ENVIRONMENTAL METABOLOMICS & BIOMONITORING OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN WILD EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*) IN GEORGIA, USA ESTUARIES

by

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(Under the Direction of Marsha C. Black)

ABSTRACT

Eastern oysters (*Crassostrea virginica*) support a large aquaculture industry and are a keystone species along the Atlantic seaboard. Native oysters are routinely exposed to a complex mixture of contaminants that increasingly includes pharmaceuticals and personal care products (PPCPs), which are designed to exert pharmacological effects at low concentrations. The biological effects of these exposures on oyster physiology is poorly understood, as there is a lack of data about the temporal and spatial variability in PPCPs bioaccumulation in Georgia's estuaries and how environmental stressors affects oyster physiology. A laboratory experiment was conducted to quantify the physiological responses of oysters exposed to fluoxetine, N,N-diethyl-meta-toluamide, 17α-ethynylestradiol, diphenhydramine, and their mixture for ten days, followed by an eight-day depuration. Exposures elicited effects based upon each chemical's postulated mechanism-of-action, as well as off-target effects. Affected metabolites were associated with Krebs cycle intermediates, fatty acids, carbohydrates, amino acid metabolism, and the urea cycle. Metabolic effects varied at each sample point, and that overall,

oysters were only able to partially recover from these exposures post-depuration. A two-year field study was conducted to determine temporal, spatial and bioaccumulation trends for 16 PPCPs in native oysters near Brunswick and Sapelo Island, Georgia. Across all samples, concentrations ranged from <LOD to 35 ng/g, with few statistically significant differences between estuaries and their respective sites. Results displayed strong seasonal and yearly trends, but was highly compound and estuary dependent, highlighting the influence of local populations on oyster bioaccumulation and potential for toxicological effects. A one-year untargeted GC-MS based environmental metabolomics study was conducted to discern seasonal and site specific changes in oyster physiology at Brunswick and Sapelo Island. These interactions make it difficult to interpret the observed physiological responses at the metabolome level, as there is a lack of baseline data for natural oyster populations. Amino acids, carbohydrates, fatty acids, and Krebs cycle metabolites were affected at both locations, although their regulation pattern varied significantly between seasons and estuaries. This research demonstrates that PPCPs are ubiquitous contaminants in Georgia's estuaries, with significant seasonal and yearly variation in bioaccumulation, as well as providing insights for future monitoring studies utilizing native oyster populations.

INDEX WORDS: *Crassostrea virginica*, Bivalves, Pharmaceuticals and personal care products, PPCPs, Metabolomics, Biomonitoring, Overall biological effect, stress, Estuaries

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DEDICATION

To Marsha Black: Thank you for being a wonderful mentor and friend. Words cannot express how much I appreciate what you have taught me about being a scientist, teacher, and an advocate. I will remember these life lessons forever.

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CHAPTER 1

Introduction & Literature Review

PPCPs in the Estuarine Environment: Importance of Estuaries

Estuaries are one of the most important environments in the coastal zone, and are one of the most biologically productive areas on earth due to the highly dynamic intermixing of freshwater and seawater. This intermixing also causes significant gradients in physical, biological, and chemical compositions, such as pH, salinity, and suspended particulate matter concentrations. Estuaries also play an important ecological role by filtering and degrading pollutants, reducing the adverse effects from off-shore storms, providing nursery habitats for many economically valuable fish and bivalve species, and providing many cultural and recreational benefits (Millennium Ecosystem Assessment, 2005; Sanger et al., 2015). Estuaries also have a large economic benefit, in that they directly and indirectly contribute tens of billions of dollars annually to communities by supporting environmental, transportation, tourism, and aquaculture industries (Barbier et al., 2011). Despite their ecological and economic benefits, it is estimated that upwards of half of saltwater marshes, and approximately a third of mangroves, coral reefs, and seagrasses around the world have already been lost or degraded (Millennium Ecosystem Assessment, 2005).

Global coastal populations are rapidly increasing, and are expected to grow by an additional two billion people over the next few decades, with approximately four billion people currently living within 60 km of the coast and over half of countries with coastlines having

between 80-100% of their total population within 100 km of the coast (Martinez et al., 2007). Additionally, 21 of the world's 33 megacities (cities with a population over 8 million) are on the coast (Li, 2003), which exerts a significant amount of stress on local infrastructure systems and the environment. Estuaries can be affected by a wide range of anthropogenic disturbances, including: urban development and tourism, waste disposal, dredging, transportation, agriculture and fishing, and as a byproduct of many of these activities, excess nutrients, pathogens, and chemical contaminants can enter estuaries from a variety of point and non-point sources (Kennish, 2002). Many of the environmental degradation issues in estuaries stem from issues related to overpopulation, loosely regulated development, and aging infrastructure systems that have the potential to both directly and indirectly influence habitat loss, ecosystem services, and both economic and recreational use of estuarine environments. Kennish (2002) identified pollution as one of the most widespread and urgent threats facing estuarine organisms.

There has been extensive research on the presence of legacy contaminants (e.g. DDT, PCBs, heavy metals, etc.) and their biological effects in estuarine organisms (Kennish, 1992); however, there has been very little research on the presence and possible adverse effects of emerging contaminants in estuaries (Gaw & Hutchinson, 2015; Granek et al., 2016). Emerging contaminants include thousands of chemicals (and their degradation products) that are released to the environment, and include contaminants such as pesticides, industrial pollutants, illicit drugs and pharmaceuticals, and personal care products. These chemicals enter coastal environments from both point sources (sewage effluents, combined sewer overflows, leaking septic tanks, manufacturing disposal and spills) and from non-point sources (sewage/storm runoff, and atmospheric disposition). Once these contaminants enter the estuarine environment, they can interact with various environmental media (i.e. water, suspended particulate matter, and

sediment) and organisms, with unknown and potentially adverse effects. To date, the environmental presence and possible biological effects of these contaminants in native bivalve populations along the coast of the state of Georgia is relatively unknown.

Economic & Ecological Importance of the Eastern Oyster

The eastern oyster (Crassostrea virginica, Gmelin 1791) is an important aquaculture species and there is an ongoing effort to increase their population levels for both aquaculture and ecological reasons, including in Georgia (MAREX – eastern oyster, 2016). C. virginica is a sessile bivalve filter-feeder that is widely distributed along the eastern seaboard of North America and has both important economic and ecological roles, despite commercial oyster harvests declining to approximately two percent of their historic harvest rates (Eastern Oyster Biological Team, 2007). C. virginica populations support a large aquaculture industry, adding an estimated $100 million dollars to the US economy in 2012 (Gomez-Chiarri et al., 2015) and by 2018, is estimated to add an estimated $1.6 million to Georgia's economy (MAREX – eastern oyster, 2016).

In addition to their important role in aquaculture, C. virginica is a keystone species that plays a key role in maintaining estuarine water quality by a highly efficient filter feeding process (Zu Ermgassen et al., 2012), and their oyster reefs provide essential habitat for a wide variety of estuarine species. They can filter upwards of 10 liters/hour/gram of dry tissue weight (Langdon and Newell, 1996) and can be exposed to contaminants from ingested organic and inorganic material, in addition to those from the filtered seawater (Bryan and Hummerstone, 1997; Philips, 1979). Due to their prodigious filtration rates, many different bivalve species have been used as biomonitors for both legacy (Cappello et al., 2013) and emerging contaminants (Fuller, 2012;

Granek et al., 2016); however, there have been no studies utilizing C. virginica simultaneously as both biomonitors for emerging contaminants and for effects-based surveillance via environmental metabolomics.

Pharmaceuticals and Personal Care Product Overview

Currently, there are over 4000 approved drug products available (U.S. Food and Drug Aministration, 2015) under various formulations, with approximately 1100 being unique prescription and over-the-counter compounds that include a large number of chemical classes and modes-of-action. Pharmaceuticals and personal care products (PPCPs) are specifically designed to be biologically active at low doses, and to target specific metabolic, enzymatic, or cell signaling mediators to produce their desired therapeutic effects and to reduce the potential for off-target interactions (Fabbri & Franzellitti 2015). Pharmaceuticals are usually not fully metabolized in humans and as much as 80-90% of these compounds are excreted as parent compounds via urine and feces (Kümmerer, 2009). After ingestion, most pharmaceuticals undergo complete or extensive Phase I and Phase II hepatic metabolism. Phase I metabolism consists of oxidation, reduction and/or hydrolysis, while Phase II involves conjugation reactions. The end goal of this metabolism is to yield polar metabolites that can be more easily excreted from the body. While it is generally assumed that these metabolites are less toxic, studies have demonstrated that these metabolites can be more biologically active than their parent compounds (Burhenne et al., 1997; Kümmerer, 2009). It is also possible that the compound is resistant to metabolism and is excreted relatively unchanged, where it remains biologically active. Currently, no country in the world regulates these contaminants in their water supplies and they are not routinely monitored in natural water bodies. However, there is evidence that these contaminants

are ubiquitous in the environment (Kolpin et al., 2002) and recently the European Union added 17 PPCPs (3 estrogens, diclofenac, 2,6-di-tert-butyl-4-methylphenol, 2-ethylhexyl-4-methoxycinnamate, 3 macrolide antibiotics, methiocarb, 5 neonicotinoids, oxadiazon and triallate) to their monitoring watch list (Barbosa et al., 2016). It is urgently required that the presence and environmental fate of PPCPs is better understood, as a wide variety of these contaminants have been shown to bioaccumulate in aquatic organisms and are considered possible causes of documented adverse effects in aquatic species (Barber et al., 2007; Kidd et al., 2007; Al-Salhi et al., 2012).

Sources of PPCPs in Estuaries

Human sewage is the primary source of PPCPs in the marine environment, primarily originating from wastewater treatment plants (WWTPs), hospital and industrial discharges, disposal of unwanted medication, and in some regions, leaking septic systems (Crago et al., 2011). Also, some PPCPs are discharged directly to estuaries and marine waters via secondary bypass or combined sewer overflows (Lubliner et al., 2010). In addition, agricultural and aquaculture practices can be a significant source of PPCPs detected in the environment, especially for antibiotics and hormones, which can be used to increase livestock's growth and reproduction (Calderón-Preciado et al., 2011). These multiple sources contribute complex contaminant mixtures to waterways, making it difficult to assign specific contaminants to a source and to determine which source(s) are contributors to any observed adverse effects. Wastewater influent contains a complex mixture of chemicals, not just PPCPs, many of which are not completely removed or degraded by conventional sewage treatment processes and have been detected in waterways receiving WWTP effluent (Schwarzenback et al., 2006; La Farre et

al., 2008). Wastewater treatment plants are designed to efficiently remove nutrients, solid waste, and pathogens, while meeting water quality standards for the receiving body of water, and are rarely designed to remove contaminants (Tijani et al., 2013). It is well documented that many PPCPs are minimally removed during the conventional activated sludge (CAS) treatment process (Verlicchi et al., 2012), which is the most widely used WWTPs treatment process in the United States and the world (Noguera-Oviedo and Aga, 2016). For example, carbamazepine, atenolol, and metoprolol are usually < 10% removed, while diclofenac is 10-39% removed, while other drugs may be released as transformation products (Herdando et al., 2006). Thus, depending on the compound, it is possible that parent and/or transformation products could be entering aquatic environments (Stackelberg et al., 2004). PPCP biodegradation in activated sludge depends on various factors, including: the physio-chemical properties of the compounds (Zeng, 2015), in situ carbon loading (Su et al., 2015), redox conditions (Stadler et al., 2015), sludge retention times (Batt et al., 2006) and the microbial community composition (Thuy & Loan, 2014). Certain compounds are removed from WWTPs via sorption to biosolids (Lubliner et al., 2010; Phillips et al., 2012), which are frequently used as an agricultural fertilizer and can potentially be a source of PPCPs in the local environment (Crago et al., 2011).

While WWTPs serve as the primary method of waste treatment in many urban areas, septic systems are the primary method for waste disposal for many households in rural, semi-rural, and suburban areas. The basic design of a septic system is a septic tank (that receives the waste from the house) and a drain field, which serves to treat the waste before it leaches into the groundwater (Kaplan, 1991). Drain fields treat the sewage waste by providing physical filtration, surface adsorption, sedimentation, and inactivation of the contaminants in the soil (Canter, 1997; Charles et al., 2005). The U.S. Environmental Protection Agency (EPA) estimates that

approximately 20% of septic systems in the United States are malfunctioning and over half of all US septic systems are older than 30 years (US EPA, 2005). Septic systems that are old and/or poorly maintained are increasingly prone to failure and fail to provide the right conditions for the effluent to be properly treated.

In many estuarine areas, septic systems may not be able to function properly due to high water tables close to the top of the soil, combined with soil that is sandy and porous (Mallin et al., 2012). These conditions allow for contaminants and waste to rapidly percolate through the soil without proper treatment (Stanford et al., 2010). These deficiencies have also led to increased levels of nutrients entering coastal waters, which have been linked to eutrophication of estuarine waters (Dederen, 1992; Smith et al., 1999), and can introduce PPCPs to estuaries (Dougherty et al., 2009; Meador et al., 2016).

PPCP's Adsorption to Suspended Solids & Sediments

PPCPs are discharged into marine and estuarine ecosystems as being dissolved in water and/or sorbed to suspended solids; however, there have been few studies on the presence of PPCPs in marine sediments. This is despite the fact that suspended solids and sediments can be an important reservoir for PPCPs (Liang et al., 2013), especially for basic PPCPs (Urase & Kikuta, 2005), and that changes in pH and salinity could possibly desorb them back into the water column (Wang et al., 2010). To date, less than 30 studies have analyzed sediment bound PPCPs in marine environments and about half of those studies did not measure water and sediment concentrations together, making comparisons difficult (Gaw et al., 2014; Arpin-Pont et al., 2016). Sediment concentrations for 62 PPCPs have been reported (Hester & Harrison, 2015), with ethinylestradiol being the most frequently reported, followed by antibiotics and

antihypertensive agents. PPCP concentrations were generally detected near 10 ng/g dry weight of sediment or less, although antibiotic concentrations over 2 mg/g wet weight were detected at a Vietnamese marine shrimp aquaculture pond that was intensively stocked (Le & Munekage, 2004).

PPCPs sorption to suspended solids is difficult to predict due to their broad range of physiochemical properties and has been shown to be compound specific (Al-Khazrajy & Boxall, 2016). For example, for basic PPCPs, sorption to suspended solids may be enhanced as the solubility of lipophilic compounds decreases with increasing salinity, thus increasing their partitioning to solids (Rodrigues et al., 2014). However, for acidic PPCPs which are expected to have a low sorption rate to sediments at alkaline pH values (Urase & Kikuta, 2005), it is expected that they will be mostly found in the dissolved water phase (Fent et al., 2006). The organic carbon content of the particles is also likely to play a role in PPCP sorption to suspended solids and sediments (Tolls, 2001; Williams et al., 2009).

Sediment sorption may decrease the potential for PPCPs to be photodegraded, as well as provide potential mechanisms for transporting sediment-bound PPCPs via water currents and tides (Stanford et al., 2010) and reduce their bioavailability (Zhao et al., 2015). The presence of suspended solids can also influence photodegradation as these particles can absorb or scatter light, which can alter a compound's potential for photodegradation (Panditi et al., 2014). The increased depth of many marine environments, compared to many freshwater environments, may also reduce photodegradation as sunlight is attenuated at depth (Challis et al., 2014; Panditi et al., 2014).

Presence of PPCPs in Seawater

PPCPs have been detected in coastal environments around the world (Emnet et al., 2015), with the majority of studies being conducted over the past six years in seawater from Europe and Asia (Gaw et al., 2014). The analysis of PPCPs in estuaries and the marine environment within the United States is relatively uncommon (Meador et al., 2016). PPCPs have been detected in effluent and marine waters in southern California (Vidal-Dorsch et al., 2012), Charleston Harbor (Hedgespeth et al., 2012), Puget Sound, Washington (Lubliner et al., 2010), in estuaries along the Texas coast (Scott et al., 2015), and in San Francisco Bay (Klosterhaus et al., 2013). There are also major research gaps about PPCPs in the marine environment for Africa, South America, and Pacific island nations.

To date, over 100 PPCPs have been detected in coastal waters, with measured concentrations ranging from <1 ng/L to the µg/L concentrations (Gaw et al., 2014). The maximum concentrations for 60% of the detected compounds exceeded the European Medicines Agency's threshold of 0.01 µg/L for predicted environmental concentrations in surface waters (Hernando et al., 2006). The most frequently reported PPCP classes were antibiotics, non-steroidal anti-inflammatories, and analgesics, which are consistent with the data from freshwater studies (Hughes et al., 2012). Generally, PPCP concentrations decrease with increasing distance between the sources and the coastline; however, there is evidence that PPCPs can travel hundreds of kilometers offshore. For example, metformin was detected 200 km off the coast of Germany (Trautwein et al., 2014) and antibiotics were detected 400 km off the Chinese coast in the Yellow Sea (Zhang et al., 2013).

Meador et al., (2016) analyzed WWTP effluent, seawater, and tissue from wild-caught juvenile Chinook salmon (Oncorhynchus tshawytscha) and Pacific staghorn sculpin (Leptocottus

armatus) for 150 PPCPs at three estuaries in Oregon. They detected 81 of their target analytes in the WWTP effluent, with 15 compounds at concentrations exceeding 1 µg/L, and detected 25 compounds in seawater. The effluent and seawater samples had 8 of 81 compounds in common. The authors calculated that the local WWTPs added between 0.8 – 6.6 kg/d of PPCPs into local receiving waters, and during "maximum design flows" from October – April, local inputs rose to over 3.5 – 16.8 kg/d (Meador et al., 2016). Fuller (2012) detected the pharmaceuticals diphenhydramine and ibuprofen in C. virginica collected from estuarine tidal creeks near Sapelo Island, Georgia. Their study found a statistically significant correlation ($p < 0.05$) between sample sites with high concentrations of pharmaceuticals in oyster tissues and septic field density. This study also concluded that water grab samples were not an effective sampling method to detect low concentrations of pharmaceuticals in rural, tidally influenced areas with no direct WWTP inputs.

PPCPs in the Estuarine Environment: Tidal & Seasonal Influences

Tidal movement and the effects of seasons are postulated to have an important role in the spatiotemporal trends of PPCP contamination in estuarine and marine waters; however, they are both understudied areas of research (Zhao et al., 2015). Tides cause a highly dynamic pattern of water movement that can induce significant physical (movement of suspended solids and sediments), chemical (e.g. pH, salinity), and biological (e.g. species distribution) gradients due to the intermixing of freshwater and seawater (Cailleaud et al., 2009). Despite their importance, its effect on PPCP contamination has been poorly studied. Zhao et al., (2015) found that in the Yangtze estuary in China, PPCP concentrations decreased from peak tide to slack tide and increased from ebb peak to ebb slack tide at the majority of their sites. For this reason, tidal

mixing can make it difficult to pinpoint sources of PPCP contamination in these environments. This is in contrast to freshwater environments without tides, where it can be easier to identify clearer relationships between potential sources of PPCPs that lie upstream or downstream of a particular site (Pascual-Aguilar et al., 2013). Another challenge of studying PPCP concentrations in estuarine and marine environments is that it can be difficult to establish a suitable reference site that is not contaminated (Ferguson et al., 2013), because these contaminants are ubiquitous in the environment.

Studies about seasonal trends in PPCP contamination have found that seasons have a mixed effect on contaminant detection, and varies greatly with the study's location. Vidal-Dorsch et al., (2012) found that there was little difference in PPCP concentrations of 56 analytes from both WWTP effluent and receiving waters in southern California. In contrast, Daneshvar et al., 2010 (River Fyris in central Sweden), Vieno et al., 2005 (River Aura, Finland), and Hedgespeth et al., 2012 (Charleston Harbor, South Carolina) found that winter months had higher detection frequencies and PPCP concentrations in both WWTP effluents and receiving waters compared to the summer season. The authors postulated that this was due to the colder temperatures inhibiting bacterial metabolism and reduced photolysis (Vieno et al., 2005). Fuller (2012) found that C. virginica in the southeastern United States displayed a seasonal bioaccumulation pattern for ibuprofen and diphenhydramine, with samples collected in the fall having a higher concentration than winter or spring samples. However, Granke et al., (2016) found that pacific oysters (Crassostrea gigas) from urban and rural estuaries in Oregon only accumulated PPCPs during the spring and summer, and did not detect any compounds in oysters during the fall. Also, for some PPCPs (e.g. DEET, antihistamines) there is likely a seasonal usage by consumers that would influence detection frequency and concentration.

PPCP Presence in Estuarine & Marine Organisms

There are three main routes that estuarine and marine organisms can be exposed to PPCPs - via their gills, contact with sediment, or through their diet. The level of their potential exposure depends on a variety of factors, including their proximity to aquaculture activities and WWTP discharges, the density and maintenance level of nearby septic sites, the dilution volume of the receiving waters, and its residency time (Nödler et al., 2014). Potential exposure levels are likely to be higher in areas receiving the greatest concentrations of PPCPs or where environmental conditions enhance the persistence of PPCPs in estuarine and marine environments (Bayen et al., 2013).

There is a currently a scarcity of data about PPCP concentrations in estuarine and marine organisms, and this data gap has been attributed to difficulties developing robust analytical methods (Klosterhaus et al., 2013). PPCPs have been detected in bivalves (Meador et al., 2016), fish (He et al., 2012), squid from a Czech fish market (Fedorova et al., 2014), dolphins (Tursiops truncates – Fair et al., 2009), and sharks (Carcharhinus leucas – Gelsleichter & Szabo, 2013), with the majority of the data being for filter-feeding bivalves (Gaw et al., 2014). Both laboratory- and field-based assays have demonstrated that certain PPCPs can bioaccumulate in bivalves, although the quantified PPCP concentrations were generally low, ranging from <1 ng/g to low µg/g (Fuller, 2012; Franzellitti et al., 2013 and Na et al., 2013; Granek et al., 2016). There is evidence that the current focus on parent PPCP compounds may be underestimating the possible adverse effects of PPCP exposure, especially as marine organisms have been shown to metabolize these compounds (McEneff et al., 2013). For example, the concentrations of the transformation products for venlafaxine were higher in the marine mussel Mytilus

galloprovincialis compared to the parent compound (Bueno et al., 2014). The data gathered from biomonitors can be used to estimate both spatial and temporal trends in contaminant exposures, provide estimates of local water quality, and estimate possible exposure scenarios for other organisms.

In coastal environments, various bivalve species have been utilized as biomonitors for PPCPs. Various oyster species (Crassostrea ssp.) have been utilized as biomonitoring organisms for PPCPs in estuaries (Fuller, 2012; Granek et al., 2016) because they are long-lived, sessile bivalves with a high filtering capacity that provides a chronic exposure to water-borne contaminants. Granke et al., (2016) sampled pacific oysters (Crassostrea gigas) and sediments over three seasons (summer and fall 2013 and spring 2014) from one site at two different estuaries (one urbanized and one rural) for PPCPs, pesticides, polybrominated diphenyl ethers (PBDEs), metals, and polychlorinated biphenyls (PCBs). The authors found that the more urbanized estuary had higher frequency of detections and concentrations for PPCPs in both oysters and sediment samples. For the majority of the detected PPCPs, concentrations ranged from not detected to < 4 µg/kg of oyster tissue to approximately 20 µg/kg in sediment samples. The authors also detected seasonal trends, with the majority of PPCPs being detected during the summer and spring, with no compounds being detected during the fall. This pattern was similar for PCBs and metals, but for unknown reasons, the rural estuary had higher concentrations of PBDEs.

For PPCPs, the acid dissociation constant (pKa) is a key physiochemical parameter that influences many of their biological and chemical characteristics. Knowledge of the pKa value for PPCPs provides an understanding of the ionic form (i.e. neutral or ionic) the molecule will have across a range of pH values. Pharmaceuticals can be classified as either acidic, neutral, or basic,

depending on their chemical structure and physiochemical properties. Wells (1988) estimated that 75% of pharmaceuticals are weak bases, 20% are weak acids, and the remainder are non-ionics, ampholytes, and alcohols. In general, acidic pharmaceuticals are neutral at pH values below their pKa and basic pharmaceuticals are neutral at pH levels above their pKa (Rendal et al., 2011). In general, pharmaceuticals that are neutral (i.e. uncharged) are able to be more rapidly absorbed across biological membranes (Palm et al., 1999) and generally show higher toxicity at pHproviding a higher fraction of the neutral chemical form (Nakamura et al., 2008; Bostrom and Berglund, 2015). Manallack (2007) estimated that approximately 63% to 95% of pharmaceutical drugs are ionizable, meaning that the pH in the receiving body of water can affect their chemical speciation (i.e. the fraction of ionic or unionized forms - Bostrom and Berglund, 2015). Seawater's higher pH values (7.8 – 8.4) compared to freshwater's (5.5 – 7.5) can influence the toxicity and bioaccumulation of pharmaceuticals by having a higher percent of the compound in the neutral form. Rendal et al., (2011) found that for certain basic pharmaceuticals (fluoxetine, norfluoxetine, propranolol, lidocaine, sertraline, and trimipramine), both the neutral form and toxicity increased the most as the pH increased from pH 6.5 to 8.5 for both algae and fish. This was in agreement with Nakamura et al., (2008), who found that as pH increased from 7 to 9, there was a 28-fold increase in toxicity for fluoxetine towards Japanese medaka (Oryzias latipes) and a 30-fold increase in the bioconcentration factor (BCF).

Biological Effects of PPCP Exposure to Estuarine Organisms

Contaminant body burdens from field-collected organisms are commonly utilized to gain an understanding of which contaminants are in a particular area (Genn, 2000). Traditionally, this has been accomplished by conventional toxicological testing, morphological assessments, and

tissue chemistry analysis, which relies on the use of biomonitoring species to act as surrogates for their ecosystem. The standard suite of ecotoxicology tests used to determine the potential effects of PPCPs on freshwater aquatic organisms lead researchers to conclude that adverse effects were unlikely due to their low measured environmental concentrations (ng – low µg - Fent et al., 2006). Thus, in the marine environment, where contaminant dilution is higher, the probability of adverse effects due to PPCPs was perceived to be even lower. However, recent evidence has called into question the validity of standard ecotoxicology tests for PPCPs because they are not sensitive to detect short- and long-term adverse effects at environmentally relevant concentrations (Aguirre-Martinez et al, 2015). It is unlikely that the concentrations that estuarine and marine organisms are exposed to would result in direct mortality; however, it is possible that they could lead to indirect mortality or reduced population fitness (Kidd et al., 2007; Santos et al., 2010). For example, several studies have speculated that minor adverse effects in immune function or growth in juvenile salmon could result in decreased survivability in their first year in the ocean (Spromberg and Meador, 2005; Meador, 2013).

Within the past decade, there has also been increasing interest in assessing the ecological risks that multiple chemical exposures pose to aquatic organisms (Meek et al., 2011). This is in part because analytical capabilities have increased to the point where contaminants could be detected at low concentrations (Kolpin et al., 2002) and multiple contaminants are routinely detected in aquatic environments (Boxall et al., 2012; Meador et al., 2016). Environmentally relevant exposure scenarios also include complex mixtures of contaminants that may have additive, synergistic, or antagonistic effects, depending on their MOA (Henry and Black, 2007; Boxall et al., 2012) and can have biological effects not predicted from their individual components (Silva et al., 2002). As noted by Escer et al., (2005) and Backhaus et al., (2011),

chemicals with similar MOAs often exhibit concentration addition toxicity when present in mixtures, and thus, for mixtures, the total load of the class should be considered when predicting their toxicity (Rand-Weaver et al., 2013). It is also difficult to predict an individual compound's potential environmental effect in a mixture because of limited or nonexistent data on their biological effect(s) (Brausch et al., 2012).

Adverse effects from mixtures detected in laboratory-based studies are usually derived from simple combinations of tightly controlled concentrations of PPCPs whose biological mechanisms are well understood (Backhaus, 2014). Further complicating efforts to extrapolate from laboratory studies to the field, is that many chemicals are likely present at concentrations below current levels of quantification; however, this does not imply that there is an absence of potential adverse effects (Schlenk et al., 2012). Unfortunately, there has been a limited number of studies assessing the toxicity of mixtures on marine organisms, and even less on the effects that mixtures containing compounds from different chemical classes have on these organisms (Hutchinson et al., 2013). Recognizing these shortfalls, there has been increasing scientific interest in developing better techniques for quantifying adverse effects from chemical exposures, especially for mixtures. This has led to the increasing use of effects-based tools in field studies (Krewski et al., 2010).

Effects-based surveillance tools can be used to improve the environmental relevance of chemical monitoring programs, including biomonitoring, by linking ecological and chemical information together to better understand the relationships between contaminant exposure and their possible physiological effects (Connon et al., 2012). These methods have a major advantage over traditional chemical monitoring because they can provide information about a contaminant's (or a chemical mixture's) MOA without a priori knowledge of their potential

toxicity or their identity (Ekman et al., 2013). The information gained from effects-based surveillance can provide a solid foundation for future effects-based monitoring studies that are more targeted in nature and seek to better define the type of adverse effect that is being observed (Bradbury et al., 2004).

In effects-based studies, biomarkers are a critical tool to address some of the limitations of traditional chemical monitoring and represent pathway- or receptor-specific observations that are chemically-induced responses at the biochemical, physiological, or morphological level of an organism (Henderson et al., 1987). Traditional biomarker studies typically rely on the detection of a small number of contaminants and their associated biomarkers; unfortunately, this method is often inadequate for fully assessing exposure and risk, especially for field-based studies. For example, some chemicals (e.g. estrogens) may be biologically active at concentrations below their current limit of analytical detection (Kuch et al., 2010). Also, exposure to some contaminants (e.g. bug spray and antihistamines), may be transient due to sporadic usage and may not be present at the time of sample collection, but could have already induced a biological effect. Furthermore, there are likely many biologically active anthropogenic chemicals that, while not on the lists of target analytes, nonetheless find their way into the aquatic environment and could cause adverse effects. More recently, “omic” technologies, such as transcriptomics, proteomics and metabolomics have been employed to provide more robust and comprehensive methods to detect chemical-induced biological effects (Ankley et al., 2011) and will be discussed in detail in subsequent sections.

Based upon their physiochemical characteristics and the large quantities entering estuarine and marine environments, many PPCPs are able to bioaccumulate in aquatic organisms and have the potential to cause adverse effects. These compounds are designed to interact with

specific molecular targets, related to their MOA, and there are an estimated 324 molecular drug targets for all classes of therapeutic drugs (Overington et al., 2006). The possibility of an adverse effect potentially occurring increases as the degree of evolutionary and functional conservation of drug targets between the target organism (e.g. humans or livestock) and the non-target organism increases (Gunnarsson et al., 2008).

This biological read-across hypothesis states that "[PPCPs] designed to act at specific mammalian targets, may have effects in non-target organisms provided that the molecular target (usually a receptor or enzyme) is conserved" (Rand-Weaver et al., 2013). A study of 12 fish species with fully sequenced genomes found that 65-86% of human drug targets were conserved in these species (Brown et al., 2014). Thus, it is reasonable to assume that many of these drugs would cause adverse effects in fish and other non-target species that have conserved drug targets. Gunnarsson et al., (2008) detected 1,318 human drug targets in seven aquatic species frequently used in ecotoxicology experiments and nine additional species with well-characterized genomes and found that receptors were not well conserved. Although, they found that enzymes were conserved, potentially suggesting that MOAs affecting enzymes would have adverse effects on a greater number of species. However, the conservation of therapeutic drug targets does not necessarily translate to conservation of function across species (Rand-Weaver et al., 2013). This could occur when conserved molecular targets govern different processes in different species (Seiler, 2002). This is especially relevant for invertebrates (including oysters), which are genetically remote from humans, but still share highly conserved metabolic features (e.g. energy metabolism, protein and DNA biosynthesis, membrane stability, etc.).

Metabolomics: Overview

There are currently over 114 million organic and inorganic substances known to mankind, with more being discovered daily (CAS, 2016), making it impossible to monitor and assess the biological effects of exposure to each individual compound, much less to their mixtures. While measuring contaminant body burdens in biomonitoring species can provide an estimate of the temporal, geographic extent and bioavailability of contaminants, their presence does not necessarily imply that an adverse effect has or will occur. Thus, effects-based tools have been developed to increase the biological relevance of chemical risk assessments (Connon et al, 2012). Metabolomics has emerged as an invaluable component of effects-based studies (Skelton et al., .2014) because it analyzes the hundreds to several thousand endogenous low molecular weight metabolites within a cell, tissue, or biofluid (termed the metabolome) in response to external stressors or stimuli (Bundy et al., 2009). This provides a profile of an organism's global metabolome response to chemical exposures or environmental changes, which can provide a better indicator of an organism's health status than a single biomarker (Miller et al., 2007).

Metabolomes are the terminal downstream product of an organism's genome, and as such, are complex systems to study because metabolites have a wide range of physical and chemical properties such as hydrophobicity/hydrophilicity, volatility, molecular weight, and size (Brown et al., 2009). Metabolites are the end products and intermediaries of enzymatic reactions in organisms and can provide information about the biochemical activity of an organism at a particular snapshot in time, under specific physiological conditions (Goodacre et al., 2004). It is currently estimated that there are about 1,500 metabolites in the human metabolome, about 7,500 in the mammalian metabolome, over 200,000 in the plant metabolome (Brown et al., 2009; Zamboni et al., 2015), and include: organic acids, amino acids, sugars, sugar alcohols, sugar

phosphates, amines, fatty acids, polar lipids, hormones, and vitamins. Metabolites have no common building blocks, other than the elements carbon, hydrogen, oxygen, nitrogen, sulphur and phosphorous, and their molecular structures and sizes are extremely diverse (Longnecker et al., 2015). As a result, there is not one single analytical platform that is capable of providing a complete analysis of an organism's metabolome (Lu et al., 2008; Roberts et al., 2012). Metabolites serve as direct signatures of biochemical activity and are therefore easier to correlate with an organism's phenotype, especially when compared to genes and proteins, which are subject to both epigenetic regulation and post-translational modifications (Patti et al., 2012), and may not manifest themselves as functional changes at the phenotype level. Thus, perturbations that affect the metabolome have a higher likelihood of also affecting an organism's phenotype (Viant, 2007).

Targeted versus Untargeted Metabolomics

All metabolomic studies (whether laboratory- or field-based) can be divided between targeted and untargeted approaches, the choice of which approach depends on the aim and scope of the research (Roberts et al., 2012; Longnecker et al., 2015). Targeted metabolomics involves measuring a predefined group(s) of chemically characterized and biochemically annotated metabolites. The advantage of this approach is that there already exists a comprehensive understanding of the involved metabolic enzymes, their kinetics, end products, and the known biochemical pathways to which they contribute (Roberts et al., 2012; Cajka and Fiehn, 2015). Targeted metabolomics works well in the laboratory or in the field when there is prior knowledge about which metabolic pathways could be perturbed; however, if this knowledge is not available, then untargeted methods should be used. Untargeted metabolomics (i.e. global) is

the comprehensive analysis of all measurable metabolites in a sample, including those that cannot be putatively identified (Cajka and Fiehn, 2015). This means that all metabolites are potentially of interest, since it is not possible to know in advance which metabolites will be affected by a treatment or different environmental conditions (Niu et al., 2014). This approach offers the opportunity for novel target discovery, as the metabolome coverage is only constrained by the sample preparation/extraction methodologies and the inherent characteristics of the chosen analytical techniques (Roberts et al., 2012; Niu et al., 2014). Advances in instrumentation and data processing has led to metabolomic studies combining both untargeted and targeted metabolomics together to increase the sensitivity and selectivity of metabolite identification (Cajka and Fiehn, 2015). Untargeted metabolomic approaches can complement targeted methods by pinpointing novel targets for more focused and quantitative follow-up experiments (Sévin et al., 2015).

Laboratory-Based Bivalve Metabolomic Studies

Laboratory-based omics studies involving PPCPs and marine bivalves are uncommon in the scientific literature. The majority of bivalve omics studies involve physiological responses to changing environmental conditions (Ellis et al., 2014; Wei et al., 2015), heavy metal pollution (Yu et al., 2016; Ji et al., 2016), or industrial pollutants (Song et al., 2016). The few bivalve omics studies involving PPCPs have observed some interesting physiological insights about their response to these exposures; however, the majority of these studies have utilized transcriptomics and proteomics, with metabolomic techniques being infrequently used (Suárez-Ulloa et al., 2013). Laboratory-based metabolomic studies have been used to elucidate how different bivalve pedigrees respond to chemical exposures. Ji et al., (2015) utilized NMR-based metabolomics to

differentiate between the metabolic responses of white and black pedigrees of the Manila clam (Ruditapes phillppinarum) after exposure to zinc, cadmium, and a mixture of both metals. The authors detected significant differences in amino acids and energy-related metabolites between the two pedigrees, that they attributed to possibly different gene expression levels between pedigrees. They also found that the white pedigree accumulated statistically significantly ($p < 0.05$) higher concentrations of cadmium, although no appreciable uptake of zinc in either pedigree or exposure scenario (single or mixture) was detected. This finding has important implications for field-based metabolomic studies and illustrates the importance of selecting organisms of the same pedigree/species and life stage to ensure accurate results.

Environmental Metabolomics Overview

While laboratory-based studies are useful and have provided valuable insights into bivalve physiology, the knowledge gained is difficult to apply to field organisms due to the presence of dynamic environmental conditions and complex chemical mixtures. Metabolomic studies involving field-collected organisms (i.e. environmental metabolomics) are relatively rare in the scientific literature (< 30 in early 2016 - Bahamonde et al., 2016) and very few of these studies have involved bivalve species (Tikunov et al., 2010; Ji et al., 2016). Environmental metabolomics applies metabolomic approaches to organisms exposed to contaminants and other environmental stressors in their natural habitat (Bundy, 2009). With this approach, the multiple and simultaneously occurring impacts and interactions between the environment, its suite of contaminants, and the organism can be examined, providing information that may be applied to develop causal relationships between the metabolome and Darwinian fitness (reproductive health, growth and survival endpoints; Viant, 2007). Although environmental metabolomics

cannot identify specific metabolic effects of an individual contaminant, it does provide information about metabolite changes within affected physiological pathways in an organism, providing analytical clues on the mixture of contaminants and their mode-of-action (MOA) at a particular time and location. Environmental metabolomics is predicated upon the idea that chemical exposures and environmental conditions perturb the metabolome of biomonitoring organisms in a manner that produces either a coincidental or residual molecular signal (Veldhoen et al., 2012).

Environmental metabolomics has been utilized to relate changes in an organism's metabolome to a specific WWTP effluent (Samuelsson et al., 2011), surrounding land use patterns (Skelton et al., 2014) and a three-day shutdown of a wood pulp mill (Davis et al., 2013). Samuelsson et al. (2011) used NMR-based metabolomics with zebrafish (Danio rerio) to identify the biological effects of exposure to WWTPs with either conventional (activated sludge) or advanced (membrane biological reactor (MBR), ozonation, and MBBR + ozonation) treatment methods. They found that the advanced treatment methods had the largest metabolic effect on lipoproteins, glucose, glutamine, and alanine metabolism compared to the conventional methods and controls (Samuelsson et al., 2011). However, the authors were unable to determine if the effects seen were adaptive or adverse; although they speculated that harmful degradation processes from the advanced treatments were adversely affecting the D. rerio.

Environmental metabolomics has a large advantage over other effects-based techniques because it can provide biologically relevant information about an organism's contaminant exposure(s) and its MOA without a priori knowledge of which contaminants are present (Ekman et al., 2013). For this reason, in situ caged experiments involving bivalves at known sites of contamination are being increasingly utilized. This has become especially popular at locations

where natural populations are not readily available (Rank et al., 2007). These studies have primarily involved the mussels Mytilus edulis and Mytilus galloprovincialis as biomonitors for various contaminant classes (Maruya et al., 2014), and only recently have these studies used omic techniques (Campillo et al., 2015a). Cappello et al., (2015b) transplanted Mytilus edulis to a site heavily polluted with petrochemicals and utilized targeted NMR-based metabolomics combined with immunohistochemical assays to quantify the biological effects of exposure related to these compounds. Compared to the control site, they found that petrochemical exposure decreased neurotransmitter (serotonin, acetylcholine, and tyrosine) concentrations in Mytilus edulis' gills, which play an important role in bivalve reproduction and feeding (Gosselin, 1961). Ji et al., (2016) conducted an untargeted NMR-based environmental metabolomics study utilizing the oyster Crassostrea sikamea collected from a metal-polluted area and a reference site. They stated that they found evidence of disruption in osmotic regulation and energy metabolism that was due to the presence of metals; however, they did not develop the necessary targeted metabolomic biomarkers for metal exposure needed to support their conclusions. Thus, the changes in the metabolome detected could be from any contaminant present causing the detected MOA and/or from different environmental conditions between sites. This paper is a good example of the care that is required when conducting environmental metabolomic studies to ensure that the conclusions are well supported.

In traditional ecotoxicology, tests to determine the biological effects of chemicals are based off of quantitative concentration–response relationships, performed on a reduced set of defined biological endpoints, which usually include growth, development, survival, and reproduction (Ankley et al., 2006; Sans-Piché et al., 2010). In environmental metabolomics, the traditional study design is to identify changes in metabolite levels without examining other

biological endpoints, which limits the biological information gained and its applicability in conducting risk assessments (Bundy et al., 2009).

Thus, to overcome these issues and maximize their interpretability and use in risk assessments, several things have been proposed to improve environmental metabolomic studies. An important aspect of these studies is that they require significant knowledge of the basic ecology of the biomonitoring organism (Hines et al., 2007). For example, parameters related to the organism's life history, developmental stages and reproductive strategies are important to know, as they will influence the organism's metabolome and subsequent physiological response(s) to chemical exposures and environmental conditions. However, in the field this can be challenging, because contaminants are present as complex mixtures, comprised of compounds with varying MOAs, and changes in the metabolome are also dependent upon the organism's natural life history traits, seasons, and environmental factors (Williams et al., 2011). Thus, it is important to know the range of the baseline (i.e. normal) metabolism of the biomonitoring organism and how it varies as a function of the above mentioned parameters.

Currently, baseline metabolism is poorly understood for the majority of biomonitoring organisms (Bundy et al., 2009). Compounding this issue is the fact that there can exist considerable metabolic variation between individuals from the same population due to changes in their local micro-environment, their genetics and other physiological parameters (e.g. time since their last meal, parasite load, etc. - Simmons et al., 2015). Dealing with this biological variability has been an issue facing environmental metabolomic research because this variation can mask the metabolomic differences between stressed and non-stressed organisms (Viant, 2007). Biological variation can mask the effect of a small number of biomarkers (Sheehan & Power, 1999; Miller et al., 2007); however, it is not an issue with metabolomics because it

measures hundreds to thousands of metabolites simultaneously. The advantage of metabolomic analysis is that there will be a subset of metabolites between stressed and non-stressed organisms that can be used to discriminate between these groups (Viant, 2007). This helps stabilize the biomarker(s) of interest by including many relevant variables; even if they are noisy, it makes it easier to gain a holistic view of the metabolome (Eriksson et al., 2001). Parsons et al, (2009) demonstrated that for NMR metabolomic studies, the inter-individual metabolic variability is generally tissue- or biofluid-dependent. Homeostatic control is generally greater in tissues, which reduces the inter-individual metabolic variability within a specific tissue, compared to biofluids (Parsons et al., 2009). Collecting organisms from uncontaminated sites has been proposed as a method for understanding a specie's baseline metabolism (Arciszewski & Munkittrick, 2015). Unfortunately, this is complicated by research that has demonstrated the challenges of finding suitable reference sites when studying contaminants such as PPCPs, due to their near ubiquitous presence in aquatic environments (Ferguson et al., 2013; Meador et al., 2016).

Integrating Environmental Metabolomics with Chemical Body Burdens

As it is analytically, physically, and financially irresponsible to detect and test the chronic toxicity all of the man-made chemicals in the environment, it is imperative that better methods be developed and validated to quantify the relationship between contaminants in the environment and their potential adverse effects in aquatic organisms. While environmental metabolomic studies can provide insights into the MOA of contaminants that are present, even if the chemical cannot be detected (Ankley et al., 2006), the challenge is to link this with conventional parameters of toxicity considered suitable for risk chemical assessments (Sans-Piche et al.,

2010). Thus, metabolomic studies are beginning to be integrated with other techniques that measure endpoints at the molecular, physiological, and population level (Bundy et al., 2008). While this type of study is still relatively uncommon, Davis et al., (2016) utilized NMR-based environmental metabolomics and PPCPs biomonitoring in cage-deployed fathead minnows (Pimephales promelas) at 18 sites across the Great Lakes basin to quantify how the metabolome changed in relation to changes in contaminant body burdens. The goal of the study was to correlate body burden data for 132 PPCPs with observed metabolomics data using partial least-squares regression analysis as a screening tool to identify contaminants most likely to cause an adverse biological effect. This method was able to reduce the number of contaminants likely to have an adverse effect by 43% - 52%, which they noted was an improvement over the traditional methods of chemical analysis in surface water samples. The authors noted that this method could be utilized to improve PPCP ecological risk assessments, be coupled with targeted omic approaches, identify unknown sources of contamination, and be used in remediation studies.

The challenges of combining environmental metabolomics into traditional field monitoring studies were demonstrated by the Canadian Environmental Effects Monitoring (EEM) program. Despite research into molecular endpoints for 65 species of fish (Barrett & Munkittrick, 2010), they have yet to include these endpoints into their monitoring programs. This is due to the lack of data about the magnitude of change at the molecular level that could be interpreted as a problem for their biomonitoring species (Bahamonde et al., 2016). A related issue the authors identified, is that for regulatory purposes, a level of biologically relevant change needs to be defined, which is dependent upon many parameters (sample size, power, the significance level, and the endpoint's variability) that are currently not well understood for metabolomics (Munkittrick et al., 2009; Bahamonde et al., 2016). Despite its many benefits and

advances in effects-based monitoring, environmental metabolomics studies are not yet at the point where they can be considered standalone experiments capable of providing the information required for regulatory purposes (Davis et al., 2013; Davis et al., 2016). To use metabolomic responses for regulatory purposes, it is necessary to demonstrate explicit linkages between developed biomarkers and adverse effects at the community and population levels (Bradbury et al., 2004). This is a substantial research gap that if filled, would provide a deeper understanding of how chronic exposure to contaminants affects marine bivalves and greatly advance ecological risk assessments for PPCPs.

Summary and Chapter Objectives

Georgia has approximately one-third of the remaining estuarine salt marshes in the US, which are some of the most biologically productive areas in the state and are simultaneously undergoing rapid degradation due to urban development. Other than habitat loss, pollution is one of the most urgent threats facing estuarine organisms. This is especially true for the eastern oyster (Crassostrea virginica, Gmelin 1791), which is a sessile filter feeder that supports a large aquaculture industry and is an ecological keystone species. One type of pollution that is garnering increasing scientific interest is pharmaceuticals and personal care products (PPCPs), which are widely used therapeutic compounds that are specifically designed to be biologically active at low doses and interact with a specific molecular target(s) (Fabbri & Franzellitti 2015). The majority of these compounds enter the marine environment via wastewater treatment plants and septic fields (Crago et al., 2011), where they can sorb to suspended particles and/or sediments, undergo abiotic and biotic transformations and depending on the compound, can bioaccumulate in aquatic organisms (Gaw et al., 2014). PPCPs are generally detected at low ng -

μg concentrations in the environment (Emnet et al., 2015), which lead researchers to conclude that adverse effects in aquatic organisms are unlikely at these concentrations (Fent et al., 2006). In the marine environment, where contaminant dilution is higher, the probability of adverse effects due to PPCPs was postulated to be even lower. While this is generally true for acute toxicity, laboratory studies have demonstrated the potential for chronic toxicity to occur after PPCP exposure(s) (Aguirre-Martinez et al., 2015), with potentially adverse effects at the population level (Kidd et al., 2007).

An additional complication is that unlike laboratory studies, which generally involve a small number of defined compounds (Backhaus, 2014), organisms in the environment can potentially be exposed to complex mixtures ranging from hundreds to thousands of compounds (Meador et al., 2016). Despite research demonstrating adverse effects in laboratory assays (Henry and Black, 2007) and contaminant body burdens in field-collected bivalves (Fuller, 2012), the goal of linking contaminant body burdens with endpoints relevant to ecological risk assessments remains elusive. For this reason, effects-based techniques, such as metabolomics, have been developed to better understand the physiological effects that an organism's life history, environment, and chemical exposure(s), (plus their interactions) have on the individual and the population (Ankley et al., 2011).

Metabolomics analyzes the hundreds to several thousand endogenous low molecular weight metabolites within an organism's cells, tissues, or biofluid in response to external stressors or stimuli (Bundy et al., 2009). Metabolite classes include: organic acids, amino acids, sugars, sugar alcohols, sugar phosphates, amines, fatty acids, polar lipids, hormones, and vitamins. Metabolomics provides a profile of the organism's global metabolomic response to chemical exposures or environmental changes, which can provide a better indicator of an

organism's health status than a single biomarker or contaminant body burdens (Eriksson et al., 2001). There have been few published laboratory-based metabolomic studies involving bivalves; however, those studies have demonstrated the utility of this approach to quantify physiological responses to changing environmental conditions (Wei et al., 2015) and contaminant exposure (Song et al., 2016). There have also been relatively few studies involving bivalves sampled directly from the environment (Tikunov et al., 2010; Ji et al., 2016). Environmental metabolomics applies metabolomic approaches to field-collected organisms that are exposed to contaminants and other environmental stressors in their natural habitat (Bundy, 2009). Although it cannot identify the specific metabolic effects of an individual contaminant, environmental metabolomics does provide information about metabolite changes within affected physiological pathways in an organism, providing analytical clues on the mixture of contaminants and their mode-of-action at a particular time and location. Currently, environmental metabolomics is not yet developed to a point where it can be considered robust enough to be a standalone experiment capable of providing the information required for regulatory purposes (Davis et al., 2016). For this reason, environmental metabolomic studies are beginning to be integrated with approaches that measure endpoints at the molecular, physiological, and population level (Bundy et al., 2008; Davis et al., 2016). Chapter one provides a foundation for chapters two – four, which are about quantifying the presence, disposition, and biological effects of PPCPs in Georgia estuaries.

Chapter two describes a laboratory-based, GC-MS metabolomic study that developed oyster metabolomic biomarkers for fluoxetine, DEET, diphenhydramine, 17α-ethinylestradiol, and their mixture for the adductor muscle. These compounds were selected because of their quantified oyster body burdens, measured water concentrations, high production and consumer use volumes and/or based upon their potential to cause adverse biological effects. The

experiment was conducted for a total of 18 days, with an initial ten-day exposure followed by an 8-day depuration period. Metabolomic samples were collected at days 0, 1, 5, 10 and after the 8-day depuration period. For individual compound exposures, we were able to link the observed biochemical responses with each chemical's postulated MOA and other off-target effects. However, the mixture's effect on the oyster's metabolome could not be well predicted from responses of the individual treatments, which agrees with other findings (i.e. Jordan et al., 2012; Jones et al., 2012; Song et al., 2016). The adductor muscle plays an essential role in storing energy in the form of proteins and carbohydrates (reviewed in Barber & Blake, 2006); as such, the major metabolic pathways affected were Krebs cycle metabolism, fatty acid β-oxidation, and amino acid metabolism. Alterations in these metabolic pathways in the adductor due to exposure to PPCPs could adversely affect the oyster's phenotype and highlights the need to study the effect of chemical mixtures in wild populations. Additionally, exposure to these PPCPs elicited several general stress responses that adversely affected the oyster's metabolome, in addition to effects from each chemical's postulated MOA. PLS-DA analysis demonstrated that the oyster's metabolome responded dynamically to these treatments over the course of the exposure and depuration periods. Based upon the number of still affected metabolites post-depuration, oysters were not able to fully recover from their exposures, regardless of treatment. This observation was confirmed by trajectory analysis, that showed while both the magnitude and trajectories varied among treatments, that overall, the oysters were only able to partially recover from these chemical exposures.

Chapter three investigates the bimonthly spatial and temporal trends in PPCPs (atenolol, acetaminophen, caffeine, methylphenidate, imidacloprid, propranolol, thiacloprid, diphenhydramine, carbamazepine, sertraline, atrazine, DEET, valsartan, norethindrone,

norgestrel and medroxyprogesterone) detected in C. virginica tissue (December 2013 – October 2015) and in seawater, (October 2014 – October 2015) in estuaries near Brunswick and Sapelo Island, Georgia. This chapter also discusses the use of bioconcentration factors (BCFs), and calculated risk quotients (RQs) in order to estimate possible exposure scenarios for local estuarine organisms. We hypothesized that PPCP concentrations would be higher at Brunswick's urban sample sites with higher nearby septic densities and WWTP inputs, compared to Sapelo Island's rural sample sites with significantly lower numbers of septic sites and no nearby WWTPs. Surprisingly, there were few statistically significant differences (based upon mean and overlapping 95% confidence intervals) in oyster body burdens both between Brunswick and Sapelo Island and within each sample sites, despite the large difference between local population sizes and PPCPs inputs into nearby waterways. We postulate that these differences are potentially due to a combination of local tidal mixing, seasonal changes in oyster physiology, variable PPCP inputs and PPCPs leaching from groundwater (especially at Sapelo Island). Analysis of seasonal trends in oyster PPCPs bioaccumulation showed that there was four general trends, where there was (1) No statistically significant changes (based on overlapping 95% confidence intervals) between any seasons (Brunswick: atrazine, atenolol, caffeine, sertraline, acetaminophen and carbamazepine; Sapelo Island: atrazine, caffeine); (2) Statistically significant changes only from Summer 2014 -> Winter 2014 (Brunswick: medroxyprogesterone, norethindrone, propranolol, imidacloprid, thiacloprid; Sapelo Island: medroxyprogesterone, norethindrone, norgestrel, imidacloprid, thiacloprid, sertraline); (3) Statistically significant changes from Summer 2014 -> Winter 2014 and Winter 2014 -> Summer 2015 (Brunswick: diphenhydramine, norgestrel; Sapelo Island: atenolol, carbamazepine, diphenhydramine) and (4) patterns for DEET, methylphenidate and valsartan that were unique to Brunswick and patterns

for DEET, propranolol, acetaminophen and acetaminophen that were unique to oysters at Sapelo Island. The overlap between select compounds likely indicates that the two distinct human populations at Brunswick and Sapelo Island, Georgia had similar usage patterns for these compounds, despite the large difference between the size of those populations. The results of the BCF and RQ illustrate that accumulation of PPCPs is a dynamic process that can vary significantly both temporally and spatially, and is highly compound specific. This has profound implications for exposures in aquatic organisms, as depending on the season, their life history and physiological status, they are likely exposed to different mixtures of contaminants that vary both within a year and from year to year.

Chapter four describes the application of environmental metabolomics to quantify the spatial and temporal trends in baseline oyster metabolism at Brunswick and Sapelo Island from October 2014 – August 2015. The goals of this study were to (1) quantify the spatial and seasonal variation in metabolic responses from natural populations of oysters present at Brunswick and Sapelo Island, Georgia and (2) assess the robustness of using the Overall Biological Effect as a measure of how exposures, environmental conditions, and normal variations in oyster physiology affect the metabolome, and to better understand baseline metabolism. This information is imperative to improve our understanding of how varying habitats, environmental conditions, and seasonal changes affect the oyster metabolome, in order to provide higher quality data for future environmental monitoring programs. This study provides evidence that untargeted GC-MS based metabolomics is an effective monitoring tool for discerning the natural and seasonal changes in the oyster metabolome (e.g. gametogenesis, utilization of anaerobic metabolism, etc.), and the highly variable physiological responses due to site-specific parameters (e.g. water quality parameters, food availability/quality, contaminants,

etc.) near Brunswick and Sapelo Island, Georgia. While amino acids, carbohydrates, fatty acids, and Krebs cycle metabolites were involved in the main metabolic pathways affected at both locations, their regulation pattern varied significantly between locations and at sample sites within them, especially at Sapelo Island. These interactions make it difficult to interpret the observed physiological responses at the metabolome level, as there is a lack of baseline data for natural oyster populations. However, the results of this study do illustrate that oyster metabolism does appear to be highly variable and dependent upon local conditions, even at sites that are in close proximity (such as Sites 1 – 3 at Brunswick), and especially for sites that are geographically isolated (i.e. Sapelo Island's sites).

CHAPTER 2

METABOLOMIC INVESTIGATIONS OF THE TEMPORAL EFFECTS OF EXPOSURE TO PHARMACEUTICALS AND PERSONAL CARE PRODUCTS AND THEIR MIXTURE IN THE EASTERN OYSTER (*Crassostrea virginica*)[1]

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[1] David W. Brew, Marsha C. Black, Marina Santos, Jackson Rodgers, and W. Matthew Henderson. Submitted to *Environmental Toxicology and Chemistry.*

Abstract

The eastern oyster (Crassostrea virginica) supports a large aquaculture industry and is a keystone species along the Atlantic seaboard. Native oysters are routinely exposed to a complex mixture of contaminants that increasingly includes pharmaceuticals and personal care products (PPCPs). Unfortunately, the biological effects of chemical mixtures on oysters are poorly understood. Untargeted GC-MS metabolomics was utilized to quantify the response of oysters exposed to fluoxetine, N,N-diethyl-meta-toluamide (DEET), 17α-ethynylestradiol (EE2), diphenhydramine, and their mixture. Oysters were exposed to 1 µg/L of each chemical or mixture for ten days, followed by an eight-day depuration period. Adductor muscle (n = 14/treatment) was sampled at days 0, 1, 5, 10, and 18. Trajectory analysis illustrated that metabolic effects and class separation of the treatments varied at each time point, and that overall, the oysters were only able to partially recover from these exposures post-depuration. Altered metabolites were associated with cellular energetics (i.e. Krebs cycle intermediates), as well as amino acid metabolism and the urea cycle. Exposure to these PPCPs also affected metabolic pathways associated with anaerobic metabolism, osmotic stress, and oxidative stress, in addition to the physiological effects from each chemical's postulated mechanism of action. Following depuration, there were fewer metabolites altered, but none of the treatments returned to their initial control values, indicating that metabolic disruptions were long-lasting. Interestingly, the mixture did not directly cluster with individual treatments in the scores plot from partial least squares discriminant analysis and many of its affected metabolic pathways were not well-predicted from the individual treatments. This research highlights the utility of untargeted metabolomics in developing exposure biomarkers for compounds with differing modes of action in bivalves.

Introduction

The presence and biological activities of pharmaceuticals and personal care products (PPCPs) in freshwater environments have been well studied over the past 15 years (Ebele et al., 2017), and in comparison, the study of PPCPs in estuaries has been limited. Over half of the world's population lives near coastal zones, and these sensitive ecological areas have large economic and recreational value (Brown et al., 2006). Exposures to PPCPs in the wild are frequently pulsed and/or transient (Wittmer et al., 2010), and in estuaries, exposure durations may vary due to tidal forces (Cailleaud et al., 2009). PPCPs primarily enter estuaries via inputs from local sources of sewage, including wastewater treatment plants and septic sites (Meador et al., 2016), in addition to runoff from impervious surfaces, landfills, and agricultural activities (Gaw et al., 2014).

Overt adverse effects from exposure to PPCPs in estuarine organisms are hypothesized as improbable, due in part to the large water volume in estuaries, and the low detected concentrations of waterborne PPCPs (Fent et al., 2006; Santos et al., 2010). However, unlike conventional contaminants, PPCPs are specifically designed to be biologically active at low doses and target specific metabolic, enzymatic, or cell signaling cascades to elicit their desired therapeutic effects (Fabbri & Franzellitti, 2015). Although the exposure concentrations of PPCPs for estuarine organisms are unlikely to result in direct mortality, these exposures could result in sublethal toxicity (Harris et al., 2011). This is particularly relevant for eastern oysters (Crassostrea virginica), which are sessile, filter-feeding bivalves that are exposed to PPCPs throughout their entire lifetime (Granek et al., 2016). This species also forms the basis of the oyster industry along the eastern coast of the United States (Tikunov et al., 2010) and as such, there is an urgent need to better understand how exposure to PPCPs affects their biochemistry.

The adductor muscle plays an essential biological role by converting stored chemical energy into movement, as it controls the gape of the oyster's shell (Tikunov et al., 2010), and it is a vital energy storage site for proteins and carbohydrates (reviewed in Barber & Blake, 2006). If exposure to PPCPs adversely affected this organ, physiological consequences, such as altered shell gape, diminished filtration, respiration, and food acquisition in these organisms could be adversely affected (Ballesta-Artero et al., 2017).

Native populations of oysters are exposed to chemical mixtures that are frequently composed of varying chemical classes that have diverse chemical structures, physio-chemical properties, and biological effects (Fabbri & Franzellitti, 2015). As noted by Escher et al., (2005) and Backhaus et al., (2011), chemicals with similar MOAs often exhibit additive toxicity when present in mixtures, although antagonistic, or synergistic effects can also occur (Meek et al., 2011), with the type of interaction dependent on the chemicals present as well as their concentrations. While literature examining chemical mixtures involving two or three compounds is present, including those with both similar and dissimilar MOAs, there have been few mixture studies involving more than three compounds (reviewed in: Altenburger et al., 2012). Unfortunately, among those studies, only a limited number of studies have assessed the toxicity of mixtures of PPCPs on marine bivalves (Juhel et al., 2017; Almeida et al., 2018; Di Poi et al., 2018). It is difficult to predict the potential biological effect of a chemical present in a mixture because of limited or nonexistent data on their biological effects in oysters (Brausch et al., 2012) and/or because many chemicals are likely present at concentrations below current levels of quantification. However, this does not imply that there is an absence of potential adverse effects (Schlenk et al., 2012). Thus, it is imperative to gain a better understanding of mixture toxicity in a laboratory setting, as prioritized by Prichard and Granek, (2016).

Towards this goal, we exposed eastern oysters to a simplified mixture of PPCPs: N, N-Diethyl-meta-toluamide (DEET), 17α-ethynylestradiol, diphenhydramine, and fluoxetine under controlled laboratory conditions. These PPCPs were selected because they were frequently detected in the environment at concentrations that could elicit adverse biological effects in aquatic organisms. Chemical concentrations measured in waterways have ranged between 0.073 – 0.831 µg/L for 17α-ethynylestradiol (Kolpin et al., 2002), and between <0.003 – 0.560 µg/L for fluoxetine (Benotti & Brownawell, 2007; Trenholm et al., 2006). Diphenhydramine has been measured up to 1.4 µg/L (Bartelt-Hunt et al., 2009) and DEET was measured between <0.030 – 24 µg/L (Dsikowitzky et al., 2014).

Recently, metabolomic analysis has been applied to ecotoxicological studies and has greatly expanded the understanding of the molecular underpinnings of how bivalves respond to both environmental and anthropogenic stressors (i.e. Tikunov et al., 2010; Lenoard et al., 2014; Roznere et al., 2017). Metabolomics analyzes how endogenous metabolites within a cell, tissue, or biofluid (termed the metabolome) respond to external stressors or stimuli (Bundy et al., 2009; Skelton et al., 2014). Metabolites serve as direct signatures of biochemical activity and are therefore easier to correlate with an organism's phenotype or pathophysiology than lower levels of biological organization. Thus, perturbations that affect the metabolome have a higher likelihood of also affecting an organism's phenotype (Viant, 2007). Research has shown that besides eliciting effects via their MOA, chemicals can also cause downstream (Webhofer et al., 2011; Jordan et al., 2012) or off-target effects (Southam et al., 2011) that can cause widespread changes at the level of the metabolome.

Based upon the critical knowledge gaps associated with understanding how chemical stressors affect oyster physiology, the objective of this study was to utilize untargeted GC/MS-

based metabolomics to elucidate the sublethal effects of exposure to these chemicals using a marine bivalve model (eastern oyster, Crassostrea virginica, Gmelin 1791) exposed for ten days to DEET, 17α-ethynylestradiol, diphenhydramine, fluoxetine, and their mixture in controlled laboratory exposures, followed by an eight-day depuration period. Our goal was to determine the biochemical pathways involved in the toxicological response and during any responses leading to compensation and/or recovery in oysters after exposure to these PPCPs.

Materials and Methods

Chemicals

All chemicals and solvents were HPLC-grade and obtained from Sigma Aldrich (St. Louis, MO). Exposures were conducted with diphenhydramine hydrochloride (purity ≥ 98%), fluoxetine hydrochloride (purity >98%), 17α-ethynylestradiol (purity ≥ 98%), and N,N-diethymeta-toluamide (DEET; purity 97.6%). Stock solutions of test compounds were created by dissolving pure compound in 100% ethyl alcohol. Working solutions were diluted in 1 L of test water to achieve the desired concentration (1 µg/L) and to ensure that the final ethyl alcohol content remained below 0.01%.

Eastern Oyster Collection and Acclimation to Laboratory Conditions

Wild eastern oysters (Crassostrea virginica; n = 350) > 75 mm in length were collected from Sapelo Island, Georgia in June 2015 and transported on ice back to the Aquatic Exposure Laboratory at the University of Georgia, Athens, Georgia, U.S.A. Oysters were acclimated to laboratory conditions for 30 days in an aerated 230-liter plastic aquarium filled with filtered/UV sterilized dechlorinated tap water with added salts (Instant Ocean, Spectrum Brands) to achieve a

salinity of 18 ± 2 ppt. Water temperature was maintained at 25°C, and the photoperiod was 16 h light/8 h dark. Oysters were fed daily with Shellfish Diet 1800 (Reed Mariculture, Campbell, CA) per the manufacturer's instructions, with each oyster receiving 0.281 mL of feed/day. To maintain optimal water quality and aid in compound depuration during acclimation, 50% water changes were conducted daily for the first week, and biweekly thereafter. Water quality parameters (pH and temperature – Oakton Instruments, Vernon Hills, Il, and salinity – Deepwater Aquatics, Orlando, FL) were measured daily, while ammonia (Mars Fishcare, Chalfont, PA) was measured twice a week.

Following acclimation, oysters were randomly assigned to twenty 38-L glass aquaria (n = 14 oysters/tank): three replicate tanks for each of five treatments (17α-ethynylestradiol, diphenhydramine, fluoxetine, DEET, and their mixture) (n = 42 oysters/treatment total), three replicate tanks for vehicle controls (ethyl alcohol, 0.01%), and two tanks for the negative controls (instant ocean only; n = 70). Oysters were acclimated in the treatment aquaria for an additional ten days prior to PPCP exposure, to allow time for the oyster's metabolome to adjust to these new conditions.

PPCPs Exposure Study

Biochemical effects were assessed at 1 µg/L for all treatments. The exposure system was static renewal, where 50% of the exposure water was renewed daily (days 1-10: test water renewal with added compound(s); days 11-18: test water renewal only, complete water change at day 11), and oysters were fed four hours after each water renewal. On exposure days 0, 1, 5, 10, and depuration day eight, two oysters were randomly selected from each replicate tank (n = 6/treatment; controls: n = 10; n = 40 total samples per sample day), measured, weighed (nearest

0.01 g), and an approximately 20 mg sample of adductor muscle was dissected and placed in 2-mL plastic microcentrifuge tubes (Fisher Scientific, Waltham, MA). Tissue samples were snap frozen in liquid nitrogen and stored at -20ºC until analysis. The remaining tissue was excised, weighed, and composited by treatment (n = 6 oysters/treatment) and control (n = 6 oysters/vehicle control, 4 oysters/control) for analyte body burden analysis. Replicate treatment tanks were combined into one sample for body burden analysis to ensure that >1 gram of tissue was used in the extraction procedure. On each sampling day, a 250-mL aliquot of water was removed from each replicate tank, placed in a glass amber bottle, covered in parafilm, and stored at 4ºC until extraction and LC-MS/MS analysis.

Analyte Body Burdens

Composite tissue samples were freeze-dried, ground into a fine powder, and the tissue weights were normalized between treatments prior to extraction. The tissue extraction procedure followed Fuller (2012). In brief, 10 mL of 3:3:1 methanol, acetonitrile, and methyl tert-butyl ether solvent mixture were added to each sample, rocked for 20 minutes, and then centrifuged for 20 minutes at 1,500 RPM. The supernatant was removed and re-centrifuged at 1,500 RPM for 5 minutes to further remove particulate material. The remaining supernatant was collected and evaporated to 0.5 mL or less under a gentle stream of nitrogen, and then diluted with 20 mL of milli-q water. SPE was performed on a Sep-Pak Florisil vacuum cartridge (3 cc, 200 mg; Waters, Milford MA). The sample solution was loaded onto the cartridge (which was preconditioned with 3 mL of methanol and 3 mL of HPLC-grade water), eluted with 6 mL of methanol, and dried under a gentle stream of nitrogen gas. Samples were reconstituted in 1 mL of 10% acetonitrile and stored at -20ºC until LC-MS/MS analysis.

Analyte Water Concentrations

Analytes were extracted from water through an extraction procedure adapted from Englert (2007). In brief, at each sample day, all replicates (n = 3) from each treatment (DEET, 17α-ethynylestradiol, diphenhydramine, fluoxetine, mixture, and controls) were pooled into one sample that was passed through a preconditioned (6 mL methanol, 6 mL diH2O) Oasis HLB 6cc 200 mg cartridge (Waters, Milford MA). After sample loading, the cartridge was eluted with 6 mL of methanol and dried under a gentle stream of nitrogen. Samples were reconstituted in 1 mL of 10% acetonitrile and stored at -20ºC until LC-MS/MS analysis.

LC-MS/MS Analysis

Samples were analyzed on an Accela HPLC coupled to a TSQ Quantum Ultra mass spectrometer (Thermo Scientific, Bellefonte, PA). Chromatographic separation was achieved on a Kinetex 3 µm C18 HPLC column (150 x 2.1 mm, particle size 1.8 µm Phenomenex, Torrance, CA). The initial mobile phase was 95% water with 0.1% formic acid (A) and 5% acetonitrile with 0.1% formic acid (B). Starting conditions were held for one minute, ramped to 95% B over 17 minutes, and held for three minutes, before returning to initial conditions of 5% B and re-equilibrating for 3 minutes (total run time of 35 minutes). The flow rate was 400 µL/min, with an injection volume of 20 µL. All analyses were conducted in selected reaction monitoring (SRM) and positive electrospray ionization mode. The SRM transitions (m/z) values and capillary voltages (V) were 192 to 119 (16 V) for DEET, 279.2 to 133 (20 V) for 17α-ethynylestradiol, 256 to 167 (10 V) for diphenhydramine, and 310 to 148 (8 V) for fluoxetine. To quantify 17α-ethynylestradiol, 400 µL of sample was derivatized by dansyl chloride, following the procedure

of Yu et al., (2011). In brief, 200 µL of sodium bicarbonate solution (100 mmol/L) and 200 µL of dansyl chloride in acetone (1 mg/L) were added to 400 µL of sample. The mixture was vortexed for one minute and derivatized for 15 minutes at 60°C prior to LC-MS/MS analysis.

Oyster Metabolite Extraction & Derivatization

Endogenous metabolites were extracted from adductor tissue through a dual phase extraction procedure adapted from Viant (2007). In brief, 20 mg of adductor tissue were homogenized and extracted utilizing milli-q water, chloroform, and methanol to separate each sample into polar and non-polar phases. Each phase was removed, dried overnight in a Savant Speed Vac Concentrator interfaced with a refrigerated vapor trap (Thermo Scientific, Waltham, MA), and stored at -20ºC until derivatization and GC-MS analysis. Polar samples were derivatized with 30 µl O-methoxyamine-HCl (Sigma-Aldrich, St. Louis, MO) at 20 mg/ml in pyridine, heated for 2.5 hours at 60°C and vortexed at 30-minute intervals. After cooling, 50 µl BSTFA (N, O-bistrifluoroacetamide) containing 10% TMS (trimethylchlorosilane - Sigma-Aldrich, St. Louis, MO) was added, the solution was heated for 1.5 h at 60°C, and was vortexed at 30 minute intervals. After derivatization, samples were transferred to microtarget inserts, placed in GC vials, and analyzed by GC-MS within 72 hours.

Metabolite Analysis by GC-MS

Derivatized adductor tissue samples were analyzed with an Agilent 7890B gas chromatograph (Agilent Technologies, Santa Clara, CA) interfaced to a Pegasus time-of-flight (TOF) mass spectrometer (LECO, St. Joseph, MI). Metabolites were separated on a Restek RTX-Sil column (30 m x 0.25 mm x 0.25 µm; Restek, Bellefonte, PA) with an initial oven temperature

of 60 °C, ramped at 8 °C/minute to 320 °C and held for 5 minutes. The injector, transfer line, and source temperatures were at 275°C, 250°C, and 200°C respectively and spectra were acquired over the mass range of 50-650 m/z.

Data Analysis – Metabolites

Chromatograms were exported as netCDF files, imported into METAlign®, and aligned based on the developer recommended parameters for fast scan analysis (Lommen, 2009). Microsoft Excel® (Microsoft, Redmond, WA) was used to filter and truncate the aligned data as described by Niu et al., (2014). Partial least squares discriminant analysis (PLS-DA) was performed with SIMCA-P+ (Version 11.0, Umetrics, San Jose, CA) following log transformation and Pareto scaling of the data (Niu et al., 2014). PLS-DA models were used to assess treatment clusters and to identify the metabolic trajectories resulting from exposure to each treatment. R2 and Q2 values were calculated as measures of the model's robustness. Models with a Q2 value less than 0.4 were considered to have insufficient predictive power and were excluded from further analysis. Student's t-tests were used to identify metabolites that varied significantly ($p \leq 0.05$) by a comparison between the control samples and treatments from each sample day. Metabolites identified as significant were putatively identified through the 2014 NIST library. The Kyoto Encyclopedia of Genes and Genomes Pathway Database (http://www.kegg.jp/kegg/pathway.html) was used to identify which biochemical pathways were affected by each treatment.

Results & Discussion

Analyte Body Burdens

Oysters exposed to fluoxetine and diphenhydramine had similar patterns of compound uptake, where body burdens increased from days one through five and decreased by day ten (Table 2.1). In comparison, the highest body burden for 17α-ethynylestradiol (EE2) and DEET exposures were at day one, and body burdens decreased at subsequent sampling periods (Table 2.1). Post-depuration, DEET was the only individual treatment with a body burden above the limit of detection (Table 2.1); however, fluoxetine's depuration day eight sample was not analyzed due to experimental error. In the mixture exposure, the accumulation patterns of these analytes were different than those observed in the individual treatments (Table 2.1), with a general trend of increasing body burdens during the exposure period, followed by a decline in body burdens post-depuration (Table 2.1). By depuration day eight, following the mixture exposure, EE2 and DEET were the only two treatments with detectable body burdens (Table 2.1). We postulate that these changes result from differences in the bioaccumulation, metabolism, excretion, and/or receptor binding for the compounds present in the mixture (Spurgeon et al., 2010). Additionally, there is evidence that PPCPs, including SSRIs, are inhibitors of several cytochrome CYP450 enzymes (Thibaut et al., 2006), and that alterations in these enzymes could affect xenobiotic metabolism and toxicity. For example, in rainbow trout (Oncorhynchus mykiss) gonadal RTG-2 cell lines exposed to a mixture of pharmaceuticals, endocrine disruptors, and fragrances, there were different induction patterns of ethoxyresorufin-O-deethylase (EROD) activity between the individual contaminants, and varying mixtures of the selected compounds (Fernández et al., 2013). While the individual treatments tested did not induce EROD activity, there was an increase in EROD measured in several of the mixture

treatments, potentially resulting from differences in xenobiotic metabolism of the individual constituents of the various mixtures (Fernández et al., 2013).

Metabolic Pathways Associated with Stress in Exposed Oysters

Thirty-one metabolites were common between each treatment (Table 2.3), and of these, six metabolites (increases in alanine, glucose, succinate, propionate – indicators of anaerobic metabolism; decreases in taurine – osmotic stress; and increases in glutamate - oxidative stress) play key roles in regulating stress responses in bivalves (reviewed in: Moore, 2001; Lannig et al., 2010; Mager et al., 2000; Liu et al., 2011; Valavanidis et al., 2006) (Table 2.2), and are often measured in bivalves following xenobiotic exposures (liu et al., 2011; González-Ortegón et al., 2013), including the PPCPs diphenhydramine (Xie et al., 2016), fluoxetine (Chen et al., 2015), and EE2 (Leonard et al. 2014). Despite detecting anaerobic metabolism frequently in oysters exposed to EE2 and the mixture treatments (Table 2.2), these oysters had detectable amounts of analytes in their body (Table 2.1), implying that the oysters were opening their shells at discrete intervals, such as during feeding, as observed in overwintering oysters (C. virginica) (Mayrand et al., 2017). Among the single treatments, osmotic stress occurred in DEET from days one – ten, at days one – five for diphenhydramine, only at day one and depuration day eight for EE2, and was not detected in fluoxetine; while oxidative stress was detected in DEET from days one – ten, from days one – five and depuration day eight in EE2, at day five in fluoxetine, and at each time point for diphenhydramine. We postulate that the observed osmotic stress (indicated by decreases in taurine) was due to treatment effects and not changes in salinity, as the 95% confidence intervals for the daily salinity measurements were non-overlapping ($p \leq 0.05$ – Data not shown).

The mixture had a unique stress response compared to each treatment individually, but its response appeared to be an amalgamation of the responses from the EE2, diphenhydramine, and fluoxetine treatments. For example, across the entire study, oysters exposed to the mixture displayed biochemical indications of anaerobic metabolism (similar to EE2), oxidative stress (similar to diphenhydramine) at each time point, and, similar to fluoxetine, did not display any signs of osmotic stress.

Trajectory Analysis

Partial Least Squares - Discriminant Analysis (PLS-DA) models were used to differentiate between each chemical's postulated mechanism of action (MOA) (Keun et al., 2004) and to assess the extent that oysters were able to recover from these exposures (Ekman et al., 2008; Ankley & Villeneuve, 2015). In addition, these models were utilized to assess the biochemical effects of this chemical mixture, based upon how mixture samples clustered in multivariate space compared to individual treatment samples (Baylay et al., 2012). Within this framework, chemicals with similar biochemical effects cluster together in multivariate space, chemicals with dissimilar effects form distinct clusters, and chemicals with non-interactive, independent effects cluster intermediate between multiple treatments (Baylay et al., 2012).

The PLS-DA models highlighted that for each treatment between days zero and one, the movement away from controls was similar (Fig. 2.3), while the relative magnitude and their position in multivariate space differed (Fig. 2.1). This suggests that the oysters were experiencing similar perturbations in biochemical effects at this time point, potentially due to the similar stress responses observed (Table 2.2). The DEET and diphenhydramine treatments, and the fluoxetine and mixture treatments had overlapping 95% confidence intervals (Fig. 2.1),

suggesting that these treatments were eliciting similar biochemical responses (Baylay et al., 2012). This was surprising for the DEET and diphenhydramine treatments, as these compounds have markedly different MOAs, targeting octopamine (Swale et al. 2014) and histamine H1 receptors (Brown and Roberts, 2001), respectively. By day five, the PLS-DA models for all treatments showed a maximum separation from their initial positions along principle component one and non-overlapping 95% confidence intervals among all treatments (Fig. 2.1). This suggests that each treatment had its largest and most unique biochemical effects at this time, likely due to each treatment's postulated unique MOA. However, by day ten, each treatment's trajectory moved towards their day zero position (Fig. 2.1), suggesting that the oysters were beginning to recover from each treatment. After eight days of depuration, none of the treatments returned to their initial day zero values, indicating incomplete recovery from the exposures (Fig. 2.3). Our results follow a trend observed in other studies, where organisms are only able to partially compensate to exposure of chemical mixtures. For example, this was demonstrated in the metabolome of male fathead minnows (Pimephales promelas) exposed to 17α-ethynylestradiol for eight days, followed by an eight-day depuration (Ekman et al., 2008), and in earthworms (Eisenia fetida) exposed to lead contaminated soil for 14 days, followed by a 14-day depuration (Tang et al., 2017).

Interestingly, while their trajectories drastically varied, the mixture and fluoxetine treatments clustered near one another in multivariate space post-depuration (Fig. 2.1), possibly because of the probable presence of norfluoxetine, fluoxetine's main biotransformation product. In mussels (Mytilus galloprovincialis) exposed to fluoxetine, norfluoxetine concentrations exceeded those of fluoxetine after 15 days of exposure (Silva et al., 2016), and were more prone to bioaccumulate than fluoxetine, while eliciting a similar biological effect (Daughton & Brooks,

2011). Although it is possible that given more time, the oysters would be able to recover completely from these exposures in a laboratory setting, it is currently unknown how accurate this prediction would be in the environment, where contaminants are introduced sporadically and for indeterminate durations (Rozman & Doull, 2000).

DEET Metabolomics – Day One

DEET is the most effective and frequently used mosquito repellant worldwide (Debboun et al., 2014), and its presumed MOA is to activate octopamine receptors (Swale et al., 2014). In mollusks, the acute stress response is mediated by the neurotransmitter norepinephrine (Adamo, 2008), which is nearly identical in structure to octopamine and can bind to its receptor (Gerhardt et al., 1997). Norepinephrine is widely distributed in invertebrate tissues and can act as a stress hormone by mobilizing energy reserves and muscle tissue as part of a flight-or-fight response (Adamo, 2008).

Supporting this presumed MOA, early exposure to DEET elicited significant changes in metabolites related to energetics. The majority of the putatively identified Krebs cycle metabolites (succinate, citrate, and fumarate) and fatty acids (butanoate, acetate, palmitic acid, and propionate) were increased on day one (Table 2.3). This indicates that to provide the metabolic fuel required by the Krebs cycle, the majority of the putatively identified fatty acids were undergoing β-oxidation to produce acetyl-CoA, NADH and FADH to meet energetic demands (Nelson et al., 2008). Increases in acetate suggests that it was being synthesized into acetyl-CoA, where it can be utilized by mitochondria as a source of immediate energy (Purmal et al., 2014). As oysters are osmoconformers, they can adjust the intracellular free amino acids concentrations in response to osmotic stress (Hosoi et al., 2003). As oysters exposed to DEET

displayed biochemical signs of osmotic stress at this time point (Table 2.2), we hypothesize that the majority of the observed changes in amino acid regulation at this time were in response to dealing with this osmotic stress. Additionally, oysters exposed to DEET appeared to utilize the glyoxylate pathway (oxalic acid and glycine) to shunt glycine into the Krebs cycle to yield succinate (Nakada et al., 1955). Glycine can also participate in glycolysis via the formation of serine and acts as a two-carbon energy source in either pathway, ultimately increasing ATP production (Nakada et al., 1955).

DEET Metabolomics - Days Five & Ten

From day five through day ten, only the DEET treatment had the majority of Krebs cycle metabolites decreased at each of these time points (Fig. 2.2). Further supporting that DEET exposure was energetically expensive, the majority of putatively identified fatty acids (glycerol, palmitic acid, butanoate, and propionate) were decreased at day five (Fig. 2.2), possibly indicating that these metabolites were becoming exhausted as a fuel source. Also, acetate was the only fatty acid to be increased at both of these time points, potentially indicating that it was heavily relied upon to synthesize acetyl-CoA (Purmal et al., 2014). Supporting our data, in common carp (Cyprinus carpio L.) exposed to subchronic concentrations of DEET, a significant decline in plasma triacylglycerols indicated an exhaustion of energy reserves during a return to homeostasis (Slaninova et al., 2014). The majority of putatively detected amino acids had also decreased during these time points (Table 2.3), indicating that they were being oxidized for metabolic fuel (Cappello et al., 2017), or were being utilized as glucose precursors (De Zwaan and Wijsman, 1976), as the majority of identified carbohydrates had decreased at these time points.

Diphenhydramine Metabolomics - Day One

Diphenhydramine is a common over-the-counter antihistamine used to treat allergies, and its MOA is inhibiting histamine binding on the histamine H1 receptor, which reduces the allergic response (Brown and Roberts, 2001). In bivalves, histamine is an important neurotransmitter and neurohormone that plays a key role in regulating gill cilia beating, and integrating sensory information (Harrison et al., 2015). At day one, decreases in metabolites involved in the Krebs cycle (citrate, fumarate, and succinate), coupled with increases in propionate (fatty acid), and the glyoxylate pathway intermediates (oxalic acid and glycine) indicate that early exposure to diphenhydramine was energetically costly (Fig. 2.2). Supporting this, increases in propionate levels could be one method of replenishing Krebs cycle intermediates via activation to propionyl-CoA to form succinyl-CoA (Brunengraber & Roe, 2006).

Similar to the DEET treatment, diphenhydramine exposure elicited biochemical indications of osmotic stress (Table 2.2); however, there were differences in the regulation pattern for the affected amino acids between these treatments (Table 2.3). The majority of amino acids were increased in the diphenhydramine treatment (Table 2.3), indicating that they were likely not being utilized as metabolic fuel and as such, these observed changes in amino acid regulation may have been in response to changes in the oyster's internal solute concentrations due to the osmotic stress. Oysters exposed to diphenhydramine also showed signs of oxidative stress at this time point, and also at subsequent time points (Table 2.2), in agreement with effects observed in crucian carp (Carassius auratus) after a 7-day exposure to 1, 5, 25, and 125 µg/L of diphenhydramine (Xie et al., 2016). There is evidence in other pharmaceutical exposures that elicit oxidative stress; a side effect is disruptions in mitochondrial respiratory chain activity, which can lead to an inhibition in ATP synthesis (reviewed in: Chan, et al., 2005). This could

provide an alternative explanation for the detected decreases in Krebs cycle metabolites in diphenhydramine at this time point. Supporting this hypothesis, chinook salmon (Oncorhynchus tshawytscha) exposed to a chemical mixture (that included both diphenhydramine and fluoxetine) showed increases in liver mitochondria respiration rates, and a reduction in total mitochondrial glutathione, suggesting a reduction in ATP synthesis, metabolic capacity, and oxidative stress was occurring, respectively (Yeh et al., 2017).

Diphenhydramine Metabolomics – Days Five & Ten

Compared to day one, oysters exposed to diphenhydramine had increases in Krebs cycle metabolites at days five and ten (Table 2.3). This regulation pattern is different than from day one responses and suggests that if oxidative stress does lead to increased liver mitochondria respiration rates and lower ATP production, then this effect appears to be transitory in bivalves, at least at the concentration tested in this study. At day five, acetate and glycerol became increased, and the majority of amino acids became decreased (Fig. 2.2), suggesting that these oysters were relying on these metabolites to provide easily accessible metabolic fuel and Krebs cycle feedstocks (Purmal et al., 2014; Cappello et al., 2017). However, as the exposed oysters had signs of osmotic stress at day five (Table 2.2), it is possible that some of the observed alterations in amino acid metabolism were due to this additional stressor. Unexpectedly, despite indications that the detected amino acids were being relied upon for metabolic fuel, there was a decrease in ornithine (urea cycle intermediate) at both days five and ten, which implies that there was a reduction in amino acid turnover (Denkert et al., 2008).

By day ten, there were widespread changes in the regulation status for the fatty acids acetate, glycerol, and butanoate and the majority of amino acids increased (Fig. 2.2). This

suggests that as the body burden of diphenhydramine decreased, there was a reduced need for metabolic fuel that could be quickly oxidized (i.e. acetate and amino acids). However, unlike at day five where none of the putatively identified carbohydrates were significantly different than the controls ($p \leq 0.05$), all of them were increased by day ten (Table 2.3). This is a classic biomarker of contaminant exposure in bivalves (Ngo et al., 2011), as these carbohydrate reserves can be utilized to provide immediate metabolic fuel to deal with a stressor (Leonard et al., 2014). While it is not clear why this was the only treatment that showed this delayed response, a possible explanation is due to biotransformation of diphenhydramine to its main active metabolite nordiphenhydramine, as well as dinordiphenhydramine and diphenylmethoxyacetic acid (Akutsu et al., 2007). These metabolites are suggested to be partially responsible for eliciting adverse biological effects (i.e. alterations in swimming, feeding, and shoaling behavior) in crucian carp (C. auratus) exposed to diphenhydramine for seven days (Xie et al., 2017).

Fluoxetine Metabolomics – Day One

Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) that is commonly prescribed to treat anxiety and depression via its MOA of increasing serotonergic activity by preventing serotonin (5-hydroxy testosterone - 5-HT) reuptake increasing 5-HT in the synaptic cleft (Wong et al., 2005). Diphenhydramine also shares a common MOA with fluoxetine, as it also affects the 5-HT reuptake transporter, although fluoxetine has a much greater specificity (Berninger et al., 2011). In bivalves, 5-HT also plays an essential role in bivalve reproductive physiology by regulating oocyte maturation, spawning, and parturition (Fong, 1998).

The only putatively identified Krebs cycle metabolites affected by fluoxetine during the exposure period (days one – ten) were succinate and citrate, both of which were decreased at day

one (Fig. 2.2). This supports the findings of Scaini et al., (2011), that fluoxetine affected mitochondrial respiratory chain activity that targeted succinate dehydrogenase and citrate synthase in Wistar rats. Decreases in these metabolites could be caused by inhibition of mitochondrial respiration, which was an adverse effect in isolated male Sprague Dawley rat liver mitochondria that were exposed to the SSRI antidepressant sertraline (Li et al., 2012). Additionally, these decreases could indicate that early fluoxetine exposure induces mitochondrial toxicity, which could lead to lower ATP levels (Li et al., 2012). Surprisingly, glutamate was not detected, suggesting that oxidative stress was not occurring, despite it being a marker of fluoxetine exposure in Wistar rats (Djordjevic et al., 2011).

The majority of the putatively identified amino acids were increased, except for glycine and glutamate and there were no signs of osmotic stress (Tables 4.2 and 4.33), indicating that the amino acids were not likely being oxidized for metabolic fuel. In zebrafish (Danio rerio) exposed to fluoxetine, there was a significant increase in transcripts regulating amino acid metabolism, and this was postulated to be in response to the fish shifting resources from producing lipids towards increasing amino acids (Wong et al., 2013). Our results did not support the conclusions of Wong et al., (2013), as most of our detected fatty acids were increased (Table 2.3), and increases in acetate, butanoate, and glycerol (Table 2.3) suggest increased need for metabolic feedstocks for the Krebs cycle (Nelson et al., 2008; Purmal et al., 2014). Corroborating our results, zebrafish (Danio rerio) larvae exposed to a low dose of fluoxetine also showed decreases in the oxidation of fatty acids (Huang et al., 2017).

Fluoxetine Metabolomics – Days Five & Ten

The majority of the identified amino acids were decreased at days five and ten in oysters exposed to fluoxetine (Fig. 2.2). Similar decreases in amino acids have been detected in zebrafish (Danio rerio) larvae exposed to fluoxetine and was suggested to be due to a reduction in aminoacyl-tRNA biosynthesis (Huang et al., 2017). This could result in an increased demand for energy from other metabolic sources to maintain homeostasis (Holbrook Jr., 1987). For example, if the detected decreases in alanine and aspartate (Table 2.3) were due to decreased amino acid synthesis, then this could adversely affect the oyster's energetic state and potentially reduce their scope for growth. This finding is supported by Hazelton et al. (2014), and Peters and Granek et al. (2016), which demonstrated that both freshwater (Lampsilis fasciola) and marine mussels (Mytilus californianus), respectively, exposed to fluoxetine had decreased energy storage and impaired algal clearance rates. Additionally, Granek et al., (2016) found that long-term exposure (107 days) to 0.3 µg/L fluoxetine caused a marked decrease in M. californianus biomass, thought to be due to the observed impaired clearance rates.

Oysters exposed to fluoxetine had increases in succinate and citrate at day five, and by day ten, only citrate changed regulation status, becoming decreased (Fig. 2.2). While succinate was decreased at day one, it increased at days five and ten, possibly implying that ATP levels initially decreased and then increased at later time points. These results could also indicate that while early fluoxetine exposure induced mitochondrial toxicity, which can lead to lower ATP levels, as has been documented in isolated rat liver mitochondria (Li et al., 2012), this effect was temporary in bivalves. Overall, the postulated increases in ATP levels over time is supported by fluoxetine's MOA, which has been demonstrated to increase Na+-K+-ATPase activity in rats (Gamaro et al., 2003).

At day five, glutamate was increased, and in mussels, fluoxetine exposure was shown to activate antioxidant defenses (i.e. catalase and glutathione-s-transferase activities – Roméo et al., 2003); however, by day ten, glutamate levels became decreased, indicating that any antioxidant effects were transient (Table 2.2). This supports research by Gonzalez-Rey & Bebianno, (2013), that found that Mediterranean mussels (Mytilus galloprovincialis) exposed to 75 ng/L of fluoxetine for two weeks also only had a transient effect on antioxidant systems. Additionally, Franzellitti et al., 2014, detected minimal antioxidant responses after M. galloprovincialis were exposed to environmentally relevant concentrations of fluoxetine for seven days. However, 4-trifluoromethylphenol, a metabolite of fluoxetine biotransformation, was demonstrated to decrease intracellular glutathione levels in rat livers (Thompson et al., 2000), and it is possible that this could be occurring in bivalves. Insufficient antioxidant protection after exposure to fluoxetine has been demonstrated to elicit lipid peroxidation in humans (Gałecki et al., 2009) and alterations in β-oxidation of fatty acids in zebrafish (Danio rerio) larvae exposed to a low dose of fluoxetine (Huang et al., 2017).

Corroborating these results, we saw extensive changes in fatty acid regulation at this time. At day five, the majority of fatty acids were decreased (potentially due to lipid peroxidation). By day ten, the majority of these metabolites had increased (Fig. 2.2), which is also after oxidative stress was no longer detected in the exposed oysters (Table 2.2). At both time points, propionate was decreased, which could reduce intermediates for the Krebs cycle (Brunengraber & Roe, 2006); increases at day ten in acetate and glycerol suggest that they were being increasingly utilized to provide metabolic fuel at this time (Nelson et al., 2008; Purmal et al., 2014). Despite fluoxetine and diphenhydramine having a shared MOA (i.e. targeting the 5-HT receptor – Berninger et al., 2011), each chemical had a distinct effect at the level of the

metabolome, as the majority of metabolites had different regulation patterns between these treatments (Fig. 2.2 & Table 2.3).

EE2 Metabolomics – Day One

The synthetic estrogen EE2 is the active ingredient in oral contraceptives and has been demonstrated to have estrogenic effects in bivalves (reviewed in: Matozzo et al., 2008). Estrogen receptors in bivalves are different from their mammalian counterparts, as they do not respond to estrogens (Thornton et al., 2003), although estrogen exposure does upregulate this receptor (Ciocan et al., 2010), potentially through a ligand dependent factor in non-genomic signaling pathways (Tran et al., 2016). Estrogen exposure can also rapidly (i.e. seconds to minutes) cause non-genomic effects to occur that affect intracellular signaling, kinase cascades (i.e. nuclear factor-kappa β), ion fluxes, intracellular calcium levels, and the immune response (Ascenzi et al., 2006). In bivalves, these non-genomic effects result in a reduction in glycogen, glycolytic intermediates, and fatty acids due to endocrine-immune responses (Leonard et al. 2014). Supporting the results of Leonard et al., (2014), we detected an increase in glucose at this time (Table 2.3). This response is likely a product of degraded glycogen stores, one of the initial responses that bivalves undergo as they switch to anaerobic metabolism.

At day one, EE2 exposure caused reductions in acetate and glycerol, and an increase in butanoate and propionate (Fig. 2.2), to promote synthesis into acetyl-CoA (Purmal et al., 2014) and undergo β-oxidation (Nelson et al., 2008), respectively, to provide fuel into the Krebs cycle. Increased propionate could be in response to replenishing Krebs cycle intermediates via activation to propionyl-CoA to form succinyl-CoA (Brunengraber & Roe, 2006). This supports

the detected increases in the Krebs cycle metabolites citrate and fumarate; however, there was an observed decrease in succinate (Fig. 2.2).

Additionally, oxidative stress was detected, which is in agreement with unionid mussels (Lampsilis fasciola) exposed to environmentally relevant concentrations of EE2 (Leonard et al., 2014). Lipid peroxidation can be caused by oxidative stress, which, via activation of Ca2+-dependent phospholipase A2 (a known target of estrogen in mussels (Mytilus galloprovincialis)), can lead to destabilization of membrane phospholipids (Canesi et al., 2004). Thus, it cannot be ruled out that lipid peroxidation also influences the changes detected in fatty acid regulation in this study. Similar to the DEET and diphenhydramine treatments, oysters exposed to EE2 had biochemical signs of osmotic stress at this time point (Table 2.2); however, the amino acids were regulated differently in this treatment, compared to the other two treatments (Table 2.3). As such, we cannot assign with any certainty which changes in amino acid regulation were due to the osmotic stress and which were a result of their use as metabolic fuel.

EE2 Metabolomics – Days Five & Ten

From day five to day ten, oysters exposed to EE2 displayed metabolic signs of anaerobic metabolism (Table 2.2) and most of the putatively identified Krebs cycle intermediates (fumarate, succinate, and citrate), carbohydrates (glucose, mannose, and galactose), and alanine were increased as a result (Fig. 2.2). During anaerobic metabolism, bivalves switch from utilizing classical glycolysis to instead breaking down glycogen to produce glucose and phosphoenolpyruvate (PEP). PEP is carboxylated to oxaloacetate, which via malate dehydrogenase, is quickly reduced to malate. Malate can be decarboxylated to pyruvate, which via a transamination reaction accumulates as alanine, a well-established marker of anaerobic

metabolism in bivalves (Lannig et al., 2010). The reduction of fumarate to succinate and the formation of succinyl-CoA, which can be converted to propionate, are additional indicators of anaerobic metabolism in EE2-exposed bivalves (Hochachka and Mustafa, 1972).

Our results were different than those of fathead minnows (Pimephales promelas) exposed to 10 and 100 ng/L 17 α-Ethynylestradiol, which resulted in an increase in lactate, which can indicate an impairment in mitochondrial function (Ekman et al., 2008). This has been observed in mice mitochondria exposed to E2, where upon exposure, there was a decrease in Krebs cycle function, which can induce lactate dehydrogenase for the production of lactate to provide oxidized NAD+ (Grimbert et al., 1993). However, supporting our results, estradiol-17β exposure increased enzymes involved with the Krebs cycle in Indian freshwater mussels (Channa punctatus); this was postulated to support increased energetic demands needed for the increased synthesis of vitellogenin, a hallmark of estrogen exposure (Sehgal & Goswami, 2001).

At day five, most of the identified fatty acids were decreased (except hexadecanoic acid and butanoate); however, by day ten, the majority of these metabolites were increased (Fig. 2.2). This suggests that EE2 exposed oysters depended on their lipid reserves as an energy source during this time, potentially to account for increased reliance on gluconeogenesis due to anaerobic metabolism (Hochachka & Somero, 2002). Supporting our results, unionid mussels (Lampsilis fasciola) exposed to EE2 utilized their lipid reserves to compensate for glycogen degradation to provide immediate metabolic fuel in the form of glucose (Leonard et al., 2014). Between days five and ten there was a reduction in the majority of the putatively identified amino acids (Fig. 2.2), which likely indicates their use as metabolic fuel due to the increased energetic demands that necessitated utilizing the oyster's lipid reserves. This phenomenon has been observed in response to xenobiotic exposures, including herbicides (Tuffnail et al., 2009),

hydrocarbons (Fasulo et al., 2012), and pharmaceuticals (Cappello et al., 2017). Additionally, ornithine (urea cycle intermediate) was increased during this time (Table 2.3), which usually indicates a higher turnover of amino acids (Denkert et al., 2008), and could be due to the postulated increase in vitellogenin synthesis (Sehgal & Goswami, 2001).

Mixture Metabolomics – Day One

Within the past decade, there has been an increasing interest in assessing the ecological risks that multiple chemical exposures pose to aquatic organisms (Meek et al., 2011). Exposure to chemical mixtures may have additive, synergistic, or antagonistic effects, depending on their MOA (Henry and Black, 2007; Boxall et al., 2012) and can have biological effects not predicted from their individual components (Jordan et al., 2012). While biological responses sometimes overlapped between the mixture and individual treatments (e.g. fluoxetine and mixture treatments at day one), differences in metabolite regulation that were unique to the mixture were also detected (Table 2.3). Similar responses have been detected in goldfish (Carassius auratus) exposed to a mixture of organic pollutants (Jordan et al., 2012), in nematodes (Caenorhabditis elegans) exposed to a mixture of nickel and the pesticide chlorpyrifos (Jones et al., 2012), and in green mussels (Perna viridis) exposed to a mixture of benzo(a)pyrene and DDT (Song et al., 2016).

The stress response for the mixture treatment appeared to be an amalgamation of changes identified in the individual treatments (Table 2.2). In addition to the similarities observed in the metabolic pathways associated with stress, there were additional similarities between the mixture and fluoxetine treatments. For example, succinate and citrate were the only Krebs cycle metabolites that were affected at day one, postulated to be due to fluoxetine's effect on

mitochondrial respiratory chain activity (Scaini et al., 2011). This finding also supports the PLS-DA model, which showed that there was a similar clustering in multivariate space between these two treatments at this time (Fig. 2.1). However, unlike in the individual fluoxetine treatment, where these Krebs cycle metabolites were decreased, succinate and citrate were increased in the mixture (Fig. 2.2), suggesting that despite a similar effect on succinate dehydrogenase and citrate synthase (Scaini et al., 2011), the downstream effects on these metabolites were unique. Upregulation of these Krebs cycle metabolites implies an increase in the oyster's energetic requirements, compared to the individual fluoxetine treatment, and supporting this, propionate, oxalic acid, and glycine were increased. Additionally, similar to the DEET and fluoxetine treatments, exposure to the mixture appeared to require an immediate reserve of energy, as the majority of carbohydrate levels were decreased, and acetate was increased (Table 2.3). Also, the decreases in aspartate, proline, serine, and isoleucine indicate their oxidation for metabolic fuel (Gabbott, 1976), or as glucose precursors (De Zwaan and Wijsman, 1976).

Mixture Metabolomics – Days Five & Ten

From days five to ten, oysters exposed to the mixture showed biochemical effects indicative of large energetic demands, as indicated by the changes in Krebs cycle metabolites and its feedstocks. For example, at day five, citrate, succinate, and fumarate from the Krebs cycle increased, as well as oxalic acid from the glyoxylate pathway, glucose, and the fatty acids acetate, butanoate, and propionate (Fig. 2.2). These findings imply that to increase metabolic fuel entering the Krebs cycle, fatty acids were likely undergoing β-oxidation (Brunengraber et al., 2006; Nelson et al., 2008), acetate and glucose were being utilized to provide immediate metabolic fuel (Purmal et al., 2014; Leonard et al., 2014), and that the glyoxylate pathway was

being used to shunt glycine into the Krebs cycle (Nakada et al., 1955). However, by day ten, most of these metabolites, except for oxalic acid, glucose, and propionate, were decreased, indicating that the majority of their lipid reserves were depleted by day ten (Fig. 2.2).

Additionally, several amino acids were decreased from days five through ten (Table 2.3), likely in response to their use as Krebs cycle feedstocks or as glucose precursors to support the observed anaerobic metabolism (De Zwaan and Wijsman, 1976; Gabbott, 1976 - Table 2.2). This decrease in amino acids was more extensive than what was detected in the individual treatments (Fig. 2.2) and implies that responding to the mixture required larger energetic reserves than the individual treatments. Similar to the EE2 treatment at days five and ten, exposure to the mixture caused an increase in ornithine (urea cycle intermediate - Table 2.3), suggesting a high turnover in amino acids, potentially due to the postulated increase in vitellogenin synthesis due to EE2 exposure (Sehgal & Goswami, 2001).

Depuration Day Eight – All Treatments

Supporting the trajectory analysis results, the majority of putatively identified metabolites were still affected by exposure to each treatment post-depuration (Table 2.2). However, across all treatments, the number of affected metabolites decreased post-depuration (Table 2.3), implying that depuration reduced some of the treatment's biochemical effects, although the metabolome of the affected oysters was slow to recover. Among the individual treatments, there were distinct differences among the affected metabolites (Table 2.3), suggesting that even after a depuration period, each treatment's postulated MOA still has an effect on the oyster's metabolome. For example, in the DEET treatment, it appeared that the biochemical effects from the postulated fight-or-flight response and its concurrent large requirement for energy remained,

as the decreases in metabolites involved with the Krebs cycle (fumarate and citrate), and the glyoxylate pathway (oxalic acid and glycine) continued post-depuration (Fig. 2.2). Conversely for oysters exposed to diphenhydramine, these same metabolites were all increased (Table 2.3), which suggests that these pathways were being increasingly utilized to provide metabolic energy as the oysters returned to homeostasis. A similarity among the single treatments was that, post-depuration, the detected carbohydrates and most of the amino acids became increased (Table 2.3). This suggests that, as part of their recovery, the oysters relied more on carbohydrates as an energy source, rather than amino acids. However, compared to the other treatments, there were more amino acids decreased in the fluoxetine treatment, which we believe was likely due to the increased energetic requirements due to the probable presence of norfluoxetine.

Biochemical indicators in oysters exposed to the mixture reveal a continuing demand for energy post-depuration, which was larger and not well predicted by the individual exposures (Table 2.3). Most of the metabolites from the Krebs cycle remained decreased from day ten to depuration day eight (Fig. 2.2), and interestingly, glucose, acetate, and amino acids, all sources of readily available energy (Leonard et al., 2014; Purmal et al., 2014), were decreased at this time, likely indicating that they were depleted as sources of metabolic fuel. Possibly to counteract this, the concurrent increases in both propionate and oxalic acid may indicate an increase in Krebs cycle feedstocks via propionate metabolism (Brunengraber & Roe, 2006) and the glyoxylate pathway (Nelson et al., 2008), respectively. Together, these findings suggest that oysters exposed to mixtures of PPCPs with varying MOAs could have difficulty recovering from these types of exposures, based upon the observed increased energetic requirements.

Additionally, if these effects can be extrapolated to wild populations of oysters, then

exposure to mixtures of PPCPs could adversely affect these populations by reducing the amount of energy available for growth and reproduction.

Conclusion

In summary, we used untargeted metabolomic profiling and multivariate modeling to quantify how the biochemistry of eastern oysters exposed to individual PPCPs and their mixture changed over time. For individual compound exposures, we were able to link the observed biochemical responses with each chemical's postulated MOA and other off-target effects. However, the mixture's effect on the oyster's metabolome could not be well predicted from responses of the individual treatments, which agrees with other findings (i.e. Jordan et al., 2012; Jones et al., 2012; Song et al., 2016). The adductor muscle plays an essential role in storing energy in the form of proteins and carbohydrates (reviewed in Barber & Blake, 2006); as such, the major metabolic pathways affected were Krebs cycle metabolism, fatty acid β-oxidation, and amino acid metabolism. Alterations in these metabolic pathways in the adductor due to exposure to PPCPs could adversely affect the oyster's phenotype and highlights the need to study the effect of chemical mixtures in wild populations. Additionally, exposure to these PPCPs elicited several general stress responses that adversely affected the oyster's metabolome, in addition to effects from each chemical's postulated MOA. PLS-DA analysis demonstrated that the oyster's metabolome responded dynamically to these treatments over the course of the exposure and depuration periods. Based upon the number of still affected metabolites post-depuration, oysters were not able to fully recover from their exposures, regardless of treatment. This observation was confirmed by trajectory analysis, that showed while both the magnitude and trajectories varied among treatments, that overall, the oysters were only able to partially recover from these

chemical exposures. Better understanding the MOA of PPCPs in bivalves, both individually and in mixtures, will provide needed insight into potential adverse effects that could be present in oysters exposed to these contaminants in situ in their natural environments.

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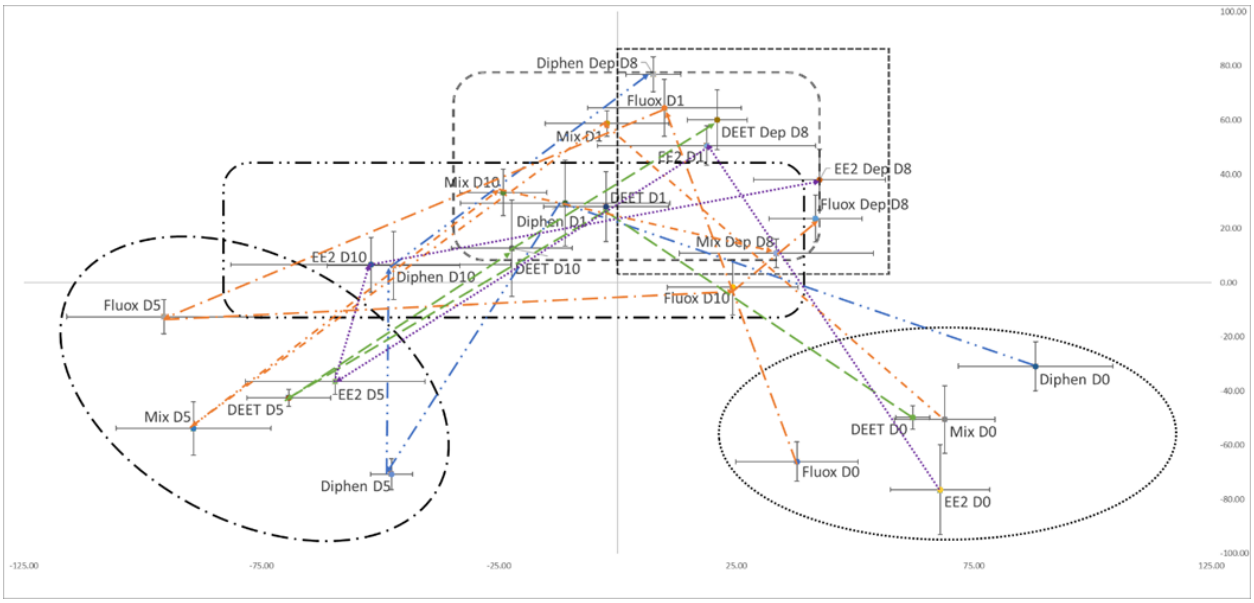
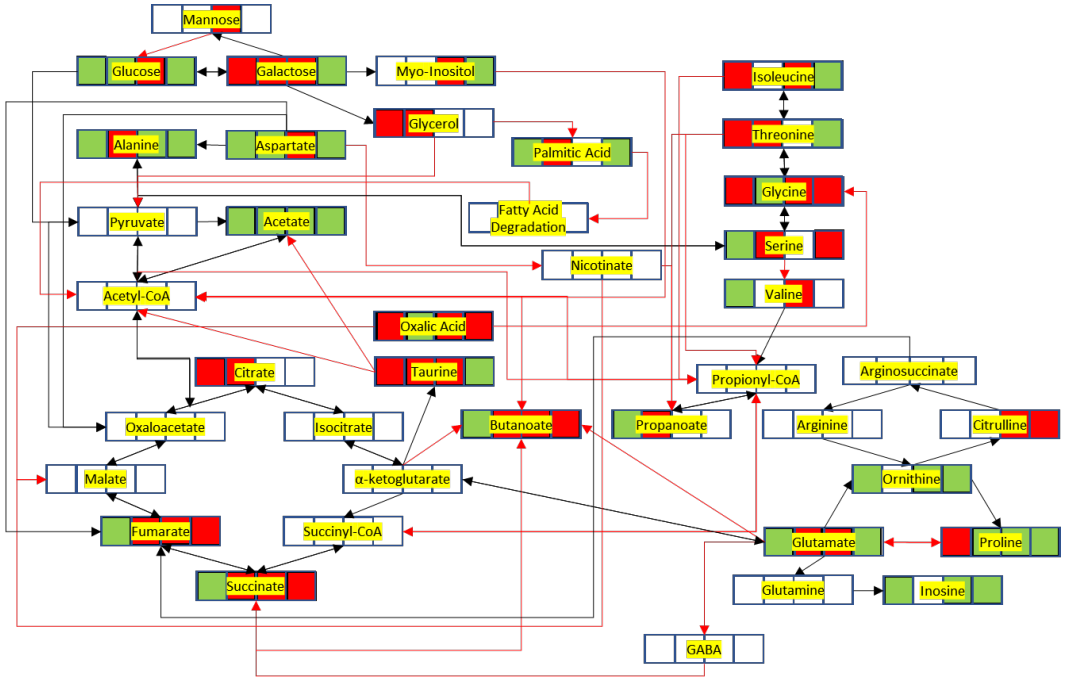
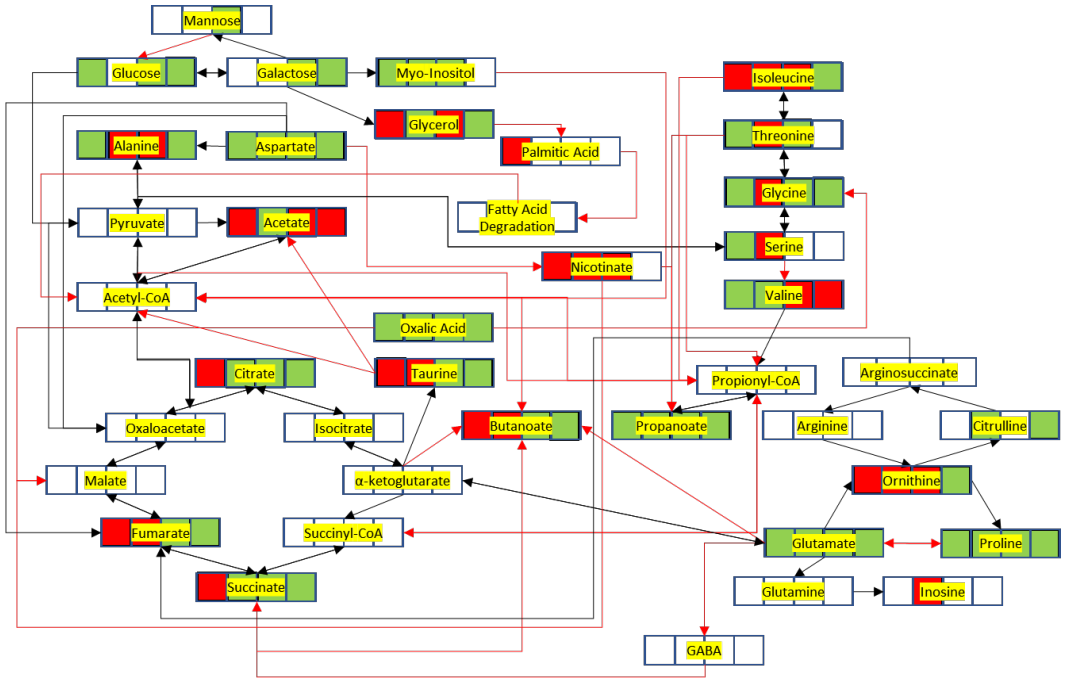


Figure 2.1: Partial least squares - discriminant analysis (PLS-DA) models of the trajectories for each individual treatment (DEET – green; diphenhydramine – dark blue; fluoxetine – orange; EE2 – purple) and the mixture (light blue) generated from oyster adductor muscle. Each point is the average score value for a given class, with its associated 95% confidence intervals.

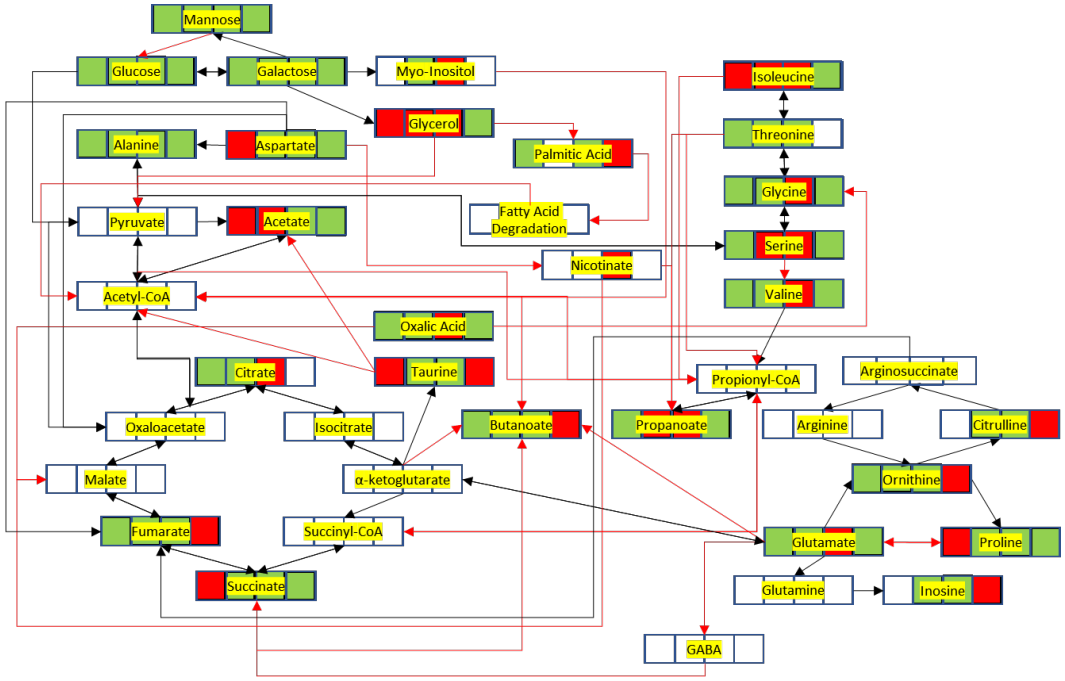
(A) DEET



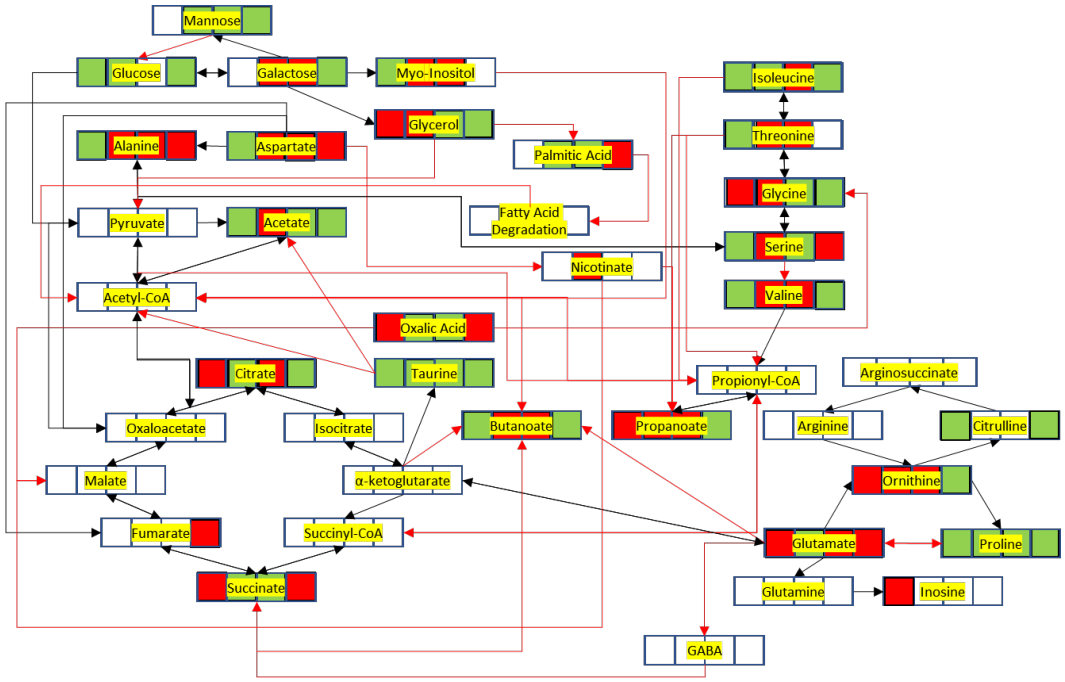
(B) Diphenhydramine



(C) EE2



(D) Fluoxetine



(E) Mixture

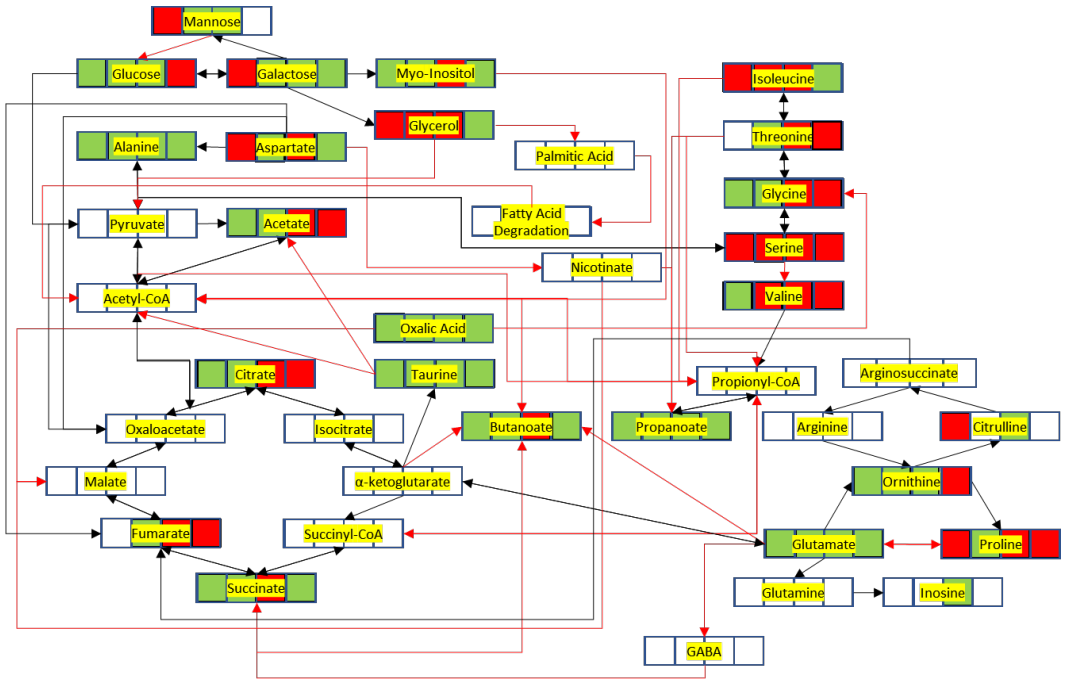


Figure 2.2A - E: KEGG Map of biochemical changes based upon fold changes of identified metabolites at each time point, (A) DEET, (B) Diphenhydramine, (C) EE2, (D) Fluoxetine and (E) Mixture. Green and red squares indicate up- and down-regulation, respectively of the selected metabolites, while solid black lines indicates a direct link, and a red line indicates an indirect link between metabolites.

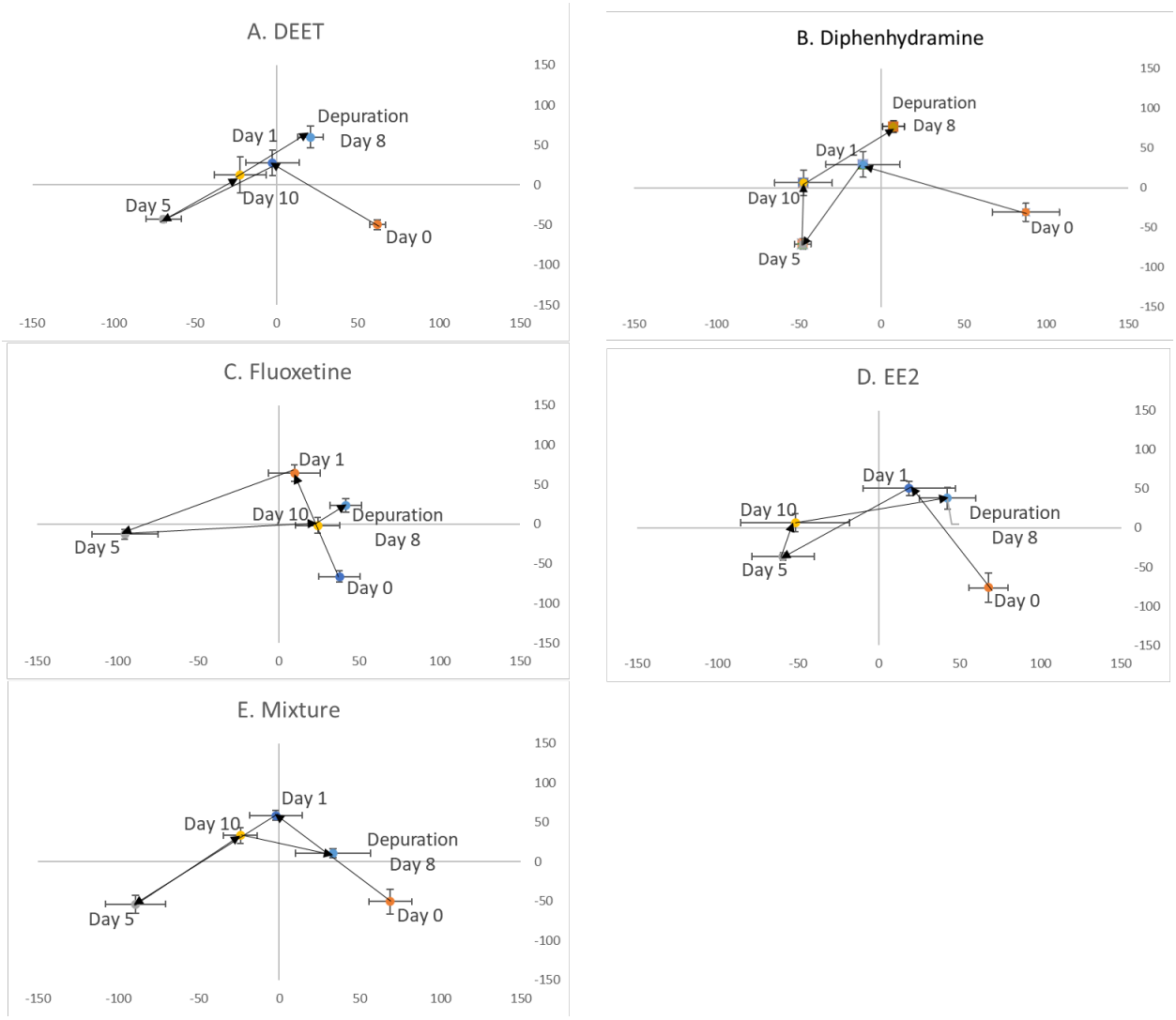


Figure 2.3: Partial least squares - discriminant analysis (PLS-DA) models showing the trajectories of the individual treatments at each time point. Each point is the average score value for a given class, with its associated 95% confidence intervals.

Table 2.1: Concentrations (ng/g dry tissue) of analytes in (A) individual treatments and (B) the mixture in whole oyster body composite samples at days 0, 1, 5, 10 and depuration day 8.

	(A) Individual Treatments				(B) Mixture Treatment			
	Diphen	DEET	Fluoxetine	EE2	Diphen	DEET	Fluoxetine	EE2
Day 0	< LOD	0.88	< LOD	< LOD	< LOD	2.33	< LOD	< LOD
Day 1	2.13	2.18	46.13	9.41	2.04	0.31	7.81	2.77
Day 5	6.41	2.14	143.56	< LOD	2.22	0.92	5.11	35.84
Day 10	2.02	1.04	41.23	< LOD	3.18	1.76	< LOD	77.31
Depuration Day 8	< LOD	0.30	N/A	< LOD	< LOD	0.53	< LOD	12.72

Table 2.2: Presence (+) or absence (-) of metabolic pathways associated with stress in oyster adductor tissue across treatments and time points.

	Anaerobic Metabolism				Oxidative Stress				Osmotic Stress			
	Day 1	Day 5	Day 10	Dep. Day 8	Day 1	Day 5	Day 10	Dep. Day 8	Day 1	Day 5	Day 10	Dep. Day 8
DEET	+	-	-	-	+	-	-	+	+	+	+	-
Diphen	+	-	-	+	+	+	+	+	+	+	-	-
EE2	+	+	+	+	+	+	-	+	+	-	-	+
Fluoxetine	+	-	-	-	-	+	-	-	-	-	-	-
Mixture	+	+	+	-	+	+	+	+	-	-	-	-

Table 2.3: Metabolites putatively identified as varying significantly ($p \leq 0.05$) via Student's t-test as being present in oyster adductor muscle across (A) individual treatments and (B) the mixture and time points (↑ = upregulation; ↓ = down-regulation; - = metabolite not sig. different from controls).

(A)	DEET				Diphenhydramine				EE2				Fluoxetine			
Compound	Day 1	Day 5	Day 10	Dep. Day 8	Day 1	Day 5	Day 10	Dep. Day 8	Day 1	Day 5	Day 10	Dep. Day 8	Day 1	Day 5	Day 10	Dep. Day 8
Krebs Cycle																
Fumarate	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	-	-	-	↓
Succinate	↑	↓	↓	↓	↓	↑	↑	↑	↓	↑	↑	↑	↓	↑	↑	↓
Citrate	↓	↓	-	-	↓	↑	↑	↑	↑	↑	↓	-	↓	↑	↓	↑
Carbohydrates																
d-Glucose	↑	↑	↓	↑	↑	-	↑	↑	↑	↑	↑	↑	↑	↑	-	↑
d-Galactose	↓	↓	↓	↑	-	-	↑	↑	↑	↑	↑	↑	-	↓	↓	↑
d-Mannose	-		↓	-	-	-	↑	-	↑	↑	↑	↑	-	↑	↑	↑
Fatty Acids																
Acetate	↑	↑	↑	↑	↓	↑	↓	↓	↓	↓	↑	↑	↑	↓	↑	↑
Butanoate	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↑	↓	↓	↑
Glycerol	↓	↓	-	-	↓	↑	↓	↑	↓	↓	↓	↑	↑	↓	↑	↑
Palmitic Acid	↑	↓	-	↑	↓	-	-	-	↑	-	↑	↓	-	↑	↑	↓
Propanoate	↑	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓	↑	↓	↓	↓	↑
Glyoxylate Cycle																
Oxalic Acid	↓	↑	↓	↓	↑	↑	↑	↑	↑	↑	↓	↑	↓	↑	↑	↓
Amino Acids																
Alanine	↑	↓	↑	↓	↑	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓
Aspartate	↑	↑	↓	↑	↑	↑	↑	↑	↓	↑	↑	↑	↑	↓	↓	↓
Dimethylglycin	-	-	-	-	-	-	-	-	-	-	-	-	↑	↑	-	-
Glutamate	↑	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓	↑	↓	↑	↓	↓
Glycine	↓	↑	↓	↓	↑	↓	↑	↑	↑	↑	↓	↑	↓	↓	↑	↑
Isoleucine	↓	-	↓	↑	↓	↓	↓	↑	↓	↓	↓	↑	↑	↑	↓	↑
Lysine	-	-	-	-	-	↑	-	↑	-	-	-	-	-	-	-	-
Proline	↓	↑	↑	↑	↑	↑	↑	↑	↓	↑	↑	↑	↑	↑	↓	↑
Sarcosine	-	-	-	-	-	-	-	-	↓	-	-	-	-	-	-	-
Serine	↑	↓	-	↓	↑	↓	-	-	↑	↓	↓	↑	↑	↓	↑	↓
Taurine	↓	↓	↓	↑	↓	↓	↑	↑	↓	↑	↑	↓	↑	↑	↑	↑
Threonine	↓	↓	-	↑	↑	↓	↑	-	↑	↑	↑	-	↑	↓	-	-
Valine	↑	-	↓	-	↑	↑	↓	↓	↑	↑	↓	↑	↑	↓	↓	↑
Urea Cycle																
Citrulline	-	-	-	↓	-	↑	-	↑	-	↑	↑	↑	-	-	↑	-
Ornithine	-	-	↑	↑	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↑	-
Urea	-	-	-	-	-	-	-	-	↓	↑	↑	-	↑	↑	↑	↑
Miscellaneous																
Inosine	↑	↓	↑	↑	-	↓	-	-	-	↑	↑	↓	↓	-	-	-
Myo-inositol	-	-	↓	↑	↑	↑	↑	-	-	↑	↓	-	↑	↓	↓	-
Nicotinate	-	-	-	-	↓	↓	↓	-	-	-	↓	-	-	↑	-	-

(B)	Mix			
Compound	Day 1	Day 5	Day 10	Dep. Day 8
Krebs Cycle				
Fumarate	-	↑	↓	↓
Succinate	↑	↑	↓	↑
Citrate	↑	↑	↓	↓
Carbohydrates				
d-Glucose	↑	↑	↑	↓
d-Galactose	↓	↑	↑	↑
d-Mannose	↓	↑	↑	-
Fatty Acids				
Acetate	↑	↑	↓	↓
Butanoate	↑	↑	↓	↑
Glycerol	↓	↓	↓	↑
Palmitic Acid	-	-	-	-
Propanoate	↑	↑	↑	↑
Glyoxylate Cycle				
Oxalic Acid	↑	↑	↑	↑
Amino Acids				
Alanine	↑	↑	↑	↑
Aspartate	↓	↑	↓	↑
Dimethylglycine	-	-	-	-
Glutamate	↑	↑	↑	↑
Glycine	↑	↑	↓	↓
Isoleucine	↓	↓	↓	↑
Lysine	-	-	-	-
Proline	↓	↑	↓	↓
Sarcosine	-	-	-	-
Serine	↓	↓	↓	↓
Taurine	↑	↑	↑	↑
Threonine		↑	↓	↓
Valine	↑	↓	↓	↓
Urea Cycle				
Citrulline	↓	-	↑	-
Ornithine	↑	↑	↑	↓
Urea	-	-	-	-
Miscellaneous				
Inosine	-	↑	-	-
Myo-inositol	↑	↑	↓	↑
Nicotinate	-	-	-	-

CHAPTER 3

BIOMONITORING OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS USING WILD EASTERN OYSTERS (CRASSOSTREA VIRGINICA) IN GEORGIA, USA ESTUARIES[1]

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[1] David W. Brew, Marsha C. Black, Madison Frazier, Rosemary Pearson-Clarke, Marina Santos, Jackson Rodgers, and W. Matthew Henderson. To be submitted to *Environmental Toxicology and Chemistry*.

Abstract

As coastal populations continue to grow, anthropogenic sources of pharmaceuticals and personal care products (PPCPs) in estuaries will be an increasingly complex problem, especially as these compounds are designed to exert significant effects on physiological systems at low concentrations. Despite these potential risks, the temporal and spatial variability in PPCPs contamination and their bioaccumulation in estuarine organisms is poorly understood. Targeted analysis of 16 analytes was conducted in whole-body tissue samples from wild eastern oysters (Crassostrea virginica), and seawater collected at Brunswick and Sapelo Island, Georgia. Oyster samples were collected bimonthly from December 2013 through October 2015, and seawater samples were collected from October 2014 through October 2015, when bioconcentration factors (BCFs) and risk quotients (RQs) for each compound were also calculated. Across all oyster samples, concentrations ranged from <LOD to 35 ng/g, with few statistically significant differences between estuaries and within their respective sites – likely due to differences between water dilution/concentration at Brunswick/Sapelo, respectively, and tidal mixing. Oyster tissue concentrations, BCFs and RQs displayed strong seasonal and yearly trends, but was highly compound and estuary dependent, highlighting the influence of local populations on oyster bioaccumulation and potential toxicological effects. This research demonstrates that PPCPs are ubiquitous contaminants in Georgia's estuaries, with significant seasonal and yearly variation in bioaccumulation patterns, and are likely exerting adverse effects in aquatic organisms.

Introduction

Estuaries are one of the most biologically productive areas on earth due to the highly dynamic intermixing of freshwater and seawater. They also play an important ecological role by filtering and degrading pollutants, reducing the adverse effects from off-shore storms, providing nursery habitats for many economically valuable fish and bivalve species and provide many cultural and recreational benefits (Board, 2005; Sanger et al., 2015). Estuaries also provide significant economic benefits in that they, directly and indirectly, contribute tens of billions of dollars annually to communities by supporting environmental, transportation, tourism and aquaculture industries (Barbier et al., 2011). Despite their ecological and economic benefits, it is estimated that upwards of half of the saltwater marshes, and a third of mangroves, coral reefs and seagrasses around the world have already been lost or degraded (Board, 2005). Estuaries can be affected by a wide range of anthropogenic disturbances, including urban development and tourism, waste disposal, dredging, transportation, agriculture and fishing, and as a byproduct of many of these activities, excess nutrients, pathogens, and chemical contaminants can enter estuaries from a variety of point and non-point sources (Kennish, 2002). Many of the environmental degradation issues in estuaries stem from issues related to overpopulation, loosely regulated development and aging infrastructure systems that have the potential to both, directly and indirectly, influence habitat loss, ecosystem services and both economic and recreational use of estuarine environments. Kennish (2002) identified pollution as one of the most widespread and urgent threats facing estuarine organisms.

There has been extensive research on the presence of legacy contaminants (e.g., DDT, PCBs, heavy metals) and their biological effects in estuarine organisms (reviewed in Kennish, 2002); however, very little research has been conducted on the presence and possible adverse

effects of pharmaceuticals and personal care products (PPCPs) in estuaries (reviewed in Gaw & Hutchinson, 2015; Granek et al., 2016). This research gap is especially pertinent for pharmaceuticals, as they are specifically designed to be biologically active at low doses and to target specific metabolic, enzymatic or cell signaling mediators to produce their desired therapeutic effects based upon their mechanism-of-action (MOA) (ECETOC, 2007; Fabbri & Franzellitti 2015). Human sewage is the primary source of PPCPs in the marine environment, primarily originating from wastewater treatment plants (WWTPs), hospital and industrial discharges, disposal of unwanted medication, and in some regions, leaking septic systems (Crago et al., 2011), in addition to discharges via secondary bypass or combined sewer overflows (Lubliner et al., 2010). In addition, these compounds can undergo various transformation reactions (i.e. due to hydrolysis, photolysis, oxidation, bacterial degradation and metabolization) that can lead to the formation of numerous secondary products, which may be more persistent and/or toxic than their parent compound (Dévier et al., 2011). Thus, due to the incomplete removal and continuous release, PPCPs are considered to be "pseudo-persistent" in the environment.

In many rural, semi-rural and suburban areas, septic systems are the primary method for waste disposal. Septic systems consist of a septic tank (that receives the waste from the house) and a drain field, which treat the sewage waste by providing physical filtration, surface adsorption, sedimentation and inactivation of the contaminants in the soil (Canter, 2019). However, in many estuarine areas, septic systems may not be able to function properly due to high water tables close to the top of the soil that is sandy and porous (Mallin & McIver, 2012). These conditions allow for contaminants and waste to rapidly percolate through the soil without proper treatment (Stanford et al., 2010). These deficiencies have also led to increased levels of

nutrients entering coastal waters, which has been linked to eutrophication (Dederen, 1992), and can introduce PPCPs to estuaries (Dougherty et al., 2010). This is especially relevant for the Georgia coast, as a study found that approximately 63% of septic systems in McIntosh County, Georgia (which surrounds Sapelo Island) were found in areas of high pollution susceptibility (Walker & Payne, 2003). These multiple sources contribute complex contaminant mixtures to waterways, making it difficult to assign specific contaminants to a source and to determine which source(s) are contributors to any observed adverse effects.

The eastern oyster (Crassostrea virginica, Gmelin 1791) is an important aquaculture species, and there is an ongoing effort to increase their population levels for both aquaculture and ecological reasons, including in Georgia (UGA Marine Extension, 2016). C. virginica is a sessile bivalve filter-feeder that is widely distributed along the eastern seaboard of North America and has both critical economic and ecological roles. C. virginica populations support a large aquaculture industry, adding an estimated 100 million dollars to the US economy in 2012 (Gómez-Chiarri et al., 2015) and an estimated $1.6 million to Georgia's economy in 2018 (UGA Marine Extension, 2016). Unfortunately, commercial oyster harvests have declined to approximately two percent of their historical harvest rates (Eastern Oyster Biological Team, 2007). Marine pollution has contributed to these declines worldwide, and pharmaceuticals and personal care products (PPCPs) have been demonstrated to cause adverse effects in bivalves in laboratory testing when tested individually (e.g. Fabbri, E., & Franzellitti, 2016; Bebianno et al., 2017). Unfortunately, only a limited number of studies have assessed the toxicity of mixtures of PPCPs on marine bivalves (Franzellitti et al., 2015 Juhel et al., 2017; Almeida et al., 2018; Di Poi et al., 2018; Brew et al., 2019a), despite these exposures mimicking real exposure scenarios for wild oysters (albeit in simplified laboratory conditions). While it is difficult to predict the

potential biological effect(s) of a chemicals present in mixtures because of limited data on their biological effects in oysters (Brausch et al., 2012), low concentrations that are likely present at or below current levels of quantification, and a lack of data about seasonal and yearly trends in their environmental presence. However, this does not imply that adverse effects could not occur (Schlenk et al., 2012). Thus, it is important to understand which PPCPs are present in native oyster populations to better inform future ecotoxicological studies and environmental risk assessments. PPCPs (diphenhydramine, ibuprofen, and naproxen) have been detected in eastern oysters near a relatively unpopulated portion of the Georgia coast near Sapelo Island, at concentrations ranging from below the limit of quantification to approximately 60 ng/g, with strong seasonal trends in their bioaccumulation (Fuller, 2012). Besides this study, relatively little is known about either the spatial or temporal patterns in CECs contamination along the Georgia coast or their possible biological effects on marine organisms.

While researchers have demonstrated PPCPs contamination in estuaries around the world (see Hedgespeth et al., 2012; Jiang & Fang, 2014; Moreno-González et al., 2015; Meador et al., 2016; Cantwell et al., 2017), there are outstanding questions about spatio-temporal patterns and seasonal changes in PPCPs contamination in eastern oysters along the Georgia coast. Thus, we conducted an approximately two-year PPCPs biomonitoring program (December 2013 – October 2015) in native oysters sampled from estuaries near Brunswick, Georgia and Sapelo Island, Georgia, in order to better understand how bioaccumulation patterns in oysters varied seasonally and yearly and to identify any relationships with human populations, septic tank densities and location within each estuary. Additionally, measured PPCPs concentrations in seawater samples from October 2014 – October 2015) were used to estimate spatio-temporal patterns in bioconcentration factors and how they varied between Brunswick and Sapelo Island, Georgia.

Materials & Methods

Chemicals & Analyte Selection

All chemicals were >98% pure, solvents were HPLC-grade and obtained from Sigma Aldrich (St. Louis, MO).

Study Site Selection

Four study sites were selected along the Brunswick River to coordinate with a University of Georgia Marine Extension study examining the effects of land use and septic tank densities on water quality (Walker et al., 2003). These sites were near Brunswick, Georgia (population of metropolitan area approximately 100,000 people) and include sites with oyster beds subjected to effluents (via tidal influences) from three local wastewater treatment plants (WWTP - combined monthly limit of 19 MGD – BGJWSC, 2019) and from septic fields in local housing areas (Table 3.3.2). Three of the Brunswick sites (Sites 1 – 3) were located along a continuous stretch of the river and are influenced by both septic fields and local WWTPs. Plantation Creek was the presumed reference site due to its location away from the main river channel (Figure 1).
Our second study location was Sapelo Island, Georgia, a small barrier island located approximately 30 miles northwest of Brunswick, Georgia, with controlled (ferry) access and a small population (60-100 people, including island visitors) that is entirely serviced by septic tanks. There is only one WWTP in McIntosh County that services the city of Darien. Septic fields are the only method to treat sewage waste for the rest of the county, including Sapelo Island. Although limited construction of new housing is currently occurring on the island, most dwellings in Hog Hammock (the only community on Sapelo Island) pre-date the 1970's and, because Sapelo Island is accessible only by ferry and has no road access to the mainland, poor to

no septic tank maintenance is likely. On Sapelo Island, two sample sites (Oakdale Creek and South End Creek) were selected, plus a presumed reference site at Cabretta Creek, due to it having the lowest density of septic tanks among the three sites (Table 3.3.2). Oakdale Creek was selected to overlap with the Fuller (2012) study, and South End Creek is adjacent to UGA's Marine Institute.

Oyster & Water Collection

Eastern oysters (Crassostrea virginica) were sampled from natural oyster beds at Brunswick and Sapelo Island, Georgia bimonthly from December 2013 through October 2015. At each sample site per month, fifteen oysters were collected during low tide (5 harvest size - >75 mm in length; 5 medium size – 50 mm - 75 mm; 5 small size - <50 mm), and stored on ice until they were dissected at either the UGA Marine Extension Lab in Brunswick, Georgia or at the UGA Marine Institute at Sapelo Island, Georgia (total of 12 Brunswick and 9 Sapelo Island samples per month; grand total of 252 samples). Whole body homogenates (minus ~50 mg of adductor muscle dissected for an environmental metabolomics study – Brew et al., 2019b) were collected from each oyster, and were stored on ice until arrival at the Aquatic Toxicology Lab at the University of Georgia, where they were stored at -20 °C until extraction. Water quality parameters (pH, dissolved oxygen (DO, mg/L), salinity (ppt) and conductivity (µm/cm) were measured at each site during each sample collection trip (Table 3.3.1). Water quality (dissolved oxygen (DO), salinity and conductivity) was measured with a YSI Pro 2030 meter (YSI, Yellow Springs, OH). pH was measured with a field pH probe (Oakton Instruments, Vernon Hills, Il). At Sapelo Island, salinity was measured with a refractometer (Deepwater Aquatics, Orlando, Fl). From October 2014 through October 2015, two 1-liter seawater samples were also collected from

each site (total of 8 Brunswick and 6 Sapelo Island samples per month) and stored on ice until arrival at the Aquatic Toxicology Lab at the University of Georgia. Samples were then stored at 4 °C until extraction within 48 hours. Prior to extraction, each sample was vacuum filtered with a 0.45 μm membrane filter (FisherBrand, Fisher Scientific, Hampton, NH) to remove particulate matter.

Water Sampling

Analytes were extracted from water through an extraction procedure adapted from Englert (2007). In brief, Oasis HLB 6cc 200 mg SPE cartridges (Waters, Milford MA) were conditioned with 6 mL of methanol, followed by 6 mL of Milli-Q water. Water samples were loaded onto SPE cartridges using a vacuum manifold at a rate of approximately 5-10 mL/min. After loading, the SPE cartridges were rinsed with 12 mL of Milli-Q water, dried under vacuum, eluted with 6 mL of methanol and blown to dryness under a gentle stream of nitrogen. Both water samples were combined into one sample and reconstituted in 1 mL 10% acetonitrile and stored at -20ºC until LC-MS/MS analysis.

Analyte Body Burdens

All five samples per size category were composited into a single sample, freeze-dried, ground into a fine powder, and the tissue weights were normalized between treatments prior to extraction. The tissue extraction procedure followed Fuller (2012). In brief, 10 mL of 3:3:1 methanol, acetonitrile, and methyl tert-butyl ether solvent mixture were added to each sample, rocked for 20 minutes and then centrifuged for 20 minutes at 1,500 RPM. The supernatant was removed and re-centrifuged at 1,500 RPM for 5 minutes to further remove particulate material.

The remaining supernatant was collected and evaporated to 0.5 mL or less under a gentle stream of nitrogen, and then diluted with 20 mL Milli-q water. SPE was performed on a Sep-Pak Florisil vacuum cartridge (3cc, 200 mg; Waters, Milford MA). The sample solution was loaded onto the cartridge (which was preconditioned with 3 mL methanol and 3 mL HPLC-grade water), eluted with 6 mL methanol, and dried under a gentle stream of nitrogen gas. Samples were reconstituted in 1 mL 10% acetonitrile and stored at -20ºC until LC-MS/MS analysis.

LC-MS/MS Analysis

Samples were analyzed on an Accela HPLC coupled to a TSQ Quantum Ultra mass spectrometer (Thermo Scientific, Bellefonte, PA) for 13 PPCPs (atenolol, acetaminophen, caffeine, methylphenidate, propranolol, diphenhydramine, carbamazepine, sertraline, DEET, valsartan, norethindrone, norgestrel and medroxyprogesterone) and 3 pesticides (atrazine, Imidacloprid, thiacloprid). Chromatographic separation was achieved on a Kinetex 3 µm C18 HPLC column (150 x 2.1 mm, particle size 1.8 µm Phenomenex, Torrance, CA). The initial mobile phase was 95% water with 0.1% formic acid (A) and 5% acetonitrile with 0.1% formic acid (B). Starting conditions were held for one minute, ramped to 95% B over 17 minutes, and held for three minutes, before returning to initial conditions of 5% B and re-equilibrating for 3 minutes (total run time of 35 minutes). The flow rate was 400 µL/min, with an injection volume of 20 µL. All analyses were conducted in selected reaction monitoring (SRM) and positive electrospray ionization mode. The SRM transitions (m/z) values and capillary voltages (V) were: 267.2 to 145 for atenolol, 152.1 to 110 for acetaminophen, 195 to 138 for caffeine, 234 to 84 for methylphenidate, 256 to 209 for imidacloprid, 260 to 183 for propranolol, 253 to 126 for thiacloprid, 256 to 167 for diphenhydramine, 237 to 194 for carbamazepine, 306 to 159 for

sertraline, 216 to 174 for atrazine, 192 to 119 for DEET, 436 to 235 for valsartan, 299 to 109 for norethindrone, 313 to 245.3 for norgestrel and 345 to 123 for medroxyprogesterone. For compounds with concentrations under the limit of detection (LOD), the measured environmental concentration was set at the LOD.

Data Analysis – Analytes

Statistical analysis was conducted using Excel 2013 (Microsoft, Redmond, WA) on each analyte' s LC-MS/MS generated data using effect size measures (mean analyte concentrations ± 95% confidence intervals) to compare (A) body burden differences between harvest, medium and small sized oysters; (B) body burdens between oyster populations at Brunswick and Sapelo Island; (C) differences in oyster bioaccumulation within each estuary; and (4) seasonal changes in oyster PPCPs body burdens. Confidence limits were selected to estimate the effect size of the measured concentrations both between and within each estuary, in order to better assess differences that are potentially ecologically relevant, not just statistically significant (i.e. null hypothesis testing) (Di Stefano, 2004; Newman, 2008). Four samples (June 2014 – Site 2 small; December 2014 – Site 2 harvest & Cabretta medium; and February 2015 – Site 3 small) were not analyzed due to experimental error.

Data Analysis – Bioconcentration Factors

Bioconcentration factors (BCF) were calculated as the analyte concentration in oysters (ng/g dry weight) divided by the analyte concentration in water (ng/mL), A BCF ratio > 1000 indicates that the compound is readily accumulated from water and that uptake of the chemical exceeds xenobiotic metabolism and elimination (Di Poi et al., 2016).

Data Analysis – PPCPs Risk Assessments

Risk quotients (RQs) for the PPCPs detected at Brunswick and Sapelo Island were calculated by the methods outlined in (Minguez et al., 2016). The predicted no-effect concentration (PNEC) for each of the target analytes (derived from Minguez et al., 2016 or the ECOTOX database) was divided by the highest measured oyster concentration for each sample month. An RQ score of < 0.01 indicates that the analyte has no predicted ecological risk, analytes with $0.01 \leq RQ \leq 0.1$ have low risk, analytes with $0.1 \leq RQ \leq 1$ have medium risk and a RQ score ≥ 1 is indicative of high predicted ecological risk (Hernando et al., 2006). Following the methods of Minguez et al. (2016) and Part D (2006), an assessment factor of 10,000 was used to extrapolate from freshwater PNEC values to seawater.

Results & Discussion

Differences in PPCPs accumulation between Brunswick & Sapelo Island, Georgia

Across all 248 samples collected from Brunswick and Sapelo Island, valsartan (angiotensin II receptor blocker), diphenhydramine (antihistamine), norgestrel, and norethindrone (progestin hormones frequently used as contraceptives) had the highest mean concentrations (± 95% confidence intervals (CI)), ranging from 7.4 ng/g to 34.2 ng/g dry weight, followed by medroxyprogesterone (progestin hormones), sertraline (selective serotonin reuptake inhibitor), acetaminophen (nonsteroidal anti-inflammatory drug - NSAID), propranolol (beta-blocker), caffeine (central nervous system stimulant), imidacloprid (neonicotinoid pesticide) with concentrations ranging between 2-5 ng/g dry weight. Thiacloprid (neonicotinoid pesticide), atenolol (beta-blocker), DEET (insect repellant), atrazine (triazine herbicide), methylphenidate

(ADHD medication) and carbamazepine (anti-epileptic drug) had concentrations of <2 ng/g dry weight (Table 3.4). While atrazine, imidacloprid, and thiacloprid are not pharmaceuticals or personal care products, they provide an estimate of compounds that enter estuaries from primarily freshwater inputs from residential and agricultural runoff (reviewed in Cuevas & Costa, 2018). While this study identified these analytes in oysters, a major limitation of this study was not quantifying their transformation products, which may be more persistent and/or toxic than their parent compound (Dévier et al., 2011). For example, the NSAID diclofenac produces several phototransformation products, with relatively unknown environmental presence, even though they are approximately five times more toxic to green algae (Schmitt-Jansen et al., 2007). Unfortunately, low concentrations of a parent compound is not synonymous with reduced risk, as their degradation leads to a multitude of transformation products with a relatively unknown behavior in the environment.

For each of the three sample sizes (harvest, medium and small) collected per site/sample month, 95% CI were used to analyze the effect of size on PPCPs accumulation (Table 3.3). For seven out of the twelve sampled months (December 2013, June 2014, August 2014, October 2014, December 2014, April 2015, and June 2015) there were statistically significant differences in total PPCP bioaccumulation (based upon non-overlapping 95% CI) between sizes, most frequently between small-sized and harvest-sized oysters (Table 3.3). Interestingly, oysters at Sapelo Island only had statistically significant differences between sizes in June 2015, which highlights potential estuary specific variables that influence oyster bioaccumulation patterns. Except for December 2013, small-sized oysters consistently had significantly higher mean PPCPs body burdens than the larger sized oysters (Table 3.3). Surprisingly few studies have examined the effect of size on PPCPs accumulation in marine bivalves,, with the majority of

studies examining heavy metals or organic contaminants in freshwater mussels (primarely PCBs, PAHs, and organochlorine pesticides – Bruner et al., 1994Macisaac et al., 1996). The results from these studies show that there is weak evidence for the relationship between mussel size and organic contaminant cncentrations. However, a field study employing biomarkers (metallothioneins, EROD activity, lipid peroxidation, DNA strand breaks, and vitellogenin-like protein disruption) measured in quagga mussels (Dreissena bugensis) found that larger sized mussels from a freshwater lake in Hungary were likely exposed to more contaminants (compared to smaller mussels) due to higher respiration rates (Ács et al., 2016). These differing results highlight the need for future research into this area, especially in marine bivalves, as they reveal the potential for varying toxicicological responses at different oyster life stages.

We hypothesized that oyster PPCPs body burdens would be significantly higher at Brunswick, Georgia due to the presence of more sources of and larger quantities of PPCPs entering the local environment, compared to Sapelo Island; however, our hypothesis is not well supported by the data. Surprisingly, across all of the selected compounds, there were only 37/192 instances where there was a statistically significant difference (based upon non-overlapping 95% CIs) between sample sites at the two estuaries (Table 3.5 & Table 3.9). Although Brunswick did have larger mean body burdens (ng/g dry weight) at the majority of sampled time points (157/192 comparisons), but there were only 11 instances where there were statistically significant differences (based upon non-overlapping 95% CIs) between oyster body burdens at Brunswick and Sapelo Island (Table 3.5). Within these 11 instances, oysters at Brunswick always had larger mean body burdens and six of those differences were from DEET and atrazine (three each – various sample months), with the remainder (propranolol, diphenhydramine, caffeine, sertraline, and carbamazepine) being statistically different only during the December

2013 sample period (Table 3.5). Granek et al. (2016) found similar results, where there was not a clear pattern of pharmaceutical, pesticide, PBDE congeners and PCB congeners in Olympia oysters (Ostrea lurida) collected from an urbanized and a non-urbanized estuary in Oregon, USA. Similar to our study, PPCPs have been detected in estuaries around the world, with the majority of measured concentrations ranging from <1 ng/L to the µg/L concentrations (reviewed in Gaw et al., 2014).

We postulate that there were few statistical differences in oyster body burdens due to the large differences between the size of the sampled waterways. The sampled oyster beds at Sapelo Island were on small estuarine tidal creeks with shallow depths. Mean ebb and flood discharges ranged up to 10 m3/s on South End Creek (Gilroy et al., 2005), a Sapelo Island sample site. In comparison, the Brunswick River is a much larger system that is also tidally influenced by multiple tributaries that contribute to overall water discharge. Actual discharge information is not available as there are no monitoring stations along the stretch of the Brunswick River near our sample sites. However, due to the overall size of the river, discharges are expected to be significantly greater than those measured in tidal creeks at Sapelo Island. Thus, to achieve similar PPCPs concentrations in oysters from the Brunswick River versus the small tidal creeks at Sapelo Island, the presumed exponentially larger PPCPs inputs into the Brunswick River must be extremely diluted by the large water volume in the river. Conversely smaller inputs of PPCPs at Sapelo Island would be concentrated by the smaller size of the tidal creeks.

Supporting this theory, caffeine was only one of two analytes measured in seawater that had significantly different concentrations between Brunswick and Sapelo Island (Supplementary Table 12). Mean caffeine concentrations at Brunswick were 13.97 ng/L ± 4.59 (95% CI), compared to 144.71 ng/L ± 145.54 (95% CI) at Sapelo Island, which is paradoxical considering

the large discrepancy between nearby populations and how caffeine is considered a tracer of domestic sewage (Proesch & Puchert, 1998). However, it does lend credence to the idea that there is a substantial dilution effect for PPCPs at Brunswick and a concentrating effect in the small tidal creeks at Sapelo Island. While standard ecotoxicology tests determined that adverse effects of PPCP exposure to freshwater aquatic organisms was unlikely due to their low measured environmental concentrations (ng to low µg) (Fent et al., 2006), thus, in the marine environment, where contaminant dilution is higher, the probability of adverse effects due to PPCPs was perceived to be lower. While there is increased evidence that these tests were not sufficiently sensitive to detect short- and long-term adverse effects at environmentally relevant concentrations (reviewed in Aguirre-Martínez et al., 2015), it is possible that chronic exposures over an oyster'ss entire lifespan (approximately 25 – 30 years (Martin, 1987)) could lead to adverse effects (reviewed in Fabbri & Franzellitti, 2016).

We hypothesized that sample sites farther away from human sewage inputs would have lower concentrations of PPCPs, which was not well supported by the data. The presence of caffeine had been utilized as a marker of human contamination in numerous studies (Buerge et al., 2003; Campanha et al., 2015), and while there was not a statistically significant difference (based upon non-overlapping confidence intervals) between caffeine concentrations at any of the sample sites, there was a positive trend between higher mean concentrations and septic tank density (Table 3.2 & Table 3.3). Additionally, the presumed reference sites at both Brunswick (Plantation Creek) and Sapelo Island (Cabretta Creek) were selected due to the lower densities of nearby septic sites and distance from nearby WWTP outfalls (Brunswick only) (Fig. 3.1 and Table 3.2). However, there were only 24/192 instances where oysters at Plantation Creek and 6/192 instances at Cabretta Creek had were significantly different body burdens (based upon

means and non-overlapping 95% CIs) than the other sample sites (Table 3.9). Although, research by Brew et al. (2019b) found that oysters from both of these reference sites had significantly higher overall biological effect (OBE) scores (a measure of the range/variability in an organism's metabolism and the effects of stress on their metabolome) than oyster populations at non-reference sites. These higher OBE scores suggests that oysters at the presumed reference sites were comparatively healthier and could indicate an increased ability to compensate under stressful conditions.

This supports findings by Ferguson et al, (2013) that highlighted the challenges in finding suitable reference sites when studying contaminants (such as PPCPs), due to their near-ubiquitous presence in aquatic environments. However, our results contrasted with Cantwell et al, (2017) and Jiang & Fang, (2014), which did find a clear spatial trend for PPCPs concentrations in Narragansett Bay, Rhode Island and in coastal Taiwan, respectively. These diverging results could be partially accounted for by the large volume of freshwater river inputs near their study sites in the Narragansett Bay (with approximately one-third of total river flow composed of WWTP effluent) and the location of the city Kaohsiung (population approximately 2.8 million people) and several large nearby river systems in the Taiwan study. We postulate that tidal movement at both Brunswick and Sapelo Island played an important role in the spatio-temporal trends of PPCPs contamination in these estuaries. Tides cause a highly dynamic pattern of water movement that can induce significant physical (movement of suspended solids and sediments), chemical (e.g., pH, salinity changes), and biological (e.g. species distribution) gradients due to the intermixing of freshwater and seawater (Cailleaud et al., 2009). Zhao & Zhang, (2015) found that in the Yangtze estuary in China, PPCP concentrations decreased from peak tide to slack tide and increased from ebb peak and ebb slack tide at the majority of their

sites. For this reason, tidal mixing can make it difficult to pinpoint sources of PPCP contamination in these environments.

Additionally, groundwater research at Sapelo Island, near the Cabretta Creek site, demonstrated that the tidal marshes in that area are influenced, via groundwater flow, by water and soil conditions in nearby upland areas, and that this effect can be exacerbated by high tides, rainfall and storm surges (Wilson et al., 2011). The authors also noted that, due to the prevailing soil type and chemistry in this area, contaminants from poorly performing septic sites could leach into groundwater from septic fields situated over 50 m from the marsh (Wilson et al., 2011). Research by Evans & Wilson, (2017) illustrated that at Sapelo Island, there exists a wide variation in geomorphology from marshlands to upland areas overlying the freshwater aquifer and that there can be extensive groundwater discharge into the marsh environment, further influencing PPCP movement to and within local tidal creeks. Thus, we postulate that if PPCPs are entering the groundwater at Sapelo Island, predominately from the septic sites at the Hog Hammock community and the University of Georgia's Marine Institute (Fig. 1), they could then spread throughout the freshwater aquifer and be discharged into nearby marshes. However, future research needs to be conducted to test the validity of this hypothesis.

Seasonal Changes in Oyster Bioaccumulation Patterns

To characterize the seasonal changes in oyster bioaccumulation, sample months were grouped into four groups (Had Winter 2013: December 2013/February 2014; Summer 2014: April/June/August/October 2014; Winter 2014: December 2014/February 2015; Summer 2015: April/June/August/October 2015) based upon measured water temperatures (Table 3.1). Months with water temperature <15 °C were considered to be winter sample months, while months with

temperatures >20 °C were considered summer months. These temperature groupings were selected to correlate with gamete development (winter months) and spawning season for oysters in Georgia's estuaires (March/April – October, with water temperatures > 20 °C and salinity > 10 practical salinity units - Heffernan et al., 1989). Using these seasonal groups, our hypothesis that seasonal changes would influence PPCP bioaccumulation was tested by comparing the overlap in the 95% CIs between each seasonal group (i.e. Winter 2013 -> Summer 2014; Summer 2014 -> Winter 2014; Winter 2014 -> Summer 2014) for each estuary (Table 3.6). This analysis showed that seasonal trends in PPCP accumulation between Brunswick and Sapelo Island had four general trends (bold analytes indicate overlap between estuaries): (1) No statistically significant changes (based on overlapping 95% CIs) between any seasons (Brunswick: atrazine, atenolol, caffeine, sertraline, acetaminophen and carbamazepine; Sapelo Island: atrazine, caffeine); (2) Statistically significant changes only from Summer 2014 -> Winter 2014 (Brunswick: medroxyprogesterone, norethindrone, propranolol, imidacloprid, thiacloprid; Sapelo Island: medroxyprogesterone, norethindrone, norgestrel, imidacloprid, thiacloprid, sertraline); (3) Statistically significant changes from Summer 2014 -> Winter 2014 and Winter 2014 -> Summer 2015 (Brunswick: diphenhydramine, norgestrel; Sapelo Island: atenolol, carbamazepine, diphenhydramine) and (4) patterns for DEET, methylphenidate and valsartan that were unique to Brunswick and patterns for DEET, propranolol, acetaminophen and acetaminophen that were unique to oysters at Sapelo Island (Table 3.6).

Surprisingly, while there were few statistical differences between the mean analyte concentrations (± 95% CIs) in oysters at Brunswick and Sapelo Island (Table 3.5), there was overlap in the bioaccumulation trends for 7/16 of the selected analytes (atrazine, caffeine, diphenhydramine, imidacloprid, medroxyprogesterone, norethindrone, and thiacloprid) between

the two estuaries. This likely indicates that the two distinct human populations at Brunswick and Sapelo Island, Georgia had similar usage patterns for these compounds, despite the large difference between the size of those populations. For example, imidacloprid's seasonal pattern is likely related to its highly seasonal usage as a neonicotinoid pesticide, while similarities for diphenhydramine are likely due to its use as an antihistamine for allergy relief during spring/summer months, and medroxyprogesterone and norethindrone are frequently prescribed progestin birth control medication and are used long-term once prescribed.

Additionally, there are additional factors that can influence seasonal changes in PPCP bioaccumulation including, environmental factors (i.e. monthly rainfall, tidal movement, storm surges, salinity, and pH changes), seasonal changes in the processing time in wastewater treatment plants (Brunswick) and septic sites (Brunswick and Sapelo Island) (Sun et al., 2014), and the rate that oysters can accumulate and metabolize these compounds (Granek et al., 2016 & Sericano et al., 1996). For example, there were several analytes at Brunswick with no statistical differences between the seasons (atrazine, atenolol, caffeine, sertraline, acetaminophen, and carbamazepine), which could indicate that they are high use compounds/consistently used in the local area, and/or that they are inefficiently metabolized by oysters. Alternatively, at Sapelo Island, only atrazine and caffeine lacked seasonal differences (Table 3.6). Other studies examining PPCP contamination in estuaries have shown a mixed effect on their seasonal detection that varied with the study's location. For example, Vidal-Dorsch et al. (2012) found few statistical differences in 56 PPCPs from both WWTP effluent and receiving waters in southern California. In contrast, Hedgespeth et al. (2012) found higher PPCP detection frequencies and concentrations in both WWTP effluents and receiving waters in winter months compared to the summer season in Charleston, South Carolina. Similar to our study, Fuller

(2012) found that C. virginica at Oakdale Creek on Sapelo Island had higher diphenhydramine body burdens in the fall, compared to winter or spring samples (Table 3.6). Granek et al. (2016) analyzed Pacific oysters (Crassostrea gigas) for a suite of legacy contaminants, metals, pesticides and 59 PPCPs, and detected only five PPCPs (diphenhydramine, erythromycin-H2O, sulfadiazine, virginiamycin M1 and naproxen) in an urban and rural estuaries in Oregon. They found that PPCPs accumulation varied between summer 2013 and spring 2014, and no compounds were detected in oysters during the fall 2013 sample period.

Yearly Changes in Oyster Bioaccumulation Patterns

Lastly, we analyzed how the mean oyster body burdens for each analyte varied from (A) Winter 2013 compared to Summer 2014, and (B) Winter 2014 to Summer 2015, in order to determine if the detected bioaccumulation trends was consistent across sample years. At Brunswick, 9/16 compounds had a consistently higher mean during either Winter 2013 and Winter 2014 (atenolol, carbamazepine, norethindrone, and thiacloprid) or Summer 2014 and Summer 2015 (DEET, diphenhydramine, imidacloprid, propranolol and sertraline); while at Sapelo Island, there were 10/16 compounds with consistently higher means during either Winter 2013 and Winter 2014 (atenolol and medroxyprogesterone) or Summer 2014 and Summer 2015 (carbamazepine, DEET, diphenhydramine, imidacloprid, methylphenidate, norethindrone, propranolol, and sertraline) (Table 3.7). The remaining compounds (Brunswick: acetaminophen, atrazine, caffeine, medroxyprogesterone, methylphenidate, norgestrel, and valsartan; Sapelo Island: acetaminophen, atrazine, caffeine, norgestrel, thiacloprid and valsartan) were inconsistent in having higher mean body burdens during winter or summer across the years sampled (Table

3.7). This suggests that the bioaccumulation and potential for adverse effects from a variety of chemical classes will vary between years.

There is evidence that exposure to chemical mixtures can, depending upon the toxicokinetics and toxicodynamics for each of the compounds comprising the mixture, result in differences in the bioaccumulation, metabolism, excretion, and/or receptor binding, which would alter the toxicological effect, compared to exposure to each compound individually (reviewed in Spurgeon et al., 2010). This is particularly relevant in the field where organisms are likely exposed to compounds well below their lab study derived or calculated effective concentration 50% (EC50) or the even below the compound's no observed effect concentration (NOEC), yet they may still produce adverse effects (reviewed in Alterburger et al., 2003). In Pacific oysters (Crassostrea gigas), a CYP Clan 2 gene (CYP450356A1) has been identified (de Toledo-Silva et al., 2008) and is postulated to play an important role in xenobiotic metabolism, as it was upregulated after exposure to untreated domestic sewage (Medeiros et al., 2008). Research by Miao et al. (2011) identified 9 different CYP families (CYP450 family 1, 2, 3, 4 – clan 7, 7 – clan mitochondrial, 12, 13, 17 – clan 3, and 24) in the scallop Chlamys farreri, with the majority of identified transcripts being assigned to the CYP2 family, which plays an important role in xenobiotic metabolism in bivalves. While efforts are underway to sequence C. virginica's genome (Gómez-Chiarri et al., 2015), research has not been conducted to identify CYP450 enzymes in this species, however, based upon the findings from other bivalves, they are likely present. This has important ramifications for this study. For example, norethindrone was demonstrated to inhibit CYP17 activity in carp (Cyprinus carpio) ovaies (Fernandes et al., 2014), sertraline exposure can inhibit CYP2D6 in humans (Hemeryck & Belpaire, 2002), and analgesics and anti-inflammatory drugs (e.g. diclofenac and ipuprofen – not analyzed in this study) have

been demonstrated to inhibit CYP1A1 and CYP1A2 isoforms in rainbow trout (Oncorhynchus mykiss) (Thibaut & Porte, 2008; Gomez et al., 2011). Since these compounds are co-occurring in oysters (Table 3.3), it is likely that they are interacting toxicologically, by influencing the rates of inhibition and induction of CYP enzymes, and thus, could be increasing or decreasing toxicity (reviewed in Spurgeon et al., 2010).

Rainfall can also strongly influence contaminant exposure to aquatic organisms, as there is expected to be increased dilution and surface runoff after rainfall (Xu et al., 2013). In coastal Georgia, rain is most abundant in the summer and early fall, with half of the annual precipitation generally occurring between June and September (CARTER, 1959). At Brunswick, there was an increase in the number of rainy days in 2015, compared to 2014, although there was less rainfall overall during 2015 (55 days, 562.61 mm rainfall from Jan – October 2015, compared to 43 days, 709.68 mm rainfall in all of 2014 - Brunswick, GA Weather, 2019). However, at Sapelo Island, the rainfall trends were different, with there being less rainy days during the 2015 study period, and increased rainfall, compared to 2014 (54 days, 943.61 mm rainfall from Jan – October 2015, compared to 61 days, 535.69 mm rainfall in all of 2014 - Meteorological Monitoring, 2019). Despite these differences in rainfall between Brunswick and Sapelo Island, it is likely that rainfall patterns were an additional influence on oyster body burdens, but to what extent remains uncertain.

Oysters also undergo large-scale changes the seasonal regulation and abundance of amino acids, carbohydrates, and lipids due to complex relationships between growth, reproduction, food quality and stressors (Zandee et al., 1980; Marin et al., 2003; Mayrand et al., 2017; Brew et al., 2019b), and these could have important implications for contaminant uptake. For example, tissue lipid levels vary significantly over the course of a year, generally increasing during winter

months and decreasing during spawning season (Dernekbaşı et al., year; Brew et al., 2019b). These changes in lipid concentrations could also influence the seasonal accumulation and depuration patterns of lipophilic contaminants. Also, during summer months, when water temperatures are increased and dissolved oxygen (DO) levels are correspondingly low (Table 3.3.1), oysters can switch from aerobic to anaerobic metabolism to meet their energetic demands (Isani et al., 1989). This results in a series of changes in the utilized energetic pathways, and as their valves are closed during this time, they have minimal interactions with their external environment (reviewed in de Zwaan & Eertman, 1996). Metabolomic analysis provided evidence that during summer 2015, oysters at South End Creek and Cabretta Creek at Sapelo Island were undergoing long-term anaerobic metabolism, while oysters at Plantation Creek and Site 1 at Brunswick were utilizing short-term anaerobic metabolism (Brew et al., 2019b). Potentially, this would lead to a reduction in the oysters' potential exposure to waterborne contaminants, while simultaneously allowing those contaminants already present in oyster tissues to undergo xenobiotic detoxification via CYP450 enzymes (reviewed in Zhang et al., 2016). Although this would likely increase the body burdens of transformation products from PPCPs (and other contaminants) during this time, which may be more persistent and/or toxic than their parent compounds (Dévier et al., 2011). However, as this process is dynamic and relies upon environmental cues (i.e., predominately increased water temperatures and low DO levels) that vary from year-to-year (Table 3.3.1), we would not expect to see this trend occur to the same extent each year. This could explain, in part, the differences in the mean compound concentrations between summer 2014 and summer 2015 (Table 3.3).

Bioconcentration Factors

Bioconcentration factors (BCFs) are commonly used to predict the bioaccumulation of PPCPs and the potential for toxicity that these contaminants pose to aquatic organisms (Klosterhaus et al., 2013). In this study, seawater grab samples were collected bimonthly from October 2014 – October 2015 and used to calculate monthly BCFs for each compound. Compounds with a BCF value >1000 are considered to be readily bioaccumulated. However, a potential weakness of this approach is that it is unknown how representative these water samples are of the long-term trends of PPCPs present at each site. Across all three sizes (harvest, medium and small) at Brunswick and Sapelo Island, the BCF varied by season and was highly compound dependent (Table 3.8). For all harvest and medium sized oysters, February – June 2015 was characterized by a lack of compounds with BCF > 1000, except for norethindrone, with small sized oysters at both estuaries having more compounds with BCF > 1000 during this time (Table 3.8).

Norethindrone had the highest frequency of a BCF > 1000 across all sizes and estuaries (40/42 instances), with norgestrel (15/42 instances) and medroxyprogesterone (21/42 instances) also having high BCFs, which potentially could result in endocrine disruption due to progestin exposure. While adverse effects in bivalves are still relatively unexplored (Croll & Wang, 2007; Cruzeiro et al., 2019), sex steroids are known to fluctuate seasonally in bivalves as part of their reproductive cycle (Reis-Henriques & Coimbra, 1990), and that injections of estradiol, progesterone and testosterone induced oogenesis and spermatogenesis in Yezo scallops (Mizuhopecten yessoensis) (Varaksina & Varaksin, 1991). Exposure to increased concentrations of natural progesterone was found to inhibit spawning in female Atlantic deep-sea scallops (Placopecten magellanicus), while enhancing spawning in male scallops (Wang & Croll, 2006).

This could have important implications for natural oyster populations, by altering the timing of egg and sperm release, progestin exposure could reduce spawning success, and potentially lead to a decline in local oyster populations.

Additionally, both estuaries had differences in BCF scores between October 2014 and October 2015 (Table 3.8), which illustrates that yearly variation is an important parameter to consider in ecotoxicological studies and effects-based studies in the field. This supports research by Brew et al., 2019a, which demonstrated that eastern oysters (C. virginica) exposed to a mixture of DEET, diphenhydramine, fluoxetine and 17α-ethynylestradiol had unique physiological responses (compared to the individual treatments) over a ten-day exposure followed by an eight day depuration period. The authors postulated that this was driven by differing rates of bioaccumulation, metabolism, excretion, and/or receptor binding for the compounds comprising the mixture, leading to responses that varied in comparison with the individual treatments. By extension, in natural populations of oysters, exposure scenarios of multiple compounds and concentrations would likely result in physiological responses that varied unpredictably over the course of a year (and between years), based upon the chemical mixture they are exposed to (Table 3.6 and 3.7), and variations in their local environment (Brew et al., 2019b).

Analyte Risk Quotients

The use of risk quotients (RQs) has been well established in conducting environmental risk assessments (ERAs), and is defined as the ratio between the measured environmental concentration (MEC) to the predicted no-effect concentration (PNEC) (Hernando et al., 2006). PNEC values were derived from Minguez et al. (2016) and Part D (2006), with an assessment

factor of 10,000 used to extrapolate from freshwater PNEC values to seawater. An RQ score of < 0.01 indicates that the analyte has no predicted ecological risk, analytes with $0.01 \leq RQ \leq 0.1$ have low risk, analytes with $0.1 \leq RQ \leq 1$ have medium risk, and a RQ score ≥ 1 is indicative of high predicted ecological risk (Hernando et al., 2006). Fig. 3.2 shows the calculated RQ ratios from October 2014 through October 2015, and 10/16 analytes (atenolol, diphenhydramine, caffeine, imidacloprid, thiacloprid, carbamazepine, methylphenidate, atrazine, DEET and medroxyprogesterone) were considered to be at no risk for adverse effect across all sample months at both estuaries. Valsartan, norethindrone, and norgestrel had RQ scores that varied between no risk and low risk depending on the sample month; while acetaminophen and sertraline had the highest RQ ratios, and the severity varied significantly by season and higher RQ ratios at Brunswick, compared to Sapelo Island (Fig. 3.2). Surprisingly, while the RQ identified sertraline and acetaminophen as high-risk compounds (Fig. 3.2), this contrasted with the BCF scores, where these compounds were only considered a bioaccumulator once, and twice, respectively (Fig. 3.2). As the seasonality of this data shows, the potential for adverse effects from PPCPs is a highly dynamic process that cannot be fully explained by bioaccumulation data alone, and supports integrating these types of studies with effects-based monitoring in order to examine endpoints that are toxicologically relevant.

Conclusion

In this study, we investigated the extent that native oyster populations bioaccumulated 13 frequently utilized PPCPs and 3 pesticides bimonthly from December 2013 through October 2015 at Brunswick and Sapelo Island, Georgia. In addition, we calculated the bioconcentration factors (BCFs) and risk quotients (RQs) to assess the potential risk that these compounds pose to

aquatic organisms from October 2014 through October 2015. Surprisingly, there were few statistically significant differences (based upon mean and overlapping 95% CIs) in oyster body burdens between Brunswick and Sapelo Island and between sample sites at each estuary, despite the large difference between local population sizes and PPCPs inputs into nearby waterways. We postulate that these differences are potentially due to a combination of local tidal mixing, seasonal changes in oyster physiology, variable PPCP inputs and PPCPs leaching from groundwater/septic sites (especially at Sapelo Island). Analysis of seasonal trends in oyster PPCPs bioaccumulation showed that there was four general trends in compound accumulation, that likely reflected local usage patterns. The results of the BCF and RQ illustrate that accumulation of PPCPs is a dynamic process that can vary significantly both temporally and spatially and is highly compound specific. This has profound implications for exposures in aquatic organisms, as depending on the season, their life history and physiological status, they are likely exposed to different mixtures of contaminants that vary both within a year and from year to year. This illustrates a limitation of these types of biomonitoring studies, where the detected concentrations are a poor indicator of the potential for a toxic response (Hollert et al., 2005), and the need to integrate these studies with biological endpoints.

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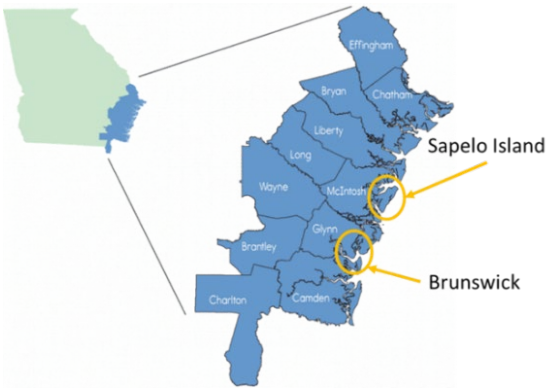
this article are those of the authors and do not necessarily represent the views or policies of the USEPA.



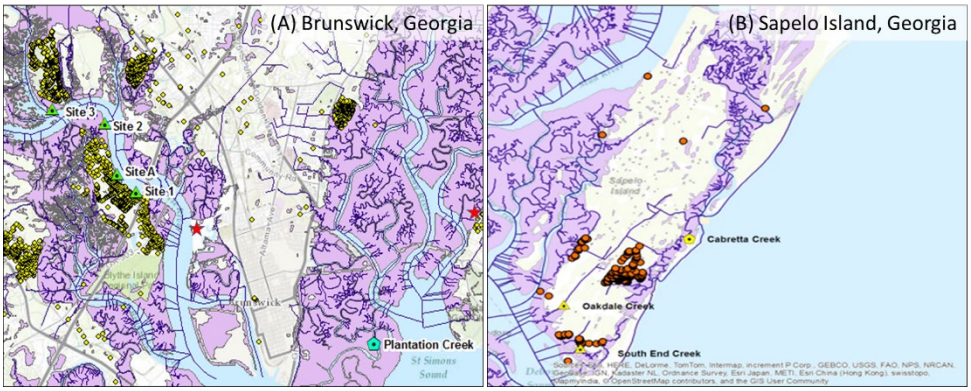


Figure 3.1: Maps showing locations of Brunswick and Sapelo Island and close up views of the sample sites at (A) Brunswick, Georgia and (B) Sapelo Island, Georgia Colored circles represent septic sites and the stars at Brunswick represent the location of wastewater treatment plants.

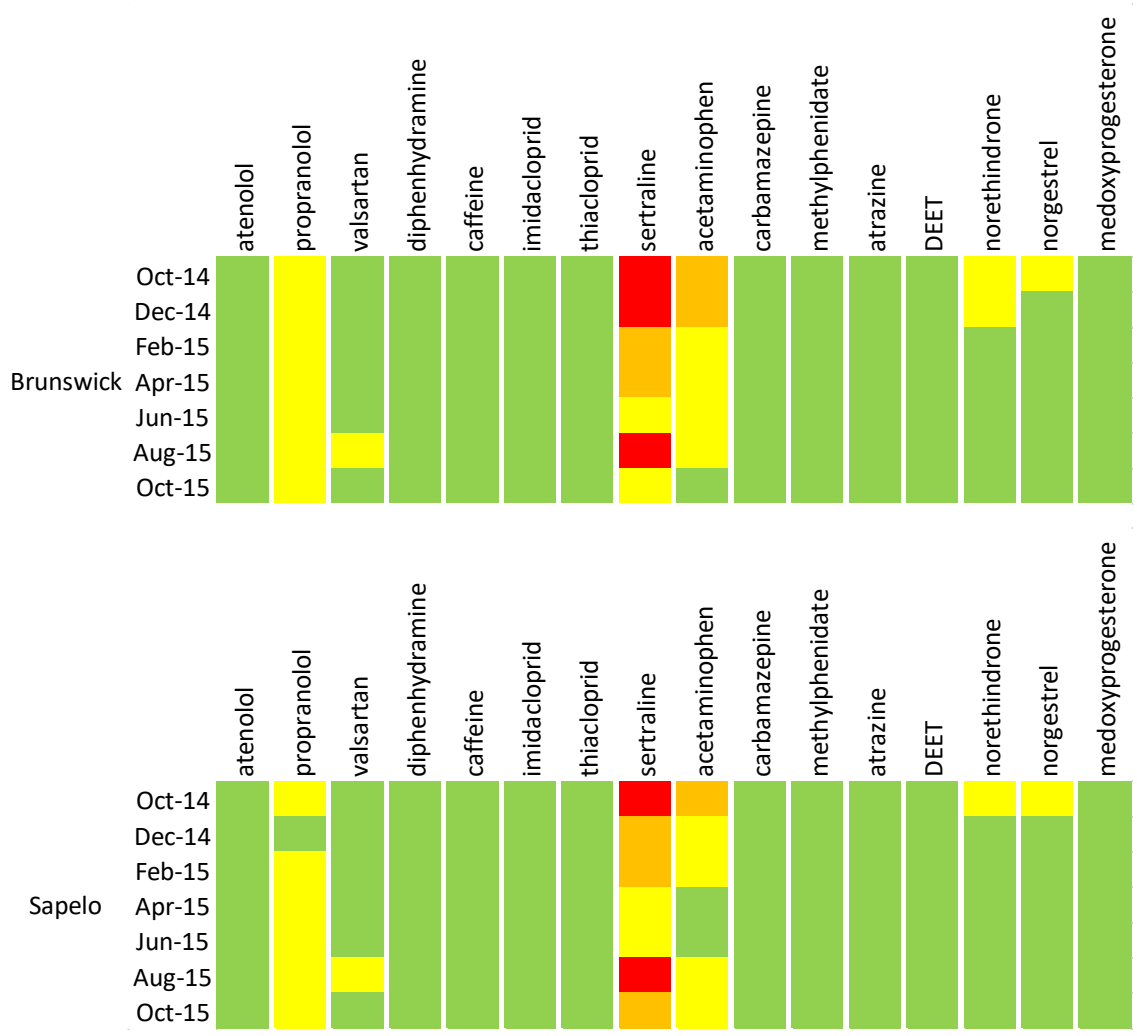


Figure 3.2: Calculated risk quotients (RQs) for each of the detected analytes from October 2014 through October 2015 at Brunswick and Sapelo Island, Georgia. Green = no predicted ecological risk, yellow = low risk, orange = medium risk and red = high risk.

Table 3.1: Bimonthly water quality measurements at (A) Brunswick, Georgia and (B) Sapelo Island, Georgia from December 2013 through October 2015.

(A) Brunswick		Site	Temp (°C)	pH	Dissolved Oxygen (mg/L)	Salinity (ppt)	Conductivity (µm/cm)
2013	December	Plantation	14.78	7.68	5.88	27.14	42900
		Site-1	16.74	7.48	5.88	24.78	39400
		Site-2	17.27	7.57	5.76	25.29	40100
		Site-3	17.52	7.61	5.92	25.51	40300
2014	February	Plantation	12.4	7.95	10.67	22.00	24230
		Site-1	14.5	7.72	9.75	19.69	23480
		Site-2	14	7.74	9.80	20.49	24025
		Site-3	12.7	7.77	11.18	20.86	23699
	April	Plantation	21.4	7.16	5.76	15.00	22300
		Site-1	23.7	6.84	5.90	8.00	13938
		Site-2	22.8	6.90	4.71	10.00	16740
		Site-3	23.5	7.08	5.79	10.00	17155
	June	Plantation	28.09	7.16	5.22	27.33	42400
		Site-1	29.87	7.11	5.01	20.54	32700
		Site-2	30.2	7.13	5.21	21.44	34000
		Site-3	29.25	7.19	5.27	21.50	34100
	August	Plantation	29.7	7.58	4.23	30.52	46700
		Site-1	30.52	7.34	2.91	28.08	43300
		Site-2	30.49	7.35	2.82	28.51	43900
		Site-3	31.06	7.44	3.38	29.48	45200
	October	Plantation	24.19	7.41	4.79	28.83	44700
		Site-1	23.56	7.36	4.13	26.38	41400
		Site-2	24.45	7.39	4.52	26.91	42000
		Site-3	25.13	7.43	4.91	27.01	42200
	December	Plantation	13.03	7.83	7.69	27.33	43400
		Site-1	12.66	7.52	6.65	26.61	42300
		Site-2	12.86	7.54	6.81	27.04	42900
		Site-3	13.46	7.58	6.92	27.28	43200
2015	February	Plantation	11.12	7.58	8.48	21.68	35200
		Site-1	11.5	7.49	8.39	17.81	29300
		Site-2	11.77	7.52	8.64	18.82	30900
		Site-3	12.2	7.59	8.89	19.30	31600
	April	Plantation	23.11	7.40	4.83	24.46	38400
		Site-1	24.3	7.19	4.46	19.44	31300
		Site-2	24.78	7.23	4.64	20.54	32900
		Site-3	25.37	7.31	4.94	21.60	34300
	June	Plantation	28.41	7.38	4.20	28.14	43500
		Site-1	28.27	7.22	4.27	22.94	36200
		Site-2	28.48	7.26	4.24	23.15	36700
		Site-3	29.53	7.38	5.21	23.54	36900
	August	Plantation	31.28	7.51	3.99	32.95	50000
		Site-1	31.53	7.28	3.25	22.89	36100
		Site-2	32.56	7.38	3.68	25.26	39300
		Site-3	33.07	7.48	4.35	25.43	39500
	October	Plantation	25.34	7.38	4.40	25.67	40200
		Site-1	25.43	7.04	3.37	19.05	30800
		Site-2	23.93	7.15	3.78	19.97	32000
		Site-3	24.95	7.28	4.77	15.46	24200

(B) Sapelo Island		Site	Temp (°C)	pH	Dissolved Oxygen (mg/L)	Salinity (ppt)	Conductivity (µm/cm)
2013	December	Cabretta	12.60	7.80	7.38	35.00	33872
		South End	12.30	7.80	7.07	31.00	32928
		Oakdale	14.50	8.87	7.66	29.00	31694
2014	February	Cabretta	10.80	7.51	8.72	33.00	27729
		South End	11.00	7.32	8.75	21.00	25592
		Oakdale	11.40	8.80	7.95	7.00	7223
	April	Cabretta	22.50	7.60	5.15	28.00	38097
		South End	23.30	7.42	4.92	15.00	25350
		Oakdale	25.10	6.66	3.42	0.00	2603
	june	Cabretta	28.80	7.65	4.71	30.00	47581
		South End	29.80	7.58	5.23	25.00	41791
		Oakdale	30.10	7.80	7.29	20.00	36567
	August	Cabretta	30.90	7.32	3.46	30.00	49068
		South End	32.10	7.36	3.66	28.00	48543
		Oakdale	33.30	7.22	3.05	21.00	42932
	October	Cabretta	22.60	7.68	6.29	34.00	43000
		South End	22.20	7.34	4.24	30.00	38500
		Oakdale	22.70	7.17	2.68	20.00	27270
	December	Cabretta	13.00	7.65	7.62	35.00	35533
		South End	12.00	7.40	7.30	32.00	31269
		Oakdale	13.20	7.19	6.87	25.00	25640
2015	February	Cabretta	9.10	8.03	8.83	35.00	28127
		South End	8.10	7.57	8.70	30.00	24861
		Oakdale	9.70	7.34	9.32	20.00	15375
	April	Cabretta	24.90	7.56	5.90	30.00	41250
		South End	25.40	7.54	6.40	25.00	31427
		Oakdale	25.30		3.97		17068
	June	Cabretta	28.70	7.45	3.45	34.50	48976
		South End	30.70	7.89	5.80	27.00	45084
		Oakdale	28.10	7.24	1.72	21.00	30320
	August	Cabretta	30.60	7.93	4.12	33.00	55192
		South End					
		Oakdale	31.80	7.61	3.81	30.00	52320
	October	Cabretta	24.70	7.52	3.40	33.00	46975
		South End	24.80	7.24	3.14	20.00	28160
		Oakdale	25.50	7.46	2.45	26.00	41508

Table 3.2: Septic tank density at specified distances (m) from each sample site at Brunswick, Georgia and Sapelo Island, Georgia, and distance (km) to local WWTP plants at Brunswick. There is not a WWTP located at Sapelo Island.

	Site	500m	1000m	1500m	2000m	2500m	Approx. Distance to WWTP (Km)
Brunswick	site-1	40	155	344	429	502	10.0
	site-2	0	8	44	81	211	4.1
	site-3	13	101	158	220	330	5.0
	Plantation	0	0	0	0	0	6.6
Sapelo Island	Oakdale	0	1	2	25	48	N/A
	Cabretta	0	0	0	0	12	N/A
	South End	8	24	31	31	31	N/A

Table 3.3: Mean and 95% confidence limits of all selected compounds for harvest, medium and small sized oysters, grouped by estuary and sample month at Brunswick and, Sapelo Island, Georgia from December 2013 through October 2015.

			Size	mean	95% CL	Lower 95% CI	Upper 95% CI
Brunswick	December	2013	Harvest	22.55	18.53	4.02	41.08
				14.19	9.94	4.25	24.12
				22.77	21.25	1.52	44.03
				12.07	11.65	0.42	23.72
			Medium	21.61	11.52	10.08	33.13
				11.29	8.77	2.51	20.06
				11.99	10.27	1.72	22.27
				5.23	3.03	2.20	8.26
			Small	25.65	21.46	4.20	47.11
				8.20	6.54	1.66	14.74
				12.44	11.09	1.35	23.54
				5.20	3.02	2.18	8.23
Brunswick	February	2014	Harvest	5.78	5.24	0.54	11.02
				5.27	4.46	0.81	9.72
				5.00	4.30	0.71	9.30
				5.08	4.49	0.60	9.57
			Medium	7.22	5.83	1.39	13.05
				5.38	4.81	0.57	10.19
				6.33	5.44	0.89	11.77
				6.01	5.07	0.94	11.07
			Small	10.51	9.68	0.84	20.19
				19.86	17.83	2.04	37.69
				17.11	14.91	2.20	32.02
				10.74	9.82	0.91	20.56
Brunswick	April	2014	Harvest	2.40	1.30	1.10	3.71
				3.54	2.30	1.24	5.84
				2.35	1.00	1.35	3.36
				1.84	0.71	1.13	2.55
			Medium	6.04	7.28	-1.23	13.32
				2.65	1.51	1.14	4.17
				6.78	9.91	-3.13	16.68
				2.93	1.71	1.22	4.64
			Small	4.92	3.58	1.34	8.50
				7.36	10.10	-2.75	17.46
				18.76	18.60	0.16	37.37
				8.03	6.29	1.74	14.32
Brunswick	June	2014	Harvest	4.25	2.63	1.62	6.88
				5.45	4.47	0.98	9.92
				3.78	2.54	1.23	6.32
				4.23	2.63	1.59	6.86
			Medium	3.74	2.42	1.32	6.16
				5.72	4.08	1.64	9.80
				10.60	10.12	0.49	20.72
				4.37	2.83	1.54	7.20
			Small	8.10	4.95	3.15	13.05
				13.69	6.81	6.88	20.50
				20.10	13.39	6.71	33.49
Brunswick	August	2014	Harvest	4.48	2.60	1.88	7.08
				4.29	2.62	1.68	6.91
				4.48	2.56	1.92	7.05
				5.24	3.61	1.62	8.85
			Medium	7.15	5.26	1.89	12.41
				6.62	3.33	3.30	9.95
				9.39	7.14	2.25	16.53
				5.13	2.62	2.51	7.75
			Small	15.33	14.19	1.14	29.52
				11.19	7.01	4.17	18.20
				16.50	8.49	8.01	24.99
				13.76	8.14	5.62	21.90
Brunswick	October	2014	Harvest	5.16	2.71	2.46	7.87
				12.59	9.25	3.34	21.84
				5.57	3.09	2.47	8.66
				8.82	7.18	1.64	16.00
			Medium	7.62	5.38	2.23	13.00
				16.18	11.65	4.53	27.83
				37.60	37.14	0.47	74.74
				10.69	6.53	4.15	17.22
			Small	21.42	13.84	7.58	35.25
				42.22	31.38	10.84	73.60
				26.06	15.10	10.97	41.16
				15.61	8.93	6.67	24.54

			Size	mean	95% CL	Lower 95% CI	Upper 95% CI
Sapelo Island	December	2013	Harvest	7.16	3.85	3.31	11.01
				11.21	8.62	2.59	19.83
				7.26	4.87	2.39	12.13
			Medium	15.60	14.21	1.39	29.81
				5.33	2.97	2.36	8.30
				4.86	3.08	1.78	7.94
			Small	6.76	4.04	2.72	10.80
				6.18	4.23	1.95	10.41
				4.85	3.08	1.77	7.94
Sapelo Island	February	2014	Harvest	4.69	4.13	0.56	8.82
				5.99	5.55	0.45	11.54
				5.82	4.44	1.38	10.26
			Medium	7.06	6.42	0.63	13.48
				8.05	8.57	-0.52	16.61
				5.15	4.72	0.43	9.87
			Small	28.68	33.57	-4.89	62.25
				8.89	8.19	0.70	17.08
				15.61	20.33	-4.72	35.94
Sapelo Island	April	2014	Harvest	2.88	1.70	1.18	4.58
				2.30	1.29	1.01	3.59
				2.10	0.68	1.42	2.78
			Medium	2.67	1.46	1.21	4.13
				2.07	0.86	1.21	2.94
				2.54	1.00	1.54	3.54
			Small	10.55	8.35	2.20	18.91
				2.90	1.25	1.65	4.14
				2.06	0.70	1.36	2.75
Sapelo Island	June	2014	Harvest	3.43	2.17	1.26	5.59
				4.29	2.62	1.66	6.91
				4.22	2.64	1.58	6.86
			Medium	4.39	2.61	1.78	7.00
				4.20	2.64	1.56	6.84
				3.92	2.58	1.34	6.50
			Small	9.59	6.32	3.27	15.91
				9.48	7.22	2.26	16.70
				4.72	2.74	1.98	7.46
Sapelo Island	August	2014	Harvest	4.23	2.63	1.59	6.86
				4.31	2.63	1.67	6.94
				4.23	2.63	1.59	6.86
			Medium	4.64	2.61	2.03	7.25
				7.28	5.33	1.95	12.61
				4.51	2.68	1.83	7.19
			Small	6.69	7.01	-0.32	13.70
				11.21	7.31	3.90	18.52
				10.96	7.52	3.44	18.48
Sapelo Island	October	2014	Harvest	5.68	3.21	2.47	8.89
				6.13	5.32	0.81	11.46
				6.95	4.71	2.24	11.66
			Medium	10.13	4.40	5.73	14.52
				22.83	20.11	2.71	42.94
				12.79	8.18	4.61	20.97
			Small	34.42	32.21	2.21	66.63
				18.97	12.67	6.30	31.64
				23.83	19.63	4.20	43.46
Sapelo Island	December	2014	Harvest	1.25	1.26	-0.02	2.51
				1.26	1.26	0.00	2.52
				1.25	1.26	-0.01	2.52
			Medium	1.26	1.26	0.00	2.52
				1.23	1.04	0.19	2.28
			Small	3.46	2.42	1.03	5.88
				1.98	1.47	0.51	3.45
				4.19	2.74	1.45	6.93
Sapelo Island	February	2015	Harvest	0.50	0.49	0.01	0.99
				0.30	0.17	0.13	0.47
				0.29	0.17	0.12	0.46
			Medium	0.77	0.79	-0.02	1.56
				0.36	0.22	0.14	0.58
				0.31	0.17	0.14	0.49
			Small	2.36	2.38	-0.02	4.73
				2.53	2.94	-0.41	5.48
				0.86	0.94	-0.08	1.80

Brunswick	December	2014	Harvest	1.28	1.27	0.02	2.55
				1.29	1.26	0.04	2.55
				0.99	1.25	-0.27	2.24
			Medium	2.75	1.98	0.77	4.73
				2.07	1.60	0.47	3.66
				1.22	1.04	0.18	2.26
				1.24	1.24	0.00	2.48
			Small	7.49	4.35	3.15	11.84
				16.74	10.82	5.92	27.56
				21.20	29.58	-8.38	50.78
				3.59	3.07	0.52	6.66
Brunswick	February	2015	Harvest	0.48	0.37	0.11	0.86
				0.36	0.20	0.16	0.56
				0.33	0.18	0.15	0.51
				0.28	0.17	0.11	0.45
			Medium	0.52	0.42	0.11	0.94
				1.01	1.14	-0.13	2.15
				1.33	1.82	-0.50	3.15
				0.68	0.60	0.08	1.28
			Small	2.36	2.21	0.16	4.57
				2.03	2.59	-0.56	4.61
				1.42	1.06	0.35	2.48
Brunswick	April	2015	Harvest	0.35	0.19	0.16	0.54
				0.32	0.18	0.14	0.50
				0.85	0.82	0.03	1.68
				0.50	0.48	0.01	0.98
			Medium	0.85	0.52	0.33	1.37
				0.91	0.74	0.17	1.66
				0.48	0.37	0.11	0.85
				0.43	0.32	0.12	0.75
			Small	3.70	4.06	-0.36	7.76
				1.77	1.17	0.60	2.94
				0.87	0.74	0.13	1.61
				0.76	0.44	0.31	1.20
Brunswick	June	2015	Harvest	0.56	0.58	-0.02	1.14
				0.32	0.25	0.07	0.57
				0.22	0.19	0.04	0.41
				0.32	0.26	0.07	0.58
			Medium	0.56	0.66	-0.10	1.22
				2.06	3.36	-1.29	5.42
				0.54	0.58	-0.04	1.12
				0.87	0.84	0.04	1.71
			Small	1.06	1.11	-0.06	2.17
				1.99	2.73	-0.75	4.72
				4.30	5.03	-0.73	9.33
				1.54	1.27	0.27	2.82
Brunswick	August	2015	Harvest	8.23	6.26	1.97	14.48
				9.48	7.96	1.52	17.43
				8.68	9.02	-0.34	17.71
				9.29	11.30	-2.01	20.60
			Medium	11.47	8.45	3.02	19.92
				8.16	6.05	2.11	14.20
				6.68	5.36	1.32	12.04
				4.93	3.89	1.04	8.83
			Small	33.16	25.79	7.37	58.95
				20.53	18.54	1.99	39.07
				69.93	83.96	-14.03	153.89
				28.80	39.65	-10.85	68.46
Brunswick	October	2015	Harvest	0.90	1.13	-0.23	2.03
				0.31	0.24	0.06	0.55
				0.18	0.11	0.07	0.29
				0.18	0.11	0.06	0.29
			Medium	0.47	0.35	0.13	0.82
				0.26	0.20	0.06	0.46
				0.14	0.07	0.07	0.21
				0.28	0.22	0.06	0.50
			Small	1.01	0.88	0.13	1.88
				0.98	0.94	0.04	1.92
				0.59	0.54	0.05	1.12
				1.49	2.02	-0.53	3.52
Sapelo Island	April	2015	Harvest	0.33	0.16	0.17	0.49
				0.29	0.17	0.12	0.46
				0.28	0.17	0.11	0.45
			Medium	0.59	0.46	0.12	1.05
				0.34	0.19	0.15	0.53
				0.33	0.20	0.13	0.53
			Small	0.61	0.44	0.17	1.04
				0.54	0.34	0.20	0.88
				0.42	0.31	0.11	0.73
Sapelo Island	June	2015	Harvest	0.26	0.20	0.06	0.46
				0.10	0.07	0.03	0.17
				0.26	0.22	0.04	0.48
			Medium	0.60	0.69	-0.08	1.29
				0.63	0.80	-0.17	1.42
				0.31	0.25	0.06	0.56
			Small	0.63	0.46	0.17	1.09
				1.08	1.20	-0.12	2.28
				1.56	2.08	-0.52	3.65
Sapelo Island	August	2015	Harvest	6.71	5.78	0.93	12.48
				6.37	5.82	0.55	12.20
				8.95	7.42	1.54	16.37
			Medium	6.65	5.77	0.87	12.42
				5.41	4.34	1.06	9.75
				6.63	5.80	0.84	12.43
			Small	21.98	20.65	1.33	42.63
				27.79	38.83	-11.04	66.62
				16.24	16.80	-0.57	33.04
Sapelo Island	October	2015	Harvest	0.93	0.88	0.05	1.81
				0.33	0.29	0.04	0.61
				0.43	0.35	0.08	0.78
			Medium	0.54	0.43	0.11	0.97
				0.89	0.82	0.06	1.71
				1.23	1.18	0.04	2.41
			Small	0.83	0.91	-0.08	1.73
				2.03	2.00	0.03	4.03
				1.01	0.89	0.12	1.90

Table 3.4: Mean and 95% confidence limits of selected compounds from oysters (n = 248) at all sample sites and months at both Brunswick, Georgia and Sapelo Island, Georgia. Samples are in order (left to right) based upon mean body burden concentrations (ng/g).

	valsartan	norgestrel	norethindrone	diphenhydramine	medroxyprogesterone	sertraline	acetaminophen	imidacloprid	propanolol	caffeine	thiacloprid	atenolol	methylphenidate	DEET	atrazine	carbamazepine
mean	26.68	22.40	18.06	9.89	4.23	4.19	3.98	3.40	3.17	2.87	1.71	1.67	1.49	1.48	0.88	0.20
95% CI	7.59	4.60	3.64	2.48	0.72	1.61	0.77	0.82	0.44	0.57	0.38	0.43	0.27	0.25	0.14	0.04
Lower 95% CI	19.09	17.79	14.42	7.41	3.52	2.58	3.21	2.58	2.73	2.30	1.33	1.24	1.22	1.23	0.75	0.16
Upper 95% CI	34.27	27.00	21.70	12.37	4.95	5.80	4.76	4.22	3.61	3.44	2.10	2.10	1.77	1.72	1.02	0.24

Table 3.5: Summary of mean ± 95% confidence intervals (CI) for each compound, stating if statistically significant differences (based upon non-overlapping 95% CI) existed between sample sites at each estuary and if mean ± 95% CI were significantly different between Brunswick, and Sapelo Island, Georgia.

	norethindrone				norgestrel			
	Statistically Sig. Diff. Between Sites?		Differences Between	Higher	Statistically Sig. Diff. Between Sites?		Differences Between	Higher
	Brunswick	Sapelo	Estuaries?	mean?	Brunswick	Sapelo	Estuaries?	mean?
Dec-13	Yes	No	No	Brunswick	Yes	no	no	Brunswick
Feb-14	No	No	No	Brunswick	no	no	no	Sapelo
Apr-14	No	No	No	Brunswick	no	no	no	Brunswick
Jun-14	No	No	No	Brunswick	no	no	no	Brunswick
Aug-14	No	No	No	Brunswick	Yes	no	no	Brunswick
Oct-14	Yes	No	No	Brunswick	no	no	no	Sapelo
Dec-14	No	No	No	Brunswick	Yes	no	no	Sapelo
Feb-15	No	No	No	Brunswick	no	no	no	Sapelo
Apr-15	No	No	No	Brunswick	no	no	no	Brunswick
Jun-15	No	No	No	Brunswick	no	no	no	Brunswick
Aug-15	No	No	No	Brunswick	no	no	no	Brunswick
Oct-15	Yes	No	No	Sapelo	no	no	no	Sapelo
	medroxyprogesterone				atenolol			
	Statistically Sig. Diff. Between Sites?		Differences Between	Higher	Statistically Sig. Diff. Between Sites?		Differences Between	Higher
	Brunswick	Sapelo	Estuaries?	mean?	Brunswick	Sapelo	Estuaries?	mean?
Dec-13	no	Yes	no	Brunswick	no	no	no	Brunswick
Feb-14	no	no	no	Brunswick	no	no	no	Brunswick
Apr-14	no	no	no	Brunswick	no	no	no	Brunswick
Jun-14	no	no	no	Brunswick	no	no	no	Brunswick
Aug-14	no	no	no	Brunswick	Yes	no	no	Brunswick
Oct-14	no	no	no	Brunswick	no	Yes	no	Brunswick
Dec-14	no	no	no	Brunswick	Yes	no	no	Btrunswick
Feb-15	no	no	no	Brunswick	no	no	no	Brunswick
Apr-15	no	no	no	Brunswick	no	Yes	no	Brunswick
Jun-15	no	no	no	Sapelo	no	no	no	Brunswick
Aug-15	no	no	no	Brunswick	no	no	no	Brunswick
Oct-15	no	no	no	Sapelo	no	no	no	Sapelo

	propanolol				valsartan			
	Statistically Sig. Diff. Between Sites?		Differences Between	Higher	Statistically Sig. Diff. Between Sites?		Differences Between	Higher
	Brunswick	Sapelo	Estuaries?	mean?	Brunswick	Sapelo	Estuaries?	mean?
Dec-13	Yes	no	Yes	Brunswick	Yes	no	no	Brunswick
Feb-14	no	Yes	no	Sapelo	no	no	no	Sapelo
Apr-14	no	no	no	Brunswick	no	Yes	no	Brunswick
Jun-14	no	no	no	Brunswick	no	no	no	Brunswick
Aug-14	no	no	no	Brunswick	no	no	no	Brunswick
Oct-14	Yes	no	no	Sapelo	Yes	no	no	Brunswick
Dec-14	Yes	no	no	Brunswick	no	no	no	Brunswick
Feb-15	no	no	no	Sapelo	no	no	no	Sapelo
Apr-15	no	no	no	Brunswick	Yes	no	no	Brunswick
Jun-15	no	no	no	Brunswick	no	no	no	Brunswick
Aug-15	no	no	no	Brunswick	no	no	no	Brunswick
Oct-15	no	no	no	sapelo	no	no	no	Sapelo

	caffeine				imidacloprid			
	Statistically Sig. Diff. Between Sites?		Differences Between	Higher	Statistically Sig. Diff. Between Sites?		Differences Between	Higher
	Brunswick	Sapelo	Estuaries?	mean?	Brunswick	Sapelo	Estuaries?	mean?
Dec-13	Yes	no	Yes	Brunswick	Yes	no	no	Brunswick
Feb-14	Yes	no	no	Brunswick	no	no	no	Brunswick
Apr-14	no	no	no	Brunswick	no	no	no	Brunswick
Jun-14	no	no	no	Brunswick	no	no	no	Brunswick
Aug-14	no	no	no	Brunswick	no	no	no	Brunswick
Oct-14	no	no	no	Brunswick	no	no	no	Brunswick
Dec-14	no	no	no	Brunswick	no	no	no	Brunswick
Feb-15	no	no	no	Brunswick	no	no	no	Brunswick
Apr-15	no	no	no	Brunswick	no	no	no	Brunswick
Jun-15	no	no	no	Brunswick	no	no	no	Brunswick
Aug-15	no	no	no	Brunswick	no	no	no	Brunswick
Oct-15	no	no	no	Brunswick	no	no	no	Brunswick

	thiacloprid				acetaminophen			
	Statistically Sig. Diff. Between Sites?		Differences Between	Higher	Statistically Sig. Diff. Between Sites?		Differences Between	Higher
	Brunswick	Sapelo	Estuaries?	mean?	Brunswick	Sapelo	Estuaries?	mean?
Dec-13	Yes	no	no	Brunswick	Yes	no	no	Brunswick
Feb-14	no	no	no	Brunswick	no	no	no	Brunswick
Apr-14	no	no	no	Brunswick	no	no	no	Brunswick
Jun-14	no	no	no	Brunswick	no	no	no	Brunswick
Aug-14	no	no	no	Brunswick	no	no	no	Brunswick
Oct-14	no	no	no	Brunswick	no	no	no	Brunswick
Dec-14	no	no	no	Brunswick	no	no	no	Brunswick
Feb-15	no	no	no	Brunswick	no	no	no	Sapelo
Apr-15	no	no	no	Brunswick	no	no	no	Brunswick
Jun-15	no	no	no	Brunswick	no	no	no	Brunswick
Aug-15	no	no	no	Brunswick	no	no	no	Brunswick
Oct-15	no	no	no	Brunswick	no	no	no	Sapelo

	methylphenidate				carbamazepine			
	Statistically Sig. Diff. Between Sites?		Differences Between	Higher	Statistically Sig. Diff. Between Sites?		Differences Between	Higher
	Brunswick	Sapelo	Estuaries?	mean?	Brunswick	Sapelo	Estuaries?	mean?
Dec-13	no	no	no	Brunswick	Yes	no	Yes	Brunswick
Feb-14	no	no	no	Brunswick	no	no	no	Brunswick
Apr-14	no	no	no	Brunswick	no	no	no	Brunswick
Jun-14	no	no	no	Brunswick	no	no	no	Brunswick
Aug-14	no	no	no	Brunswick	no	no	no	Brunswick
Oct-14	no	no	no	Sapelo	no	no	no	Brunswick
Dec-14	no	no	no	Brunswick	no	no	no	Brunswick
Feb-15	no	no	no	Sapelo	no	no	no	Sapelo
Apr-15	no	no	no	Brunswick	no	no	no	Brunswick
Jun-15	no	no	no	Sapelo	no	no	no	Sapelo
Aug-15	no	no	no	Brunswick	no	no	no	Brunswick
Oct-15	no	no	no	Sapelo	no	no	no	Sapelo

	DEET				atrazine			
	Statistically Sig. Diff. Between Sites?		Differences Between	Higher	Statistically Sig. Diff. Between Sites?		Differences Between	Higher
	Brunswick	Sapelo	Estuaries?	mean?	Brunswick	Sapelo	Estuaries?	mean?
Dec-13	Yes	no	no	Brunswick	no	no	no	Brunswick
Feb-14	no	no	no	Brunswick	Yes	no	no	Brunswick
Apr-14	no	no	no	Brunswick	no	no	Yes	Brunswick
Jun-14	no	no	no	Brunswick	no	no	no	Brunswick
Aug-14	no	no	Yes	Brunswick	no	no	no	Brunswick
Oct-14	no	no	no	Brunswick	no	no	no	Brunswick
Dec-14	no	no	Yes	Brunswick	Yes	no	Yes	Brunswick
Feb-15	no	no	no	Sapelo	no	no	no	Brunswick
Apr-15	no	no	Yes	Brunswick	no	Yes	Yes	Brunswick
Jun-15	no	no	no	Brunswick	no	no	no	Brunswick
Aug-15	no	no	no	Brunswick	no	no	no	Sapelo
Oct-15	Yes	Yes	no	Sapelo	Yes	no	no	Sapelo

	sertraline				diphenhydramine			
	Statistically Sig. Diff. Between Sites?		Differences Between	Higher	Statistically Sig. Diff. Between Sites?		Differences Between	Higher
	Brunswick	Sapelo	Estuaries?	mean?	Brunswick	Sapelo	Estuaries?	mean?
Dec-13	Yes	no	Yes	Brunswick	Yes	no	Yes	Brunswick
Feb-14	no	no	no	Brunswick	no	no	no	Brunswick
Apr-14	no	no	no	Sapelo	no	no	no	Brunswick
Jun-14	no	no	no	Brunswick	no	no	no	Sapelo
Aug-14	no	no	no	Brunswick	no	no	no	Brunswick
Oct-14	no	no	no	Brunswick	no	no	no	Brunswick
Dec-14	Yes	no	no	Brunswick	no	no	no	Brunswick
Feb-15	no	no	no	Sapelo	no	no	no	Brunswick
Apr-15	no	no	no	Brunswick	no	no	no	Brunswick
Jun-15	no	no	no	Brunswick	no	no	no	NA
Aug-15	no	no	no	Brunswick	no	no	no	Sapelo
Oct-15	no	no	no	Sapelo	no	no	no	Sapelo

Table 3.6: Compound means with associated 95% confidence intervals (CI) for (A) Brunswick, Georgia and (B) Sapelo Island, Georgia for each season (Winter 2013, Summer 2014, Winter 2014 and Summer 2015). Seasonal changes in PPCPs bioaccumulation in oysters was tested by comparing the overlap in the 95% CI between each seasonal group (i.e. Winter 2013 -> Summer 2014; Summer 2014 -> Winter 2014; Winter 2014 -> Summer 2014) for each estuary.

Estuary	Compound	Season	mean	95% CI	Lower 95% CI	Upper 95% CI
(A) Brunswick	medroxyprogesterone	Winter 2013	7.09	2.83	4.26	9.92
		Summer 2014	7.73	2.15	5.59	9.88
		Winter 2014	4.28	3.31	0.97	7.59
		Summer 2015	1.05	0.57	0.48	1.63
	norethindrone	Winter 2013	30.74	9.64	21.10	40.38
		Summer 2014	30.70	13.70	17.00	44.40
		Winter 2014	10.39	6.33	4.06	16.72
		Summer 2015	8.97	3.27	5.70	12.24
	norgestrel	Winter 2013	50.07	16.58	33.49	66.65
		Summer 2014	42.66	13.16	29.50	55.82
		Winter 2014	0.34	0.11	0.24	0.45
		Summer 2015	6.94	2.18	4.76	9.12
	DEET	Winter 2013	1.01	0.33	0.68	1.34
		Summer 2014	2.35	0.85	1.50	3.20
		Winter 2014	1.25	0.47	0.78	1.71
		Summer 2015	1.82	0.78	1.04	2.60
	atrazine	Winter 2013	1.31	0.52	0.79	1.83
		Summer 2014	1.50	0.50	1.01	2.00
		Winter 2014	1.14	0.55	0.59	1.69
		Summer 2015	0.49	0.14	0.36	0.63
	atenolol	Winter 2013	5.43	2.07	3.36	7.50
		Summer 2014	2.26	1.41	0.84	3.67
		Winter 2014	0.93	0.59	0.34	1.52
		Summer 2015	0.36	0.23	0.13	0.59
	propanolol	Winter 2013	3.28	1.66	1.62	4.94
		Summer 2014	5.01	1.34	3.67	6.35
		Winter 2014	1.53	0.68	0.85	2.22
		Summer 2015	2.91	0.77	2.14	3.67
	valsartan	Winter 2013	52.25	14.82	37.42	67.07
		Summer 2014	18.99	5.63	13.36	24.62
		Winter 2014	19.84	21.00	-1.16	40.84
		Summer 2015	34.55	31.48	3.07	66.03
	diphenhydramine	Winter 2013	12.53	5.98	6.55	18.51
		Summer 2014	12.63	4.57	8.06	17.21
		Winter 2014	1.11	1.61	-0.51	2.72
		Summer 2015	12.26	7.98	4.28	20.24
	caffeine	Winter 2013	4.25	1.17	3.08	5.42
		Summer 2014	5.59	2.27	3.32	7.87
		Winter 2014	2.68	1.67	1.01	4.36
		Summer 2015	1.44	0.72	0.72	2.17
	imidacloprid	Winter 2013	3.62	1.74	1.88	5.36
		Summer 2014	7.94	3.21	4.74	11.15
		Winter 2014	0.91	1.02	-0.11	1.93
		Summer 2015	1.33	0.46	0.86	1.79
	thiacloprid	Winter 2013	4.22	2.26	1.96	6.48
		Summer 2014	3.35	1.12	2.23	4.47
		Winter 2014	0.43	0.38	0.05	0.80
		Summer 2015	0.19	0.14	0.05	0.33
	sertraline	Winter 2013	3.18	0.93	2.24	4.11
		Summer 2014	4.80	1.78	3.02	6.58
		Winter 2014	1.76	1.33	0.43	3.09
		Summer 2015	8.87	7.85	1.03	16.72
	acetaminophen	Winter 2013	4.97	1.41	3.55	6.38
		Summer 2014	8.08	2.62	5.46	10.70
		Winter 2014	3.21	2.51	0.70	5.71
		Summer 2015	1.54	0.78	0.75	2.32
	carbamazepine	Winter 2013	0.30	0.08	0.23	0.38
		Summer 2014	0.20	0.05	0.16	0.25
		Winter 2014	0.15	0.09	0.06	0.24
		Summer 2015	0.08	0.04	0.04	0.12
	methylphenidate	Winter 2013	0.75	0.38	0.37	1.13
		Summer 2014	2.60	0.49	2.10	3.09
		Winter 2014	2.09	1.51	0.58	3.60
		Summer 2015	0.68	0.52	0.15	1.20

Estuary	Compound	Season	mean	95% CI	Lower 95% CI	Upper 95% CI
(B) Sapelo Island	medroxyprogesterone	Winter 2013	5.26	1.98	3.28	7.24
		Summer 2014	5.74	0.93	4.81	6.67
		Winter 2014	1.28	0.97	0.31	2.26
		Summer 2015	0.78	0.37	0.41	1.15
	norethindrone	Winter 2013	20.83	10.05	10.78	30.89
		Summer 2014	32.91	11.45	21.46	44.36
		Winter 2014	5.61	2.98	2.63	8.59
		Summer 2015	6.23	2.38	3.86	8.61
	norgestrel	Winter 2013	36.62	13.44	23.19	50.06
		Summer 2014	34.46	15.06	19.40	49.52
		Winter 2014	0.67	0.61	0.06	1.28
		Summer 2015	3.25	2.52	0.73	5.77
	DEET	Winter 2013	0.95	0.32	0.63	1.27
		Summer 2014	1.48	0.52	0.96	2.00
		Winter 2014	0.76	0.28	0.48	1.04
		Summer 2015	0.91	0.15	0.76	1.07
	atrazine	Winter 2013	0.79	0.25	0.53	1.04
		Summer 2014	0.81	0.25	0.56	1.07
		Winter 2014	0.48	0.23	0.25	0.71
		Summer 2015	0.46	0.13	0.33	0.59
	atenolol	Winter 2013	3.32	1.12	2.20	4.44
		Summer 2014	2.44	1.12	1.33	3.56
		Winter 2014	0.45	0.22	0.22	0.67
		Summer 2015	0.08	0.03	0.05	0.12
	propanolol	Winter 2013	1.48	0.50	0.98	1.98
		Summer 2014	3.95	1.19	2.77	5.14
		Winter 2014	1.47	0.99	0.48	2.46
		Summer 2015	2.92	0.88	2.03	3.80
	valsartan	Winter 2013	51.83	28.05	23.79	79.88
		Summer 2014	14.65	4.79	9.86	19.44
		Winter 2014	6.15	3.43	2.72	9.58
		Summer 2015	21.23	19.04	2.19	40.28
	diphenhydramine	Winter 2013	5.41	1.89	3.52	7.30
		Summer 2014	10.47	4.48	5.99	14.95
		Winter 2014	0.35	0.38	-0.03	0.73
		Summer 2015	12.95	9.96	2.99	22.90
	caffeine	Winter 2013	2.52	0.48	2.05	3.00
		Summer 2014	3.06	1.45	1.61	4.52
		Winter 2014	1.36	0.53	0.83	1.89
		Summer 2015	1.09	0.33	0.76	1.42
	imidacloprid	Winter 2013	2.95	1.62	1.33	4.57
		Summer 2014	6.12	2.07	4.05	8.19
		Winter 2014	0.12	0.09	0.03	0.22
		Summer 2015	0.69	0.53	0.16	1.22
	thiacloprid	Winter 2013	2.07	0.80	1.28	2.87
		Summer 2014	2.51	0.83	1.67	3.34
		Winter 2014	0.27	0.22	0.05	0.49
		Summer 2015	0.13	0.11	0.03	0.24
	sertraline	Winter 2013	2.16	0.47	1.69	2.63
		Summer 2014	3.49	1.55	1.94	5.04
		Winter 2014	0.83	0.52	0.31	1.34
		Summer 2015	2.61	1.68	0.93	4.29
	acetaminophen	Winter 2013	4.50	2.11	2.39	6.61
		Summer 2014	5.80	2.54	3.27	8.34
		Winter 2014	1.59	0.73	0.86	2.31
		Summer 2015	0.76	0.23	0.53	0.99
	carbamazepine	Winter 2013	0.23	0.10	0.13	0.34
		Summer 2014	0.48	0.19	0.29	0.67
		Winter 2014	0.03	0.01	0.02	0.03
		Summer 2015	0.12	0.05	0.07	0.17
	methylphenidate	Winter 2013	0.55	0.49	0.06	1.04
		Summer 2014	2.68	0.71	1.97	3.38
		Winter 2014	0.04	0.01	0.03	0.05
		Summer 2015	1.76	0.82	0.94	2.58

Table 3.7: Summary of higher mean oyster body burdens (ng/g) between Winter 2013 (W2013) or Summer 2014 (S2014), and Winter 2014 (W2014) or Summer 2015 (S2015) across all sample sites at (A) Brunswick and (B) Sapelo Island, Georgia.

(A) Brunswick	Higher mean concentrations?	
Compound	W2013 or S2014	W2014 or S2015
acetaminophen	S2014	W2014
atenolol	*W2013*	*W2014*
atrazine	S2014	W2014
caffeine	S2013	W2014
carbamazepine	W2013	W2014
DEET	*S2014*	*S2015*
diphenhydramine	*S2014*	*S2015*
imidacloprid	*S2014*	*S2015*
medroxyprogesterone	S2014	W2014
methylphenidate	S2014	W2014
norethindrone	W2013	W2014
norgestrel	W2013	S2015
propanolol	*S2014*	*S2015*
sertraline	*S2014*	*S2015*
thiacloprid	W2013	W2014
valsartan	W2013	S2015

(B) Sapelo	Higher mean concentrations?	
Compound	W2013 or S2014	W2014 or S2015
acetaminophen	S2014	W2014
atenolol	*W2013*	*W2014*
atrazine	S2014	W2014
caffeine	S2014	W2014
carbamazepine	S2014	S2014
DEET	*S2014*	*S2015*
diphenhydramine	*S2014*	*S2015*
imidacloprid	*S2014*	*S2015*
medroxyprogesterone	W2013	W2014
methylphenidate	S2014	S2015
norethindrone	S2014	S2015
norgestrel	W2013	S2015
propanolol	*S2014*	*S2015*
sertraline	*S2014*	*S2015*
thiacloprid	S2014	W2014
valsartan	W2013	S2015

Table 3.8: Bioconcentration factors (BCFs) for harvest, medium and small sized oysters across all sample sites at (A) Brunswick, and (B) Sapelo Island, Georgia. An “x” represents a BCF score >1000.

	oyster size	month	atenolol	propranolol	valsartan	diphenhydramine	caffeine	imidacloprid	thiacloprid	sertraline	acetaminophen	carbamazepine	methylphenidate	atrazine	DEET	norethindrone	norgestrel	medoxyprogesterone
(A) Brunswick	harvest	Oct-14	x	x	x		x	x	x		x		x	x		x	x	x
		Dec-14	x		x				x		x		x			x		x
		Feb-15																
		Apr-15														x		
		Jun-15														x		
		Aug-15			x	x		x	x	x			x			x	x	x
		Oct-15												x		x		
	medium	Oct-14	x	x	x		x	x	x	x	x		x	x		x	x	x
		Dec-14	x		x				x		x		x			x	x	x
		Feb-15														x		
		Apr-15														x		
		Jun-15														x		
		Aug-15	x	x	x			x	x	x			x			x	x	x
		Oct-15												x				
	small	Oct-14	x	x	x		x	x	x	x	x		x	x		x	x	x
		Dec-14	x		x		x	x	x	x	x		x	x		x	x	x
		Feb-15		x							x		x			x		
		Apr-15	x	x							x					x		x
		Jun-15		x												x		x
		Aug-15	x	x	x	x		x	x	x	x		x			x	x	x
		Oct-15		x			x							x		x		
(B) Sapelo Island	harvest	Oct-14	x		x	x		x	x		x		x	x		x	x	x
		Dec-14	x		x				x		x		x			x		x
		Feb-15														x		
		Apr-15														x		
		Jun-15													x	x		
		Aug-15	x	x	x	x		x	x				x			x	x	x
		Oct-15		x										x		x		
	medium	Oct-14	x		x	x	x	x	x		x	x	x	x		x	x	x
		Dec-14	x		x				x		x		x			x		x
		Feb-15														x		
		Apr-15														x		
		Jun-15														x		
		Aug-15			x	x		x	x				x			x	x	x
		Oct-15		x										x		x		
	small	Oct-14	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x
		Dec-14	x		x				x	x	x		x			x	x	x
		Feb-15		x							x		x			x		
		Apr-15														x		
		Jun-15													x	x		
		Aug-15		x	x	x		x	x	x	x		x			x	x	x
		Oct-15		x										x		x		x

Table 3.9: Sample sites at (A) Brunswick and (B) Sapelo Island that were identified as having oysters with statistically significant differences (based upon non-overlapping 95% confidence intervals) in compound body burdens (ng/g).

Estuary	Sample Month	norethindrone	DEET	propanolol	caffeine	sertraline	norgestrel	atrazine	valsartan	imidacloprid	acetaminophen	atenolol	diphenhydramine	thiacloprid	carbamazepine
(A) Brunswick	December 2013	Site 2 & Site 1	Site 1, Plantation & Site 1	Site 2 & Site 1; Site 1, Site 3 & Plantation	Site 1 & Site 3; Sites 1 - 3 & Plantation	Plantation, Site 2, Site 3 & Site 1	Plantation, Site 1 & Site 3		Plantation & Site 1	Plantation & Site 1, Site 2	Plantation & All Other Sites		Plantation, Site 1 & Site 3	Plantation, Site 2 & Site 1	Site 2-3, Plantation & Site 1; Plantation & Site 2
	February 2014				Plantation & Site 3			Plantation, Site 3 & Site 1							
	August 2014						Site 3 & Site 2					Site 1, Site 2 & Site 3			
	October 2014	Plantation & Site 2		Plantation & Site 1					Plantation & Site 3						
	December 2014			Site 2 & Site 3; Plantation & Site 2		Plantation & Site 1	Plantation & Site 2	Plantation & Site 2				Plantation & Site 2			
	April 2015								Site 3 & Site 1						
	October 2015	Site 3, Plantation & Site 1	Site 2, Plantation & Site 1												

	DEET	propranolol	valsartan	atrazine	atenolol
February 2014		South End & Cabretta			
April 2014			Oakdale & South End		
(B) Sapelo Island October 2014					South End & Cabretta
April 2015				South End & Cabretta	
Octpber 2015	South End & Cabretta				Oakdale & Cabretta

CHAPTER 4

ENVIRONMENTAL METABOLOMICS FOR IN SITU MONITORING OF EASTERN OYSTERS AFFECTED BY STRESSORS FROM POINT AND NONPOINT SOURCES ALONG COASTAL GEORGIA, U.S.A.[1]

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[1] David W. Brew, Marsha C. Black, Madison Frazier, Rosemary Pearson-Clarke, Marina Santos, Jackson Rodgers, and W. Matthew Henderson. To be submitted to *Environmental Toxicology and Chemistry*.

Abstract

In the wild, eastern oysters (Crassostrea virginica) routinely adjust their physiology according to the numerous stressors present in their environment. However, the underlying biochemical processes at the level of the metabolome that represent these responses are poorly understood. We utilized untargeted GC-MS metabolomics to assess the metabolome of native oysters collected from oyster beds along the Brunswick River in Brunswick, GA (four sites) and small tidal creeks at Sapelo Island, GA (three sites). Adductor muscle metabolites were extracted and analyzed bimonthly from October 2014 –August 2015. While amino acids, carbohydrates, fatty acids, and Krebs cycle metabolites were the main metabolic pathways affected at both estuaries, their regulation pattern varied significantly between sample sites, especially at Sapelo Island. These differences are likely due to natural and seasonal changes in the oyster metabolome (due to gametogenesis, spawning, anaerobic metabolism, stress responses), and the highly variable physiological responses due to site-specific parameters (changes in water quality parameters, food availability/quality, contaminants) present near the sample sites. The presumed reference sites had significanntly higher OBE scores than those from non-reference sites, suggesting that these oysters populations were healthier compared to those from non-reference sites. Overall, the results of this study do support using the oyster adductor muscle in environmental metabolomic studies and provide baseline data for future monitoring studies utilizing natural populations of oysters.

Introduction

There is growing interest in utilizing omics technologies to elucidate the risks that anthropogenic pollutants pose to marine organisms in their natural environment, as the presence or absence of a chemical provides limited information about its potential toxicity. Thus, it is critical that the responses of natural populations of organisms to changing environmental conditions (both naturally occurring and anthropogenic) be better understood, if omics technology is going to gain wider usage for regulatory purposes (Bahamonde et al., 2016). Metabolomics is the system-wide study of endogenous metabolites within a cell, tissue, or biofluid and how they interact with physiological processes, stressors, or stimuli (Bundy et al., 2009; Skelton et al., 2014), with environmental metabolomics applying this concept to naturally occurring populations (Lankadurai, et al., 2013). However, this application of metabolomics is complicated by both abiotic and biotic stressors, and an organism's life history traits that can induce a multitude of changes at the level of the metabolome, which limits the ability to assign a cause and effect as a result of these uncontrollable conditions (Bahamonde et al., 2016).

Since the start of the Mussel Watch Program in the 1970s, bivalves have been utilized in environmental monitoring programs (Goldberg, 1980; Livingstone, 1993; Lau et al., 2004), with the eastern oyster (Crassostrea virginica) being an ideal bivalve species for biomonitoring studies along the eastern coast of North America (Eastern Oyster Biological Review Team, 2007; Brew et al., 2019a). Oysters are sessile, filter feeders that are exposed to both suspended solids and pollutants, and as such, they can provide an ideal representation of the stressors present at a site and their potential biological effects. The adductor muscle was sampled due to its function as a large energy storage site for proteins and carbohydrates (reviewed in Barber & Blake, 2006) and

its control of shell gape (Tikunov et al., 2010). Due to its pivotal role in oyster physiology, contaminant exposures that adversely affected this organ could reduce respiration/filtration rates and food acquisition, which could reduce the oyster's overall fitness (Ballesta-Artero et al., 2017). For marine bivalves, their physiological responses are strongly dependent upon a variety of biotic and abiotic parameters (not including potential toxicological responses from contaminants), such as age, reproductive status, salinity, dissolved oxygen concentrations, temperature, food availability, and predation, which makes it difficult to isolate and interpret any biological effects exerted by exposure to pollutants (Sheehan and Power, 1999; Camus et al., 2004; Manduzio et al., 2004; Giarratano et al., 2011).

In the environment, natural populations of oysters have an unknown exposure history for anthropogenic contaminants, natural stressors (i.e. food availability/quality, disease/parasite exposures, sub-optimal water quality parameters) and life history traits (i.e. age, reproductive statues, etc.) that elicit effects at the level of the metabolome, in addition to those due to normal seasonal physiological processes – (e.g. reproductive changes, utilizing anaerobic metabolism, etc.). As noted by Bahamonde et al. (2016), it is difficult to link cause-and-effect relationships in environmental metabolomic field studies, as the majority of field conditions are uncontrollable and ill-defined. Part of this challenge is that both normal physiological processes and chemical exposures elicit biological effects that change temporally (Depledge, 1994), as there is a latent period before a biological response becomes observable, followed by maximal induction, initial recovery and, possibly, complete recovery (Wu et al., 2005). This response is further complicated by the fact that oysters are exposed to chemical mixtures in the environment, where changes in the bioaccumulation, metabolism, excretion, and/or receptor binding for the compounds present in a mixture can alter chemical exposures and effects (Spurgeon et al. 2010).

For example, when C. virginica were exposed to an environmentally relevant concentration of fluoxetine, DEET, 17α-ethynylestradiol, diphenhydramine, and their mixture, their metabolic response in the mixture treatment was generally unique compared to the individual treatments (Brew et al., 2019a). The authors suggested this was due to differences in xenobiotic metabolism of the individual contaminants comprising the mixture, which supports findings in rainbow trout (Oncorhynchus mykiss) gonadal RTG-2 cell lines exposed to a mixture of pharmaceuticals, endocrine disruptors, and fragrances (Fernández et al., 2013).

To better understand the effects that different habitats have on the oyster metabolome, samples sites were selected at Brunswick (n = 4) and Sapelo Island (n = 3), Georgia. The city of Brunswick has a large metropolitan area with a population of approximately 100,000 people, with the sample sites being subjected to effluents (via tidal influences) from three local wastewater treatment plants (WWTP) and from septic fields in local housing areas (Table 4.2). Brunswick has three listed Superfund sites within its boundaries, with the largest one being the LCP Superfund site. At this site, there have been various industries since 1919, most recently a chlor-alkali chemical manufacturing facility from 1955 – 1994. Extensive environmental contamination from PCBs (predominately Arolcor 1268 – a highly chlorinated PCB formulation), mercury, lead, and petroleum-related contaminants has been detected in the surrounding surface water, groundwater, soils, sediments, mammals, birds, fish, and invertebrates (Kannan & Tanabe, 1997; Kannan et al., 1998). In addition, pharmaceuticals and personal care products have been documented in the same population of oysters sampled in this study and in nearby environmental media (sediment, suspended solids, and water) (Brew et al., 2019b). The second study location was Sapelo Island, Georgia, a small, mostly undeveloped barrier island approximately 30 miles northeast of Brunswick, Georgia, with controlled (ferry)

access and a small population (60-100 people, including island visitors) that is entirely serviced by septic tanks. Sapelo Island hosts NOAA's Sapelo Island National Estuarine Research Reserve (NERR) and the University of Georgia's Marine Institute (UGAMI). Historically, predominant land use in Sapelo Island was agricultural starting in the 1800s, but since 1976, environmental and ecological research at NERR and UGAMI has become more prevalent (Wirth et al., 2014). Septic fields are the only method to treat sewage waste in the county (McIntosh County, Georgia). While industrial inputs into local estuaries are expected to be minimal, pharmaceuticals and personal care products have been detected in oyster and water samples near Sapelo Island, Georgia (Fuller, 2012; Brew et al., 2019b).

The main goal of the present study was to (1) quantify the spatial and seasonal variation in metabolic responses from natural populations of eastern oysters (C. virginica) present at Brunswick and Sapelo Island, Georgia; (2) compare metabolic signatures from oyster populations present in a large, highly industrialized city (Brunswick) versus those from a rural area with a low population (Sapelo Island); and (3) assess the robustness of using the Overall Biological Effect as a measure of how exposures, environmental conditions, and normal variations in oyster physiology affect the metabolome, and to better understand baseline metabolism. This information is imperative to improve our understanding of how varying habitats, environmental conditions, and seasonal changes affect the oyster metabolome, in order to provide higher quality data for future environmental monitoring programs.

Materials & Methods

Study Design

At Brunswick, four study sites were selected (Site 1: 31.195097. -87.552545; Site 2: 31.21909, -81.556975; Site 3: 31.218510, -81.573572; Plantation Creek (presumed reference site): 31.138272, -81.446598). Sites 1 – 3 were located along a continuous stretch of the river (approximately 3.94 km from Site 1 to Site 3). The presumed reference site (Plantation Creek) was located at a smaller tidal creek off of the Brunswick River, and was located approximately 17.8 km from Site 1 (Fig. 4.1). On Sapelo Island, three study sites were selected (Oakdale Creek: 31.412528, -81.2862213; South End Creek: 31.395559, -81.280020; Cabretta Creek (presumed reference site): 31.438051, -81.238525). The Oakdale Creek site was selected to overlap with a previous study (Fuller 2012). South End Creek was located adjacent to the University of Georgia's Marine Institute, and Cabretta Creek was the presumed reference site (Fig. 4.1), with one septic tank within 500 m (Table 4.2). Ideally, the presumed reference site would have zero nearby septic sites (Table 4.2), and oyster beds located on the northern edge of the island met that condition; however, bimonthly sampling could not be done there due to unpredictable road conditions.

Experimental Design

Adult eastern oysters (C. virginica) were sampled bimonthly from natural oyster beds at each site from October 2014 through August 2015. At each sample period, five oysters over 75 mm in length were collected from each site (total of 30 samples per site, and a total of 210 samples overall). Approximately 20 mg were subsampled from each adductor muscle for biochemical analysis, with the remainder of the tissues collected and analyzed for PPCPs (Brew

et al., 2019b). Samples were immediately frozen in liquid nitrogen to arrest metabolic activity and subsequently stored at -20°C at the Aquatic Toxicology lab at the University of Georgia. Water quality parameters (pH, dissolved oxygen (DO, mg/L), salinity (ppt), and conductivity (µm/cm)) were measured at each site during each sample collection trip (Table 4.1). Water quality (dissolved oxygen (DO), salinity, and conductivity) was measured with a YSI Pro 2030 meter or TYSI xxx meter (YSI, Yellow Springs, OH). pH was measured with a field pH probe (Oakton Instruments, Vernon Hills, IL). At Sapelo Island, salinity was measured with a refractometer (Deepwater Aquatics, Orlando, FL).

Adductor Tissue Metabolite Extraction & Derivatization

Endogenous metabolites were extracted from adductor tissue through a dual phase extraction procedure adapted from Viant (2007) and described in detail by Brew et al. (2019a). In brief, 20 mg of adductor tissue were homogenized and extracted utilizing Milli-Q water, chloroform, and methanol to separate each sample into polar and non-polar phases. Each phase was removed, dried overnight in a Savant Speed Vac Concentrator interfaced with a refrigerated vapor trap (Thermo Scientific, Waltham, MA), and stored at -20ºC until derivatization and GC-MS analysis. Polar samples were derivatized with 30 µl O-methoxyamine-HCl (Sigma-Aldrich, St. Louis, MO) at 20 mg/ml in pyridine, heated for 2.5 hours at 60°C, and vortexed at 30-minute intervals. After cooling, 50 µl BSTFA (N,O-bistrifluoroacetamide) containing 10% TMS (trimethylchlorosilane - Sigma-Aldrich, St. Louis, MO) was added, the solution was heated for 1.5 h at 60°C, and was vortexed at 30 minute intervals. After derivatization, samples were transferred to microtarget inserts, placed in GC vials, and analyzed by GC-MS within 72 hours.

Metabolite Analysis by GC-MS

Derivatized adductor tissue samples were analyzed with an Agilent 7890B gas chromatograph (Agilent Technologies, Santa Clara, CA) interfaced to a Pegasus time-of-flight (TOF) mass spectrometer (LECO, St. Joseph, MI). Metabolites were separated on a Restek RTX-Sil column (30 m x 0.25 mm x 0.25 µm; Restek, Bellefonte, PA) with an initial oven temperature of 60 °C, ramped at 8 °C/minute to 320 °C and held for 5 minutes. The injector, transfer line, and source temperatures were at 275°C, 250°C, and 200°C respectively and spectra were acquired over the mass range of 50-650 m/z.

Data Analysis – Metabolites

Chromatograms were exported as netcdf (.AIA) files, and imported into Metalign® for preprocessing and alignment based upon developer recommended parameters for scan analysis (Lommen, 2009). Microsoft Excel® (Microsoft, Redmond, WA) was used to filter and truncate the data according to Niu et a. (2014) and data were then uploaded into Metaboanalyst® 4.0. The uploaded data were filtered via mean intensity value, and they were log transformed and pareto scaled. Partial least squares - discriminant analysis (PLS-DA) was performed to identify outliers with a Hotelling's T2 test at the 95% confidence interval (Jackson, 2005), which were removed from subsequent analysis. ANOVA (p-value ≤ 0.05) and Fisher's LSD post-hoc analysis were used to determine which features varied significantly (Chong et al., 2018) between months for each site and significantly significant features were identified via the NIST 2014 library. The Kyoto Encyclopedia of Genes and Genomes Pathway Database (http://www.kegg.jp/kegg/pathway.html) was used to identify the biochemical pathways that were affected by each treatment. From the adductor muscle, a total of 87 polar metabolites were

identified and classified into 7 main metabolite classes. The largest class was amino acids, (18 metabolites), followed by carbohydrates (16 metabolites), Krebs cycle (7 metabolites), fatty acids (6 metabolites), nucleotides (3 metabolites), signaling molecules (2 metabolites), and miscellaneous (35 metabolites). The identified features were subjected to heat map analysis utilizing Euclidean distance and ward clustering to quantify how the identified metabolites varied by sample site and sampled months (Chong et al., 2018).

Data Analysis - Overall Biological Effect (OBE)

The Overall Biological Effect (OBE) score provides a relative benchmark for comparing both the magnitude and natural variation in an organism's metabolome. The OBE score was derived for each oyster following the methods of Ekman et al. (2018), where for each oyster per site and month, the absolute value of the intensity for each metabolic feature was summed (Supplementary Material). The mean OBE score was calculated for each sample site per sample month (± 95% confidence intervals) to assess differences among sample sites and months (Fig. 4.4).

Results & Discussion

Overall Biological Effect (OBE)

Estuaries are complex and continually fluctuating ecosystems that provide vital habitats for a large variety of species, and as such, pose unique adaptive challenges for resident oyster populations, especially as their endogenous biochemistry is determined by their biochemical processes (e.g. gametogenesis, spawning, stress responses) and interactions with local environmental conditions (e.g. temperature, salinity, food quality/availability). The Overall

Biological Effect (OBE) was utilized to estimate the range in an oyster's normal biological variability at heavily-industrialized sites (Brunswick, Georgia) and rural sites (Sapelo Island, Georgia). Utilizing the OBE meets several important goals outlined for the validation and interpretation of biomarker responses, including: (1) identifying the range of natural variability in an organism's biological response (Depledge & Fossi, 1994) and their temporal variations (Ringwood et al., 1999); and (2) understanding how organism metabolic responses at sites with chronic and/or acute stressors deviate from their normal variability, compared to responses of organisms at reference locations (Marjan et al., 2014).

During the current study, we found that both the presumed reference sites (Brunswick: Plantation Creek; Sapelo Island: Cabretta Creek) had OBE scores that were several orders of magnitude larger than those from non-presumed reference sites and had more pronounced changes between sample months (albeit different between the two sites); however, none of the changes were statistically significant (mean ± 95% CI - Fig. 4.4). The significantly higher OBE scores at both of these sites suggest that these oyster populations were healthier compared to those oysters at non-reference sites. Their higher metabolic capability and variability (as evidenced by their higher mean OBE scores and wider 95% CI) could indicate an increased ability to adjust and compensate under stressful conditions. Additionally, higher OBE scores could indicate better water quality conditions and/or higher food availability or quality present at the presumed reference sites. This has been observed in previous bivalve studies where that, even within the same coastal zone, significant site-specific variations can exist in biochemical composition, condition index, and growth rates and that these differences can be driven by the availability and quality of food (Camacho et al., 1995; Hernández-Otero et al., 2014; Irisarri et al., 2015).

At Brunswick, sites 1 – 3 were located in a gradient along the Brunswick River (Fig. 4.1), and as such, we expected them to have similar OBE scores; however, at Sapelo Island, each sample site was located at a unique tidal creek (Fig. 4.1), and thus, we expected the OBE scores to reflect unique site-specific parameters. This hypothesis was well supported, as at Brunswick, Site 2 and 3 had overlapping 95% confidence intervals (indicating statistically similar OBE scores), and neither Oakdale Creek nor South End Creek had overlapping error bars (indicating not statistically similar OBE scores - mean ± 95% CI – Fig. 4.4). Additionally, as both Oakdale Creek and South End Creek had higher mean OBE scores than oysters at Sites 1-3 (Fig. 4.4), this suggests that these sites had better habitat conditions than those found at Brunswick. Research has shown that eastern oysters (C. virginica) display seasonal changes in their metabolic rates, with lower rates of metabolism observed during winter, compared to summer months (Li et al., 2007; Pernet et al., 2007); however, our data didn't support this, as Plantation Creek at Brunswick was the only site with the expected trend of lower metabolic rates during winter months (i.e. December 2014 and February 2015 – Fig. 4.4). Future research is required to better understand how abiotic and biotic parameters contribute to an OBE score, but our results demonstrate the sensitivity of the OBE in measuring differences in oyster metabolism among reference and non-reference sites.

Metabolic Pathways Associated with Gametogenesis & Amino Acid Metabolism

In bivalves, the reproductive cycle is one of the most dominating seasonal physiological processes to occur (de Zwaan & Eertman, 1996). In Georgia's coastal waters, oysters spawn from March/April to October (Heffernen et al. 1989) when the water temperature is at or above 20ºC and salinity is higher than 10 psu (Eastern Oyster Biological Review Team, 2007). In our

study, these water conditions were met between March/April through October 2015, and we presume that the oysters were spawning during this time (Table 4.1). Over the course of a year, the regulation and levels of amino acids, carbohydrates, and lipids (e.g. fatty acids) varies significantly due to complex relationships between growth, reproduction, food quality, and stressors (Zandee et al., 1980; Marin et al., 2003; Baek et al., 2014; Dernekbaşı et al., 2015; Mayrand et al., 2017), and as such, the following sections will focus on these metabolite classes. Gamete development occurs during winter months (i.e. December 2014 - February 2015 in our study), and during this developmental stage, amino acids serve as substrates for gluconeogenesis and the Krebs cycle intermediates α-ketoglutarate, fumarate, succinyl-CoA, and oxaloacetate (Fig. 4.5). Studies on Mytilus spp. in European waters (Kube et al., 2007) and Perna viridis in estuaries near Hong Kong (Lau et al., 2004) showed a strong seasonal component to levels of amino acids (in whole-body homogenates) and proteins (in gills), respectively, with higher concentrations observed during winter months, and lower levels in spring and summer. However, other researchers have found different trends in whole body homogenates from razor clams (Ensis siliqua) collected from the Sabo estuary in Portugal with significant variability in amino acid levels in both colder and warmer months, including peaks in February and June and minimums in December and April (Baptista et al., 2014). Thus, it appears that a variety of factors (e.g. location, season, species, and tissue type analyzed) contribute to the responses detected in amino acid metabolism. Additionally, amino acids serve as osmolytes to protect against fluctuating extracellular salinity levels while maintaining cellular volume (Somero and Bowlus, 1983) and anaerobic metabolism (De Zwaan & Wijsman, 1976). Amino acids can also be utilized as an energy source under stressful conditions, such as starvation (Sheedy et al., 2016),

exposure to heavy metals (Ji et al., 2015), hydrocarbons (Fasulo et al., 2016), and pharmaceuticals and personal care products (Leonard et al., 2014).

In our study, only oysters at Plantation Creek and Site 1 in Brunswick and South End Creek at Sapelo Island observed the expected trend in amino acid regulation (high levels in winter and lower levels in spring/summer), similar to Kube et al. (2007), with the remainder of the sites either:

(1) Having minimal changes in monthly amino acid (Site 2 - Brunswick) – Overall, oysters at Site 2 had the least amount of seasonal variation in amino acid regulation among all the sites and had the lowest number of affected metabolites in any category (Figs. 3.2 & 3.3). Due to the essential roles amino acids have in maintaining bivalve homeostasis and gametogenesis, this low response could indicate that oysters at Site 2 had diminished reproductive output due to a lack of available energy. However, similar to the other sites, there was a decrease in glycine levels during winter months, which is correlated to gonadal development in Mytilus spp. (Kube et al., 2007). Further research needs to be conducted to better relate reproductive output/success with metabolic endpoints in bivalves to better understand this phenomenon.

(2) Having the majority of amino acids decreased throughout winter, and increased later in the year (Site 3 - Brunswick) – The unexpected behavior of amino acids at Site 3 implies the presence of one or more stressor(s) that directly affect amino acid metabolism (i.e. decreases in glutamate suggesting increased glutathione synthesis for antioxidant defense) - (Cappello et al., 2017 & Leonard et al., 2014), and/or that the amino acids were being oxidized during this time to provide metabolic fuel (De Zwaan & Wijsman, 1976), which could be stressor related (Brew et al., 2019a). Supporting the decrease in amino acid metabolism during gametogenesis, ornithine

(urea cycle intermediate) was decreased, which indicates that less amino acid turnover was occurring during this time at Site 3 (Fig. 4.2D; Denkert et al., 2008).

(3) Having increases in amino acid metabolism during winter months that lasted through June 2015 (Cabretta and Oakdale Creek – Sapelo Island) - At Sapelo Island, the pattern for amino acid metabolism varied more between sites (likely as each site was located on a unique tidal creek – Figs: 3.3A – 3.3B), which are likely the result of variations in gametogenesis in individual oysters, as they spawn for the majority of the year. Similar behavior has been observed in C. virginica collected from the coastal lagoons in the Gulf of Mexico (Rogers & Garcia-Cubas, 1981), and Crassostrea corteziensis from the Southern Gulf of California in Mexico (Páez-Osuna et al., 1993), which was postulated to be a result of individual variation in the timing of gameotogenesis. While it is difficult to associate a specific cause and effect to these varied changes in amino acid metabolism between locations and sites, our results concur with biomarker research in the green mussel (Perna viridis) near Hong Kong (Lau et al., 2004). They found that environmental and individual biological factors (e.g. variability not related to exposure to chemical pollutants) naturally vary on a seasonal cycle, which can complicate interpreting results from field studies.

A potential influence on amino acid levels is salinity, (Kube et al., 2007) and in bivalves, taurine levels are actively adjusted during salinity changes to handle osmotic stress (Liu et al., 2011). However, taurine was only detected at two sites (Brunswick: Site 3 & Sapelo Island: South End – data not shown), and while salinity levels did vary seasonally, all of the study sites displayed similar temporal trends (Table 4.1). Thus, there is little evidence that salinity and osmotic stress were large contributors to the detected effects in amino acid metabolism. While we are unsure of the exact cause of these differing responses in amino acid metabolism, we

speculate that there is a complex interaction occurring between gametogenesis, local environmental conditions, and stressors that affect amino acid metabolism.

Overview of Relevant Oyster Physiology – Role of Carbohydrate Metabolism on Gametogenesis

In addition to amino acids, carbohydrates are another important energy source for bivalves during both their reproductive cycle (Camacho et al., 2003), and during periods of stress, (Aru et al., 2017) and as such, they also display strong seasonal trends. For example, glycogen stores comprise over 50% of the carbohydrate reserves in bivalves (Baek et al., 2014), and play a key role in physiology. In C. virginica, glycogen provides energy for both growth and gametogenesis (Mathieu and Lubet, 1992), with levels generally decreased during winter (Greenway & Storey, 1999 & Mayrand et al., 2017). As such, we would expect that glucose levels would increase during winter months, as the oysters are undergoing glycogenolysis during this time (Fig 3.5). This situation reverses in summer, where the adductor muscle undergoes glycogen synthesis, which results in glucose uptake via gluconeogenesis (Greenway & Storey, 1999).

Glycogenolysis during winter months is generally accompanied by increases in Krebs cycle metabolites (e.g., acetate, acetoacetate, citrate, fumarate, pyruvate, succinate), indicating an increased mobilization of energy reserves during this time (Cappello et al., 2013). This pattern was observed in oysters at Brunswick, with increased levels of Krebs cycle metabolites during February and April 2015 (compared to December 2014 – Figs. 3.2 & 3.3). This finding supports previous research that C. virginica in New Brunswick, Canada increasingly rely on their energy reserves during later winter months, as food availability is generally low during this time (Mayrand et al., 2017). In comparison, oysters at Sapelo Island had increased levels of Krebs

cycle metabolites during early winter (December 2014), compared to February and April 2015 (Fig. 4.3). This implies that oysters at Brunswick and Sapelo Island have differing qualities of energy reserves, and likely relates to the differing habitat fitness between sample locations. In our study, this expected trend in glucose levels (glycogen levels were not analyzed), was generally not observed, as glucose levels were infrequently increased (Brunswick: Plantation creek, Sapelo Island: Cabretta creek, Oakdale creek – Figs. 3.2 & 3.3) during the timeframe when we expect gametogenesis to be occurring (December 2014 – March/April 2015). Research by Mann, 1979 showed that in C. gigas, there is a period of storage metabolism resulting in an increase in carbohydrates (corresponding to summer season), before gametogenesis begins during winter, leading to a depletion in carbohydrates during the gamete development phase. This model better explains our data, especially for Brunswick's sites (except for Site 2, which had minimal variation in carbohydrate levels), which generally had decreases in carbohydrate metabolism during the winter months through April 2015, after which, most carbohydrate levels increased (Fig. 4.2). At Sapelo Island, each of the three sites had a unique pattern of carbohydrate metabolism, which as noted with the amino acids, may be due to the differences in timing of gametogenesis in individual oysters (Páez-Osuna et al., 1993).

Additionally, we hypothesize that differences in carbohydrate metabolism between oysters at Brunswick and Sapelo Island could potentially be due to the presence of PCBs (predominately Aroclor 1268) in estuaries near Brunswick, Georgia. While we did not analyze oyster tissues for PCBs, blue crab (Callinectes sapidus) collected from this area had lipid-normalized mean concentrations of 197 µg/g total PBC congeners (Kannan et al. 1998). PCBs have been detected at Sapelo Island in sediment (mean PCBt concentrations = 0.205 ng/g dry weight (dw), standard error (SE) 0.637 ng/g, compared to 79.3 ng/g dw, SE 2.47 ng/g dw), and

fish (mean PCBt concentrations = 3.90 ng/g wet weight (wet weight), SE 0.577, compared to 141 ng/g ww, SE 0.478 ng/g ww - Wirth et al., 2014), with sediment concentrations not exceeding published sediment toxicity guidelines (Long et al., 1995). However, at Brunswick, all of the sampled fish exceeded the lower threshold for non-cancer risks (based upon U.S. EPA consumption guidelines – U.S. EPA, 2000), and only two fish collected from Sapelo Island exceeded the same threshold (Wirth et al., 2014). Laboratory studies with eastern oysters (C. virginica) demonstrated that exposure to 0.35 and 3.5 μg of PCBs (1:1 mixture of Aroclor 1242, 1254 and 1260) inhibited lipid metabolism in the adductor muscle, indirectly reducing the amount of glycogen available to support gametogenesis by redirecting it towards maintenance metabolism and lipid conversion (Vale et al., 1998; Encomio & Chu, 2000). If this was occurring in oysters at Brunswick (although PCB concentrations were not quantified in these oysters), then it is plausible that exposure to PCBs could elicit the observed decreases in carbohydrate metabolism (especially at Sites 2 and 3).

Overview of Relevant Oyster Physiology – Gametogenesis & Fatty Acid Metabolism

Overwintering C. virginica tend to reduce their feeding rates during exposure to colder water temperatures, and utilize endogenous lipids and fatty acids (FAs) to supply energy during this time (Mayrand et al., 2017). In addition, oysters can convert their glycogen stores into lipids to supply energy for the developing gametes (Fig. 4.5; Encomio & Chu, 2000) and utilize lipid β-oxidation to produce Krebs cycle feedstocks (i.e. Acetyl-CoA, NADH and FADH) (Nelson et al., 2008). Lipid levels tend to vary significantly during the year, with their levels increasing during gametogenesis until spawning occurs, at which time they generally decrease (Dernekbaşı et al., 2015; Baptista et al., 2014), which is opposite of the expected trend in carbohydrate metabolism

(Greenway & Storey, 1999). While the two long-chain FAs considered to be essential for bivalves - eicosapentaenoic acid and docosahexaenoic acid (Lane, 1989) – were not detected in this study, the short-chain FAs acetate (C2:0), butyrate (C4:0), and propanoate (C3:0), medium-chain FA gluconic acid (C6:0), and the long-chain FAs palmitic acid (C16:0) and stearic acid (C18:0) were detected (Figs. 3.2 & 3.3).

The putatively detected fatty acids from almost all sites (exceptions: Site 3 at Brunswick and Cabretta Creek at Sapelo Island) generally followed the expected trend in fatty acid metabolism, with the majority of them increasing from December 2014 through April 2015 (Figs. 3.2 & 3.3). Gabbott (1983) found that during gametogenesis, oysters convert glycogen into lipid reserves, which supports our observations and provides evidence that this metabolic process is resilient. However, it is possible that lipid metabolism in oysters at Brunswick is also affected by PCB exposure, especially at Site 3, where oysters had the lowest fatty acid levels, and inhibition in lipid metabolism is a marker of PCB exposure (Encomio & Chu 2000). In bivalves, food availability and quality has a large effect on the FA profile, with differences in natural phytoplankton communities in the field (Budge et al., 2001; Irisarri et al., 2014), and in laboratory conditions (Albentosa et al., 1994; González-Fernández et al., 2016), changing the proportions of FAs present. As noted previously, it is plausible that differing amounts of food availability and quality at each unique tidal creek at Sapelo Island (Fig. 4.1B) could influence the unique effects detected in the oyster metabolome from each site. Additionally, research has demonstrated that different bivalve populations can have a distinct genetic susceptibility to pollutants, which can cause difficulties in making comparisons between populations due to differences in biomarker responses (Astley et al., 1999). For example, variation in an individual's biomarker response may be due to their intrinsic susceptibility, which can be due to

inherited genetic mutations (Schlenk, 1999), prior contaminant exposures (Brew et al., 2019a), and the overall health status/developmental stage of the individual (i.e. due to abiotic and biotic conditions - Schlenk, 1999; Dissanayake et al. 2008).

Overview of Relevant Oyster Physiology – Anaerobic Metabolism

During the summer months, increased water temperature and corresponding low dissolved oxygen (DO) levels are large stressors on oyster physiology (Table 4.1), with summer mortality events more likely to occur at temperatures above 30 ºC (Eastern Oyster Biological Review Team, 2007). Additionally, most oyster populations are intertidal and regularly experience daily periods of aerial exposure (year-round), when oysters generally have minimal contact with their environment but are more susceptible to stress from ambient air temperatures (Willson & Burnett, 2000; Mayrand et al., 2017). To survive in these low oxygen situations, oysters have evolved to switch from aerobic to anaerobic metabolism to meet their energetic demands (Isani et al., 1989). Initially, glycolysis and aspartate are the main substrates for anaerobic metabolism (aspartate – succinate pathway), and during long-term anaerobic metabolism, phosphoenolpyruvate shunts carbon towards succinate, propanoate, and acetate production (Fig. 4.5). This series of reactions leads to a general increase in alanine and succinate levels, a decrease in aspartate and ATP levels (Isani et al., 1989), with sustained levels of anaerobic metabolism leading to a reduction in metabolic rate (De & Putzer, 1985). This reduced metabolism leads to a lower ATP consumption, a reversed Pasteur effect (i.e. lowered glucose consumption - de Zwaan & Eertman, 1996), and could, in part, explain the differences observed in carbohydrate metabolism during June and August 2015 at Plantation Creek and Site 2 (Brunswick) and at Oakdale (Sapelo Island) (Figs 3.2A, 3.2C, 3.3B). Suppression in oyster

metabolism could also be observed in the OBE scores, which had the general trend of being reduced during summer months (Fig. 4.4). While the overall trends on OBE scores varied between sites, there were several sites (i.e. Brunswick: Site 1, Site 2; Sapelo Island: Cabretta, Oakdale) where there was a decrease in OBE scores between June 2015 and August 2015 (Fig. 4.4).

Further complicating this response, is that depending on energetic needs (e.g. if increased by a stressor(s)), bivalves can also utilize terminal oxidoreductases that can undergo a reductive condensation reaction with pyruvate and an amino acid to form opines (i.e. octopine, alanopine, strombine, and tauropine) to increase rates of energy production (Livingstone et al., 1981; Hammen, 1983; Livingstone, 1993; Llanso & Diaz 1994). This shift in metabolic pathways utilized for anaerobic metabolism can cause a shift in end-products accumulated (either succinate and/or propionate or pyruvate derivatives). Additionally, bivalves can utilize aerobic and anaerobic pathways simultaneously (Livingstone & Bayne, 1974), as deeply located tissues have a higher likelihood of using anaerobic pathways, compared to superficially located ones (Akberali & Trueman, 1985).

Unsurprisingly for field-collected organisms, there was a wide variation in metabolic responses detected involving Krebs cycle metabolites, that while they cannot be assigned to a specific(s) cause, there was evidence that anaerobic metabolism occurred during summer months. South End Creek and Cabretta Creek at Sapelo Island were the only sites with oysters displaying the classic markers of sustained anaerobic metabolism occurring (in June 2015 and August 2015, respectively), with both sites having lower OBE scores at these time points, supporting the argument that sustained levels of anaerobic metabolism can lead to a reduction in metabolic rate (De & Putzer, 1985). While sustained anaerobic metabolism was not detected in

oysters collected from the other sites, changes in the aspartate-succinate pathway (compared to winter months) at Plantation Creek (June 2015) and Site 1 (June and August 2015 – Figs. 3.2A – 3.2B) could indicate that oysters at these sites were either briefly or had recently begun utilizing anaerobic metabolism. There was a slight reduction in OBE scores at Plantation Creek from April to June 2015 and also at Site 1 from June to August 2015 (Fig. 4.4), supporting the findings that these oysters were utilizing anaerobic metabolism. The varying responses in anaerobic metabolism from these oyster populations further demonstrates that anaerobic metabolism is a dynamic process that is influenced by a variety of biological processes (i.e. glycogenolysis, toxicological responses, etc.) and external factors (e.g. season, water temperature, DO, food availability/quality, etc.), whose interactions and effects on the oyster metabolome are poorly understood in wild oyster populations.

Conclusion

This study provides evidence that untargeted GC-MS based metabolomics is an effective monitoring tool for discerning the natural and seasonal changes in the oyster metabolome (e.g. gametogenesis, utilization of anaerobic metabolism, etc.), and the highly variable physiological responses due to site-specific parameters (e.g. water quality parameters, food availability/quality, contaminants, etc.) near Brunswick and Sapelo Island, Georgia. While amino acids, carbohydrates, fatty acids, and Krebs cycle metabolites were involved in the main metabolic pathways affected at both locations, their regulation pattern varied significantly between locations and at sample sites within them, especially at Sapelo Island. These interactions make it difficult to interpret the observed physiological responses at the metabolome level, as there is a lack of baseline data for natural oyster populations. However, the results of this study do

illustrate that oyster metabolism does appear to be highly variable and dependent upon local conditions, even at sites that are in close proximity (such as Sites 1 – 3 at Brunswick), and especially for sites that are geographically isolated (i.e. Sapelo Island's sites). Both the presumed reference sites had significanntly higher OBE scores than those from non-reference sites, suggesting that these oyster populations were healthier compared to those oysters at non-reference sites. While more research needs to be conducted in utilizing the OBE in field studies, there is evidence that it has utility in identifying the range in an organism's biological responses and quantifying how different sites effect their metabolism. Overall, the results of this study do support using the oyster adductor muscle in environmental metabolomic studies and provide baseline data for future monitoring studies utilizing natural populations of oysters.

Acknowledgments

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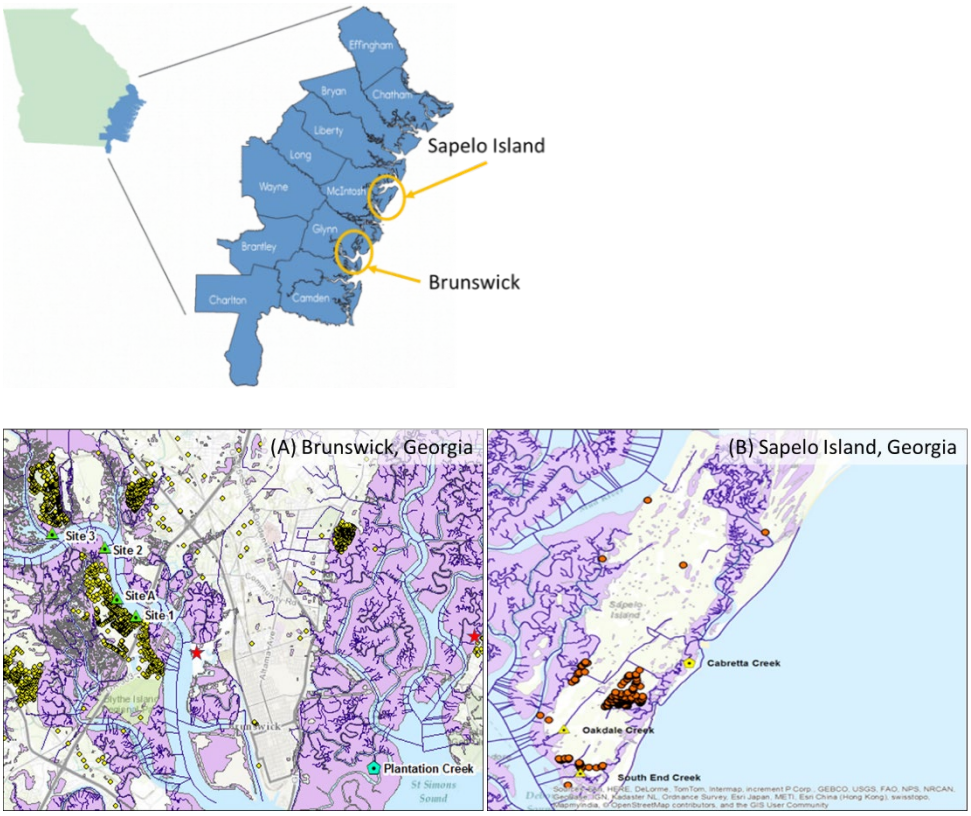
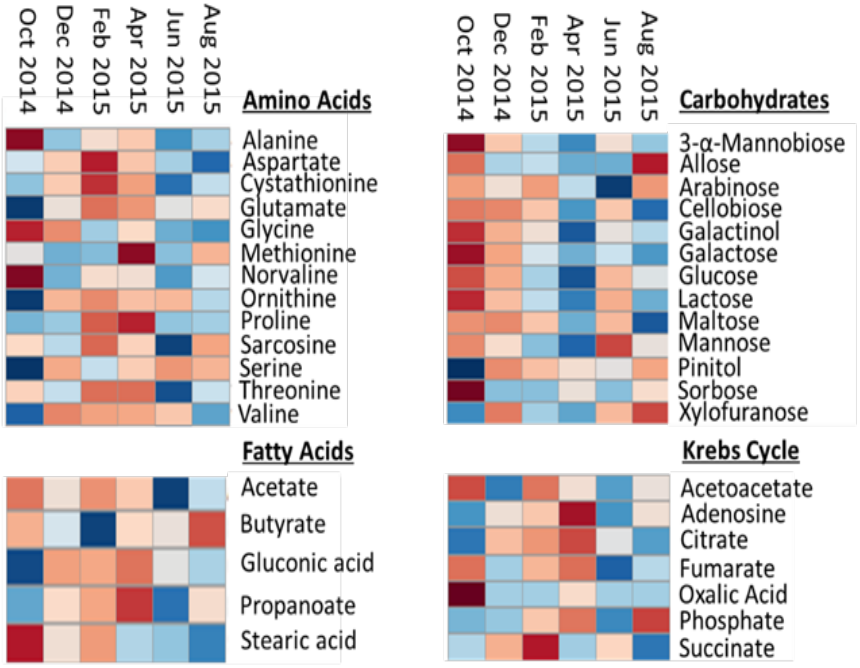
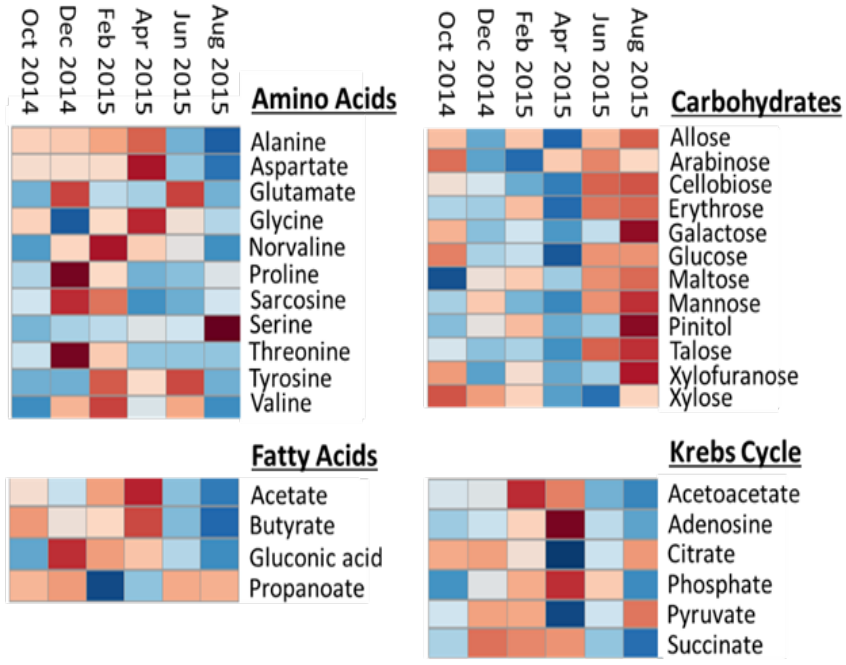


Figure 4.1: Maps showing locations of Brunswick and Sapelo Island and close up views of the sample sites at (A) Brunswick, Georgia and (B) Sapelo Island, Georgia Colored circles represent septic sites and the stars at Brunswick represent the location of wastewater treatment plants.

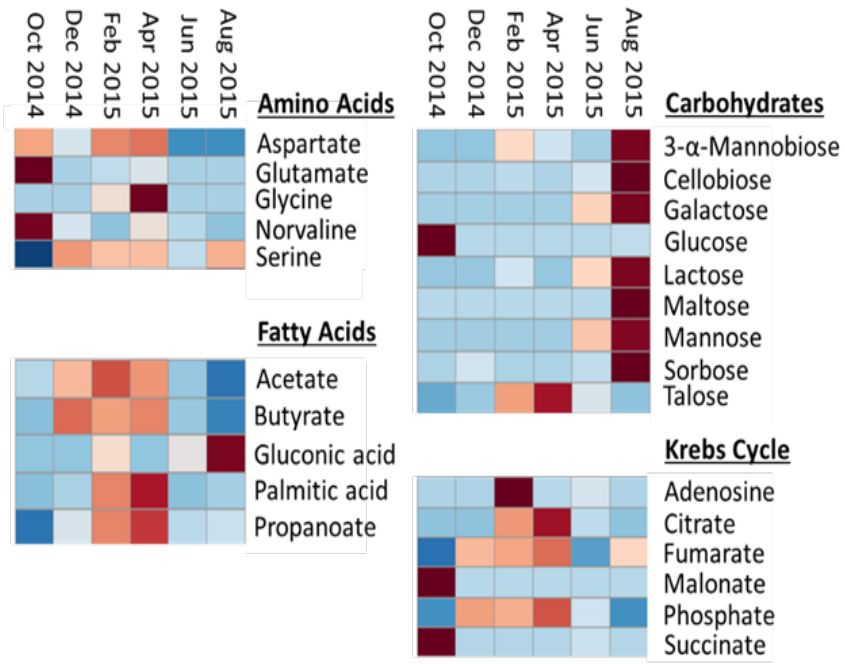
A (Plantation Creek)



B (Site 1)



C (Site 2)



D (Site 3)

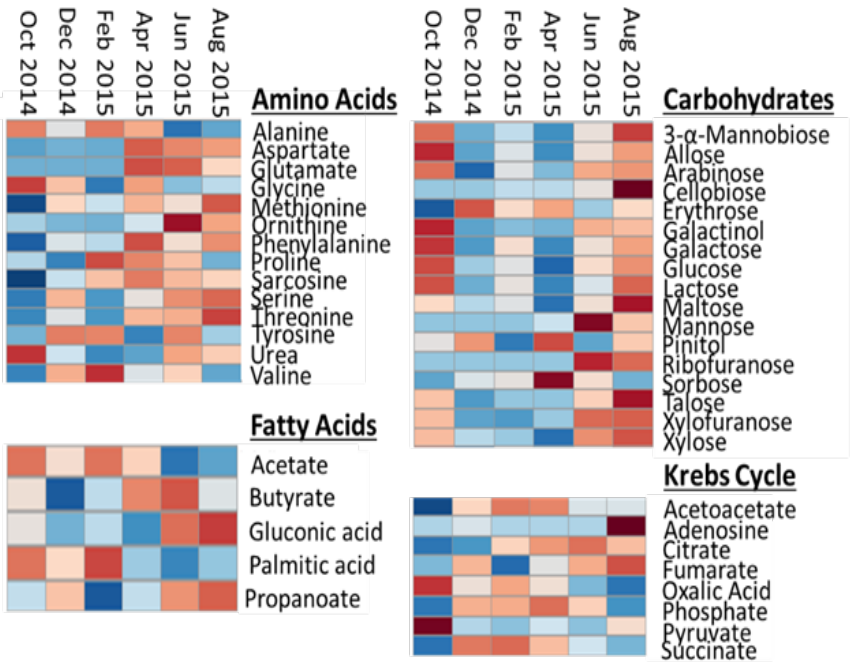
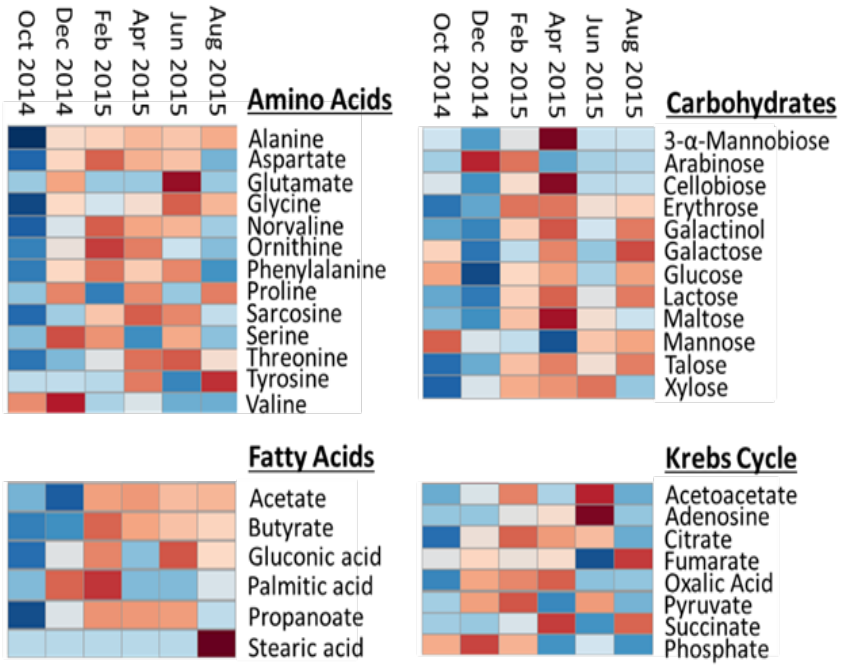
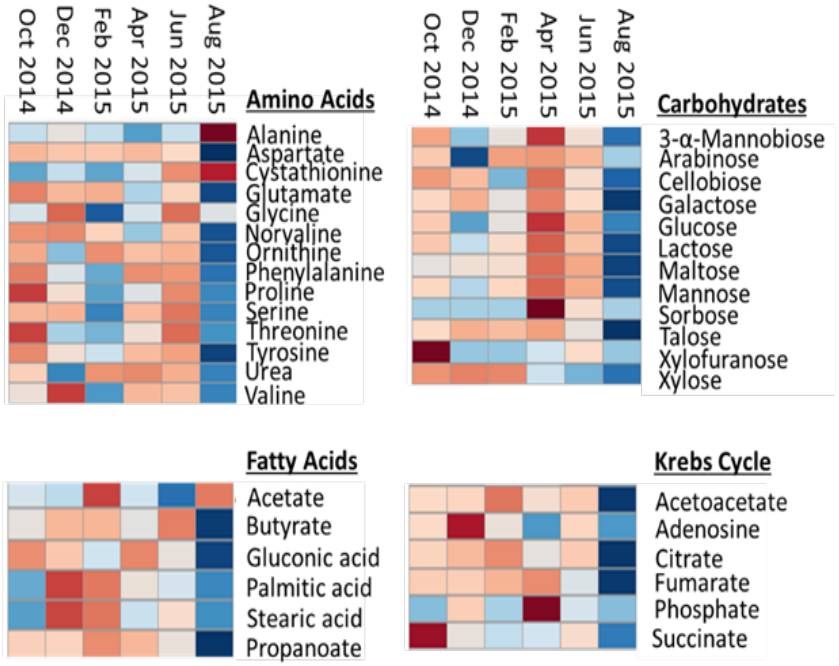


Figure 4.2A – 4.2D: Heat maps showing relative abundances of metabolites that were identified as being statistically significant different (p-value ≤ 0.05) at Brunswick's sample sites (2A = Plantation Creek – presumed reference site; 2B = Site 1; 2C = Site 2; 2D = Site 3). The blue color represents decreased metabolite abundance and red indicates increased abundance.

A (Cabretta)



B (Oakdale Creek)



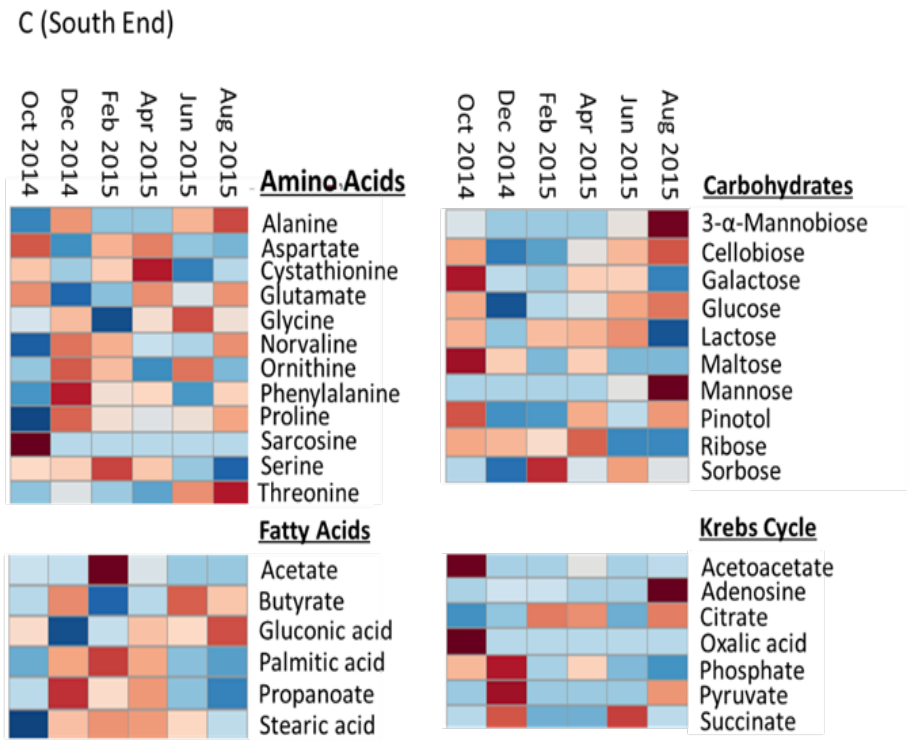


Figure 4.3A – 4.3C: Heat maps showing relative abundances of metabolites that were identified as being statistically significant different (p-value ≤ 0.05) at Sapelo Island's sample sites (3A = Cabretta Creek – presumed reference site; 3B = Oakdale Creek; 3C = South End Creek). The blue color represents decreased metabolite abundance and red indicates increased abundance.

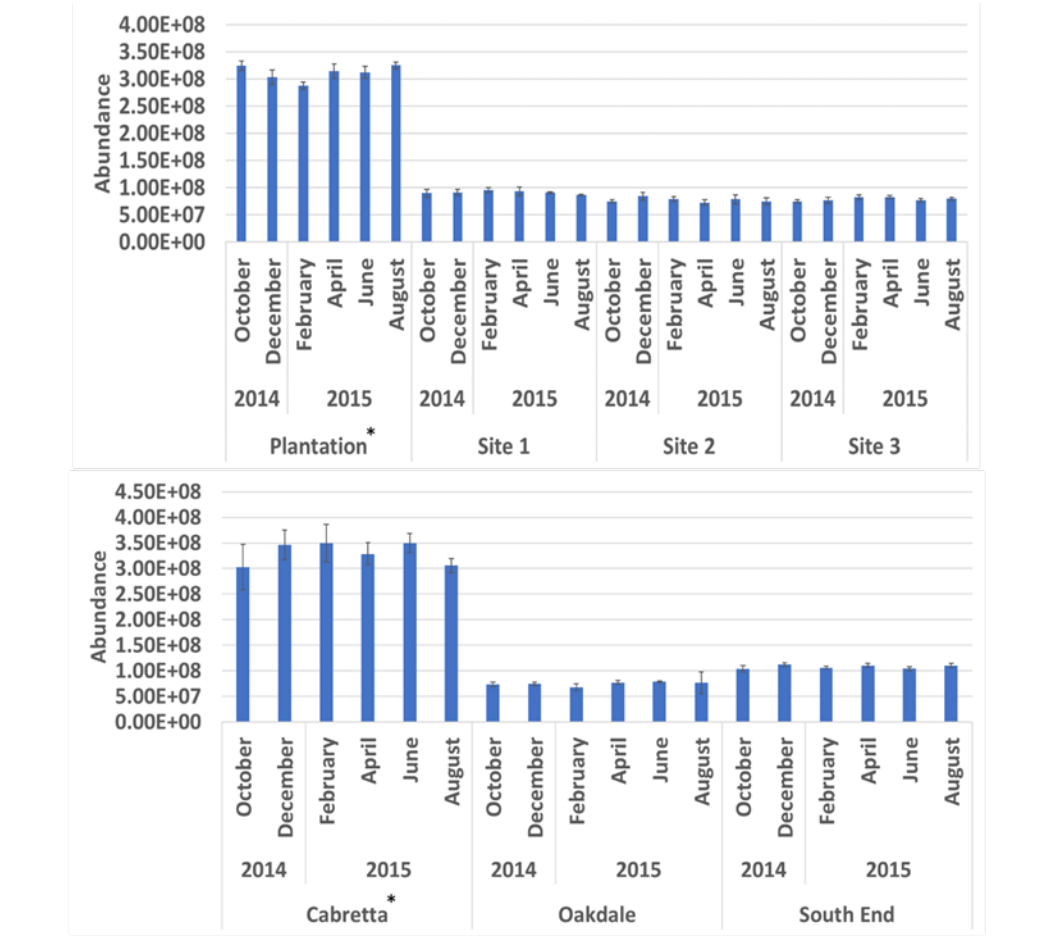


Figure 4.4: Mean Overall Biological Effect (OBE) scores from oysters at (A) Brunswick and (B) Sapelo Island, ± 95% confidence intervals. * indicates presumed reference site.

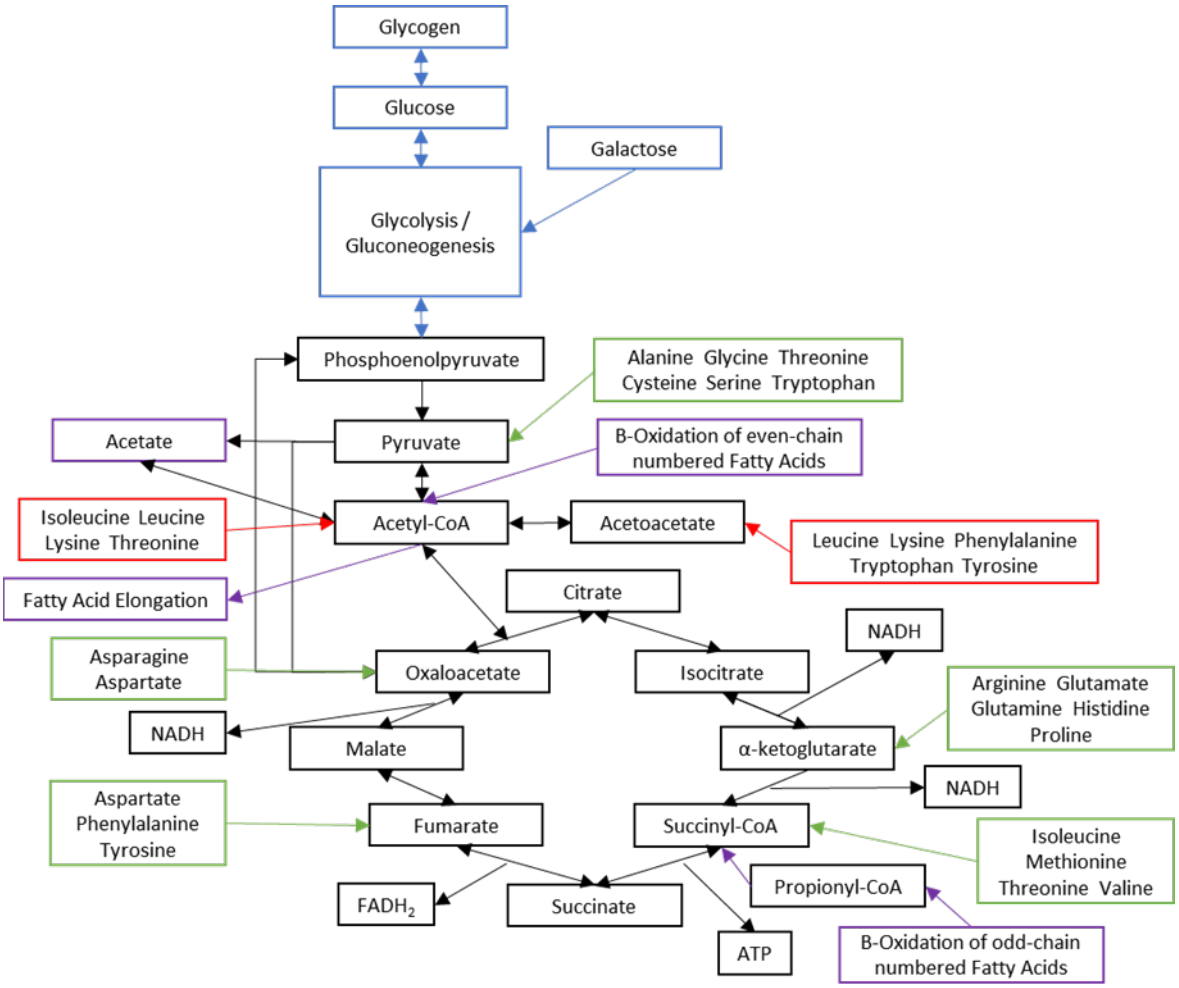


Figure 4.5: Metabolic map of relationships between amino acid, carbohydrate, fatty acid and Krebs cycle metabolism, adapted from figures in Voet et al. (2013). Black boxes = Krebs cycle pathway; red boxes = ketogenic amino acids; green coxes = glucogenic amino acids; blue boxes = carbohydrates and purple boxes = fatty acids.

Table 4.1: Water quality parameters measured bimonthly from October 2014 through October 2015 at (A) Brunswick and (B) Sapelo Island, Georgia

(A)

Year	Month	Site	Temp (°C)	pH	Dissolved Oxygen (mg/L)	Salinity (ppt)	Conductivity (µs/cm)
2014	October	Plantation Creek	24.19	7.41	4.79	28.83	44700
	December		13.03	7.83	7.69	27.33	43400
	February		11.12	7.58	8.48	21.68	35200
	April		23.11	7.4	4.83	24.46	38400
2015	June		28.41	7.38	4.2	28.14	43500
	August		31.28	7.51	3.99	32.95	50000
	October		25.34	7.38	4.4	25.67	40200
2014	October	Site 1	25.13	7.43	4.91	27.01	42200
	December		13.46	7.58	6.92	27.28	43200
	February		12.2	7.59	8.89	19.3	31600
	April		25.37	7.31	4.94	21.6	34300
2015	June		29.53	7.38	5.21	23.54	36900
	August		33.07	7.48	4.35	25.43	39500
	October		24.95	7.28	4.77	15.46	24200
2014	October	Site 2	24.45	7.39	4.52	26.91	42000
	December		12.86	7.54	6.81	27.04	42900
	February		11.77	7.52	8.64	18.82	30900
	April		24.78	7.23	4.64	20.54	32900
2015	June		28.48	7.26	4.24	23.15	36700
	August		32.56	7.38	3.68	25.26	39300
	October		23.93	7.15	3.78	19.97	32000
2014	October	Site 3	23.56	7.36	4.13	26.38	41400
	December		12.66	7.52	6.65	26.61	42300
	February		11.5	7.49	8.39	17.81	29300
	April		24.3	7.19	4.46	19.44	31300
2015	June		28.27	7.22	4.27	22.94	36200
	August		31.53	7.28	3.25	22.89	36100
	October		25.43	7.04	3.37	19.05	30800

(B)

Year	Month	Site	Temp (°C)	pH	Dissolved Oxygen (mg/L)	Salinity (ppt)	Conductivity (µs/cm)
2014	October	Cabretta	22.6	7.68	6.29	34	43000
	December		13	7.65	7.62	35	35533
	February		9.1	8.03	8.83	35	28127
	April		24.9	7.56	5.9	30	41250
2015	June		28.7	7.45	3.45	34.5	48976
	August		30.6	7.93	4.12	33	55192
	October		24.7	7.52	3.4	33	46975
2014	October	Oakdale	22.7	7.17	2.68	20	27270
	December		13.2	7.19	6.87	25	25640
	February		9.7	7.34	9.32	20	15375
	April		25.3	7.63	3.97	29	17068
2015	June		28.1	7.24	1.72	21	30320
	August		31.8	7.61	3.81	30	52320
	October		25.5	7.46	2.45	26	41508
2014	October	South End	22.2	7.34	4.24	30	38500
	December		12	7.4	7.3	32	31269
	February		8.1	7.57	8.7	30	24861
	April		25.4	7.54	6.4	25	31427
2015	June		30.7	7.89	5.8	27	45084
	August		31.3	7.72	4.56	31	52646
	October		24.8	7.24	3.14	20	28160

Table 4.2: Septic tank density at specified distances (m) from each sample site. Distance (km) to local wastewater treatment plants (WWTPs) from each sample site in Brunswick, Georgia. There is not a WWTP in McIntosh County, that would affect Sapelo Island, Georgia.

	Septic Tank Density					
Site	500m	1000m	1500m	2000m	2500m	Distance to WWTP (km)
Plantation	0	0	0	0	0	10.0
Site 1	40	155	344	429	502	4.1
Site 2	0	8	44	81	211	5.0
Site 3	13	101	158	220	330	6.6
Cabretta	1	1	1	1	12	N/A
South End	8	24	31	31	31	N/A
Oakdale	0	1	2	25	48	N/A

Chapter 5

Concluding Remarks

Georgia has approximately one-third of the remaining estuarine salt marshes in the US, which are some of the most biologically productive areas in the state and are simultaneously undergoing rapid degradation due to urban development. Other than habitat loss, pollution is one of the most urgent threats facing estuarine organisms. This is especially true for the eastern oyster (Crassostrea virginica, Gmelin 1791), which is a sessile filter feeder that supports a large aquaculture industry and is an ecological keystone species. One type of pollution that is garnering increasing scientific interest is pharmaceuticals and personal care products (PPCPs), which are widely used therapeutic compounds that are specifically designed to be biologically active at low doses and interact with a specific molecular target(s) (Fabbri & Franzellitti 2015). The biological effects of these exposures on oyster physiology is poorly understood, complicating this fact is the lack of data about the temporal and spatial variability in PPCPs bioaccumulation in Georgia's estuaries and how other environmental stressors affects oyster physiology. The studies in this dissertation investigated the extent of pharmaceutical and personal care product (PPCP) contamination in eastern oysters (Crassostrea virginica) in estuaries at Brunswick and Sapelo Island, Georgia, and via metabolomics, potential adverse effects from these exposures in a laboratory and field study.

Wild oysters at Brunswick and Sapelo Island, Georgia accumulated low ng/g dry weight of 13 PPCPs (atenolol, acetaminophen, caffeine, methylphenidate, propranolol,

diphenhydramine, carbamazepine, sertraline, DEET, valsartan, norethindrone, norgestrel and medroxyprogesterone) and three pesticides (atrazine, imidacloprid, and thiacloprid) sampled bimonthly from December 2013 – October 2015. Surprisingly, there were few statistically significant differences (based upon mean and overlapping 95% confidence intervals) in oyster body burdens both between Brunswick and Sapelo Island and within each sample sites - despite the large difference between local population sizes and PPCPs inputs into nearby waterways. We postulate that these differences are potentially due to a combination of local tidal mixing, seasonal changes in oyster physiology, variable PPCP inputs and PPCPs leaching from groundwater (especially at Sapelo Island). While this study identified these analytes in oysters, a major limitation of this study was not quantifying their transformation products (i.e. due to hydrolysis, photolysis, oxidation, bacterial degradation and metabolization), which may be more persistent and/or toxic than their parent compound (Dévier et al., 2011). Thus, low concentrations of a parent compound is not synonymous with reduced risk, as their degradation leads to a multitude of transformation products with relatively unknown behaviors in the environment. For this reason, we highly recommend that future biomonitoring studies examine the environmental presence and effects of transformation products, as they are likely a significant source of adverse effects to aquatic organisms.

The results of the seasonal bioaccumulation data, bioconcentration factors, and risk quotients illustrate that accumulation of PPCPs is a dynamic process that can vary significantly both temporally and spatially, and is highly compound specific. This has profound implications for exposures in aquatic organisms, as depending on the season, their life history and physiological status, they are likely exposed to different mixtures of contaminants that vary both within a year and from year to year. Within the past decade, there has also been increasing

interest in assessing the ecological risks that multiple chemical exposures pose to aquatic organisms (Meek et al., 2011). This is in part because analytical capabilities have increased to the point where contaminants could be detected at low concentrations (Kolpin et al., 2002) and multiple contaminants are routinely detected in aquatic environments (Boxall et al., 2012; Meador et al., 2016).

While adverse effects from mixtures detected in laboratory-based studies are usually derived from simple combinations of tightly controlled concentrations of PPCPs whose biological mechanisms are well understood (Backhaus, 2014), this is on contrast to environmentally relevant exposure that contain a complex mixtures of contaminants that may have additive, synergistic, or antagonistic effects, depending on their MOA (Henry and Black, 2007; Boxall et al., 2012) and can have biological effects not predicted from their individual components (Silva et al., 2002). For these reasons, we conducted a laboratory-based, untargeted GC-MS metabolomic study to developed oyster metabolomic biomarkers for fluoxetine, DEET, diphenhydramine, 17α-ethinylestradiol, and their mixture. The experiment was conducted for a total of 18 days, with an initial ten-day exposure followed by an 8-day depuration period, with metabolomic samples being collected at days 0, 1, 5, 10 and after the 8-day depuration period. For individual compound exposures, we were able to link the observed biochemical responses with each chemical's postulated MOA and discovered other off-target effects. However, the mixture's effect on the oyster's metabolome could not be well predicted from responses of the individual treatments, which agrees with other findings (i.e. Jordan et al., 2012; Jones et al., 2012; Song et al., 2016). Metabolites involved with Krebs cycle metabolism, fatty acid β-oxidation, and amino acid metabolism were affected by each treatment, although their regulation pattern varied based upon treatment, and sample day.

Additionally, exposure to these PPCPs elicited several general stress responses that adversely affected the oyster's metabolome. PLS-DA analysis demonstrated that the oyster's metabolome responded dynamically to these treatments over the course of the exposure and depuration periods. Based upon the number of still affected metabolites post-depuration, oysters were not able to fully recover from their exposures, regardless of treatment. Trajectory analysis results indicating that the oysters were able to only partially recover from these exposures has implications for wild oyster populations, as this lab study would represent a best-case scenario for recovery to occur. As during the depuration period, all chemical exposures were halted, which is not realistic for native oysters. This raises an interesting question about how the oyster metabolome would respond to a "pulsed" exposure in the lab (especially for a "pulsed" mixture exposure), and the insights this would provide that could be used to increase our knowledge of how oysters respond to a more realistic exposure scenario. Further complicating efforts to extrapolate from laboratory studies to the field, is that many chemicals are likely present at concentrations below current levels of quantification; however, this does not imply that there is an absence of potential adverse effects (Schlenk et al., 2012). Recognizing these shortfalls, there has been increasing scientific interest in developing better techniques for quantifying adverse effects from chemical exposures, especially for mixtures. This has led to the increasing use of effects-based tools in field studies (Krewski et al., 2010).

Effects-based surveillance tools can be used to improve the environmental relevance of chemical monitoring programs, including biomonitoring, by linking ecological and chemical information together to better understand the relationships between contaminant exposure and their possible physiological effects (Connon et al., 2012). These methods have a major advantage over traditional chemical monitoring because they can provide information about a

contaminant's (or a chemical mixture's) MOA without a priori knowledge of their potential toxicity or their identity (Ekman et al., 2013). The information gained from effects-based surveillance can provide a solid foundation for future effects-based monitoring studies that are more targeted in nature and seek to better define the type of adverse effect that is being observed (Bradbury et al., 2004). This approach was used to quantify the spatial and temporal trends in baseline oyster metabolism at Brunswick and Sapelo Island from October 2014 – August 2015. This information is imperative to improve our understanding of how varying habitats, environmental conditions, and seasonal changes affect the oyster metabolome, in order to provide higher quality data for future environmental monitoring programs. This study provides evidence that untargeted GC-MS based metabolomics is an effective monitoring tool for discerning the natural and seasonal changes in the oyster metabolome (e.g. gametogenesis, utilization of anaerobic metabolism, etc.), and the highly variable physiological responses due to site-specific parameters (e.g. water quality parameters, food availability/quality, contaminants, etc.) near Brunswick and Sapelo Island, Georgia. While amino acids, carbohydrates, fatty acids, and Krebs cycle metabolites were involved in the main metabolic pathways affected at both locations, their regulation pattern varied significantly between locations and at sample sites within them, especially at Sapelo Island. These interactions make it difficult to interpret the observed physiological responses at the metabolome level, as there is a lack of baseline data for natural oyster populations.

This is a major limitation for effect-based studies involving oysters, as there is a limited understanding of baseline metabolism, how it varies over the course of a year and how chemical mixtures affect the metabolome in natural populations. Relating to the other dissertation chapters, the utility of environmental metabolomic studies would be vastly improved if there was

a better understanding of how gonad development and spawning affects the oyster metabolome, as the reproductive cycle is one of the most dominating seasonal physiological processes to occur (de Zwaan & Eertman, 1996). If this was better understood, then it would be easier to discriminate between natural metabolic variation and those due to contaminant mixtures. The challenges of combining environmental metabolomics into traditional field monitoring studies were demonstrated by the Canadian Environmental Effects Monitoring (EEM) program. Despite research into molecular endpoints for 65 species of fish (Barrett & Munkittrick, 2010), they have yet to include these endpoints into their monitoring programs. This is due to the lack of data about the magnitude of change at the molecular level that could be interpreted as a problem for their biomonitoring species (Bahamonde et al., 2016). A related issue the authors identified, is that for regulatory purposes, a level of biologically relevant change needs to be defined, which is dependent upon many parameters (sample size, power, the significance level, and the endpoint's variability) that are currently not well understood for metabolomics (Munkittrick et al., 2009; Bahamonde et al., 2016). Despite its many benefits and advances in effects-based monitoring, environmental metabolomics studies are not yet at the point where they can be considered standalone experiments capable of providing the information required for regulatory purposes (Davis et al., 2013; Davis et al., 2016). To use metabolomic responses for regulatory purposes, it is necessary to demonstrate explicit linkages between developed biomarkers and adverse effects at the community and population levels (Bradbury et al., 2004). This is a substantial research gap that if filled, would provide a deeper understanding of how chronic exposure to contaminants affects marine bivalves and greatly advance ecological risk assessments for PPCPs.

As it is analytically, physically, and financially irresponsible to detect and test the chronic toxicity of all of the man-made chemicals in the environment, it is imperative that better methods

be developed and validated to quantify the relationship between contaminants in the environment and their potential adverse effects in aquatic organisms. While environmental metabolomic studies can provide insights into the MOA of contaminants that are present, even if the chemical cannot be detected (Ankley et al., 2006), the challenge is to link this with conventional parameters of toxicity considered suitable for risk chemical assessments (Sans-Piche et al., 2010). Thus, metabolomic studies are beginning to be integrated with other techniques that measure endpoints at the molecular, physiological, and population level (Bundy et al., 2008). While this type of study is still relatively uncommon, Davis et al., (2016) utilized NMR-based environmental metabolomics and PPCPs biomonitoring in cage-deployed fathead minnows (Pimephales promelas) at 18 sites across the Great Lakes basin to quantify how the metabolome changed in relation to changes in contaminant body burdens. The goal of the study was to correlate body burden data for 132 PPCPs with observed metabolomics data using partial least-squares regression analysis as a screening tool to identify contaminants most likely to cause an adverse biological effect. This method was able to reduce the number of contaminants likely to have an adverse effect by 43% - 52%, which they noted was an improvement over the traditional methods of chemical analysis in surface water samples. Applying these techniques to future biomonitoring and effects-based studies with oysters would greatly expand our understanding of their baseline physiology and how they respond to a wide variety of stressors, and would greatly expand their utility as estuarine biomonitors.

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