EFFECTIVENESS OF OYSTER SPAT STICK COMMUNITIES IN REMOVING PHARMACEUTICAL CONTAMINANTS FROM TIDAL CREEKS

by

SCARLETT LOUISE FULLER

(Under the Direction of Marsha C. Black)

ABSTRACT

Pharmaceuticals are biologically active molecules, designed to affect biological processes at very low doses. Exposure to these compounds poses risks to both aquatic organisms and human health. Faulty septic tanks in coastal areas may release these chemicals into aquatic environments. Field and controlled laboratory studies explored the accumulation of five pharmaceuticals by oysters (*Crassostrea virginica*). Three sites in Coastal Georgia showed significant ($p < 0.05$) seasonal variation of pharmaceutical and lipid concentrations in oyster tissue. Controlled laboratory studies indicated a significant ($p < 0.05$) increase in uptake over 96 h exposure, as well as microbial interactions. The presence of an algal food source significantly ($p < 0.05$) decreased ibuprofen concentrations in oysters. This study confirms the ability of oysters to assimilate pharmaceuticals associated with human sewage pollution and may vary by seasonal fluctuations in lipid content.

INDEX WORDS: Pharmaceuticals, *Crassostrea virginica*, Septic tanks, Ibuprofen, Diphenhydramine

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DEDICATION

To my mom, who imparted a love of learning, a dare to dream and the will to achieve. And to my papa and granny, for all those bedtime stories.

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CHAPTER 1

INTRODUCTION

The detection of pharmaceutically active compounds (PhACs) in aquatic environments has created a need to study the fate and impact of these chemical compounds on aquatic organisms and human health as well as to determine effective removal methods from contaminated sites. In particular, sessile marine organisms like oysters (e.g., *Crassostrea virginica*, Gmelin, 1791) are vulnerable to constant, low dose exposure over their entire life cycle. In addition to aquatic toxicity, human health may be at risk of exposure from either consuming contaminated shellfish or from direct contact with contaminated waters. The research presented herein looks at five common pharmaceutical compounds (naproxen, ibuprofen, ethynylestradiol, triclosan and diphenhydramine HCl) and the effectiveness of using experimental oyster reefs to remove these chemicals from contaminated waters. The literature review in Chapter 2 discusses how human pharmaceuticals make their way into the environment as well as the detection, occurrence and fate of these chemicals in aquatic systems. Current research is reviewed and presented as it relates to target PhACs in aquatic environments; oysters (*C. virginica*) as remediation tools and biomonitoring species; and marine tidal creek systems' susceptibility to human sewage contamination via leaky/faulty septic systems. This literature review is followed by a statement of research goals, hypotheses and an outline of experimental approach.

Chapter 3 describes and evaluates the use of oyster spat stick communities in remediating tidal creeks in Coastal Georgia that are likely contaminated with septic waste. The implications of human pharmaceutical detection in oyster tissue are also discussed. In addition, a controlled laboratory study designed to determine effects of food and microbial presence on pharmaceutical uptake by oysters is described.

Chapter 4 reviews and summarizes the findings and conclusions for both field and controlled laboratory components of this study.

CHAPTER 2

LITERATURE REVIEW

Introduction

Human pharmaceuticals are biologically active molecules, designed to affect biological processes at very low doses. The consumption of pharmaceuticals is expected to increase in the future due to higher standards of living and increased life expectancies, which often require the use of more drugs (Kümmerer, 2010). Pharmaceuticals ingested by humans are not always completely metabolized by the body and are therefore excreted unchanged or as polar conjugates of the parent compound (Thomas, 2002). It is at this step that human pharmaceuticals make their journey to the environment. The risks of exposure of these low concentrations of biologically active compounds lies primarily with aquatic organisms in receiving waters due to the evolutionary conservation of pathways and receptor targets among different organisms. However, risks posed to humans should not be overlooked. Risks to human health may include exposures to endocrine-active compounds and hormones (Hannah et al., 2009), anticancer drugs which are toxic even at low doses and development of antibiotic resistant bacteria (Kümmerer, 2009; Christensen, 1998). The quantity of pharmaceuticals reaching surface waters can depend on metabolic and excretion rates in both humans and animals. Improper disposal of unwanted prescription drugs (e.g. down the drain) bypasses metabolic breakdown altogether, releasing chemicals directly into wastewater flows. Degredation abilities in the environment and in wastewater treatment plants (WWTP) also affect the quantity of

pharmaceuticals reaching surface waters. In rural coastal environments, septic systems are the most likely input source for human sewage. Just as pharmaceuticals are released from WWTP effluent, septic systems may release human PhACs into coastal aquatic environments. Oysters are a common, sessile aquatic species that are able to filter large quantities of water. Their filter feeding characteristics provide direct contact with contaminated waters and make them ideal as a sentinel species. Because of these characteristics oysters may also provide a bioremediation tool for estuaries contaminated by human pharmaceuticals.

Pharmaceutically Active Compounds (PhACs) in Aquatic Environments

The presence of PhACs in aquatic environments is an emerging field of environmental research (Daughton & Ternes, 1999; Halling-Sørensen et al., 1998; Thomas, 2002). Due to technological advances in analytical chemistry and better detection methods, researchers have been able to detect low levels (ng/L to μg/L) of PhACs in both surface and ground water (Daughton & Ternes, 1999). The effects of PhACs on the environment are only recently being investigated, and understanding risks associated with exposure is an issue of great complexity. PhACs can be introduced into the environment in a variety of ways (Figure 2.1). They may be introduced via industrial and sewage treatment effluent, leaking septic tanks, improper landfill disposal or runoff from land applied biosolids, although the major source seems to be effluent from sewage treatment plants (Thomas, 2002). Sewage treatment plants receive waste from a host of sources such as private homes, hospitals and industries which provides a constant supply of PhACs in wastewater. The manufacture of pharmaceuticals is a billion dollar industry

with the highest waste generation per mass of product produced for any commercial sector (Slator & Savelski, 2007). Because of this huge waste generation, pharmaceuticals may comprise a large amount of sewage received by treatment plants due to their manufacturing processes (Daughton & Ternes, 1999). Human pharmaceutical use and subsequent excretion further adds to the chemical composition of wastewater. Wastewater treatment plants and septic systems were not originally designed to treat or remove PhACs. They were primarily designed to deactivate harmful bacteria and viruses and to remove solids in wastewater before returning it back to the environment. Wastewater treatment plants often vary in design and level of treatment processes (primary, secondary, tertiary treatments). Sometimes infrastructure is old and failing, which may result in direct release of untreated sewage into the environment. Wastewater treatment differs from that of drinking water treatment in that generally, ozonation, activated carbon filtration and reverse osmosis are employed for additional purity in drinking water (Daughton & Ternes, 1999). These additional measures used in drinking water treatment are often adequate to remove most PhACs from raw water (Daughton & Ternes, 1999), although some studies have detected low nanogram per liter concentrations of pharmaceuticals in drinking water samples (Heberer et al., 1996; Ternes, 2000, 2001a,b; Ternes et al., 2002).

Human pharmaceuticals are often excreted unchanged or only slightly transformed as conjugated polar molecules (Thomas, 2002). The sewage treatment process can allow conjugates to be cleaved, thereby allowing the original molecule to be released back into the environment via wastewater effluent. Some PhACs are susceptible to microbial degradation or photodegradation while others persist in the environment.

Parent forms and metabolites alike may negatively impact aquatic organisms. The fate of PhACs during wastewater treatment have previously been described as falling into one of three categories including: (1) mineralization to carbon dioxide and water; (2) not readily degradable due to their lipophilic nature and therefore retained in the sludge; or (3) metabolized to a more hydrophilic but still persistent form of the parent lipophilic compound, which passes through the WWTP and ends up in receiving waters (Halling-Sørensen, et al., 1998). Treated effluents are discharged into rivers, large lakes and oceans and may contain forms that could affect resident aquatic organisms if the metabolites are biologically active.

Non-point sources of PhACs may include agricultural uses such as veterinary drugs, feed additives in livestock breeding or biologically deactivated sludge used as fertilizer. In rural areas, municipal sewer systems may not be available, making private septic systems and land applied sewage disposal methods necessary. Rainwater runoff from such sites can introduce PhACs directly into aquatic environments (Bloetscher & Plummer, 2011; Meyer et al., 2011; Pedersen et al., 2005; Topp et al., 2008). Figure 2.1 shows the various point and non-point sources for PhACs to be introduced into an aquatic environment.

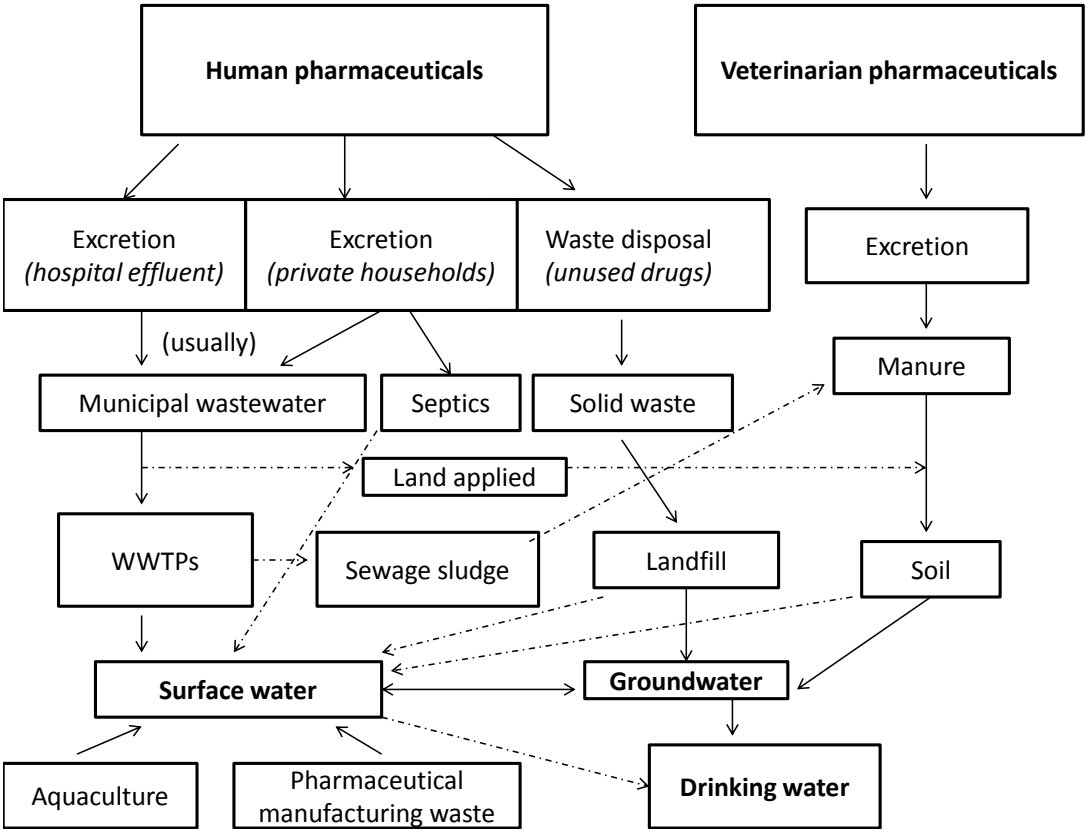

Figure 2.1 Possible point and non-point sources for PhACs to be introduced into an aquatic environment (modified from Thomas, 2002)

PhACs have been studied in many places around the globe including: Austria, Brazil, Canada, Croatia, England, Germany, Greece, Italy, Spain, Switzerland, the Netherlands and the U.S. In the U.S. alone, more than 80 different prescription PhACs have been detected at concentrations up to µg/L levels in sewage, surface and groundwater sources (Thomas, 2002). A study of 139 U.S. streams revealed that 80% of streams sampled contained organic wastewater contaminants (OWCs) such as pharmaceuticals and hormones (Kolpin et al., 2002). Out of those 139 streams, researchers detected an average of seven and a maximum of 36 OWCs per sampling

location. Non-prescription drugs were detected in more than 80% of samples (Kolpin et al., 2002). Multiple PhACs are frequently detected within the same water body. Therefore, increased toxicity due to additive or synergistic effects adds another layer of complexity to this issue. Some advancements has been made in the arena of aquatic toxicology as it relates to PhACs, however there is limited information on the toxicological effects of entire suites of human pharmaceuticals on aquatic organisms exposed over their entire life cycle. Studies have shown that pharmaceutical cocktails may affect the chloroplasts of common river microalgae, *Pseudokirchneriella subcapitata* (Vannini et al., 2011). Mixture toxicity of an anti-inflammatory cocktail was found in *Daphnia magna* even at concentrations at which the single substances showed no or only very slight effects (Cleuvers, 2004). Henry and Black (2007) found additive toxicity of five selective serotonin reuptake inhibitors (SSRIs) on reproduction by *Ceriodaphnia dubia*, suggesting that the SSRIs have a similar mechanism of action. Results from these and other studies indicate that environmental risk assessments should consider that toxic effects of multiple pharmaceuticals in aquatic organisms may increase for each compound in a mixture. Due to limited knowledge about the presence and effects of PhACs mixtures in aquatic ecosystems there is a great need for more research in this area.

Ibuprofen and Naproxen: Mode of Action and Environmental Presence

Naproxen (Figure 2.2) and ibuprofen (Figure 2.3) are both classified as non-steroidal anti-inflammatory drugs (NSAIDs) and generally work by blocking the body's inflammation pathway as well as pain and fever responses (Figure 2.4). NSAIDs represent a class of drugs with analgesic, antipyretic and anti-inflammatory properties.

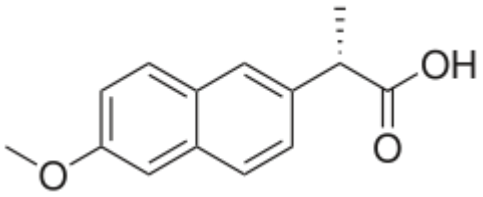

Figure 2.2 Molecular structure of naproxen

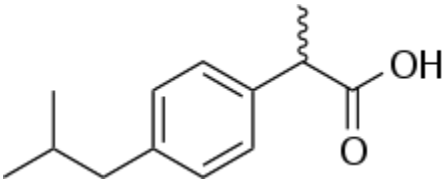

Figure 2.3 Molecular structure of ibuprofen

Naproxen and ibuprofen are both non-specific cyclooxygenase (COX) inhibitors (Van Hecken et al., 2000). COX-1 is important in cell protection of the body's gastric system by producing prosanoids that protect cells against acidic conditions commonly found in the gastrointestinal tract. Inhibition of COX-1 may lead to gastrointestinal ulcers. COX-2 is induced by inflammatory stimuli. Inhibition of COX-2 prevents the body from producing prostaglandins and therefore further inflammatory responses (Van Hecken, et al., 2000).

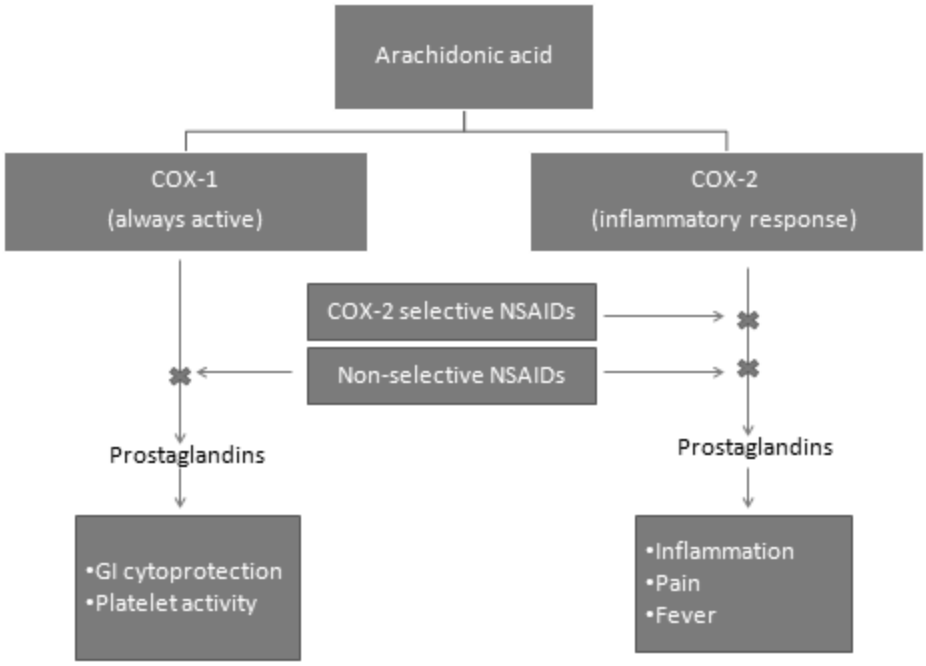

Figure 2.4 NSAID mechanism of action (modified from Vane & Botting, 1995)

The detection of naproxen and ibuprofen in the environment may be due to their ubiquitous use as over-the-counter pain relievers/anti-inflammatory. Naproxen has been on the market since 1976 and was approved for over-the-counter use in 1994 by the U.S. Food and Drug Administration (U.S. FDA). Likewise, ibuprofen was made available in the U.S. in 1974. Concentrations of ibuprofen in surface waters have been reported at the µg/l level (Kolpin et al., 2002). Ibuprofen is not well metabolized by the human body and therefore can be excreted in similar form to the parent compound (Buser et al., 1999). The combination of widespread use and lack of metabolism by the body can lead to significant environmental inputs of ibuprofen. Another factor that may contribute to its persistence in the environment is its relatively long half-life of 50 days (Tixier, 2003). In a survey of 14 Canadian WWTPs, ibuprofen was detected at concentrations as high as 75

µg/L in untreated sewage and 24.6 µg/L in treated WWTP effluent (Metcalfe et al., 2003).

Aquatic organisms exposed to NSAIDs show similar inhibition of COX activity as intended in humans. COX-1 activity has been shown to play an important role in the development of zebra fish and some mammals (Cha et al., 2005). COX mediated production of prostaglandins has been shown to be important for ovulation in mammals and fishes (Gaytn et al., 2006; Sorbera et al., 2001). Other biological changes observed include temporal changes in spawning of Japanese medaka (*Oryzias latipes*) after six weeks of exposure to ibuprofen (Flippin et al., 2007). As exposure concentrations of ibuprofen increased, the Japanese medaka spawned less frequently, but produced more eggs when they did spawn (Flippin et al., 2007). Temporal changes in spawning could be very detrimental to fish species, especially species such as cod and salmon that rely on highly coordinated spawning rituals for reproduction and survival (Scott, 2006). Another study suggests that PhACs like naproxen can cause adverse effects on the immune system of bivalves (Gagné et al., 2006) which are extremely important species in coastal estuary environments.

A study on the fate of naproxen and ibuprofen in a Swiss lake suggested that sedimentation might be the main elimination route for ibuprofen, while naproxen is more likely eliminated through biodegradation and phototransformation (Tixier, 2003). However, one study showed phototransformed byproducts of naproxen to be more toxic to *Daphnia magna* and *Vibrio fischeri* than the parent compound (DellaGreca, 2003).

17α-Ethynylestradiol: Mode of Action and Environmental Presence

17α-Ethynylestradiol (EE2) (Figure 2.5) is a synthetic steroid and a major component of human contraceptive drugs. Estrogens are also widely prescribed and used as hormone replacement therapy drugs.

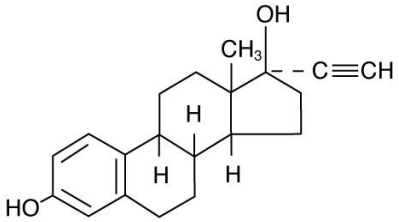

Figure 2.5 Molecular structure of 17α-Ethynylestradiol

The detection of estrogens in aquatic environments has raised concern in recent years due to their increase in use as a therapeutic drug and oral contraceptive and their endocrine disrupting nature (Daughton & Ternes, 1999; Kolpin et al., 2002; Matozzo et al., 2008). Humans and animals excrete considerable amounts of natural estrogens. For example, pregnant women may excrete up to 5 mg/day of 17β-estradiol (E2) (Khanal et al., 2006). Additionally, large amounts of estrogens can leach into groundwater or runoff into surface waters from confined-animal feeding operations (CAFOs) or from poultry farms. Because manure and poultry litter are sometimes land applied for disposal or use as fertilizer, estrogen-laden fertilizers can directly enter surface waters during rain events. Like humans, many animal species use chemical signals (hormones) to regulate important internal functions such as growth, development and reproduction. Substances that alter normal hormone functions have been coined "endocrine disrupting chemicals" (EDCs).

EDCs have been linked to negative reproductive and developmental effects in non-target organisms, such as feminization of males, decreased fertility, hermaphroditism and stunted growth in some species (Baronti et al., 2000; Gagné et al., 2001; Jobling et al., 1996; Metcalfe et al., 2001; Pelley, 2003; Pickering & Sumpter, 2003).

Although estrogens are found predominantly in females, they are also present in males as an active metabolic product of testosterone. Humans excrete on average 2.7 mg/L of estrogen per day in their urine (Zuo et al., 2006). Estrogens are believed to be excreted primarily as inactive conjugates of glucuronic and sulphuric acids. Conjugates themselves do not cause endocrine disrupting activity until they are cleaved by microorganisms found naturally in surrounding environments. Once cleaved, these conjugates revert to free estrogenic compounds capable of hormonal activity (Zuo, et al., 2006). Estrogens are degraded by bacteria present in WWTPs and to a somewhat lesser degree by microbes naturally occurring in aquatic environments (Khanal et al., 2006). Once in the environment EE2 may also undergo direct and indirect photochemical degradation (Zuo, et al., 2006), although it is unknown whether or not these byproducts are also capable of endocrine disruption.

Low levels (ng/L) of estrogens have been detected in water bodies all over the world. EE2 was detected at levels of 4.7 ng/L in the Acushnet River Estuary in Massachusetts, where there was concern of their potential effects on fish and lobster populations due to feminization (Zuo, et al., 2006). In Germany, EE2 has been detected at 5 ng/L in rivers and 9 ng/L in WWTP effluent (Daughton & Ternes, 1999; Kuch & Ballschmiter, 2001). Concentrations of EE2 have also been measured in surface waters in France and the Netherlands. In coastal waters and rivers EE2 was detected at 0.1-4

ng/L and 2.7-4.5 ng/L in WWTP effluents released to surface waters (Belfroid et al., 1999; Cargouët et al., 2004). Analysis of WWTP effluents from the United Kingdom revealed EE2 levels at 7 ng/L. Studies concluded that environmentally relevant concentrations of natural steroids were enough to stimulate male trout to produce female egg protein vitellogenin (Routledge et al., 1998). The United States Geological Survey's (U.S.G.S) landmark study of 139 streams in 30 states revealed EE2 levels from an average of 73 ng/L to a maximum of 813 ng/L in surface waters (Kolpin et al., 2002).

The effects of estrogenic compounds like EE2 on non-target aquatic organisms are only recently being understood. A few studies of WWTP discharge into rivers have detected intersex fish and feminization effects (Harries et al., 1996; Hashimoto et al., 2000). Additionally, fathead minnow (*Pimephales promelas*) populations in one Canadian lake study were experimentally exposed to EE2 (5-6 ng/L), causing death in male fish due to kidney damage. This left the male fish weakened and unable to maintain reproduction levels necessary to prevent the population from disappearing (Pelley, 2003). Another exposure study of synthetic estrogen to fathead minnows (*Pimephales promelas*) reported population collapse due to feminization of male fish (Kidd et al., 2007).

Estrogenic Effects on Bivalves

Although estrogens have been shown to have endocrine disrupting effects in several vertebrate species, less is known about invertebrate endocrinology (de Fur et al., 1999; Porte et al., 2006). Invertebrates are known to bioaccumulate EDCs in their tissues (Le Curieux-Belfond et al., 2005; Morcillo & Porte, 2000) and snails have exhibited

sensitivity to estrogens at low concentrations found in the environment (Jobling et al., 2003). However, the vertebrate model of estrogen in hormonal regulation of reproduction has been applied to marine invertebrates such as mussels and bivalves with mixed results. Vitellogenesis describes the process by which vitellins (cleaved products of vitellogenins) are taken up by the ovary as an energy source for the egg yolk in reproduction (Puinean et al., 2006). Under natural conditions, oocytes secrete 17β-estradiol (a naturally occurring estrogen) to activate the target organ responsible for vitellogenesis. It has been shown that 17β-estradiol is major factor in the control of vitellogenesis in oysters and therefore, likely to be altered when bivalves are exposed to estrogen contaminated environments (Li et al., 1998). Li et al. (1998) observed significantly increased oocyte diameter and vitellin protein levels in female oysters (*Crassostrea gigas*) that were injected with 5 mg of estradiol directly into the gonads. In contrast, a laboratory study conducted on the marine mussel *Mytilus edulis* exposed to 200 ng/L of 17β-estradiol in seawater over 10 days detected no significant change in vitellogenin gene expression even though estradiol uptake was detected in mussel tissue (Puinean et al., 2006). These results may have been due to reduced affinity for estrogen receptors or unresponsiveness of estrogen receptors and vitellogenin genes to estrogens in this particular species of mussel (Puinean, et al., 2006). Other estrogenic effects have been reported in freshwater mussel species. *Elliptio complanata* exposed in situ and under controlled experimental conditions to municipal effluents experienced significantly increased vitellogenin levels after exposure (Gagné et al., 2001). Additionally, mussels downstream of the WWTP effluent exhibited higher vitellogenin levels, decreased shell growth and increased total and soft tissue weights (Gagné et al., 2001).

Due to the sessile nature of bivalves, they are particularly vulnerable to estrogen contaminated waters from runoff, wastewater treatment effluent and leaky septic systems. Exposure to estrogens throughout their life cycle could cause population dynamics to shift if feminization of whole populations were to occur. This, in turn, could cause a cascade effect on entire ecosystems that depend on this keystone species.

Triclosan: Mode of Action and Environmental Presence

Triclosan (Figure 2.6) is a common microbial disinfectant and anti-fungal compound. It has been in use for more than 30 years (approved by U.S. FDA in 1973) and is present in a wide array of personal care products such as soaps, deodorants, toothpastes, shaving creams and mouthwashes (Jones et al., 2000). It is also infused in an increasing number of consumer products such as kitchen utensils, toys, bedding, socks and trash bags (Daughton & Ternes, 1999). Due to its presence in many common household products, triclosan can easily be directly discharged into WWTP influent as well as personal septic systems and subsequently receiving waters if not thoroughly degraded. It may also be released into the environment via wastewater treatment effluent infused with household, hospital and industrial waste and byproducts or in sewage sludge (Singer et al., 2002). Leachate from improperly lined landfills may also provide a route of entry into the environment, as well as run-off from land applied wastewater disposal.

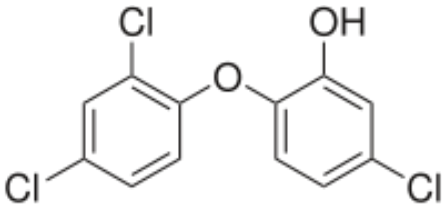

Figure 2.6 Molecular structure of Triclosan

Once delivered to the environment via discharges of WWTP effluent, triclosan is known to have endocrine disrupting capabilities associated with thyroid hormone gene expression, which disrupts amphibian development (Veldhoen et al., 2006). Triclosan has also been suggested to have estrogenic effects due to similar molecular structure as many synthetic estrogenic compounds (Ishibashi et al., 2004). Preliminary data from studies with the freshwater mussel *Mytilus galloprovincialis* suggest triclosan may alter immune cell signaling and digestive gland functions (Canesi et al., 2007).

Triclosan acts as an anti-microbial by inhibiting lipid synthesis of bacteria resulting in the rupturing of their cell membranes (Canesi et al., 2007). Due to the widespread use of antibiotics and products infused with antibiotics, bacterial resistance has become an important topic of research. Bacteria retain their ability to resist antibiotics even after its environmental presence has been removed. If natural bacterial communities are altered, it could also have negative impacts for organisms higher on the food chain that rely on bacteria as a food source or in symbiotic relationships (Daughton & Ternes, 1999; Wilson et al., 2003). Microbial communities are also important in many natural processes including degradation of chemicals, nutrient cycling and photosynthesis. Alteration of microbial communities due to triclosan exposure could have monumental effects on the environment.

Human risks of exposure to triclosan include accumulation in human breast milk (Toms et al., 2011; Wang et al., 2011; Ye et al., 2008). Triclosan has also been detected in the bile of fish exposed to wastewater effluent as well as wild fish downstream of wastewater treatment plants (Adolfsson-Erici et al., 2002). In the U.S.G.S. survey of 139 streams, triclosan was detected in 57% of streams sampled at average concentrations of 0.14 μg/L and a maximum concentration of 2.3 μg/L (Kolpin et al., 2002).

Like most PhACs, triclosan is only partially degraded in wastewater treatment processes. It can be photodegradated; reactions with sunlight may form other toxic compounds such as chlorophenols and dioxins (Aranami & Readman, 2007). Triclosan may also adsorb to particulate matter in the water column, which may settle out causing the formation of pockets of sediment containing the chemical. One study detected triclosan in 30-year old core sediment samples collected from a Swiss lake, suggesting that triclosan is very slow to degrade in sediment (Singer et al., 2002). Marianne et al. (2004) determined that lipid-based concentrations of methyl triclosan (a transformation byproduct) in fish were significantly higher than concentrations in lake water, suggesting that significant bioaccumulation of the chemical could occur. Acute toxicity data for triclosan in multiple species (*Daphnia magna, Pimephales promelas, Lepomis macrochirus and Oncorhynchus mykiss*) indicated that environmentally relevant concentrations are not directly toxic (NOEC 34.1 μg/L and LOEC 71.3 μg/L) (Orvos et al., 2002). However, algae (*Scenedesmu subspicatus*) was determined to be the most susceptible organism (Orvos et al., 2002) due to population declines which could have negative impacts to organisms who rely on this algal food source.

Diphenhydramine Hydrochloride (HCl): Mode of Action and Environmental Presence

Diphenhydramine HCl (referred to from now on as diphenhydramine) is a commonly used over-the-counter antihistamine (Benadryl™) (Figure 2.7). Diphenhydramine works by blocking histamine, which is the body's response to allergens. It is also sometimes used as a sedative or hypnotic.

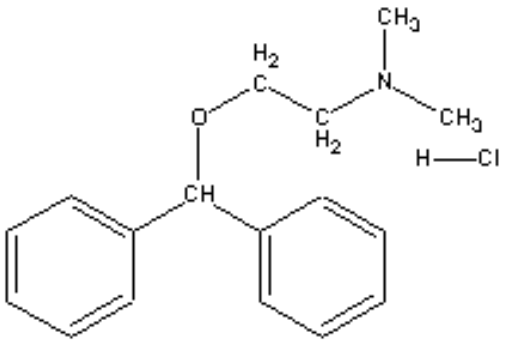

Figure 2.7 Molecular structure of diphenhydramine HCl

Diphenhydramine has been reported in raw drinking water samples at a maximum concentration of 0.023μg/L (Focazio et al., 2008). Although little research is available on the ecotoxicology of diphenhydramine, one study reported no significant impact on *Daphnia magna* survival and reproduction when exposed to concentrations 5 times greater than the environmentally relevant concentration (0.023 μg/L) (Meinertz et al., 2010). In this same study, mean time to death became significant only after the daphnids were exposed to concentrations exceeding 62 μg/L (Meinertz et al., 2010), which is more than 2,500 times the previously reported environmental concentration. However, it is important to note that standardized toxicity tests such as this do not represent an

organism's continuous exposure to pharmaceutical contaminants over its entire life cycle, nor do they include additive or synergistic effects of suites of pharmaceuticals often detected in surface waters. Fish (*Catostomus insignis, Micropterus salmoides, Cyprinus carpio, Amia calva, Catostomus commersonii* and *Ictiobus bubalus*) have been found to bioaccumulate diphenhydramine in their tissues (mean 1.4 ng/g, max 2.5 ng/g) and liver (mean 0.5 ng/g, max 11.1 ng/g) (Ramirez et al., 2009).

As with most other PhACs, diphenhydramine enters the environment via WWTP effluent. In Nebraska, diphenhydramine was detected in several stream samples upstream and downstream of WWTPs (Bartelt-Hunt et al., 2009). In upstream samples, diphenhydramine concentrations varied from 7.7 ng/L to 18 ng/L and was attributed to surface runoff and the ability of diphenhydramine to persist in the environment. Downstream of WWTPs, concentrations ranged from 22.5 ng/L to 2,520 ng/L suggesting diphenhydramine concentrations increased as a result of loading from WWTP discharges. In Omaha, Nebraska, diphenhydramine was also detected in wastewater treatment effluent at concentrations of more than 500 ng/L (Bartelt-Hunt et al., 2009). The occurrence of diphenhydramine in drinking water has been studied by the U.S.G.S and the Centers for Disease Control and Prevention (CDC). They found the drug present in 25% of raw water samples, although it was below the level of reporting which is 0.0148 μg/L (Stackelberg et al., 2004). Fortunately, it was not detected in finished water samples.

Land application of biosolids is another possible route of entry for PhACs into aquatic environments. A study of leaching and soil dissipation of several pharmaceuticals found that diphenhydramine, along with other chemicals, is likely to

persist in biosolids-incorporated soil layers for an extended period of time (Wu et al., 2010). Only low concentrations (ng/L) of diphenhydramine were detected in the leachate from amended soils, likely due to sorption to dissolved organic matter. The study concluded that diphenhydramine has a very limited mobility through soils (Wu et al., 2010).

Oysters as Potential Biomonitors

In marine tidal ecosystems oysters are a superior organism to monitor for water quality due to their wide distribution, sessile nature, filter feeding habits and human consumption. Their inability to escape chemically polluted waters is marked by a complex life cycle, culminating in the eventual setting of veliger larva on suitable substrates after about 20 days (Wallace, 2001). This sessile stage allows oysters to become non-target receptors of pharmaceutically laden releases. Exposure to PhACs can not only alter endocrine and other functions in oysters causing possible population declines (Li et al., 1998; Matthiessen & Gibbs, 1998; Oberdörster & Cheek, 2001), but human health could also be at risk of exposure by consuming contaminated shellfish. Oysters may act as an indicator species of human waste contamination through the detection of commonly used human pharmaceuticals and other hydrophobic organic chemicals in their tissues (Huckins et al., 2004). For example, oyster bioaccumulation and transformation of 17β-estradiol could be studied as a potential biomarker of endocrine disruption (Le Curieux-Belfond et al., 2005).

Oysters as Bioremediators

Bioremediation refers to the use of living organisms to remove or detoxify chemical or elemental pollutants in the environment (Gifford et al., 2007). Most bioremediation research has focused on bacteria, plants and algae. The term zooremediation has been coined for the use of animals for bioremediation, but has seen limited use due to ethical concerns and human consumption of many aquatic species. However, aquatic species like oysters have the ability to hyperaccumulate, stabilize and degrade pollutants (Gifford et al., 2007). Zooremediation models should be cost effective to justify and enhance their use. A model organism for zooremediation is not only an effective remediation tool but it also should provide some economic value. Several aquatic organisms have been identified as potential zooremediators thus far, including oysters, sponges, edible mollusks, gastropod mollusks, bryozoans and ascidians (Gifford et al., 2007; Nelson et al., 2004).

The potential for zoostabilization and zootransformation/degradation is a focus of this research. Zoostabilization refers to the use of animals to inhibit pollutant migration within the environment and involves maintaining or supplementing wild populations without actually harvesting animal biomass (Gifford et al., 2007). Zootransformation/degradation refers to the use of animals to degrade organic pollutants to less toxic compounds and also excludes the harvest of animal biomass (Gifford et al., 2007).

The use of oyster species like *C. virginica* to improve water quality has the potential to meet the many requirements of a successful zooremediation model. Gifford et al. (2007) devised a checklist for candidate zooremediation species that included the

following traits: ability to accumulate pollutants, resistance to toxicity, non-invasive, rapid growth rate, sedentary, ease of culture, known population dynamics, known uptake dynamics, knowledge of carrying capacity and understanding of disease risks. Much is already known about the life history and ecology of *C. virginica* and studies have already shown that *C. virginica* can effectively improve water quality in small tidal creeks. Total suspended solids (TSS) and chlorophyll *a* concentrations were reduced after *C. virginica* beds were transplanted into tidal creeks in North Carolina (Nelson et al., 2004). And, as previously discussed, the Pacific oyster, *C. gigas*, has the ability to bioaccumulate estrogens in their soft tissues (Le Curieux-Belfond et al., 2005). However, some adverse responses to the use of *C. virginica* as a bioremediation species have been noted and include: increases in ammonium concentrations downstream of transplanted oyster reefs and deposition of feces and pseudofeces, resulting in accumulation of finer-grained materials in treated channels (Nelson et al., 2004). Thorough cost-benefit analyses are needed when considering these types of bioremediation tools.

Filtration rates of *C. virginica* may dictate its effectiveness as a bioremediation tool. There is some debate on the filtration rates of oysters. The Chesapeake Bay Foundation reports that *C. virginica* filters an average of 5 liters (1.3 gallons) per h, whereas others report the rate as high as 36 liters per h (Brusca & Brusca, 1990). One laboratory experiment showed normal pumping rates for *C. virginica* to be 6.8 liters per h (Riisgård, 1988). Loosanoff (1958) hypothesized that the pumping rate of oysters was dependent on temperature. He concluded that the maximum pumping rate of 13 liters per h occurred between 28.1 and 32.0°C. Regardless of the discrepancies in filtration rate of oysters, one fact remains -- that they are very efficient filter feeders with the ability to

greatly increase water quality. Gaps in the research indicate a need for more investigations on the mechanisms of oyster endocrinology as well as their ability to uptake and remove PhACs in aquatic environments, which was a focus of this research.

Septic Systems in Coastal Environments

In rural areas, on-site septic systems are a primary source of wastewater treatment and removal due to limited infrastructure and funding. While WWTPs in the U.S. are required to meet stringent EPA and state treatment requirements that require sophisticated equipment, most septic systems undergo little to no monitoring once they are installed and construction designs are generally based on 100 year old models (Cotteral & Norris, 1969).

McIntosh County, GA, is a rural coastal community that has one WWTP that services only the City of Darien (*City of Darien and McIntosh County Joint Comprehensive Plan,* 2007). The remainder of McIntosh County's 14,000 residents requires the use of on-site septic systems (U.S. Census, 2011). In addition to aging septic systems, population growth over the past decade has led to an increase in the number of new septic systems located adjacent to coastal waters (Walker et al., 2003). As of 2007, McIntosh County had experienced rapid growth in population and housing, putting a proportionally higher demand on services such as wastewater treatment (*City of Darien and McIntosh County Joint Comprehensive Plan,* 2007). With no WWTP service outside the City of Darien, on-site septic systems are the only wastewater treatment option. Population growth in the county is predicted to continue increasing through the year 2030 (*City of Darien and McIntosh County Joint Comprehensive Plan*, 2007). In 2002, a GIS

inventory of 1,056 septic tanks located adjacent to salt marshes, creeks and rivers in McIntosh County, GA, revealed that 5% were dysfunctional, 63% were located in areas susceptible to high pollution due to sandy soils and 75% occurred in the 100-year floodplain (Walker et al., 2003). Additionally, 18% of septic systems were located in water recharge areas and 111 septic systems were located within the 25 ft buffer required by state law. Non-point source pollution is the primary cause of impaired surface waters in Georgia (GA Environmental Protection Division). Faulty or leaking septic systems add to this threat to water quality in Coastal Georgia and can be a source of introduction of PhACs into the environment.

Sewage contamination in surface waters has generally been detected experimentally using bacteria commonly found in human waste (e.g. *E. coli*, fecal coliform, etc.). However, chemical indicators of human sewage pollution can be a viable alternative to microbial methods (Bruner et al., 1994; Huckins et al., 2004). They tend to require less sample preparation and analysis times, are specific to human sewage pollution and are not subject to regrowth in the environment (Glassmeyer et al., 2005; Hagedorn & Weisberg, 2009). Ibuprofen, naproxen and diphenhydramine have been previously regarded as potential human chemical markers in the emerging field of fecal source tracking (Comeau et al., 2008; Glassmeyer et al., 2005; Hagedorn & Weisberg, 2009).

Research Objectives and Outline

The purpose of this research was to determine the effectiveness of using oyster spat stick communities to monitor and remediate tidal creeks that are likely contaminated

with human sewage pollutants via leaky/faulty septic systems. Five PhACs (EE2, triclosan, naproxen, ibuprofen and diphenhydramine) were chosen as model contaminants for the study. The effects of these PhACs have been discussed and their presence is known to be in vicinities of human waste disposal, either from wastewater treatment plants or private septic systems. Finding effective and environmentally sound methods to remediate PhAC contaminated waters is important to both human and environmental health. If found effective, experimental oyster spat stick communities can be a renewable and inexpensive remediation tool. Oysters may also be used as biomonitors of human sewage contamination through detection of human pharmaceuticals in their tissues. Additionally, understanding oyster uptake of these types of pollutants adds to the knowledge base of the fate and transport of these emerging contaminants of concern. I hypothesize that the Eastern oyster *C. virginica* will remove selected chemical markers of human sewage contamination from seawater within tidal creeks. Removal of marker chemicals would correlate with increased tissue concentrations of these agents in *C. virginica* and efficiency of removal of chemicals from seawater would increase with exposure time until a steady state would occur. These hypotheses were tested in a series of field sampling events over a two year time span and validated with a controlled laboratory experiment.

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CHAPTER 3

EFFECTIVENESS OF OYSTER SPAT STICK COMMUNITIES IN REMOVING PHARMACEUTICAL CONTAMINANTS FROM TIDAL CREEKS[1]

[1]Scarlett L. Fuller and Marsha C. Black. To be submitted to *Marine Pollution Bulletin*.

Abstract

Pharmaceuticals are biologically active molecules, designed to affect biological processes at very low doses. They enter surface waters primarily through effluent discharges from wastewater treatment plants; however, septic systems may also act as a route of entry in rural underserved coastal areas. Exposure to these compounds may pose risks to non-target aquatic organisms, as well as human health. Oysters (*Crassostrea virginica*, Gmelin, 1791) represent ubiquitous, sentinel filter feeders with the ability to sequester such organic compounds. Field studies explored the accumulation of five pharmaceuticals by oysters at three sites in Coastal Georgia, while a controlled laboratory study was conducted to determine uptake abilities of oysters exposed to an algal food source and microbes, in addition to the five pharmaceuticals. Field studies showed significantly lower ($p < 0.05$) ibuprofen (IBU) concentrations in oyster tissues in December (2011) than March (2012) or August (2011) and seasonal tissue concentrations correlated with lipid content in tissues. Diphenhydramine (DPH) tissue concentrations were significantly ($p < 0.05$) lower in March (2012) than August (2011). Oyster bioaccumulation of IBU and DPH concentrations proved to be a more effective tool for monitoring pharmaceuticals in tidal creeks than collection of traditional water grab samples because of the low and intermittent exposure concentrations. Controlled laboratory studies indicated a significant ($p < 0.05$) increase in accumulation of ibuprofen between 12 and 96 h of exposure as well as microbial interactions. In the presence of an algal food source oysters accumulated significantly ($p < 0.05$) lower IBU concentrations in oysters. These results suggest that oysters may preferentially feed on algae food sources without sorbed chemicals or that the presences of food increases metabolism and

elimination of chemicals. This study confirms the ability of oysters to assimilate pharmaceuticals associated with human sewage pollution and concentrations may vary by seasonal fluctuations in oyster lipid content and the presences of an algal food source and/or microbes. In addition, oysters may preferentially feed to avoid chemical exposure or metabolize and eliminate faster with the presence of food.

INDEX WORDS: Pharmaceuticals, Biomonitoring, *Crassostrea virginica*, Septic tanks, Ibuprofen, Diphenhydramine,

Introduction

The presence of pharmaceutically active compounds (PhACs) in aquatic environments is a recent and emerging field of environmental research (Daughton & Ternes, 1999; Halling-Sørensen et al., 1998; Thomas, 2002) that poses human health and environmental concerns. Due to technological advances in analytical chemistry and better detection methods, researchers are now able to detect low levels (ng/L to μg/L) of PhACs in both surface and ground water (Daughton & Ternes, 1999). The effects of PhACs on estuarine and marine waters are only recently being investigated and understood (Buser et al., 1998; Thomas, 2002; Weigel et al. 2002; Zuo et al., 2006). PhACs can enter the environment in a variety of ways. They may be introduced via industrial and sewage treatment effluent, leaking septic tanks, improper landfill disposal or runoff from land applied biosolids, although the major source seems to be sewage treatment effluent (Buser et al., 1999; Thomas, 2002; Weigel et al., 2002; Zuccato et al., 2000). Effluent from wastewater treatment plants (WWTPs) and on-site septic systems provide primary routes of entry of PhACs into the environment. In addition, improper disposal of unused and expired pharmaceuticals directly into sewer or septic systems are all too common, and provide another direct source of PhACs into wastewater. Pharmaceuticals such as non-steroidal anti-inflammatory drugs (NSAIDs) are often excreted only slightly transformed or unchanged as conjugated polar molecules (Farré, 2007; Thomas, 2002; Zuccato et al., 2000). The sewage treatment process can allow conjugates to be cleaved, thereby allowing the original PhAC to be released to the environment via wastewater effluent. Chronic human and veterinary use of PhACs may provide a continuous source of contamination into the environment because variable

amounts, up to 95% of the administered dose, may be excreted as unmetabolized compounds and subsequently discharged into wastewater (Castiglioni et al., 2005; Calamari et al., 2003; Dollery, 1991; Farré et al., 2007; Zuccato et al., 2000).

Although WWTP effluent is a well-documented source of pharmaceutical contamination into aquatic environments (Bartelt-Hunt et al., 2009; Belfroid et al., 1999; Brown et al., 2007; Buser et al., 1999; Kolpin et al., 2002), on-site septic systems are a primary source of wastewater treatment and removal in rural locales due to limited funding and infrastructure. McIntosh County, GA, is a rural coastal community that has one WWTP, servicing only the City of Darien, GA (*City of Darien and McIntosh County Joint Comprehensive Plan*, 2007). The remainder of McIntosh County's 14,000 residents requires the use of on-site septic systems (U.S. Census, 2011). In addition to aging septic systems, population growth over the past decade has led to an increase in the number of new septic systems located adjacent to coastal waters (Walker et al., 2003). As of 2007, McIntosh County has experienced rapid growth in population and housing, putting a proportionally higher demand on services such as wastewater treatment (*City of Darien and McIntosh County Joint Comprehensive Plan*, 2007). With no WWTP service outside the City of Darien, on-site septic systems are the only wastewater treatment options. Population growth in the county is predicted to continue increasing through the year 2030 (Georgia Department of Community Affairs).

In 2002 a GIS inventory of 1,056 septic tanks located adjacent to salt marshes, creeks and rivers in McIntosh County, GA, revealed that 5% were dysfunctional, 63% were located in areas susceptible to high pollution and 75% occurred in the 100-year floodplain (Walker et al., 2003). Additionally, 8% of septic systems were located in

water recharge areas. Faulty or leaking septic systems add to the non-point source pollution threat to water quality in Coastal Georgia and can be a source of introduction of PhACs into the environment.

In marine tidal ecosystems of Coastal Georgia, oysters (*Crassostrea virginica*) are a superior organism to monitor and mitigate water quality due to their wide distribution, sessile nature and filter feeding habits. Human consumption of oysters requires regular water quality monitoring of shellfish harvesting grounds and oyster tissue itself to ensure human health is not at risk of exposure to pathogens or chemical contaminants. *C. virginica* are economically and ecologically important to Coastal Georgia. They provide economic benefits to the community through harvesting and consumption. Ecological benefits include reducing algae, nutrients and suspended solids, as well as providing fish habitat. However, their inability to escape chemically polluted waters allows oysters to become non-target organisms of PhAC exposures. Exposure to PhACs can not only alter normal metabolic and reproductive functions in oysters causing possible population declines, but human health could also be compromised by the consumption of contaminated shellfish. Oysters may act as an indicator species of human waste contamination through the detection of commonly used pharmaceuticals in their tissues. Oysters may also provide a remediation tool for contaminated estuaries by further metabolizing or sequestering PhACs released from human sewage pollution.

The first objective of this study was to determine the ability of experimental oyster spat stick communities to sequester commonly used pharmaceuticals (naproxen, ibuprofen, ethynylestradiol, triclosan and diphenhydramine HCl) from the water column at three sites in Coastal Georgia. Secondly, a controlled laboratory study was conducted

to determine the uptake kinetics of these chemicals in oysters under varying conditions of food availability and microbial presence to mimic conditions in the field that may influence accumulation of PhACs

Materials and Methods

Field Study

Site Selection

Three sites were selected within the Sapelo Sound (Fig 3.1) located in McIntosh County, GA. This area is characterized by little freshwater input other than rain runoff, and is extremely rural, with only a single wastewater treatment plant serving the City of Darien, which was remote from all study sites. The sites represented a gradient of potential human sewage contamination previously determined by a GIS inventory of near-water septic systems (Walker, et al., 2003). Study sites included: 1) Eagle Creek (31.48844, -81.30429) which served as a reference site with no inhabitants or septic inputs located directly upland; 2) Four Mile Island (31.55256, -8130072), an area of intermediate contamination from human sewage located along the Julienton River with 25 septic systems directly upgradient; and 3) Oak Dale Creek (31.412783, -81.2864), an area with high incidence of failing septic systems with 34 suspect systems located upland (Fig 3.1).

Experimental Spat Stick Construction and Deployment

Experimental oyster spat sticks were constructed from 1 inch PVC pipe dipped in a cement slurry as previously described by Manley et al. (2008). Spat sticks were arranged in a 1 m x 1 m square configuration containing 36 spat sticks (6 x 6) in triplicate

(108 spat sticks per site) within each tidal creek. Deployment of spat sticks in May 2010 corresponded temporally to the spawning behavior of a native oyster species (*C. virginica*). By August 2010, spat were successfully recruited to the experimental reef.

Sampling method

Quarterly water and oyster sampling was conducted at each site beginning in August 2011 and ending in March 2012. Three one-liter water grab samples were collected in solvent rinsed amber glass bottles on ebbing tides. Oyster spat recruited to spat sticks (subsequently referred to as "spat") and naturally occurring oysters (subsequently referred to as "adult") were harvested and placed in separate ziploc bags. Both water and oyster samples were stored on ice until they were returned to the laboratory at UGA where they were held at 4 °C until processing, always within 48 h of collection. Water quality parameters including: pH, salinity, dissolved oxygen and temperature were measured at each site using a YSI multi-parameter sonde (Model 550, Yellow Springs, OH). Rainfall in the watershed was recorded using data from the Brunswick station of The University of Georgia's Automated Environmental Monitoring Network (www.georgiaweather.net).

Water sample preparation

Within 36 h of collection water samples were filtered with 0.7 μm TCLP glass fiber filters (Pall Life Sciences, West Chester, PA), acidified to approximately pH 2 using 3 M sulfuric acid (Farré, 2007). Samples were extracted using solid phase extraction (SPE) with Supel™ -Select HLB Resin cartridges (200 mg resin /6 ml capacity) placed on an SPE vacuum manifold system (Supelco Analytical, Bellefonte, PA, USA). Prior to sample extraction, HLB cartridges were conditioned with 6 ml of methanol followed by

12 ml ultra pure water (Milli-Q, Millipore). HLB cartridges were then loaded with one-liter water samples and passed through at a flow rate of no more than 5 ml/minute. After each water sample passed through the HLB cartridge, approximately 12 ml of ultra pure water was passed through to bring the pH back to neutral (~7). Sample cartridges were then allowed to dry on the SPE vacuum manifold for 30 minutes. Once dry, samples were stored at -20 °C until further analysis.

Oyster sample preparation

Oyster samples were rinsed with ultra pure water to remove marsh mud and debris prior to being shucked. Within 48 h of collection all tissue was eviscerated from oyster shells. Approximately 3 adult oysters or 6-8 spat (as defined above) were pooled by site (approximately 10 g wet weight for each pooled sample) and three pooled samples were prepared from each site. Oyster tissues were then placed in glass scintillation vials and stored at -20 °C. Lastly, frozen oyster tissues were freeze dried (Labconco Freeze Dry System, Kansas City, MO) and stored in the dark, at room temperature until extraction.

Chemicals

Pharmaceutical standards of 17α-ethynylestradiol (EE2), naproxen (NAP), ibuprofen (IBU), triclosan (TRI), diphenhydramine hydrochloride (DPH) and meclofenamic acid were obtained from TCI America (Portland, OR), Enzo Life Sciences (Plymouth Meeting, PA), EMD Chemicals, Inc. (San Diego, CA), Spectrum (Gardena, CA) and MP Biomedicals, LLC (Solon, OH) respectively. All pharmaceuticals were at least 98% pure. Chemical characteristics and properties are presented in Table 3.1.

All solvents used were HPLC grade. Isotopically (Table 3.2) labeled standards of TRI, NAP, EE2 and IBU were purchased from Cambridge isotope Laboratories, Inc (Andover, MA). The isotopically labeled standard for DPH was obtained from CDN Isotopes, Inc (Quebec, Canada).

Water extraction method

The HLB cartridges (with extracted water samples) were thawed and rehydrated with 5 ml ultra pure water prior to elution. Samples were then eluted with 10 ml methanol into solvent-rinsed (acetonitrile) 10 ml centrifuge tubes. Methanol was allowed to flow through via gravity until completion, then vacuum was applied to ensure removal of all solvent from each cartridge. All eluted samples were spiked with 10 µg/ml of each isotopically labeled standard and blown to dryness using a nitrogen evaporation device. Dried samples were reconstituted in 1 ml 10% acetonitrile (in Milli-Q water). Samples were vortexed and transferred into 1 ml LC-MS amber glass vials for analysis.

Oyster extraction method

Freeze dried oyster samples were weighed to the nearest 0.0001 g and placed in a 20 ml glass (solvent rinsed) centrifuge tube. Tissues were extracted using 8 ml of 3:3:1 methanol, acetonitrile, and methyl tertiary-butyl ether (MTBE) solvent and placed on a rotary extractor for 10 minutes. Next, samples were centrifuged for 20 minutes at 1,500 RPGs to pellet out large particulates. The supernatant was decanted into a 10 ml centrifuge tube and blown dry to 0.5 ml or less using a nitrogen evaporation device. The remaining supernatant was diluted to 20 ml and passed through a Sep-Pak Florisil three cm^3 vacuum cartridge for cleanup (500 mg sorbent per cartridge, 50-200 µm particle size, Waters Corporation, Milford, MA). This cleanup step was added to reduce matrix effects

common with tissue extractions. The florisil cartridge was then eluted with 6 ml methanol, blow to dryness and reconstituted in 1 ml 10% acetonitrile. Samples were transferred into LC-MS 1 ml amber glass vials for analysis.

Analysis of PhACs with LC-MS/MS

Pharmaceuticals in oyster tissue and water extracts were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The instrument used was Varian LC with a 320MS triple quad mass selective detector in Electrospray Interface (ESI) mode. Target pharmaceuticals were separated using an Agilent ZORBAX Eclipse Plus C18 analytical column (2.1x150mm, 3.5 μm). Column temperature was maintained at 35 °C. The initial mobile-phase consisted of 10% acetonitrile (ACN) held constant for 1.5 min, followed by a linear gradient to 100% ACN in 8.5 min, after which the mobile phase composition was held at 100% ACN for 10 min. The last 8.5 min of the program were to allow the column to re-equilibrate to the starting eluent (10% ACN). The flow rate was 0.2 ml/min, and 10 μl of extracts were injected. The system was operated in multi-reaction monitoring (MRM) mode, and reactions that yielded the best intensity for analytes were selected for monitoring (Table 3.3). Optimum declustering potential (DP) and collision energy (CE) were selected on an individual basis (Table 3.1). Collision cell gas (argon) was maintained at a pressure of 1.8 mTorr. Quantitation of water and oyster samples was performed with an external standard curve prepared by direct injection of standards at concentrations ranging from 0.0001 to 1 μg/l.

Prior to analysis all samples were spiked with isotopically labeled surrogate standards (Table 3.2). A series of external standards were prepared with different concentrations of the target analytes and fixed concentrations of isotope surrogates. The

concentrations of the analytes were determined by comparing the response to each analyte in the samples to the responses to each analyte in the external standards over the range of a calibration curve. These response data were adjusted according to the relative ratios of the responses to the stable isotope surrogates in the sample and external standard. This approach adjusts the quantitative data to compensate for efficiencies of extraction <100% and enhancement or inhibition of the signal due to the effects of the sample matrix. Blanks were prepared by spiking ultra-pure water with labeled surrogates, and extracting and analyzing them as described previously. The limits of quantitation (LOQs) were estimated as the second lowest point in the linear calibration curve prepared by analysis of the external standards, for which the signal to noise ratio for the analytes in the native samples was >10.

Lipid extraction

A modified Bligh and Dyer (1959) method was used to extract total lipids from oyster samples. Freeze-dried, homogenized oyster tissues were weighed out to the nearest 0.0001 g and placed in solvent rinsed centrifuge tubes. Six ml of 2:1 chloroform:methanol (HPLC grade) were added to each tube and the tissue-solvent mixture was homogenized. Homogenates were centrifuged for 5 minutes (1,000 RPM) and the supernatant (solvent) was removed, measured and placed in a clean centrifuge tube. Sodium chloride (NaCl) was added at 20% of the measured solvent volume, vortexed (30 s) and centrifuged for 5 minutes. The aqueous supernatant layer was discarded and the remainder was poured into a previously combusted and weighed aluminum pan. The solvent was evaporated on a heating block at 60 °C for 15 minutes. Weigh pans containing total lipids were placed in a dessicator for 30 minutes and then

weighed to determine lipid weight. Percent lipid was calculated using lipid weight per gram of oyster tissue extracted.

GIS analysis

Septic tank densities were calculated for each sample site from a GIS inventory of near-water septic systems collected by the University of Georgia's Marine Extension Service (Walker et al., 2003). Septic tank densities were compared to detected levels of pharmaceutical contamination to determine a correlation between densities of septic tanks and pharmaceutical contamination. Map construction and GIS analyses were conducted using ArcGIS10 (ESRI, Redlands, CA).

Controlled laboratory study

The controlled laboratory study was conducted at the University of Georgia's Marine Extension Service Laboratory on Skidaway Island in Savannah, GA. Oysters for the study were harvested from a public shellfish harvesting ground, Oyster Creek (31.995264; -80.922244) located in Chatham County, GA. To ensure minimal background levels of contaminants, oysters were depurated in filtered natural seawater from the Skidaway River for 24 h in a 600 gallon tank under flow-through conditions. Seawater was filtered through a 10 μm bag filter, sand (Pentair Tagelus TA-50/500) and sterilized with ultraviolet (UV) light (SmartUV Sterilizer, model #02130, Emperor Aquatics, Pottstown, PA). After depuration, 18 oysters were placed in each solvent rinsed aquaria filled with 113.6 liters of filtered, sterilized seawater (as previously described). Oysters were fed 100 μL of Shellfish Diet (2×10^6 cells/ml, Reed Mariculture), an algal food source, once per day. Daily water quality parameters were

recorded for each tank, including temperature, dissolved oxygen, ambient temperature, pH (YSI multi-parameter sonde, model 550, Yellow Springs, OH) and ammonia (API ammonium test kit, Mars Fishcare Inc., Chalfont, PA).

The experiment was designed to determine the uptake and removal of pharmaceuticals from the overlying water column over a 96 h exposure. To imitate field conditions and gain a better understanding of characteristics that might alter chemical uptake by oysters, combinations of treatments were used and included: chemicals only (IBU, DPH, NAP, TRI, EE2), chemicals plus food, chemicals plus microbes (*Enterococcus faecalis*) and chemicals plus food and microbes. Control tanks contained food and oysters, microbes only, food only and chemicals only. Each treatment was run in triplicate.

The laboratory exposure was conducted for 96 h with static renewal at 48 h (Table 3.4). Each treatment was initially seeded to a final concentration of 50 µg/L solution for each target chemical. Treatments containing microbes were seeded with approximately 1 x 10^5 colony forming units (CFUs)/ml *E. faecalis*. Tanks with treatments requiring food were fed daily aliquots of 100 µL of Shellfish Diet. For each treatment 500 ml water samples were collected at 0 h, 48 h pre-renewal, 48 h post-renewal and 96 h. An additional 200 ml of water was collected every 12 h and composited into two 1-liter samples (composite 1 = 0 – 48 h pre-renewal; composite 2 = 48 h post-renewal – 96 h). Water samples were extracted and analyzed as described for field water samples. Oyster samples (3 individuals) were collected at times 0, 12, 24, 48 and 96 h and extracted and analyzed as described for field oyster samples.

Statistical analysis

Data from both field sampling and controlled laboratory studies were log transformed to obtain normal distributions, subjected to normality tests and analysis of variance (ANOVA). Critical differences between exposed and unexposed groups were determined with Tukey's Simultaneous Tests. Pearson Product Moment Correlation Coefficients were calculated to determine relationships between environmental parameters. Each target chemical was analyzed separately for significance. Concentrations below detection levels were assigned non-zero values of one-half the detection limit for each target analyte. Statistical analyses were conducted using Minitab® Statistical Software (version 15.1.30.0, State College, PA 2007). All statistical analyses were considered significant at $p < 0.05$.

Results

Environmental parameters

Water temperatures ranged from a low of 13.8 °C (Oak Dale Creek, December 2011) to a high of 33.1 °C (Oak Dale Creek, August 2011). The pH ranged from a low of 6.84 (Oak Dale Creek, December 2011) to a high of 7.73 (Eagle Creek, December 2011; 4-Mile Island, March 2012). Salinity ranged from a low of 16.68 ppm (Oak Dale Creek, March 2012) to a high of 35.62 ppm (4-Mile, August 2011). Water quality parameters were similar between sites within each sampling month (Table 3.5). Salinity was negatively correlated with lipid content ($p < 0.001$) (Table 3.6) and positively correlated with diphenhydramine concentrations in oysters. Dissolved oxygen levels ranged from a low of 2.00 mg/l (Oak Dale Creek, March 2012) to a high of 7.12 (Eagle Creek, December 2011). Dissolved oxygen was negatively correlated with IBU concentration in

oysters ($p = 0.003$) and lipid content, but was positively correlated with pH and salinity (both $p < 0.001$) (Table 3.4). Rainfall in the watershed ranged from 0.23 – 2.64 cm per week. Pearson's correlation coefficient indicated an inverse relationship between rainfall and DPH oyster concentrations ($p = 0.019$), water temperature ($p < 0.001$), and salinity ($p < 0.001$). Rainfall was positively correlated with lipid content ($p = 0.023$) and pH ($p = 0.005$) (Table 3.6).

Field data: Chemical concentrations detected in water samples

Concentrations of target pharmaceuticals in one-liter water samples were consistently below detection levels and therefore are not reported here. The one exception was a single detection of IBU (0.157 μg/l) at 4 - Mile Island in March 2012.

Field data: Chemical concentrations measured in oysters

Of the five target chemicals analyzed in this study (NAP, IBU, EE2, TRI and DPH), only two chemicals, IBU and DPH, were detected with adequate frequency for analysis in field samples. Mean differences between populations of spat and adult oysters were determined to be insignificant; therefore, data were pooled by sample time and location for statistical analysis.

ANOVA analyses revealed significant ($p < 0.001$) differences between sampling months for oyster lipid content (percent lipid, dry weight basis). December (2011) lipid concentrations were significantly ($p < 0.001$) lower than March (2012). We observed lower lipid concentrations in oysters collected in December (2011), versus August (2011), but the relationship was not significant ($p = 0.0739$). There were no differences

between lipid normalized and non-normalized concentrations of pharmaceuticals in oyster tissues; therefore, data presented are not normalized for lipid content.

Mean IBU concentrations in oysters ranged from a minimum of 0.003 μg/g (4-Mile Island/Eagle Creek, December 2011) to a maximum of 0.050 μg/g (4-Mile Island, March 2012) (Figure 3.2). ANOVA revealed significant ($p < 0.001$) differences in mean IBU concentrations between sampling months. Specifically, pairwise comparisons indicated that the mean IBU concentrations were significantly ($p < 0.002$) lower in December (2011) than in August (2011) and March (2012). In December (2011) and March (2012), oyster tissue concentrations of IBU were greater in Oak Dale Creek than in Eagle Creek ($p=0.063$).

Mean DPH concentrations in oysters ranged from a minimum of 0.001 μg/g (Oak Dale Creek, March 2012) to a maximum of 0.003 μg/g (Eagle Creek, August 2011). Pairwise comparison of DPH concentrations verses months indicated a significant ($p < 0.05$) difference in the means between March (2012) and August (2011), with oysters accumulating significantly higher concentrations of DPH in August. December (2011) concentrations were not significantly different than March (2012) or August (2011) and there were no differences in DPH concentrations in oysters between sites.

Naproxen was only detected in oyster tissues at two times in the duration of this experiment. Both detections occurred in the March (3/1/12) at 4-Mile Island (0.001 μg/g) and Oak Dale Creek (0.002 μg/g).

Our laboratory study provided initial data on the ability of oysters to accumulate three widely-used over-the-counter pharmaceuticals from the overlying water column within 96 h. Oyster tissue is a complex matrix and effectiveness of extraction and

analysis by LC-MS/MS can vary depending on chemical properties such as solubility, bioavailability and degradability. Our extraction methodologies were effective in measuring NAP, IBU and DPH in oyster tissues and exposure water. However, the same methods were not as effective in detecting TRI or EE2 in oyster tissues. From the LC-MS/MS analysis, it was determined that isotopically labeled EE2 and TRI were not adequate surrogates, thus confounding their detection in water and tissue samples. The peak response of isotopically labeled EE2 was so large that it overshadowed the response of EE2 in the samples. The opposite was true of isotopically labeled TRI, which was obscured by the matrix and therefore not detected. Triclosan and EE2, concentrations are not reported here due to lack of confidence in response standards. During extraction method development, sonication was tested as an extraction option. However, limited detection of EE2 with this method agreed with previous studies that indicated EE2's ability to be broken down by chemical bond disruption due to the energy provided by ultra sonication (Frontistis & Mantzavinos, 2012). This finding is also relevant to how solutions were added to experimental tanks. All stock solutions of chemicals used in this experiment were dissolved in ethanol, but only EE2 was dissolved by sonication. The sonication of EE2 in preparation of the stock solution may have resulted in its breakdown prior to the experiment, which likely added to the detection difficulties.

GIS Analysis of Septic Densities

GIS analysis was based on previous data from a septic system inventory of 1,056 adjacent to coastal waters in McIntosh County, GA (Walker et al., 2003). A 2,500 m

zone was allocated around each sample site. No septic systems were detected within the zone at Eagle Creek (our presumed site of low/no contamination). Our site at 4-Mile Island contained 25 septic systems within the allocated buffer and Oak Dale Creek had 35 septic systems, the highest density of septic systems among our sites. There were no dysfunctional septic tanks located within 2,500 m of Eagle Creek, however there was a single septic located just outside the 2,500 m zone that had previously been identified as dysfunctional (Walker et al., 2003). While none of the 25 septic tanks located within 2,500m of 4-Mile Island were identified as dysfunctional, there were 2 dysfunctional systems located just outside this zone. Oak Dale Creek had the highest incidence of dysfunctional septic systems located within the 2,500 m zone (~5). There were additional dysfunctional septic systems located more than 2,500 m upgradient of Oak Dale Creek (~4). In total, 53 systems were determined dysfunctional within entire sample area located in McIntosh County (Walker et al., 2003). Septic system density was positively correlated with ibuprofen concentrations in oysters ($p = 0.051$) and negatively correlated with pH ($p < 0.001$), salinity ($p = 0.03$) and dissolved oxygen ($p < 0.001$).

Controlled laboratory study: Chemical accumulation by oysters

Oysters accumulated IBU in a time dependent manner that was influenced by the presence of food and microbes (Figure 3.4). General linear modeling (GLM) of IBU concentrations in oysters versus exposures determined significant differences in the means of treatments containing food ($p < 0.05$), exposure times ($p < 0.001$) and a significant interaction between exposure time and microbes ($p < 0.02$). Ibuprofen concentrations in oysters exposed to treatments containing food were significantly lower

($p < 0.05$) than in those without food (Figure 3.4). No difference was detected between treatments with and without microbes; however there was a significant interaction effect between microbial treatment across the 96 h exposure period on IBU concentrations. Mean IBU concentrations were significantly higher at 48 ($p < 0.001$, 0.03) and 96 (both $p < 0.001$) h than at 12 or 24 h.

Oyster tissue accumulation of DPH increased over the 96-h exposure in a continuously increasing curvilinear fashion for the following treatments: control, food only and microbes only. The treatment containing food and microbes peaked at 24 h and decreased at 48 and 96 h. General linear modeling revealed no significant differences between the mean concentrations of DPH accumulated by oysters exposed to food or microbes. However, Tukey's 95% simultaneous confidence intervals determined a significant ($p = 0.05$) increase in mean DPH concentrations in oysters between 12 and 96 h for all treatments except food plus microbes, which decreased from 48 to 96 h (Figure 3.3).

Naproxen concentrations in oyster tissues across treatment groups generally increased to 48 h and decreased at 96 h. The one exception was the treatment containing chemicals and food, in which NAP continued increasing to 96 h. Modeling (GLM) showed NAP concentrations were significantly increased in oyster tissues at longer exposure durations. Significant increases in NAP tissue concentrations were observed from 12 to 48 and 96 h of exposure ($p < 0.001$) and again from 24 to 48 and 96 h ($p < 0.001$, 0.02) (Figure 3.3). No significant differences were detected between treatments containing food or microbes.

Discussion

On-site septic systems provide an important and practical alternative to rural wastewater treatment. However, unless properly maintained, they can become a source of environmental pollutants. Faulty and leaking septic systems located adjacent to coastal waters provide a non-point source entry of human sewage into the aquatic environment (Comeau et al., 2008; Harris, 1995; Meile et al., 2010; Turriciano et al., 2004). Our field study demonstrated that human pharmaceuticals enter the environment via septic system runoff into adjacent coastal waters and accumulate into the tissues of oysters (*C. virginica*). Diphenhydramine, IBU and NAP were all detected in oyster tissues in the 0.001 – 0.050 μg/g range. Although not significant, IBU concentrations in oysters were generally higher at Oak Dale Creek, the site presumed to be most contaminated (by density of septic systems at 2,500 m) and was detected more frequently in oyster tissues (75%) than any other analyte. Ibuprofen is a largely prescribed and widely used painkiller with a low degree of human metabolism (Buser et al., 1999). This observation reflects previous studies showing IBU's ability to persist in the environment (half life = 50 days; (Tixier, 2003) and confirms its potential as a chemical indicator of human sewage pollution (Buser et al., 1999; Comeau et al., 2008; Farré, 2007; Glassmeyer et al., 2005). Oyster IBU concentrations were significantly lower in December (2011) than August (2011) or March (2012). These differences in IBU concentrations between sampling months could be due to several factors including rate of input into the environment from septic systems, seasonal variation in oyster metabolism, oyster lipid content, photodegradation, sedimentation or biodegradation. Our field study determined a positive correlation between IBU concentrations and lipid content in

oysters. Additionally, lipid content was significantly lower in December (2011) than March (2012) (December lipid concentrations were not significantly different than August). Correlating with lower lipid content in December (2011), IBU concentrations in oysters were also significantly lower in December than March (2012) or August (2011). There is very little research regarding seasonal variation in pharmaceutical contaminants in estuarine environments due to septic system input; however seasonal variations have been studied in systems receiving WWTP effluent, with concentrations higher during summer when low water periods are common (Comoretto & Chiron, 2005; Loraine & Pettigrove, 2005).

Concentrations of target pharmaceuticals in field water samples were consistently below detection levels. Ibuprofen and NAP concentrations have been shown to be eliminated from the water column by adsorption to particles and subsequent sedimentation, biodegradation or photodegradation (Nakada et al., 2008; Packer et al., 2003; Vieno et al., 2005; Winkler et al., 2001). Nakada et al. (2008) found opposite trends of IBU concentrations in coastal waters, measuring higher concentrations in winter than spring. However, this observation was attributed to greater adsorption of IBU to estuarine particles in spring because of its hydrophobicity (log K_{ow} = 3.97, Table 3.1) and higher concentration of suspended solids during the spring (Nakada et al., 2008). Suspended solids were not measured during this study, but would be valuable information in future studies. Naproxen was only detected in two oyster samples (4-Mile Island and Oak Dale Creek, March 2012), which is consistent with reports of a relatively short half-life (0.7-1.4 h) and photodegradation in estuaries (Nakada, et al., 2008). Naproxen's estimated partition coefficient (log K_{ow} = 3.10, Table 3.1) is only slightly less

than IBU; however IBU is much more persistent in the environment with a half-life of 50 days (Tixier, 2003). Diphenhydramine concentrations were not significantly different between sampling months or sites. Ferrer et al. (2004) also highlighted the importance of testing sediments for pharmaceutical compounds with a log $K_{ow} > 3.0$, and suggested that DPH may be concentrated as much as a thousand times in sediments over their concentration in water. Diphenhydramine has been reported in aquatic sediments in concentrations as high as 48.6 µg/kg (Ferrer et al., 2004). Due to its use as an antihistamine, DPH inputs into the system may fluctuate seasonally with environmental allergies, although this was not observed in the current study. Triclosan was not detected in any field samples, which was surprising due to its ubiquitous use. However, it is more likely to be present in sediment than in water, due to its high log K_{ow} (5.4) and pKa (8.1) (Table 3.1). Triclosan has been found to persist in 30 year old lake sediments at concentrations as high as 53 ng/g (Singer et al., 2002).

The lipid content measured in oyster tissues was significantly lower in December (2011) than March (2012). Lipid content may affect uptake of chemicals from the water column (Bruner et al., 1994; Huckins et al., 2004), especially acidic organic compounds with hydrophobic properties like ibuprofen (log $K_{ow} = 3.97$). Other studies have shown seasonal lipid variation in pre-spawning (high lipid) and post-spawning (low-lipid) mussel populations (Bruner et al., 1994). High lipid, pre-spawning mussels had a greater bioconcentration factor (BCF) and faster uptake for hydrophobic compounds (Bruner et al., 1994). Bruner et al. (1994) also concluded that lipid affinity of a compound can be a good indicator of mussel contaminant accumulation. Comparatively, oysters sampled in this study had significantly lower percent lipid content in December (2011) (prior to the

start of gametogenesis in oysters in January; Heffernan et al., 1989) than March (2012) (prior to spawning). Our study showed higher IBU concentrations corresponded with higher lipid levels in March (2012) versus December (2011). In Coastal Georgia, oyster spawning season usually starts late March to early April and continues through early October (Manley et al., 2008). This corresponds with Bruner et al. (1994) and our findings of higher lipid content in March (2012) (pre-spawning) versus August (2011) and December (2011) (post-spawning).

Experimental oyster spat stick reefs proved successful in recruitment of spat into tidal creeks in Coastal Georgia. This allowed for consistency in our sample population and control of oyster exposure duration in the environment. Although we were able to detect human pharmaceuticals in oyster tissue from our experimental reefs, results on the effectiveness of experimental oyster reefs as a remediation tool remains inconclusive. The small size of the experimental reefs coupled with the large amount of water in the tidal creeks and significant tidal mixing did not allow proper evaluation of the effectiveness of these reefs to mitigate water quality. Other studies have shown experimental reefs to be effective in small, shallow tributaries much like Oak Dale Creek (Nelson et al., 2004).

This study also demonstrated that grab sampling methods for detection of low concentrations of pharmaceuticals is not effective in rural, tidally influenced environments with no direct WWTP effluent input. Wastewater treatment plants provide a concentrated and consistent stream of chemical laden effluent while septic system input can be sporadic and subtle. Other studies have suggested that integrative sampling may be more advantageous in detecting pharmaceutical contaminants in similar environments.

These studies used polar organic chemical integrative sampling (POCIS) or semi-permeable membrane devices (SPMDs) in lieu of or in addition to biomonitoring species (Huckins, et al., 2004; Mills, 2007). Unless there is a nearby WWTP outfall providing a concentrated source of chemical contamination, higher volumes of water are needed to extract such low concentrations of pharmaceuticals from aquatic environments. Furthermore, the total burden of pharmaceutical contaminants can be confounded by binding to organic fractions of suspended sediments, deconjugation by microbial action and/or chemical degradation by microbes and UV light (Mills, 2007). Grab sampling methods provide only a snapshot of current conditions and water is commonly filtered at a sub-micron level, which can lead to further losses of chemicals that associate with particulate matter. Passive sampling devices deployed over weeks to months should be more useful in determining low level concentrations in surface waters by providing time-weighted average concentrations (Mills, 2007).

Chemical indicators of human sewage pollution can be a viable alternative to microbial methods (Bruner et al., 1994; Huckins et al., 2004). They tend to require less sample preparation and analysis time, are specific to human sewage pollution and are not subject to regrowth in the environment (Glassmeyer et al., 2005; Hagedorn & Weisberg, 2009). Ibuprofen, NAP and DPH were detected in oysters from coastal estuaries with presumed contamination by nearby on-site septic systems and have been previously regarded as potential human chemical markers in the emerging field of fecal source tracking (Comeau et al., 2008; Glassmeyer et al., 2005; Hagedorn & Weisberg, 2009). However, chemical monitoring is not always a viable approach due to expense and expertise associated with analysis.

Our laboratory study provided initial data on the ability of oysters to accumulate three widely-used over-the-counter pharmaceuticals from the overlying water column within 96 h. Both IBU and DPH behaved similarly in oyster tissue with greater uptake over 96 h in treatments without *E. faecalis* than those with the microbe. Although this trend was not significant ($p > 0.05$), there was a significant interaction effect between the presence or absence of microbes and time points sampled for IBU concentrations. A concurrent study showed *E. faecalis* experiences natural dieoff with only 42% remaining after 24 h (Crews, 2012). In addition, after 8 h, there was a 92% reduction of *E. faecalis* in water by oysters (Crews, 2012). The significant interaction between the presence or absence of microbes and concentrations across time points seen in this study could be explained by oysters' ability to reduce microbial concentrations in water over time, particularly within 24 h. As microbial concentrations in the water column declined over time (by oyster transfer to feces/psuedofeces and natural dieoff), it may have caused the effects of microbes to differ (as indicated by modeling) between time points and vice versa. This could explain the significance ($p=0.019$) of the interaction term (microbes*time) in our statistical modeling. Microbial degradation of IBU may also play a role in higher concentrations seen in treatments devoid of microbes versus than those with microbes. If microbial concentrations had remained more stable during the experiment, we may have been able to observe the relationship between the presence or absence of microbes and chemical concentrations.

Significantly lower IBU concentrations were accumulated by oysters in treatments containing an algal food source versus those without algae. Both the presence of an algal food source and microbes (in this case *E. faecalis*) affected uptake of IBU

found in oyster tissue. Studies have shown that oysters have the ability to sort particles, ingesting food and rejecting particles that are non-nutritive or of an unfavorable size (Jorgensen, 1966; Newell and Jordan, 1983; Haven and Morales-Alamo, 1970; Wetz et al. 2002; Loosanoff, 1949). Therefore oysters may have preferentially fed on algal food sources provided and rejected organic particulates or algae with adsorbed chemicals. Alternatively, fed oysters may have metabolized and eliminated IBU at a faster rate due to the presence of food.

Naproxen uptake by oysters plateaued at 48 h before decreasing; however the presence or absence of microbes and/or algae appeared to have no effect on NAP accumulation. It is important to note that all five chemicals (IBU, DPH, NAP, TRI and EE2) were presented together in each treatment. Synergistic or additive effects could play a role in the results of this study, as it is unknown how combinations of these drugs (especially those that share a common mode of action like NAP and IBU) affect uptake and elimination of pharmaceuticals in oysters (Farre, 2007). Toxicity of one or many chemicals may alter biological fitness of oysters, thereby reducing biotransformation abilities. Future research could be directed to determine differences between pharmaceutical uptake by oysters for both individual and suites of chemicals.

To conclude, human pharmaceuticals (IBU, DPH and NAP) were detected in oyster tissues collected from tidal creeks in Coastal Georgia where there was no immediate WWTP input, only septic systems. Triclosan and EE2 were not detected in oyster tissues due to analytical limitations as well as their likely association with sediments instead of remaining within the water column. Concentrations in oyster tissues varied seasonally and correlated with oyster lipid content. Due to the hydrophobic nature

of many human pharmaceuticals, total suspended solids data would be helpful to better understand the bioavailability of these chemicals in future studies. Oyster tissue extractions proved to be a better detection medium than water; however, additional research should include integrative sampling with passive samplers alongside biomonitoring with oysters. In addition, the sporadic and intermittent characteristics of septic system discharge into adjacent waters would warrant a more frequent (monthly as opposed to seasonally) sampling schedule. Controlled laboratory studies showed the presence of an algal food source and microbes correlated with decreased tissue concentrations for IBU and could affect its accumulation by oysters in the field as well. These findings call for including data collection of algal and microbial densities to better model uptake kinetics for future field studies. The effectiveness of oyster reefs as a bioremediation tool was inconclusive due to the amount of water present in tidal creeks versus experimental reef size and tidal mixing. Overall, oysters showed the ability to accumulate human pharmaceuticals in the environment. Further research is needed to determine the fate of human pharmaceuticals after uptake by oysters as well as biological or toxicological effects.

Table 3.1 Target analyte characteristics and solubility properties

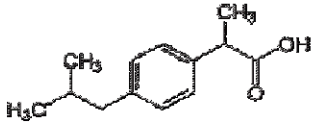
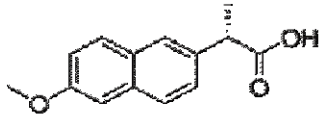
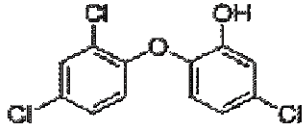
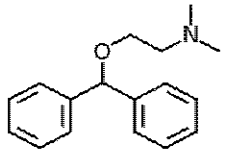
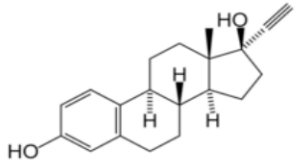
Group	Compound	Use	Structure	CAS No.	Pka	Kow
Arylpropionic acids (profens)	**Ibuprofen** 2-(p-isobutylphenyl)propionic acid	Non-steroid anti-inflammatory (NSAID)/analgesic		15687-27-1	4.91	3.97
	Naproxen 2-(6-methoxynaphthalen-2-yl) propionic acid	Non-steroid anti-inflammatory (NSAID)/analgesic		22204-53-1	4.15	3.10
Polychloro phenoxy phenol.	**Triclosan** 2,4,4'-trichloro-2'-hydroxydiphenyl ether	Anti-microbial disinfectant		3380-34-5	7.9	4.76
Amine	**Diphenhydramine HCl** N,N-dimethyl-(diphenylmethoxy) ethylamine	Anti-histamine		58-73-1	9.00	3.27
Synthetic steroid/ Estrogen	**17α-ethynylestradiol** 19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol	ovulation inhibitor		57-63-6	17.59	4.3

Table 3.2 Isotopically labeled standards for target analytes.

Compound	MW	Purity
Diphenhydramine-d_5 HCl	260	98%
EE2 (20,21-13C2)	298.4	99%
Ibuprofen (Propionic-13C3)	209.26	99%
Naproxen (Methyl-13C)	234.28	99%
Triclosan (13C12)	301.45	99%
Estradiol (3,4-13C2)	274.37	>98%
Estrone (3,4-13C2)	272.35	98.50%

Table 3.3 Multi-reaction monitoring (MRM) mode settings. Limits of detection (LOD) are based on a calibration curve, not a calculated value.

MRM Reactions monitored	Product [CE]	LOD
Diphenhydramine	256-167 [-9.0V]	0.1 µg/L
Diphenhydramine ISO	259-169 [-9.0V]	
Ibuprofen	205-161 [6.0V]	0.1 µg/L
Ibuprofen ISO	208-163 [6.0V]	
Naproxen	229-170 [7.0V]	0.1 µg/L
Naproxen ISO	233-170 [7.0V]	
17α-ethynylestradiol	295-145 [40.0V]	1 µg/L
17α-ethynylestradiol	295-159 [40.0V]	
17α-ethynylestradiol	295-183 [40.0V]	
17α-ethynylestradiol ISO	297-183 [40.0V]	
Triclosan	289-35 [6.0V]	1 µg/L
Triclosan ISO	301-35 [6.0V]	

Table 3.4 Controlled laboratory study sampling scheme.

Time Points:	0	12	24	36	48	60	72	84	96
Composite	200 ml	200 ml	200 ml	200 ml	200 ml pre/post renewal	200 ml	200 ml	200 ml	200 ml
Non-composite	500 ml				500 ml pre/post renewal				500 ml
Oysters	3	3	3		3				3
Total water collected	700 ml	200 ml	200 mL	200 ml	1.2 l	200 ml	200 ml	200 ml	700 ml

Table 3.5 Mean (±SEM) environmental parameters by seasons. Data were pooled among the three field sites (Eagle Creek, 4-Mile and Oak Dale Creek)

Season	Temp (°C)	Salinity (ppt)	DO (mg/L)	pH	Rainfall (in/wk)
August 2011	31.49±0.79	35.23±0.39	5.33±0.29	7.29±0.03	0.09
December 2011	14.78±0.53	30.50*	6.17±0.57	7.39±0.28	0.48
March 2012	18.06±0.66	25.76±4.58	4.92±1.47	7.48*	1.04

*****YSI sampling error at one site reduced sample size to n=2, therefore no SEM was calculated

Table 3.6 Field sample correlations (Pearson Product Moment) of IBU, DPH and lipid concentrations with each other and with environmental parameters and septic system density at 2,500 meters. Correlation coefficients in bold are significant at $p < 0.05$.

	DPH oyster	IBU oyster	% Lipid	Temp	pH	Salinity	DO	Rainfall
IBU oyster	-0.096							
% Lipid	0.029	**0.300**						
Temp	0.250	0.254	0.115					
pH	-0.155	-0.125	0.190	-0.235				
Salinity	**0.354**	-0.325	**-0.336**	**0.636**	0.089			
DO	0.035	**-0.399**	**-0.266**	-0.172	**0.602**	**0.760**		
Rainfall	**-0.319**	0.195	**0.308**	**-0.682**	**0.379**	**-0.718**	-0.154	
Septic 2500m	0.068	**0.267**	-0.126	0.062	**-0.514**	**-0.416**	**-0.491**	0.000

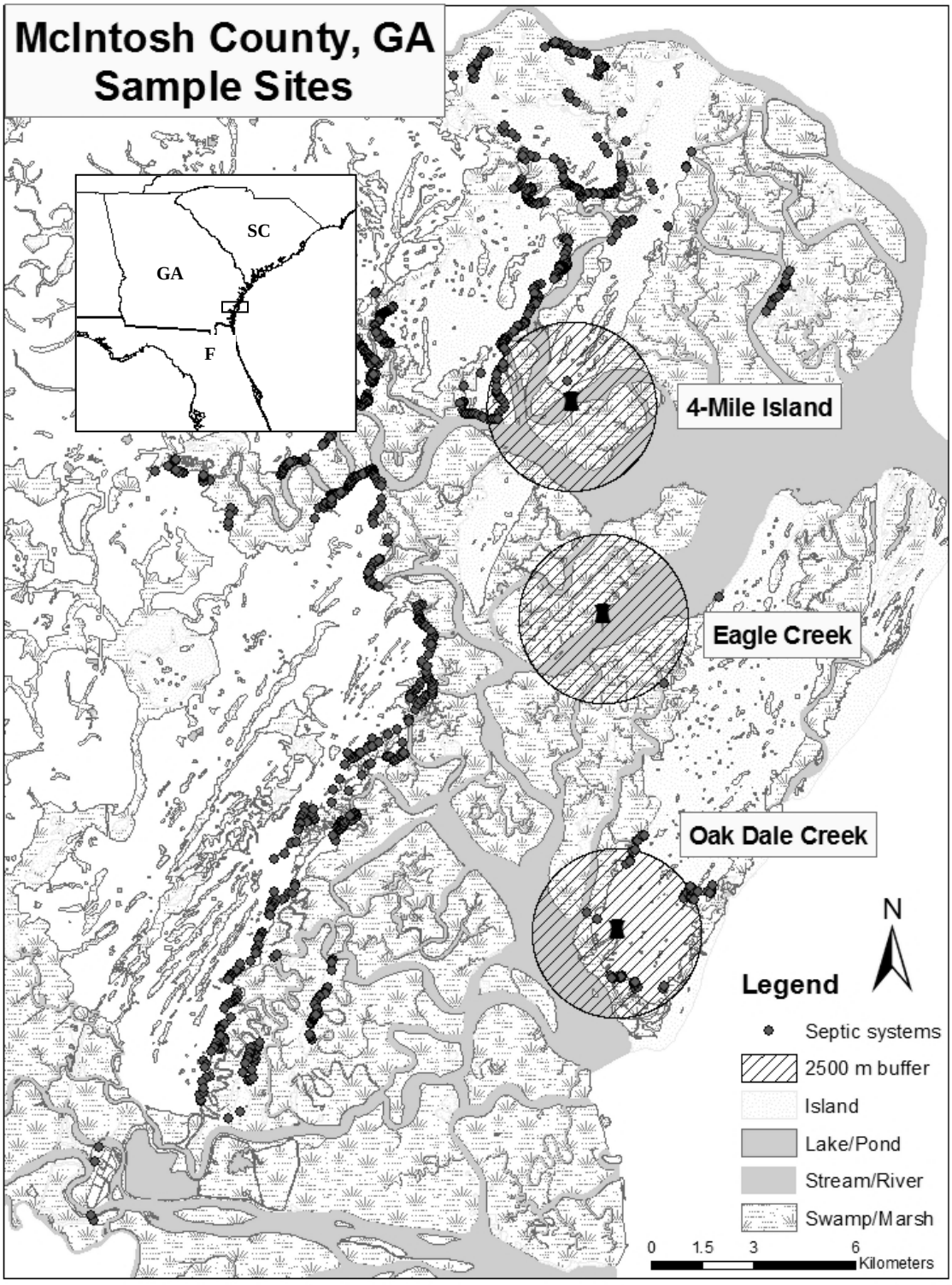


Fig 3.1 Sampling sites in McIntosh County, GA, representing a gradient of potential exposure to septic systems. Hashed circles around the sites indicate a 2,500 meter radius.

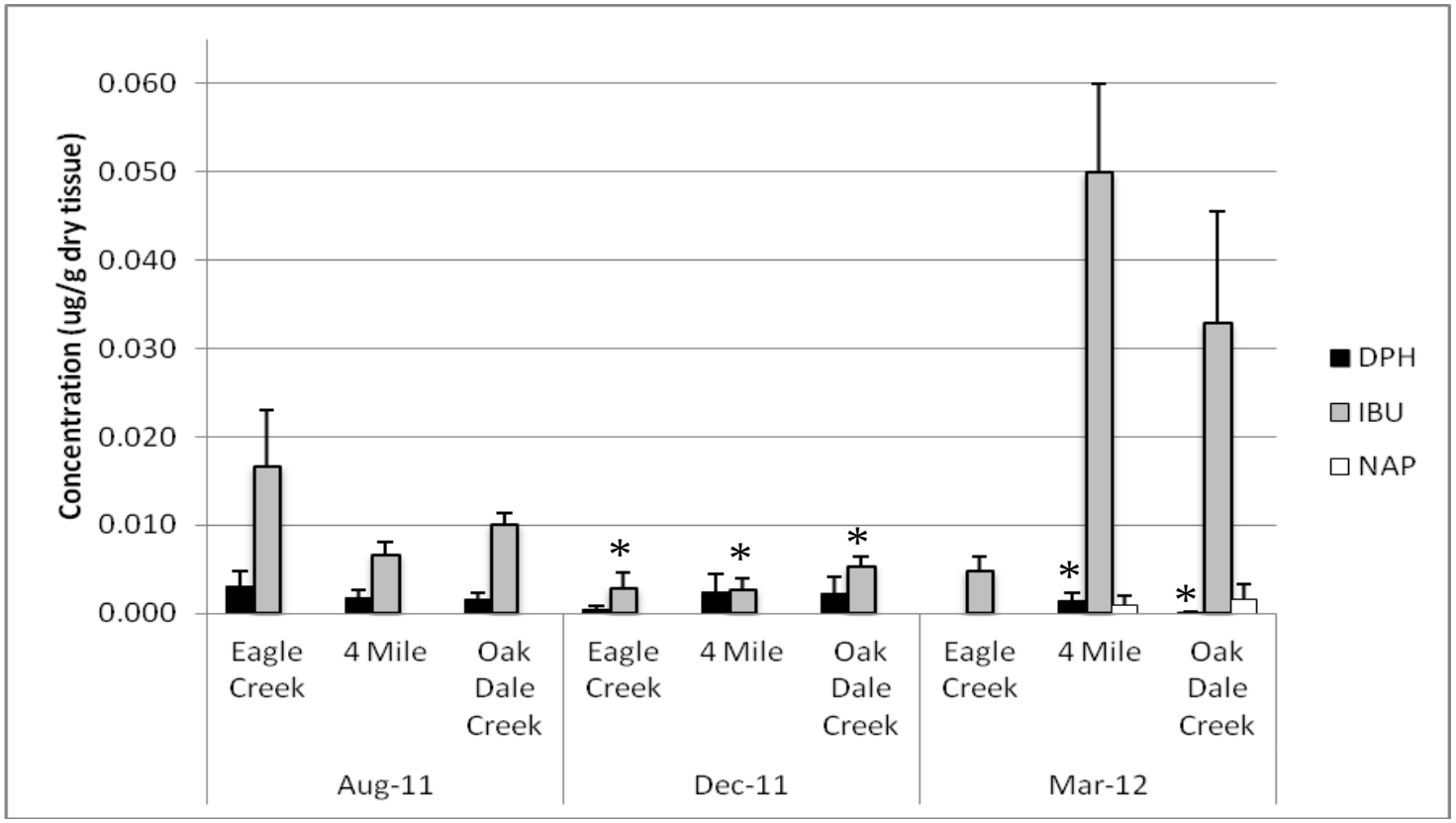


Fig 3.2 Mean (+SEM) concentrations (µg/g dry tissue mass) of diphenhydramine (DPH), ibuprofen (IBU) and naproxen (NAP) in oysters (n = 6) collected from McIntosh County, GA, by season and site. Means with astericks are significantly different by sampling month (Tukey's simultaneous test, $p < 0.05$). There were no significant differences between sites.

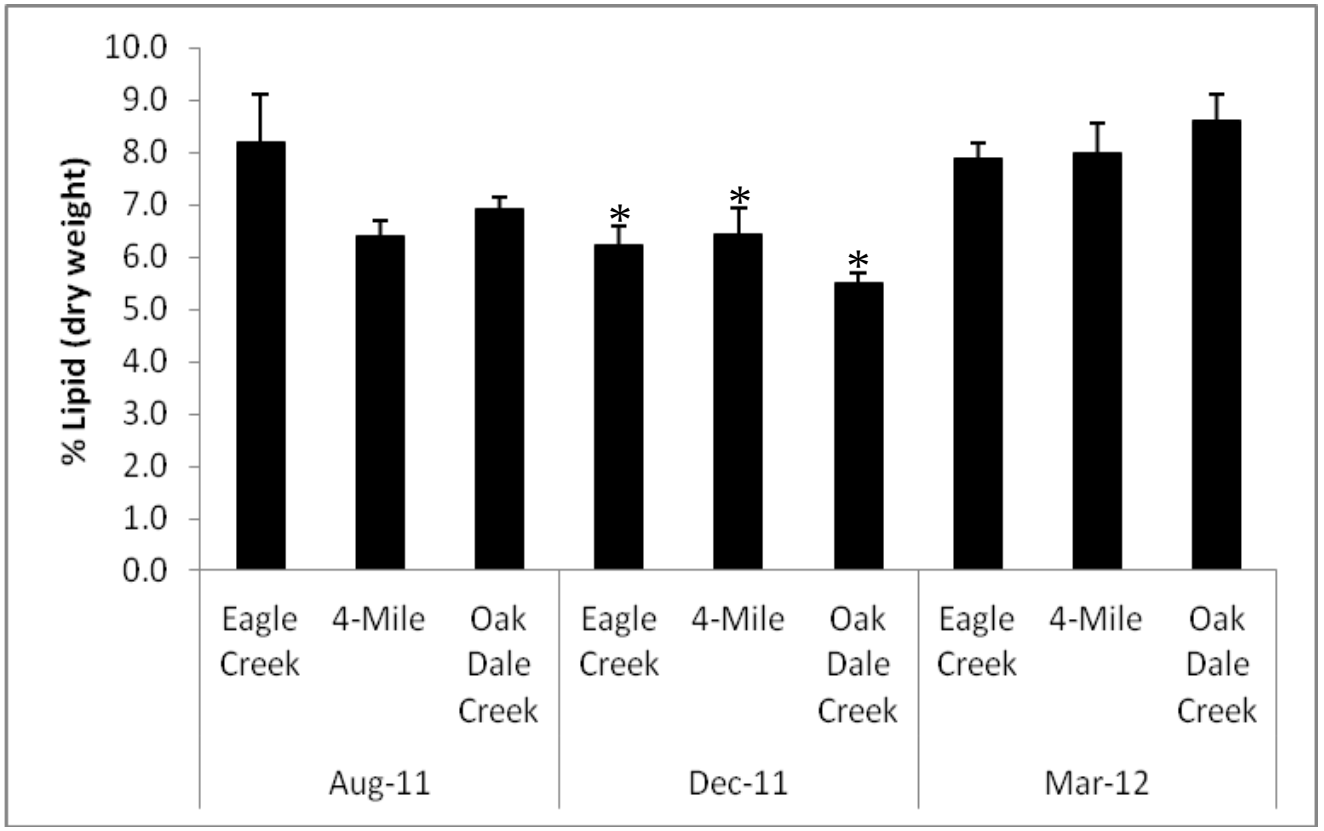


Fig 3.3 Percent lipid content (dry weight basis, ±SEM) in oysters collected from the field sites (n=6; from pooled samples prepared for analysis). Means with astericks are significantly different (Tukey's simultaneous test, $p < 0.001$) versus other months. There were no significant differences in tissue lipid content between sites.

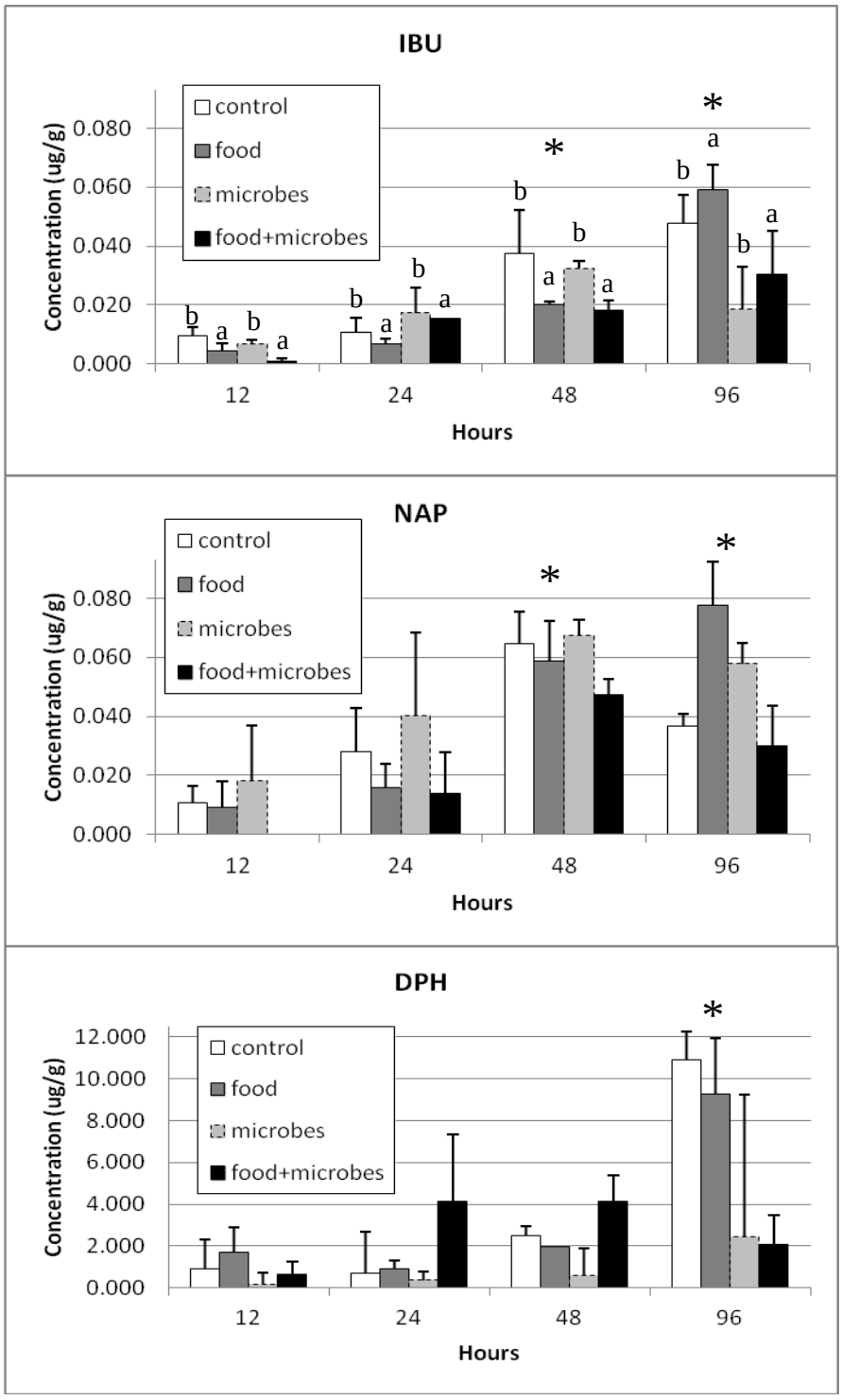


Fig 3.4 Mean (±SEM) tissue concentrations of pharmaceuticals in oysters (n=9) sampled during the 96 h controlled laboratory study. All treatments contain chemicals (IBU, NAP, DPH, TRI and EE2). "Control" treatments represent chemical only treatments. Means with different letters are significantly different (Tukey's simultaneous test, $p<0.05$). Asterisks indicate significant difference between concentrations across the time points sampled.

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CHAPTER 4

CONCLUSION

Field sampling and controlled laboratory studies were conducted to assess the effectiveness of experimental oyster (*Crassostrea virginica*) reefs in remediating human pharmaceutical contaminants from tidal creeks in Coastal Georgia. Water and oysters were collected over three sampling months (August 2011, December 2011 and March 2012). The human pharmaceuticals ibuprofen (IBU), diphenhydramine (DPH) and naproxen (NAP) were detected in oysters collected from three tidal creeks in Coastal Georgia where there was no immediate WWTP effluent input. Our results imply that septic systems were the contributory source. Analysis revealed a significant correlation between IBU concentrations in oysters and septic system density at 2,500 m. In the field, IBU was detected in the highest concentrations followed by DPH and NAP. Ibuprofen tissue concentrations varied with seasonal lipid content and were significantly less in December (2011) than March (2012) or August (2011). Diphenhydramine tissue concentrations were significantly less in March (2012) than August (2011) which could be related to bioavailability rather than oyster uptake. Oyster tissues proved to be a better detection medium than water; however, additional research should include integrative sampling alongside biomonitoring. The use of passive sampling devices may be a more effective sampling approach due to low level, intermittent releases that are characteristic of septic systems. Controlled laboratory studies were conducted to better understand the temporal aspects of accumulation and effects of a food source and microbial presence on

chemical uptake by oysters. These experiments showed that in the presence of an algal food source and microbes (*E. faecalis*), oysters accumulated lower concentrations for some compounds (IBU and DPH), but not others (NAP). The presence of microbes correlated with lower chemical concentrations detected in oysters, which may be due to microbial degradation of the compounds in the exposure water. The presence of an algal food source significantly lowered IBU concentrations detected in oyster tissues. These findings could be applied to natural settings and contribute to limiting factors affecting uptake and subsequent detection in the environment. In retrospect, determining uptake of pharmaceuticals in oysters would have been more conclusive had oysters been exposed to chemicals separately in addition to an entire suite. This way, individual effects of chemicals could have been compared to mixture effects on uptake. Additionally, data collection on total suspended solids of the natural seawater used would have been helpful in determining the extent of chemical adsorption to particulates within each tank.

The effectiveness of oyster reefs as a bioremediation tool was inconclusive due to the amount of water present in tidal creeks versus experimental reef size, tidal mixing and low concentrations at present at sporadic intervals. Overall, oysters showed the ability to accumulate human pharmaceuticals in the environment. Further research is needed to determine the fate of human pharmaceuticals after uptake by oysters as well as biological consequences of exposure.