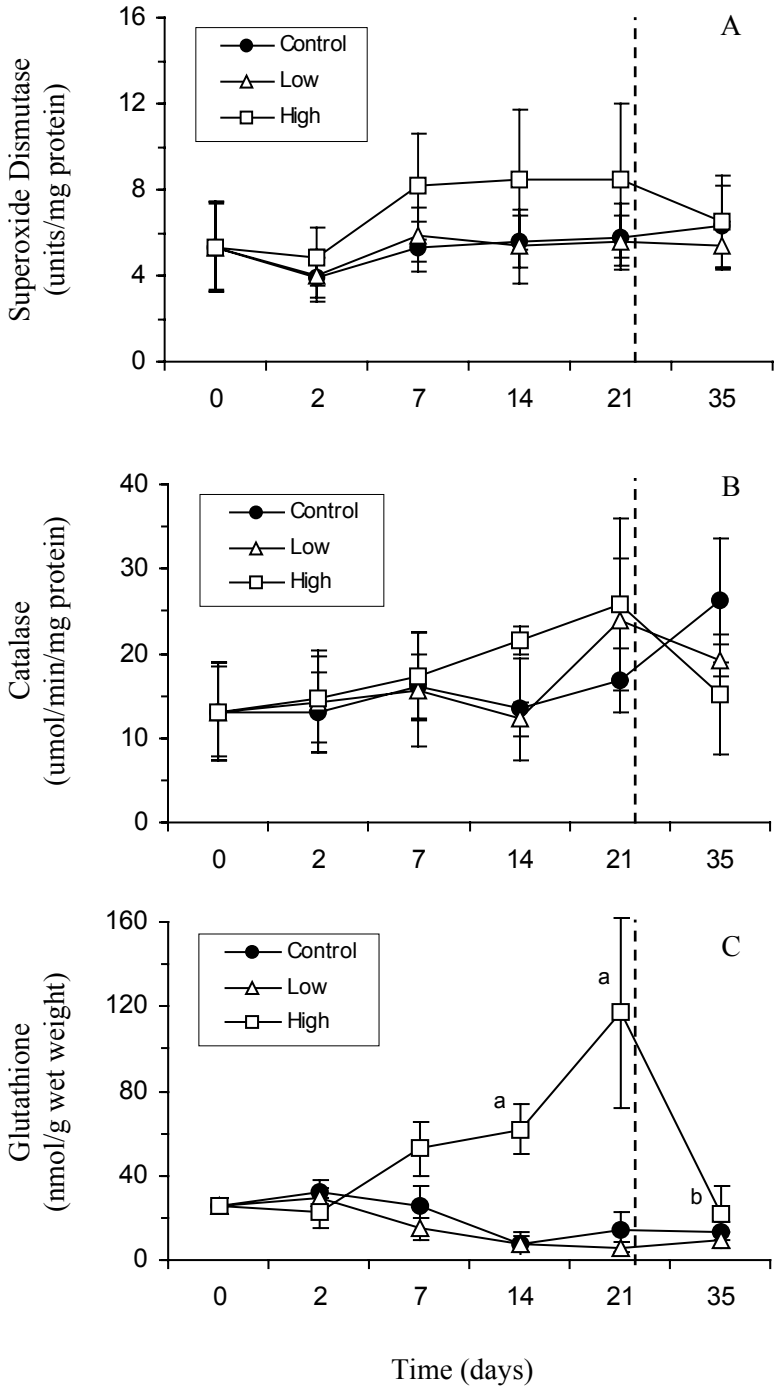

Figure 5.1. Responses of superoxide dismutase (A), catalase (B) and glutathione (C) in freshwater clams exposed to a complex mixture of turf care chemicals for 21 days, then depurated (---) for 14 days. Superscripts indicate significant differences from controls (a) or from preceding time point (b) ($P < 0.05$).

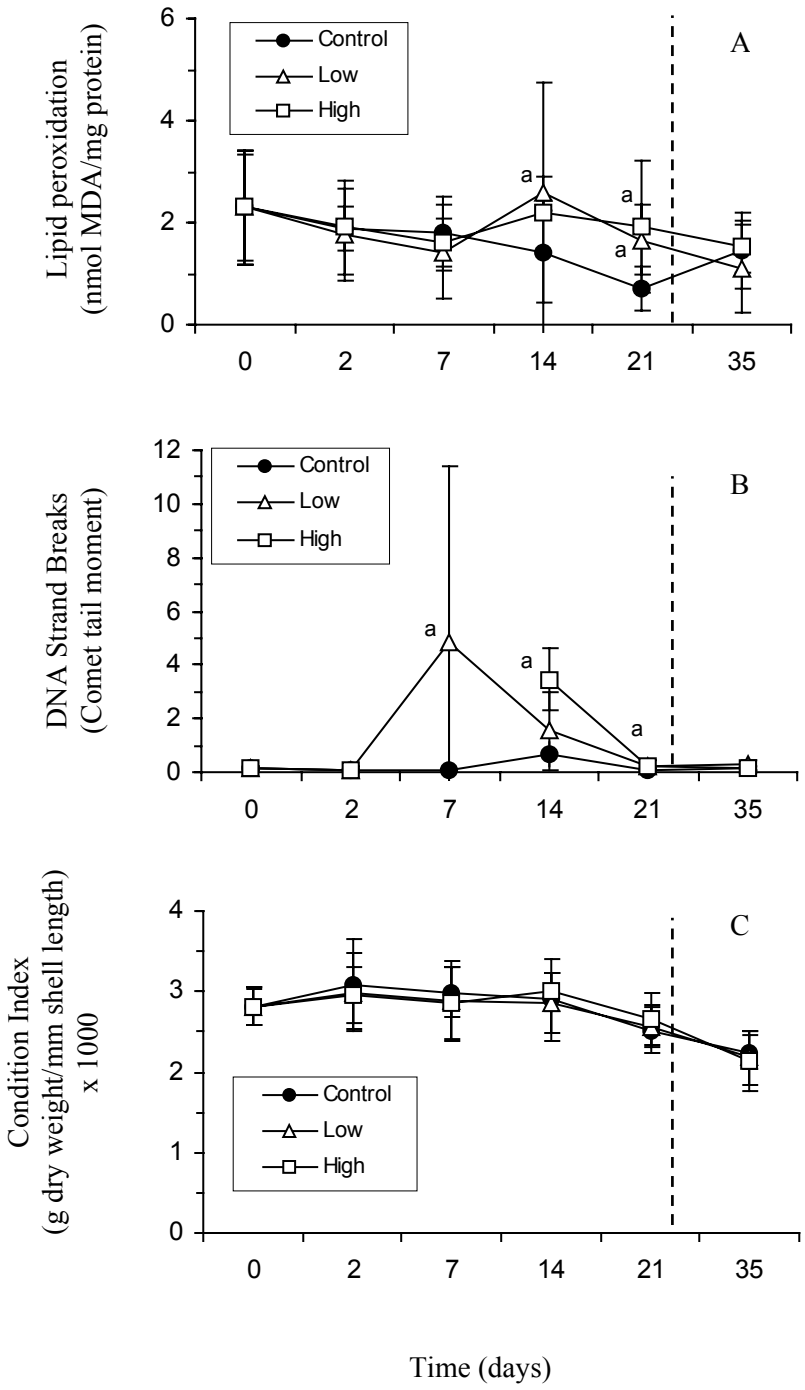

Figure 5.2. Responses of lipid peroxidation (A), DNA single-strand breaks (B) and condition index (C) in freshwater clams exposed to a complex mixture of turf care chemicals for 21 days, then depurated (---) for 14 days. Superscripts indicate significant differences from controls (a) ($P < 0.05$).

CHAPTER 6

CONCLUDING REMARKS

Protecting against water quality degradation by xenobiotics will be a priority for water resource managers worldwide during the 21st century (UNESCO 2003). Despite the recent improvements achieved through reducing concentrations of contaminants in surface waters, xenobiotics continue to pose threats to human and ecosystem health. Such threats arise because we cannot entirely eliminate accidental releases, new chemicals of unknown risks are developed daily for use in areas such as agriculture and medicine and chemicals with recalcitrant properties continue to persist in the environment regardless of restrictions on their use. Furthermore, new scientific advances in ecotoxicology are now enabling us to observe deleterious effects of chronic, low level contamination on aquatic communities that previously were undetectable. Current legislation designed to protect aquatic life from xenobiotics is dominated by laboratory-based, survivorship-oriented approaches to data collection. However, some countries like Canada and France are taking important initiatives to incorporate both sublethal measures of toxicity (e.g., molecular and physiological responses, life-history endpoints) and field-based biological assessments into usable and realistic information for water resource managers.

The studies contained within this dissertation investigated the use of oxidative stress biomarkers in freshwater clams as mechanistic tools to evaluate the impairment of stream ecosystem health by lawn care pesticides. Bivalves deployed in streams draining residential lawns and a golf course exhibited signs of oxidative stress, and an integrated bioeffects model indicated that turf contaminants contributed significantly to the etiology of adverse responses. This finding was confirmed in a subsequent laboratory study where realistic concentrations of turf contaminants induced oxidative stress in clams. However, oxidative stress in laboratory-exposed clams was not as severe as that observed in field-exposed clams.

These results suggest that turf contaminants contributed to, but were not solely responsible for, the adverse responses observed in clams from suburban and urban streams. Likely, other contaminants co-occurring in streams contributed to the adverse effects on clam health.

In addition to pesticides, watershed urbanization can increase the loading of other chemicals to streams (e.g., metals, petroleum hydrocarbons), and urbanization may also alter hydrological and geomorphological processes (Paul and Meyer 2001). Notably, Herbert (2003) observed that a hydrological alteration (e.g., increased velocity) was the primary stressor affecting leaf degradation rates in these residential streams. By on-going comparisons of our results on clams with those of project collaborators that measured community indices and ecosystem processes, we hope to identify what mechanisms are controlling perturbations in urbanized streams. Preliminary data are shown in Table 6.1 to illustrate how this may be accomplished (i.e., impairments to bivalve health would be indicative of a chemical etiology to stream degradation, while impairments to community indices and ecosystem processes integrate both chemically-mediated and habitat-related degradation mechanisms). At one residential stream (R2), only effects on bivalve health were observed possibly indicating that turf contaminants are present and bioavailable, but not at concentrations that will impair ecologically relevant stream functions. Conversely, at another residential stream (I), effects on bivalve health were not observed but community and ecosystem processes were impaired. These data may indicate that hydrological and geomorphological stressors are degrading this stream and affecting habitat quality. At the high property value streams (H1 and H2), effects on bivalve health and community and ecosystem processes were observed, possibly indicating (1) that turf contaminants are impacting ecologically relevant stream functions or (2) that multiple stressors are affecting water quality and habitat in the streams. Knowledge of what factors (e.g., chemical, hydrological, geomorphological) are impacting suburban and urban streams would benefit environmental managers whose goal is to protect these valuable natural resources.

The turf contaminants identified as problematic to freshwater clams in streams draining residential lawns and a golf course included pendimethalin and trifluralin (herbicides), chlorpyrifos

(insecticide), 4-OH-chlorothalonil (fungicide degradation product) and the metals arsenic, copper and zinc. These chemicals are often applied to turf for the care and maintenance of lawns and may have other residential uses. Chlorpyrifos was recently phased out of use as an insecticide for domestic applications in the United States (US EPA 2001), and so has the use of arsenic as a fungicide for preserving wood (US EPA 2003). Pendimethalin, trifluralin and chlorothalonil are still commonly used as pesticides, and copper and zinc continue to be added to fertilizers as essential metals. If these turf care chemicals are adversely affecting stream biota, several management options exist that may reduce their negative impact. Loading of turf chemicals to streams can be reduced through diverting storm water to retention ponds, increasing the width of riparian buffers and educating consumers against applying pesticides before rain. Additionally, reducing pesticide use via integrated pest management strategies or eliminating nonessential pesticide use would be valuable for protecting water quality in streams.

In conclusion, this research highlights the sensitivity and utility of applying suites of oxidative stress biomarkers in freshwater clams to assessments of stream ecosystem health. As was noted at a recent Bivalve Biomarker Workshop (Ringwood et al. 1999), it is time to develop strategies for incorporating biomarkers into ecological risk assessments that guide environmental management activities.

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Table 6.1. Impairments to stream ecosystem health as indicated by biomarkers of oxidative stress in clams, benthic invertebrate community structure and leaf degradation rates.

Residential Streams	Biomarkers of Oxidative Stress in Clams [1]	Benthic Invertebrate Community Structure [2]	Leaf Degradation Rates [3]
R1			
R2	X		
L			
I		X	X
H1	X	X	X
H2	X		X

[1] Symbols (X) indicate streams where oxidative stress was observed in deployed clams (Conners et al. Chapter 4)
[2] Symbols (X) indicate streams where indices of benthic community structure were poor (Jay Overmyer, The University of Georgia, personal communication)
[3] Symbols (X) indicate streams where reduced leaf degradation rates were observed (Herbert 2003)