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The Effects of Climate Change on the Ecotoxicology of Contaminants of Emerging Concern: Flame Retardants, Contemporary Use Pesticides and Pharmaceuticals Personal Care Products on the Estuarine Grass Shrimp, *Palaemontes pugio*

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DEDICATION

I dedicate this dissertation to my Mother and to Inmar Soil.

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ABSTRACT

Global Climate Change may adversely affect the environment, increasing water temperature and altered salinity which may affect the toxicity of both legacy pollutants and Contaminants of Emerging Concern (CECs). Acute, 96 hour toxicity tests with adult grass shrimp (*Palaemonetes pugio)* assessed the effects of CECs (Polybrominated Diphenyl Esther (PBDE) – 47, ibuprofen, bifenthrin, triclosan, and bifenthrin/triclosan mixtures) under Standard Conditions (20˚C, 20psu) and different Climate Change Conditions (30˚C, and/or35psu) In addition, the grass shrimp microbiome (e.g. Vibrio bacteria) were assessed following acute triclosan exposures at the Maximum Exposure Concentration (MECs) and Minimum Inhibitory Concentration (MICs) under standard conditions. Colonies of *Vibrio parahaemolyticus*, V. *vulnificus*, *V. cholerae* and total vibrios from controls and triclosan exposed grass shrimp were enumerated and then isolates from each species were then tested for multiple antibiotic resistance. Statistical tests (Probit and Chi Square Analysis) were used to estimate LC50 values and to determine significant ($p \leq 0.05$) differences (ANOVA and Dunnetts or nonparametric equivalents) in survival and levels of antibiotic resistance between controls and each CEC tested.

Results indicated that PBDE-47 under increased temperature and salinity conditions was more toxic, with a 96h LC_{50} of 31.30˚C /L compared to 201.48 µg/L under Standard Conditions (30˚C & 35 psu). Results for Ibuprofen indicated a 96-h LC_{50} under Standard Conditions (20˚C, 20 psu) of 81.89 mg/L compared to 96-h LC_{50} ranging

from 32.69 mg/ - 61.6 mg /L under different Climate Change Conditions of increased temperature and salinity (30°C; 20 or 35 psu). Bifenthrin under climate change conditions was more toxic with 96-hour LC_{50} of 96-hour LC_{50} of 43.74 ng/L compared to 53.47 ng/L under Standard Conditions. The triclosan LC_5 under Climate Change Conditions was 325 µg/L compared to an LC_{50} of 580 µg/L under Standard Conditions. The triclosan and bifenthrin mixtures based upon Climate Change Conditions approached levels that were more than additively toxic (Additive Index = 1.00) with an Additive Index of 0.99 versus 1.32 for the Standard Condition Mixture. Exposure to triclosan MEC reduced Vibrio bacteria levels and both MEC and MIC exposure levels generally increased the antibiotic resistance of several of the 11 antibiotics tested.

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LIST OF ABBREVIATIONS

CBZ.....Carbamazepine

CECs.....Contaminants of Emerging Concern

DF.....Diclofenac

EU.....European Union

GCC.....Global Climate Change

IBU.....Ibuprofen

LC50.....Median Lethal Concentrations

LOECs.....Lowest Observable Effects Concentrations

MAR.....Multiple Antimicrobial Resistance

MEC.....Maximum Exposure Concentration

MIC.....Maximum Inhibitor Concentration

NOECs.....No Observable Effects Concentrations

PBDEs……..... Polybrominated Diphyenyl Ether

PPCPs..... Pharmaceutical and Personal Care Product

TCBS..... Thiosulfate Citrate Bile Salts Sucrose

TCS ..... Triclosan

TSA ..... Tryptic Soy Agar

WWTPs ..... Wastewater Treatment Plant

CHAPTER 1

INTRODUCTION

1.1 Background: Contaminants of Emerging Concern

Thousands of organic chemicals are produced or imported annually into the U.S. and other industrialized nations (Diamond et al., 2011). More than 40,000 organic chemicals are contaminants of emerging concern (CECs) (Diamond et al., 2011). CECs encompass a wide number of different substances that are largely unregulated such as pharmaceuticals, flame retardants, contemporary use pesticides, and food additives. These compounds often have very little or limited monitoring data available for environmental media (e.g., air, water, sediment, and biota) as well as limited ecotoxicology data (Koplin et al., 2002; Synder et al., 2003, Oros et al., 2005). These compounds are contemporary, only being present in aquatic ecosystems for several years (e.g. GenX) or even decades (e. g. Polybrominated Flame Retardants) but were not previously detectable using available analytical methodologies or due to a lack of analytical standards. New developments and improvements in analytical chemistry analysis have helped to detect many CECs in environmental media, which has led to efforts to estimate their potential environmental hazards (Koplin et al., 2002; Synder et al., 2003, Oros et al., 2005). It is often very difficult to quantify the amount of these chemicals in different environmental media (e. g. air, water sediments and biota), because of a lack of analytical standards or robust methods of measurement (Koplin et al., 2002; Synder et al., 2003; Oros et al., 2005).

Because-CEC use is widespread globally, they enter water bodies throughout the world, with many sources such as industrial and sewerage wastewater treatment plant discharges, urban runoff and recycled water treatment that are only partially effective in their removal or degradation. CECs are discharged into the environment with treated wastewater effluent, recycled water, and wastewater plant sludge (Raghav et al., 2103).

The effects of CECs on human and ecosystem health are largely unknown, with little known about their environmental partitioning and effects on metabolic pathways within living organisms other than humans. Many CECs have a negative effect on endocrine systems in both vertebrates and invertebrates, disrupting hormones that modulate and control metabolism, growth and development and regulate pathways that maintain homeostasis. In addition, disruption to the endocrine system may lead to alterations in hormone levels that may potentially lead to the development of cancerous tumors, birth defects and developmental disorders (Raghav et al., 2103).

CECs are found in many products that are continuously released into the environment following manufacturing or commercial use. CECs have chemical properties that make them resistant to natural environmental degradation processes, thus they may be bioaccumulated by terrestrial and aquatic organisms, potentially causing adverse effects on ecosystem/human health. In addition, some CECs such as Pharmaceutical and Personal Care Product (PPCPs) (e.g. antibiotics) threaten human health by enhancing the antibiotic resistance of disease-causing microorganisms (Raghav et al., 2103).

Along with stress that is caused by direct CEC exposure in aquatic ecosystems, there are additional concerns about the interactive effect of stress such as temperature, salinity, and pH due to Climate Change and urbanization. While aquatic organisms

possess the ability to adapt to these natural stressors (temperature, salinity and pH) the ability of some species to adapt these natural stressors in combination with CECs may lead to significant alterations in toxicity and ecotoxicological changes in the structure and function of ecosystem services (Schiedek et al., 2007). Changes in the water quality variables are usually associated with weather and Climate Change might change the transport, transfer, deposition and fate of contaminants within ecosystems (Macdonald et al., 2005).

1.1.1 Pharmaceutical and Personal Care Product (PPCPs)

Historically, most research focused on the impact of legacy chemical pollution, especially those acutely toxic/carcinogenic pesticides and industrial contaminants which exhibit persistence in the environment or were extremely toxic. However, there are other types of bioactive chemicals, which have received little attention as potential environmental pollutants such as, the pharmaceuticals and active ingredients in personal care products that are called Pharmaceutical and Personal Care Product (PPCPs).

The PPCPs include human and veterinary prescription drugs and biologics, diagnostic agents, "nutraceuticals," fragrances, sunscreen agents, and other compounds used for medicinal purposes. These compounds can enter into the aquatic environment as complex mixtures via a number of routes but primarily by both untreated and treated sewage (Daughton and Ternes, 1999). PPCPs have a specific mode of action and many are persistent in the body (Ternes et al., 2002).

Recently PPCPs have been identified as possible toxic chemicals in aquatic environments (Martín-Díaz et al., 2009). The PPCPs are used intensively all over the world. In the European Union (EU) about 3,000 different substances are used in human

medicine such as analgesics and anti-inflammatory drugs, contraceptives, antibiotics, beta-blockers, lipid regulators, neuroactive compounds and many others. Moreover, a major number of pharmaceuticals substances are used in veterinary medicine, among them antibiotics and anti-inflammatory drugs.

In 1995, there was an establishment of the first requirement for ecotoxicity testing as a prerequisite for registration of pharmaceuticals to the European Union (EU), Directive 92/18 EEC, and the corresponding requirements for veterinary pharmaceuticals (EMEA, 1998). PPCPs have been detected in many countries in sewage treatment plant (STP) effluents, surface waters, seawater, groundwater and some drinking waters. As people use more and more drugs for a vast array of ailments, consumption may reach hundreds of tons year of pharmaceuticals within the European Union (Fent et al., 2006). Pharmaceuticals may be combined as combinatorial drugs, which are metabolized and excreted intact either totally or partially metabolized, often associated with their metabolites via urine or fecal matter. PPCPs for human use ultimately find their way into wastewater treatment plants (WWTPs) and are discharged into aquatic environments. Traditional sewage treatment is only partially effective in removing/degrading some of the more unstable compounds in wastewater streams (Brun et al., 2006). Veterinary pharmaceuticals may enter aquatic systems via manure application to fields as fertilizers and subsequent runoff, but also via direct application in aquaculture (fish farming) (Holm et al., 1995). As a result, PPCPs have been detected in surface waters of many countries like the U.S. and Europe via two main sources: sewage effluent and agricultural runoff. These compounds often occur as mixtures which may potentially lead to additive toxicity and potential combined cumulative effects (Brain and Grant, 2004).

As new pharmaceuticals are introduced to the marketplace, each one may have different modes of biochemical action, many of which are poorly understood. The primary concern about pharmaceuticals is not only high production volume of a certain pharmaceuticals, but also the environmental persistence and critical biological activity in aquatic organisms and humans (Holm et al., 1995). For example, synthetic hormones may cause endocrine disruption in fish at low levels of exposure (ng L−1) (Seiler, 2002). In addition, the contamination produced by pharmaceutical products ("green pharmacy") in surface and ground waters has also been shown as an environmental problem, as some compounds may persist as potential bioactive chemicals in the environment for up to fifteen years (Kümmerer,2009). Pharmaceuticals are treated as emerging pollutants in water bodies, as they still remain unregulated and may affect water quality and potentially impact drinking water supplies and thus impact ecosystems and human health (Fent et al., 2006; Yuan et al., 2009).

In addition, some highly used PPCPs are difficult to measure using conventional analytical methodologies, as some PPCPs have high water solubility, and this prohibits the use of conventional analytical methods for sample extraction and clean-up/preconcentration (Bosch, 1998).

The effects of PPCPs on aquatic organisms are difficult to assess as effects may occur at much lower concentrations for direct effects (acute toxicity) but chronic toxicity effects are not as obvious to discern and are more difficult to assess (Jensen and Bro-Rasmussen, 1992). PPCPs enter the aquatic environment through different ways such as treated sewage effluent, leakage from septic tanks, drugs used in agricultural practices and landfills. About 30– 90% has been estimated as prescription and nonprescription

drugs administered to humans and animals which are excreted in urine and feces as an active substance (Halling-Sørensen et al., 1998).

1.1.2 Antibiotics and Triclosan

Antibiotics are widely administered for both human and animal health, including livestock and pets. The term "antibiotic" usually includes a large number of compounds, both natural and semi-synthetic that possess antibacterial activity (Kanfer et al., 1998). Broadly, it is a chemotherapeutic agent that inhibits or abolishes the growth of microorganisms, such as bacteria, fungi and protozoa (Kümmerer and Henninger, 2003). Antibiotics play a big role in different industries such as agriculture, medicine, and livestock. Moreover, they are used to enhance growth and feed conversion efficiency in healthy livestock (Levy, 1992). Antibiotic use in aquaculture is particularly important because seafood is a common food source and aquaculture is a major part of various local economies. For example, the UN FAO shows that half of the world's seafood supply will be from aquaculture in 2020, as wild capture fisheries are in decline (Moriarty, 1997). In fact, in 2017 aquaculture production surpassed natural wild capture fisheries as the major source of seafood (NOAA, 2017). Aquaculture has seen a very rapid growth over the past 50 years. The industry has grown to around 52.5 million tons in 2008, worth US$98.5 billion and involved about 50% of the world´s food fish supply. This is due to rapid growth in the region, increased population, aquaculture practices, relaxed regulatory framework, economic growth and expanding export opportunities (Bostock. et al., 2010).

Antibiotics are often used in aquaculture for both fish and shellfish production. The main reason for using large amounts of antibiotics in shrimp aquaculture is to prevent shellfish diseases (Moriarty, 1999), especially by luminous Vibrio (*Vibrio* spp.,

especially the luminous *V. harveyi*), Bacillus bacterial species, and viruses (Baticado et al., 1990; Le et al., 2005). The problem that antibiotics pose is their ability to change microbial community structure, promoting the development of antibiotic resistant human pathogens, which may cause illness as well as death in humans (Moriarty, 1999). Antibiotics are used in aquaculture through the production cycle, primarily in the larval life stages of growth and development. The use of antibiotics in aquaculture is associated with environmental and human health problems, such as bacterial resistance, persistence and spread of the diseases in the aquatic environment, and effects on the biogeochemical composition of the sediment (Ma et al., 2006). In addition, about 25 to 75% of antibiotics leave the organisms unaltered via feces or urine, due to poor absorption by humans and animals after intake (Karthikeyan and Meyer 2006). Exposure to pharmaceutical compounds such as antibiotics for a long term at low doses in the environment may lead to adverse impacts to non-target organisms including endocrine disruption, chronic toxicity and antibiotic resistance (Andreozzi et al., 2004).

In the past, the primary focus of environmental risk assessments was on the impact of legacy chemical pollutants such as trace metals, PAHs, and pesticides, especially those chemicals which had high acute or chronic toxicity, had carcinogenic potential or which were highly bio accumulative and persistent in the environment. More recently, focus has shifted to new contemporary pollutants, termed Contaminants of Emerging Concern (CECs), including highly bioactive chemicals like pharmaceuticals and active ingredients in personal care products (PPCPs) (Daughton & Ternes, 1999, Scott et al., 2012; Muraya et al., 2013). Pharmaceuticals and personal care products (PPCPs) are one of emerging environmental contaminants, produced in tons per year

concentrations, such as, prescription drugs, veterinary drugs (antibiotics, steroids), diagnostic agents, fragrances, lotions, and cosmetics. Because of their wide uses, PPCPs are discharged in large quantities into aquatic ecosystems (Kolpin et al. 2002; Fent et al. 2006). Pharmaceuticals are used intensively throughout the world. In the European Union (EU) about 3000 different substances are used in human medicine such as analgesics and anti-inflammatory drugs, contraceptives, antibiotics, beta-blockers, lipid regulators, neuroactive compounds and many others. Similarly, in England, Germany and Australia, about hundreds of tons of drugs are used per year (Jones et al., 2002). The main concern about pharmaceutical is not only high production volume of a certain pharmaceutical, but also the environmental persistence and critical biological activity (Holm et al.,1995). Along with stress that is caused by contamination, there are other ability to produce additional stress such as temperature, salinity, and pH through deviation from the optimal conditions for an organism to function or by modifying the behavior of contaminants in exposed organisms. On the other hand, there is the ability of some species to adapt, which may lead to significant changes in the structure, function and services of ecosystems (Schiedek et al.,2007).

Antibiotics are most widely administered for animal health and livestock management. They are often used to enhance growth and feed efficiency in healthy livestock (Levy, 1992). This includes a large number of compounds, both natural and semi-synthetic that possess antibacterial activity (Kanfer et al., 1998; Scott et al., 2016). Antibiotics are broadly defined as a chemotherapeutic agent that inhibits or abolishes the growth of microorganisms, such as bacteria, fungi and protozoa (Kümmerer & Henninger 2003). Therefore, antibiotics play a major role in many different important daily uses

such as in agriculture and livestock production as well as modern medicine to treat microbial infections.

Triclosan [5-chloro-2- (2,4-dichlorophenoxy) phenol, is an antimicrobial compound that has been widely used over North America, Europe and Asia. There are many household items that contain triclosan such as soap, detergent, toothpaste, mouthwash, fabric, deodorant, shampoo and plastic additives as well as many personal care, veterinary, industrial and household products due to its effective use against many types of bacteria and certain types of fungi to prevent bacterial propagation and may eventually result in microbial cell death (Russell, 2004). Triclosan was invented over 40 years ago and its use has increased substantially over the past 25 years as the incidence of antimicrobial infections increased (Jones et al., 2000). The average concentration of triclosan in consumer products at ranges from 0.1% to 0.3% active ingredient by weight. Triclosan differs from other organochlorine compounds because it is not highly regulated and also antimicrobial has a low acute toxicity being generally well tolerated and safe (Jones et al., 2000; Sabaliunas et al., 2003). Triclosan has low water solubility, a pKa of 8.1, and a vapor pressure of 4×10^{-6} mm Hg (Merck,1983).

As consumer demand for antimicrobial products is anticipated to grow, the probable prevalence of chemicals such as TCS increases as do many concerns that the product might be harmful to human health and the environment (Chu and Metcalfe, 2007). Wastewater treatment plants (WWTPs) are the major pathway for Triclosan to enter into the aquatic environment. An estimated 14,748 POTWs provide wastewater collection, treatment, and disposal service to 238.2 million people (EPA, 2000; EPA, 2016)..Through wastewater treatment plant disinfection processes, such as

biodegradation and sorption onto particulates, TCS is removed from the aqueous phase of most systems, which results in a lower dissolved concentration of TCS being discharged into surface waters via WTP effluents (McAvoy et al., 2002). There are many factors that may affect triclosan concentrations in aquatic systems, including pH, sediment density and organic matter content, water flow, velocity and depth (Reiss et al., 2002).

Triclosan has been detected in surface water, sediment, biosolids, soils, aquatic species and humans. For example, it has been detected in 85 out of a total 139 U.S. streams sampled, with maximum and median concentrations of 2.30 µ/L and 0.14 µg/L, respectively (Kolpin et al., 2002). Triclosan is bioaccumulated in aquatic organisms due to its highly lipophilic character. It may be taken up by algae and as a result many aquatic organisms which feed on algae become exposed (Capdevielle et al., 2008). Triclosan concentrations of 181 mg/kg dry weight in marine sediments have been detected which may adversely affect marine benthic communities. With an organic carbon fraction of 2% for many sediments, this is equivalent to 137mg/L triclosan exposure in the interstitial porewater (Ho et al., 2013). Triclosan laboratory toxicity tests have been conducted with many species of freshwater and estuarine aquatic organisms (Table 1 e.g. Orvos et al., 2002; DeLorenzo et al., 2008; Elodie et al., 2017); however, very few have assessed the effects of increasing exposure temperatures and salinities that may occur as a result of future Climate Change Conditions.

1.1.2.3 Triclosan effects on the antibiotic resistance in vibrio bacteria

Antibiotics have wide usage such as medicine to treat bacterial infections in humans and animals and are widely used in agriculture to promote animal growth

(Kümmerer, 2004). Annually, the global amount of usage of antibiotics has been estimated between 100,000-200,000 tons (Kummerer, 2003). Many sources of the antibiotics have been identifies including surface water discharges of municipal wastewater treatment plants (WWTPs), agricultural runoff form confined animal feeding operations (CAFOs) and discharges from pharmaceutical manufacturers (Kolpin et al., 2002, 2004; Miao et al., 2004). More than 3,000 chemical substances have been used in human medicines, aquaculture and farming applications (Ternes et al., 2004). The main concern of antibiotics in the environment is not only their discharge and dispersion of high concentrations but also their persistence over long periods of time (Blackwell et al., 2005). Typically, most of the high concentrations measured are in areas with high human activity, whereas pristine environments usually have low concentrations of antibiotics, unless downstream of CAFOs (Baquero et al., 2008). Therefore, the highest levels of antibiotic concentrations measured are detected in highly urbanized watershed with a large concentration of hospital effluents in their wastewater treatment plant systems and resulting effluent discharges (Patrolecco et al., 2015). Antibiotic-resistant bacteria have also been detected in drinking water (Kümmerer, 2004; Salmore et al., 2006), and in ground water but at much lower concentrations far below the l mg/L range (Pruden et al., 2006; Sapkota et al., 2007). Antibiotics have been found in high concentrations in most media such as raw and treated sewerage and soils (Baquero et al., 2008). Due to the excessive use and misuse of antibiotics, increased antibiotic resistance is a growing global health concern (Huerta et al., 2013).

Vibrio bacteria are a naturally occurring saltwater bacterium commonly found in seawater and is widespread in temperate zone estuaries and adjoining coastal waters. It is

also commonly found in the microbiome of crustaceans residing in these coastal waters. They have been detected in tissues and/or organs of many marine species such as, algae and aquatic organisms (e.g., abalones, bivalves, corals, fish, shrimp, sponges, squid, and zooplankton) (Thompson & Swings,2004).The genus Vibrio includes more than 30 species, and many are pathogenic to humans and/or have been associated with water and food-borne diseases (Chakraborty et al., 1997).There are three main Vibrio spp. re *Vibrio cholera*, *V. vulnificus*, and *V. parahaemolyticus*, which are considered foodborne pathogens that cause illness through raw seafood consumption as well as wound infections. For example, the average annual incidence of all *Vibrio* infections (foodborne and wound infections) has increased by > 80% from 1996 and 2001 (Ho and Cunha 2009) by another 43% between 2012 and 2017 (CDC, 2019). Approximately, 296 cases of infection were caused by *Vibrio's* in the United States in 2000 compared to an estimated 80,000 estimated illnesses today (CDC, 2019), as reported by Vibrio surveillance system the Centers for Disease Control and Prevention (http://www.cdc.gov/). The majority of the illnesses are caused by V. parahaemolyticus from seafood consumption whereas the majority of deaths are from V. vulnificus which accounts for 85% of all deaths from seafood consumption (Scott et al., 2019). Many of these Vibrio bacteria in the southeastern US (> 99%) are antibiotic resistant (Baker-Austin et al., 2009)

Humans could be exposed to bacterial resistant by different pathways, including agricultural plants are that may be watered with surface sewage sludge or effluent as fertilizers or from livestock manure applied as a fertilizer (Perretin et al., 1997; Salyers, 2002). Bacteria are found in all environments so, there are many reasons to study this

issue, particularly the ones that are pathogenic to humans that may be taken up by shellfish. shellfish, like the Vibrios which also cause wound infections which are generally highly antibiotic resistance (Han et al., 2007). Bacterial resistant has been found in the aquatic environment (Kümmerer, 2004; Kim and Aga, 2007; Vanneste et al., 2008) and (Scott et al., 2016) developed methods to predict these hazards to the environment and humans.

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol) is a broad-spectrum antimicrobial used in medical and personal hygiene products. It occurs in the aquatic environment due to residual concentrations in wastewater effluent. About 70% to 98% of TCS is removed through biodegradation and sorption to biosolids (Bock et al. 2010). The first use of TCS in the health care industry was in 1972 and it was introduced into toothpaste in Europe in 1985. TCS can be used without a prescription and its use over more than 40 years has increased in the United States (Jones et al., 2000). About 0.1% to 0.3% of all products contain TCS (Singer et al., 2002). TCS is formulated in antibacterial skin cleansers at 1–2% w/v and in bactericidal soaps at lower concentrations (Faoagali et al. 1999). There are some physical characteristics of triclosan, such as its stability and moderately solubility in water (10 mg/l at 20 $^{\circ}$C). It is a lipophilic compound (with log Kow =4.8) which can be bioaccumulated. It may enter aquatic environments primarily via local wastewater treatment plants (WWTPs), where typically 90% to 98% is removed because of biodegradation and sorption but the remainder is discharged into environment (Bock et al. 2010). TCS may be discharged into the rivers with estimated concentrations usually ranging between 11 – 98 ng/L (Singer et al., 2002). Even though TCS is banned in hand soap in the U.S. (U.S. Food and Drug Administration, HHS 2016), it is still in use

in other personal care products (Singer et al., 2002). Colgate removed it from its toothpaste in 2019.The annual production of approximately 1,500 t, of which about 350 t (Bester 2005) and more than 450 t were applied in Europe and in the USA respectively (Halden and Paull 2005). The main function of antibiotics is killing or inhibiting the growth of microorganisms such as bacteria, archaea, viruses, protozoa, microalgae and fungi. Its mechanisms of action are by inhibition of cell wall synthesis, alteration of cell membranes, protein synthesis inhibition, synthesis of nucleic acids inhibition and metabolic or anti-competitive antagonism (Kümmerer, 2009). TCS has been detected to be effective against a wide range of gram-positive and gram-negative bacteria (Ciba 2001). Minimum inhibitory concentrations of TCS for different bacterial strains range between 10 and 3,000 µg/L (Bhargava and Leonard 1996).

1.1.4 Anti-inflammatory Compounds and Ibuprofen

Human activity is the main reason for different pollutants like, heavy metals and pesticides, which generate adverse effect on aquatic organisms. In addition, there is another concern on water ecosystems about not only heavy metals, pesticides, and other common pollutants but also increasing residual concentrations of human pharmaceuticals (Halling-Sørensen et al.,1998; Jorgensen, 2010;). However, there are other type of bioactive chemicals, which have received little attention as potential environmental pollutants such as, the pharmaceuticals and active ingredients in personal care products that are called Pharmaceutical and Personal Care Product (PPCPs) (Daughton &Ternes, 1999). A recent survey conducted by the USGS found that 84 PPCPs were detected in 38 US streams across the US (Bradley et al., 2017). In addition, the contamination produced by PPCPs in surface waters, seawater, groundwater and drinking waters has also been

shown to be a significant environmental problem, as some compounds may persist as potential bioactive chemicals in the environment for up to fifteen years (Fent et al., 2006; Yuan et al., 2009). Ebele et al. (2017) reported that while not all PPCPs are persistent, their continuous use in livestock management and use by patient results in their continuous release into the environment and thus should be considered "pseudo-persistent". Pharmaceuticals are treated as emerging pollutants in water bodies, as they still remain largely unregulated and may affect water quality and potentially impact drinking water supplies and thus impact ecosystems and human health (Fent et al., 2006; Yuan et al., 2009; Scott et al., 2102). Water catchments are now at higher risks for potential contamination from PPCPs because of increasing human population density or livestock techniques. Some examples of these pharmaceuticals include, analgesics, antibiotics, steroids, and various drugs used to treat mental illness (Fent et al., 2006; Martínez Bueno et al., 2007). PPCPs analgesics and anti-inflammatory drugs can reach detectable concentrations in the environment. Because some of these drugs are purchased without prescription, the actual consumption is certainly even higher (Stumpf et al., 1996). One group of these compounds are known as nonsteroidal anti-inflammatory drugs (NSAIDs) (Schwabe and Paffrath, 2001).

Ibuprofen (IBU) is one of the most widely distributed pharmaceuticals in the world. IBU or (+)-2-(4-isobutylphenyl) propionic acid is a non-steroidal anti-inflammatory drug (NSAID. (Onesios et al., 2007). IBU which belongs to the family of non-steroidal anti-inflammatory drugs, among pharmaceuticals chemicals it is widely uses worldwide. For example, IBU is used as analgesic, having anti-inflammatory and antipyretic effects by inhibiting the synthesis of prostaglandin (Corcoran et al., 2010).

The mode of action of IBU is unknown; however, it is a non-selective inhibitor of cyclooxygenase, an enzyme involved in prostaglandin synthesis (Cleuvers, 2004).

IBU and its metabolites like carboxyl ibuprofen and both hydroxyl ibuprofen isomers have been detected in rivers, lakes and streams (Weige et al.,2004). IBU represents a potential risk for the aquatic ecosystem, because of its bio-accumulative ability, its water-solubility, and with its low volatility (González-Naranjo et al., 2013). About, 87.5 million prescriptions for these substances were written in 2001, with a transaction volume of 1.13 billion Euros in Germany (Schwabe and Paffrath, 2001).

The continuous discharge of pharmaceuticals and personal care products (PPCPs) into environment will influence non-target organisms in term of chronic exposure. In addition, they can affect water quality, drinking water supplies and thus ecosystems and human health (Yuan et al., 2009). There are also environmental stressors such as, temperature and salinity that may produce additional stress to aquatic organisms as a result of Climate Change. This can happen through deviation from the optimal conditions for an organism to function or by change the behavior of contaminants in exposed organisms (Schiedek et al., 2007).

IBU has been observed in both WWTPs and in septic system in U.S. as well as other PPCPs (Carrara et al., 2007). Globally pharmaceutical concentrations are generally below the Predicted No-Effect Concentration (PNEC) level for most PPCPs; however effluent from sewage treatment plants may contain a variety of the pharmaceuticals, like trimethoprim, ciprofloxacin and IBU, which may often exceed the level of PNECs when uncertainty factors are applied (Anderson et al., 2012). In America and Asia, the levels

are about two times higher than the adjusted PNEC levels when compared with the concentrations in the freshwater environments in other parts of the world (Pal et al., 2010). Because wastewater treatment plants (WWTPs) are only partially effective in removing/degrading some of the more unstable compounds in water streams, some PCPPs are continually present in aquatic ecosystems and are often causing unpredictable effects on the aquatic organism (Santos et al., 2010). Aqueous environmental concentrations of PPCPs are generally in the nanogram per-liter and low microgram-per-liter range. Pharmaceutical characteristics show high reactivity with biological systems; and are often highly stable, which might cause toxic effects to aquatic organisms even at these low environmental concentrations (Hernando et al., 2006).

The contamination of the environment by PPCPs such as IBU may affect different organisms like crustacea, which are very important in aquatic ecosystems. Crustaceans such as grass shrimp play a very important role in estuarine food web within many marine and estuarine ecosystems. Grass shrimp, *P. pugio*, comprise up to 56% of pelagic macro fauna in estuarine tidal creeks (Scott et al.,1999). They serve as detritivores and as prey items for many commercially and recreationally important fish species. Therefore, they were chosen as indicator species in many laboratory toxicities tests due to relative ease of collection, obtain multiple life stages for testing and their demonstrated sensitivity to many chemical contaminants (Key et al., 2006).

1.1.2 Contemporary Use Pesticides

Contemporary Use Pesticides are economically justified poisons and regardless of their classification (e.g. insecticides, herbicides, fungicides, biocides, nematocides or rodentacides) may be used in a variety of applications including agriculture pest control,

vector control, right-of-way control and in-home construction (e.g. termiticides). Pesticides may often become both a threat to human health and aquatic life. Stone et al. (2014) reported from 2002-2011, that the exceedance of aquatic life criteria in freshwater rivers and streams in the U.S. ranged from 66% in agricultural areas to > 90% in urban areas surveyed. Only one stream in the U.S. had exceedances of Human Health Criteria compared to 17 streams in the 1992 - 2001 timeframe (Stone et al., 2014). They are often one of the main harmful factors contributing to water quality degradation in ~~the~~ estuaries and coastal environments, due to pesticide runoff associated within urban areas (Fulton et al., 1993), golf courses and agricultural operations (Bocquene at al., 1997; Scott et al., 1999.) In some countries, the agriculture sector is a high percentage of the work force and agricultural use is the highest pesticide use sector of the economy. For example, El Poniente, the eastern area of Almeria province (Southeastern Spain) has the highest density of greenhouses in the world (Requejo Liberal, 1991). About 73.2% of the workforce are employed in a primarily agricultural activity and thus agricultural pesticides may pose a significant risk to human workforce exposed to them and the environment. Insecticidal activity is generally not-species specific and poses acute and chronic toxicity risks to humans and aquatic life.

In United States, over 500 million kg of pesticides are used each year in both agricultural and urban settings (Majewshi and Caspel, 1995). In 1980s, organophosphates accounted for 65% of total insecticide usage (Larson et al., 1997). Today the greatest pesticide risks occur from contemporary use pesticides such as pyrethroids used for mosquito control for West Nile and Zika viruses and novel insecticides like Fipronil, used on golf course mole crickets. The state of California

expert Panel on CECs listed 3 contemporary use pesticides –chlorpyrifos, an organophosphate insecticide and two pyrethroids –bifenthrin and permethrin on their "Dirty Dozen" List.

Salt marshes along the Atlantic Coast of the US are habitats for common marsh fauna including both fish and invertebrates such as the grass shrimp (*Palaemonetes pugio*), which play a big role in the transfer of energy and maintenance of ecosystem health (Welsh, 1975). Coastal habitats are prone to pesticides effects due to increasing residential and urban development (residential developments, golf courses, etc.). Aquatic life like the grass shrimp inhabiting these habitats can be particularly sensitive to pesticide runoff (Key et al., 1998; Leight et al, 2005).

1.1.2.1 Pyrethroids and Bifenthrin

Global Climate Change (GCC) refers to a variety of changes in global weather patterns including any change in temperature, salinity and precipitation that may pose a threat to global biodiversity (Durack et al. 2012). Changes in water temperature and salinity may occur in aquatic habitats affected by Global Climate Change, which may alter the toxicity of many pollutants and will affect the risk assessment of many pollutants (DeLorenzo et al., 2009). Differences in the water temperature and salinity may impact the toxicity of pollutants, due in part to changes in chemical fate and transport, and because of changes in physiological response (Kennish, 2002).

Pyrethroid pesticides have widespread use in the United States in the past due to their lower mammalian toxicity than other insecticides but aquatic organisms are often quite sensitive, especially crustaceans (Oros and Werner, 2005). Pyrethroids also have

many properties such as low water solubility, high affinity for sediments and organic carbon, with hydrolysis half-lives of days to weeks in aquatic environments (Oros and Werner, 2005). They cause a concern for both pelagic and benthic species in different environments as it has been noted that the toxicity of pyrethroids is observed at the parts-per-trillion level in some crustaceans, fish, and amphibians (Oros and Werner, 2005; DeLorenzo et al. 2006; Werner and Moran 2008). Pyrethroids can enter aquatic ecosystems via spray drift, non-point source (NPS) runoff, and discharges from wastewater treatment plant effluent (USEPA, 2005). Another concern for these compounds is centered on their chemical properties because they are highly lipophilic and tend to bind to sediments, which may lead to a substantial decrease in their toxicity in aquatic environments but may also increase their persistence (Leahey, 1985).

Bifenthrin 3-[(1Z)-2-Chloro-3,3,3-trifluoro-1-propenyl]-2,2 dimethylcyclopropanecarboxylic acid (2-methylbiphenyl-3-yl) methyl ester, is a pyrethroid insecticide that is widely used in agriculture, horticulture, and for residential pest control. It is more resistant to environmental degradation than previous generations of pyrethroids (Mokry and Hoagland, 1990). It is a member of the pyrethroid ester class of insecticides, known as acaricides. Human and animal exposure to Bifenthrin often occurs through different routes such as ingestion, contact, and inhalation (EFSA, 2011). The molecular weight of Bifenthrin is 422.9 g mol/L (Laskowski, 2002) and its water solubility is 0.1 mg/L at 25°C (Fecko, 1999). Bifenthrin is not an easily volatilized compound in the atmosphere because it has a low vapor pressure of 1.8 x 10^{-7} mmHg and low Henry's law constant of about 7.20 x 10^{-3} atm m^3/ mol. Bifenthrin also tends to partition into lipids because of its high octanol–water partition coefficient (1.0 x 10^6)

(Oros and Werner, 2005). The main effect of Bifenthrin on the nervous systems of insects is by interaction with the sodium channel and disruption of the normal transmission of nerve impulses, followed by paralysis and death (EFSA, 2011). Discharge into aquatic systems may result from spills, NPS runoff, leaching, spray drift, and misapplication, which may have a negative impact on aquatic life.

The grass shrimp, *Palaemonetes pugio*, is an abundant crustacean inhabiting tidal marsh along the U.S. Atlantic and Gulf of Mexico coastlines (Anderson, 1985). Grass shrimp, *P. pugio*, comprise up to 56% of pelagic macrofauna in estuarine tidal creeks (Scott et al., 1999). They play a significant role in estuarine food webs as many species higher in the food web consume grass shrimp as prey items in their diet, including commercially and recreationally important fish species. They also serve as detritivores and are considered an important link between many species through the breaking down of organic matter subsequently used by microscopic organisms. In general, grass shrimp have been used in toxicity tests because they are relatively easy to collect and obtain multiple life stages for testing, as well as demonstrated sensitivity to many chemical contaminants (Key et al., 2006). The toxicity of most chemicals to aquatic invertebrates depends on temperature, an important environmental factor that effects the physiological mechanisms in aquatic organisms at the enzymatic and cellular levels (Cairns et al., 1975; Ward and Stanford, 1982). Temperature may also affect the ability of aquatic organisms to detoxify xenobiotics and may alter contaminant uptake, elimination, or biotransformation rates. (Cairns et al., 1975; Hooper et al., 2013).

The effects of increasing or decreasing temperature on chemical toxicity varies based on the type of chemicals. For example, many organophosphate insecticides show

increased toxicity for many invertebrates at higher temperatures. Some chemicals like pyrethroid insecticides have inverse temperature coefficients being more toxic at lower temperatures (Coats et al., 1989; Lydy et al., 1999). Fluctuations in water temperature have their greatest effect on toxicity when close to either the lower or upper thermal survival limits which may impact ecological toxicity risk estimates (Willming et al., 2013).

1.1.2.2 Mixture triclosan – bifenthrin

There are few data available on interaction of pesticides in mixture. Generally, mixtures of pesticides can produce an additive effect (sum of individual pesticide toxicities), a less than additive effect (antagonistic toxicity) or a greater than additive effect (synergistic toxicity) (Marking, 1977). Due to synergistic pesticide mixtures, there are two important factors that increase the toxicological risk to aquatic organisms, the overlap in chemical application and environmental persistence (DeLorenzo & Serrano 2006). The mixture toxicity involving binary combinations of pesticides from across classes can result in additive and greater-than additive responses (Lydy & Austin,2004). The assessment of pesticides as toxic components of more complex mixtures that include other types of contaminants is necessary (Belden et al. 2007). Therefore, it is very important to study the mixture toxicity, so the evaluation of individual component toxicity alone is not enough for determining the environmental impacts of toxicants (Fernández-Alba et al., 2002). In the past, scientists have focused on individual toxicity chemical pollutants, but there is a big need to fill the gap between the toxic effects of exposure to individual and those effects from exposure to mixtures of these chemicals (Altenburger et al. 2000; Lin et al. 2004). The mixtures of various pollutant can enter

many environments such as estuarine habitats adjacent to marinas, agricultural fields, golf courses, and residential land uses. Because of the relative persistence of pesticide compounds, aquatic organisms may be exposed to chronic, low-level pesticides as well as other contaminants (e.g. PAHs, PPCPs and trace metals). There are many contaminant mixtures that have been frequently measured in streams in the U.S. (Kolpin et al. 2002). For example, many streams in United States and Europe contain pesticides that frequently and most often occur in mixtures (Belden et al. 2007). United States surface waters contain complex mixtures of chemicals, with concentrations of individual chemicals commonly at levels not considered toxic (Kolpin et al. 2002).

Some mixtures may eb additively toxic. Mixtures of different organophosphate insecticides in combination with one another exhibited additive responses due to the similar mechanism of toxic action or when large numbers of chemicals are present (Lydy & Austin,2004). Mixtures of compounds with different mechanisms of action are hard to predict in terms of additive toxicity. In addition, Climate Change may provide additional complexity for testing mixtures as environmental stressors such as changes in temperature, salinity, and pH may produce additional stress that may modify and interact with different combinations of chemicals in the environment. Many aquatic organisms are quite capable of adapting to Climate Change stress while others are not, and this may lead to significant changes in the structure, function and services of ecosystems (Schiedek et al., 2007). Global water surface temperature has increased about 0.78°C. Since the mid-20th century, the rate of global warming has increased (IPCC, 2013). It is predicted that by the end of the 21st century, global mean surface temperature will

increase between 1.1 and 4.8 °C, and best estimates of ocean warming in the top one hundred meters are within 0.6 °C -2.0 °C (IPCC, 2013).

Mixture pesticides toxicity has been observed in aquatic organism. For instance, diazinon and chlorpyrifos, both OPs, exhibited an additive effect in *Ceriodaphnia dubia* when dosed together (Bailey et al,.1997). Key et al. (2007) observed no increase in toxicity with atrazine and fipronil in *Palaemonetes pugio*. Jin-Clark et al. (2002) studied the synergistic effects of herbicides on the toxicity of chlorpyrifos, cyanazine and atrazine alone at 1, 10, 100, or 1,000 mg/L was not very toxic to *C. tentans* in 48-h acute toxicity bioassays. DeLorenzo & Serrano (2006) studied the toxicity of irgarol, individually and in binary mixtures with three other pesticides (the fungicide chlorothalonil, and the herbicides atrazine and 2,4-D), to the marine phytoplankton species *Dunaliella tertiolecta.* Trimble and Lydy (2006) observed greater than additive toxicity with atrazine and organophosphate insecticides in *H. azteca*. Furthermore, Additive Effects were detected in coho salmon, rainbow trout and starry flounder exposed to a mixture of two anti-sapstain wood preservatives, polyphase P-100 and bardac 2280 (Farrell et al. 1998). Additive effects of large number of mixtures of organic chemicals in fathead minnows (*Pimephales promelas*) was observed (Broderius& Kahl 1985).

Normally, pharmaceuticals like other chemicals do not exist as a single contaminant in the environment, but they are found as complex mixtures with other pharmaceuticals and contaminants such as pesticides or industrial chemicals (Kolpin et al., 2002). Contaminants of emerging concern (CECs) such as pharmaceuticals and pesticides are frequently entering the aquatic environment through different

anthropogenic sources, with many adverse toxic effects on aquatic organisms and possibly humans. Pharmaceutical residues found in the aquatic environment, usually occur as mixtures, not as single compounds. There is generally limited information on the toxicological effects of pharmaceutical mixtures (Fent et al., 2006; Galuset al., 2013).

Most man-made chemicals that are found in the environment are degradation products. For example, one-third of 139 surveyed streams in the US contained 10 or more different chemicals from a broad range of chemical classes and use groups, such as synthetic hormones, other pharmaceuticals, industrial chemicals, pesticides, biocides and flame retardants. Of the streams surveyed, 20% of surveyed streams contained 10 or more compounds simultaneously (Backhaus, & Faust ,2012).

Contamination of surface and ground waters by pharmaceutical compounds has been observed and is an environmental problem (Fent et al 2006; Kümmerer, 2010). Pharmaceutical mixtures have been detected in European effluent streams as 18 of the 26 pharmaceuticals that were detected in the analysis. About 38 pharmaceuticals and personal care products were detected bio-solids (Backhaus, 2014). Because they are not regulated or are currently undergoing a regularization process, the pharmaceutical compounds are considered as emerging pollutants in water ecosystem (EU, 2015). Pharmaceutical compounds have been generally detected in the environment at low concentrations in the ng/L range (Kolpin et al. 2002). Many studies have been shown pharmaceutical compounds to be present in measurable concentrations in the aquatic environment (Daughton and Ternes, 1999; Heberer, 2002; Kümmerer, 2008).

Recently, dozens of human and veterinary pharmaceuticals have been identified in surface waters throughout the world, because of increased global pharmaceutical use

and the increased sensitivity of detection. The fate and transport for many chemicals, especially ‘‘emerging contaminants’’, such as human and veterinary pharmaceuticals, industrial and household wastewater products, and reproductive and steroidal hormones, is receiving increasing attention (Kolpin et al., 2002). Pharmaceutical compound concentration rate in the aquatic systems is based on their prevalence of use, human metabolism, possible biotic and/or a biotic transformation, and on the effectiveness of wastewater treatment. Therefore, it is possible to consider each single drug as a chemical mixture (parent compound plus metabolites plus transformation products) (Kümmerer, 2009). However, some factors like increasing environmental awareness and regulations have led to decreased concentrations of most of the legacy pollutants (heavy metals, organic contaminants, pesticides). However, there are other chemicals group which are not well studied or regulated, like pharmaceuticals (Daughton and Ternes, 1999).

1.1.3 Flame Retardants

Brominated flame retardants, or polybrominated diphenyl ethers (PBDEs) were used widely after the 1970s in different commercial technologies such as indoor decorations, textile and electronics (WHO,1997). There are three commercial formulations of PBDEs including, Penta- (over 70% of BDE-47 and BDE-99), Octa- (over 40% of BDE-183), and Deca- (over 98% of BDE-209) BDEs (Alaee et al., 2003). These different formulations are polymer additives and are not chemically bound to materials, so they leach into the surrounding environment. Widespread use has led to increases in PBDE levels in the environment resulting from spillage, emissions during production, release from consumer products, and disposal of end-of-life consumer products (Hu et al. 2010). PBDEs can enter marine ecosystems via surface runoff from

land, discharge from industry, municipal wastewater discharges and deposition from atmospheric transport. PBDEs are synthetic pollutants that cause environmental concern because they have high persistence and bioaccumulation potential (Stapleton et al. 2009). PBDEs also have many properties that promote partitioning into sediments and tissues, such as low vapor pressures and water solubility as well as high octanol–water partition coefficients (log Kow ranges from 4.87 to 9.97 for tetra- to deca- PBDEs) (Environment Canada, 2006). Because PBDEs have physiochemical properties similar those of chlorinated organic compounds, they are linked to many adverse effects such as thyroid hormone disorder, neural response deficit and cancer (Staskal et al.,2005). Therefore, PBDEs were designated as new persistent organic pollutants at the 2009 Stockholm Convention (WHO,1997). In many countries there are no restrictions on PBDEs use. For example, the global use of brominated flame retardants was about 150,000 tons in 1992 of which 40,000 tons were PBDEs (WHO,1994). Furthermore, in China, the estimated domestic production of deca-BDE was 20,000 metric tons in 2006 (Xiao, 2006). Annual use of PBDEs has increased at an estimated rate of 8%/year (Mai et al., 2005). Penta-BDE was banned in 2004 in the European Union (EU 2003) because of its high toxicity to the environment, but it is still used in many commercial products in our daily life, like electronic equipment, plastics, textiles and building materials (Chou et al. 2010). In the US, EPA is concerned that some of the component congeners of PBDEs are persistent, bio-accumulative and toxic and has initiated actions to limit the exposure and release of PBDE congeners and/or commercial products to which they have been added. These actions include: (1) significant changes in the Toxic Substance Control Act to reduce the use of PBDEs in commercial products and thus reduce environmental and humane

exposure; (2) Support and encourage the voluntary phase-out of the manufacture and import of certain PBDEs (e.g. c-decaBDE); and (3) Add PBDEs to the list of chemicals which present or may present an unreasonable risk to health or the environment (EPA, 2019).

1.3.1 PBDE-47

PBDE-47 is one of the classes of flame retardants (polybrominated diethyl ethers) found in many consumer products (Lema et al.,2007). PBDE-47 represented one of the primary PBDEs detected in environmental media and human samples (Wang et al., 2012), which makes toxicity data necessary to assess the environmental hazards and risks posed to aquatic organisms (Mhadhbi et al.,2012). PBDEs have been detected in high concentrations in both water and atmosphere substrates like soil and sediment where they remain stable and long lasting (Hale et al., 2003).

PBDEs have potential to accumulate in marine organisms and be transferred and magnified along the food chain, because they are considered lipophilic compounds, with many adverse effects on the health of aquatic ecosystems and humans (Schuhmacher et al., 2013). PBDEs have been shown to accumulate in crustaceans, fishes and marine mammals and may interfere with endocrine system function (Hale et al., 2003). It has been found that PBDE-47 was a dominant contaminant in commercial fish, with penta-PBDE as the main contaminant (Cheung et al., 2008). PBDEs may also pose a significant public health to human consumers of fish and shellfish due to potential effects on human health. For example, high concentration of PBDEs was detected in the serum of local workers from specific areas (Wang et al., 2012). Therefore, the environmental presence of polybrominated diphenyl ethers (PBDEs), especially PBDE-47, requires toxicity data

to assess the exposure hazards and toxicological risk posed by PBDE in aquatic organisms (Mhadhbi et al.,2012).

1.2 Effects of Climate Change on Chemical Contaminants

Global Climate Change is caused primarily by the increased combustion of fossil fuels which increase the amount of carbon dioxide (CO_2), discharged into the environment which will trap re-radiated sunlight containing infrared radiation (IR) reflected off the Earth's surface, The increased CO_2 acts as a greenhouse to trap IR which will lead to increased global temperatures, alterations in precipitation patterns, and rising sea level**.**

Rising sea level is particularly important in coastal communities as urbanization is an important factor for enhancing the effects of Climate Change and pollution that may change both biotic and abiotic ecosystem properties including affecting the scale of effects from urban NPS runoff. More than 50% of the U.S. and world's population lives in the coastal zone which is only 17% of the world's land area. As a result, pollution discharges and effects are much greater in coastal ecosystems than in more inland terrestrial habitats. Urbanization is one of the major factors altering the structure, function, and dynamics of earth's terrestrial and aquatic ecosystems (Grimm, 2008). Moreover, increases in human populations have been observed. For example, in 1900 the ratio was 10% of the global population who were urban residents. That percentage now exceeds 50% and will rise even more in the next 50 years (UNDE, 2006). Urbanization is considered a primary factor affecting climate as fossil-fuel, industrial, agricultural, and other land-use emissions that alter atmospheric composition as well as other greenhouse gases (CO2, CH4, N2O, tropospheric ozone, and chlorofluorocarbons) may ultimately

affect air and water quality (Solomon, 2007). It has been shown that they have effects on Global Climate Change in many aspects (Poff et al., 2002). There are many environmental factors that are affected by Global Climate Change, which cause interaction within ecosystem in a complex manner, so that alternation in nonnative species population (Rahel, 2007). Increased imperviousness associated with urbanization will enhance NPS runoff of chemical contaminants and increased coastal flooding with sea level rise will both enhance chemical contaminant exposure in estuarine and coastal ecosystems.

1.3 Climate Change Interactions on CECs

Global Climate Change may have a number of effects on the environment such as increasing water temperature and altering salinity that lead to changes in risk assessment of aquatic pollutants. Climate Change may impact the acute and chronic toxicity of pollutants because of altered chemical uptake, fate and transport, and changes in physiological response (Kennish, 2002). Pollutants enter into the environment from different sources such as runoff from agriculture, nurseries, leaching from antifouling paints, golf course runoff, and home lawns (McLusky et al., 1986). Temperature is an important variable for metabolic rate in aquatic organisms. Any change in temperature will have an effect on physiological mechanisms at the enzymatic and cellular levels (Ward, 1982, Cairns., et al 1975). Altered temperature may also modify an organism's ability to detoxify xenobiotics by altering contaminant uptake, elimination, or biotransformation rates, ultimately affecting toxicokinetic and toxicodynamic processes and toxicity (Hooper et al., 2013). Adaptation to Global Climate Change by aquatic organisms could have effects on the use and release of chemicals into the environment.

The different pollutants such as pesticides, pharmaceuticals, and veterinary medicines, timing and frequency of uses are probably different today in response to changing disease and pest pressures resulting from Global Climate Change (Boxall et al., 2009; Tirado et al., 2010). Additionally, increases in temperature and changes in moisture content are likely to alter the partitioning and/or persistence of chemicals (Bloomfield et al., 2006).

Because of Global Climate Change, altered soil characteristics (organic carbon, dustiness) and hydrology may change how contaminants are transported within a terrestrial ecosystem as well as the dilution potential of contaminants in rivers and streams. As a result of Climate Change, some extreme weather events like floods and droughts, have led to runoff transport of dioxins, metals, and hydrocarbons from contaminated areas to non-contaminated areas (Lake et al., 2005). The impact of Global Climate Change on chemical exposure and human sensitivity may not always be highly significant and may occur in either a positive or a negative direction, but cumulative impacts for multiple chemical pollutants could significantly alter risks to human health. Changes in risks are probably the most significant for chemicals where microbes, plants, and lower animals are involved in the source to receptor pathway (Balbus et al., 2013). Agrochemicals may be affected by Climate Change, because of their specificity for use on particular crops and their diffuse application and transport/fate pathways. Moreover, they are affected by Climate Change in many ways including geographical shifts in agricultural practices and changes that affect pest occurrence/distributions and pesticide transport/fate pathways (Bloomfield et al., 2006).

Global Climate Change associated with increased combustion of fossil fuels is one of the main reasons for warmer water temperatures in northern-latitude aquatic

ecosystems, which led to seasonally stressful conditions for cold-water adapted fish species, but at the same time will supply appropriate thermal conditions to allow non-native warm water fish species to thrive in these environments as they warm (Sharma et al., 2007). In this case, there will be competition for food sources between native and non-native species. For example, with increased temperature the world becomes warmer, especially in northern latitudes, and at least over the Southern Ocean and northern North Atlantic.

Climate Change could affect aquatic systems in many ways such as warming water temperatures, altered stream flow patterns, and increasing frequency of storm events. As a result, the distribution of species and the productivity of aquatic ecosystems may also change. In addition, climate-related effects such as change in thermal regimes, reduced ice cover in lakes, changed streamflow regimes, increased salinity, and increased water-development activities in the form of canal and reservoir construction may be expected to occur (Poff et al., 2002). Currently predicted effects included warmer air and seawater temperatures, increased ocean acidity, increased sea level rise, and changes to the intensity of weather disturbances, precipitation and wind patterns (Solomon, 2007). Global Climate Change will also affect thermal regimes which will result in warming of much of the Earth's surface. Increased air and water temperatures will increase adverse effects for many aquatic (Rahel, 2007).

In addition, Climate Change effects will alter precipitation rates and patterns, and will alter salinity in many environments. Increased rates of desiccation may occur which will increase observed salinity of freshwater and estuarine ecosystems (Seager, 2007).

Climate Change impacts are expected to worsen over the next decades and the consequences for future generations are largely unknown (Solomon, 2007).

The interactions of increased temperature and salinity on the ecotoxicology of CECs is largely unknown, as conventional toxicity testing for pesticides and PPCPs does not traditionally address these issues. Rather acute and chronic toxicity studies in aquatic organisms are traditionally conducted at standard temperature and salinity (20^{o}C and 20 psu) for most marine species. Thus, there is a significant need to address this potential shortfall in traditional toxicity testing so that we can better address the interactions between CECs and climate change.

1.4 Grass Shrimp *Palaemonetes pugio* as a Model Organism to Test for Interactive Effects of Climate Change on CECs

Grass shrimp live in estuaries along the Atlantic and Gulf Coasts. *Palaemonetes pugio Holthuis, P. vulgaris Say and P. intermedius Holthuis* comprise up to 56% of pelagic macrofauna in estuarine tidal creeks (Scott et al., 1988**)**. In South Carolina, grass shrimp are among the most widely distributed, abundant, and conspicuous of the shallow water benthic macroinvertebrates in our estuaries, often reaching hundreds to thousands per meter (Coen and Luckenbach, 2000). Grass shrimp inhabit southeastern marshes and tidal creeks, usually associated with beds of submerged or emergent vegetation, oyster reef habitats, oyster shell, fouling communities and woody debris (Ruiz et al., 1993). Shrimps live in different depths; they can inhabit very shallow areas near their margins at depths as great as 15.2 m (50 feet). The seasonal temperature affects the distribution of shrimp throughout the different water levels. In winter and summer when the temperature

is low and high respectively, daggerblade grass shrimp may move from shallow (prefers shallow water when cold) to relatively deeper water when warm. Grass shrimp are considered by many to be primarily detritivores, in spite of their eating a wide variety of foods, but they can be carnivorous in capture. They are also predators of meiofauna and small infauna polychaetes, oligochaetes and nematodes (Coen et al. 1981; Heck and Thoman, 1981).

1.5 Environmental Factors Affecting Grass Shrimp: Temperature, Salinity and pH

Temperature is one of most important parameters that affects metabolism in all life stages of shrimp by modification of enzymatic activity, which could affect directly the molting process, food consumption, oxygen consumption and growth (Hewitt and Duncan (2001). In addition, temperature may affect the respiratory rate of aquatic organisms. For instance, the respiratory rate increases when temperature is increased from 25°C to 30°C in *L. vannamei*, negatively affecting food consumption, growth and survival (Stimpson et al., 2005). High temperature directly could have an effect on the survival of *Marsupenaeus japonicus* juveniles due to several physiological damages, like denaturation of proteins, respiratory stress or deterioration in membrane structures. (Hewitt and Duncan (2001)

The decrease of growth in the shrimp occurs at low temperatures because it reduces their metabolic rates as a physiological strategy to reduce the effective duration of the stress (Walker et al., 2011). However, the growth in the *F. paulensis* juveniles has been negatively affected when the water temperature was below 18°C (Krummenauer et al., 2006). Changes in water temperature and salinity on aquatic habitats could be affected by Gobal Climate Change, which might alter the risk assessment of aquatic

pollutants. In addition, difference in the water temperature and salinity may impact the toxicity of pollutants, due to changed chemical fate and transport, and because of changes in physiological response (Kennish, 2002).

The range of temperatures and salinities grass shrimp can survive is wide as they are considered halotolerant (Alon, 1989). Estuaries where they reside are known to have rapid changes in salinity and temperature because of tides, evaporation and the influx of fresh water from rivers and rain. The tides process affects directly on water temperature and salinity by infusion of different water sources and indirectly by solar heating and evaporation. *P. pugio* generally avoid high temperatures by migrating to deeper waters (~15 m) (Wood, 1967). In addition, rapid changes in temperature impact heart rates in all crustaceans, so the pooling of hemolymph may be due to a reduction of heart pumping efficiency in *P. pugio* and the pressure difference between the heart and the pericardial sinus is decreased as a result of the reduction of cardiac contraction pressure (Guadagnoli et al., 2007).

Grass shrimp are eurythermal, as daggerblade grass shrimp can survive at temperatures from 5 to 38°C (41 - 101°F), but the optimal temperatures range from 18 to 25°C (65 -77 °F). Grass shrimp are considered euryhaline, having been collected from waters with salinities of 5 - 39 psu, but two freshwater species, *P. paludosus* and *P. kadiakensis*, often live in brackish waters. *P. paludosus* has a range salinity from 0 - 10 psu (Anderson, 1985). Salinity exposure to high salinity at 44 psu for 96 h is lethal to half the population. Grass shrimp are more commonly found in salinities ranging from 2 to 36 psu (Anderson, 1985). Increased or decreased salinity has an effect on oxygen consumption; *P. elegans* have shown that a sudden 9 psu increase in salinity causes a

120% increase in oxygen consumption (i.e. metabolic) rates for 3 h after the increase. While a sudden decrease of the same magnitude does not significantly affect oxygen consumption rates (Von Oertzen, 1984). The range of survival in 5 psu was very poor, but in salinities of 10 to 307 at low and moderate temperatures (20˚C and 25˚C), survival was high (Bhandiwad and Johnsen, 2010). Increasing the salinity and temperature can lower respiratory efficiency in *P. pugio*, while decrease in respiratory efficiency increases intramuscular CO2 levels, leading to low pH in the muscle fibers and alkalosis in the extracellular space (Whiteley et al., 2001).

1.6 Grass Shrimp Growth and Reproduction

Reproduction in *Palaemonete*s species in the field occurs from early spring through fall, depending upon the location (White, 1949). Grass shrimp embryo development occurs externally on the pleopods of the female. The reproductive type of grass shrimp is similar to that described for *Palaemonetes varians* (Antheunisse et al., 1968). Shortly after a female molt, one or more males may deposit spermatophores externally on her ventral thorax, and the eggs are extruded within 2 to 3 hours of mating. After fertilization eggs are passed over the spermatheca and attached with ‘glaire or cement to pleopodal setae that form the incubation chamber (Williams, 1984). The embryonic period lasts about two weeks during summer months, and is largely dependent on temperature (White, 1949). Given their ecological importance and relative hardiness in the laboratory, grass shrimp have been used widely in laboratory experiments. (Williams, 1984).

Grass shrimp larvae go through a series of developmental stages (10 zoeae and a postlarva). Juvenile grass shrimp mature when they are perhaps 1.5 to 2 months old or

about 15 to 18 mm (0.6 to 0.7 inches) long, and they live from 6 to 13 months. Usually the older individuals spawn early over wintering in the year and die by the following winter while most young of the year spawn late in the fall as adults. Because some grass shrimp populations reproduce two broods each year, length-frequency distributions may be polymodal (two or more peaks), with growth rates difficult to characterize. Growth rates vary somewhat between species, sexes, habitats and times of year. In colder coastal waters, growth patterns are different, and salinity also affects growth (Anderson, 1985).

Grass shrimp have a well-developed 'horn' or ‘rostrum’ with teeth along the dorsal and or ventral surfaces. These teeth are used to separate out the species in the genus. There are many distinguishing characteristics such as, lack of claws on the third pair of walking legs and their diminutive size, nearly larger than 5 cm (2 inches) which is used to differentiate them from commercial shrimp. In the grass shrimp, it can be separated between male from female by the presence of the ‘appendix masculina’ attached to the ‘appendix interna’ of the endopod of the second pair of pleopods. As well, the endopod of the first pleopod is larger in males versus females. In addition, there is a positive correlation between female length and egg number that has been observed as it has in many decapod crustaceans (Hines, A.H. 1982; Anger et al. 2002).

In the grass shrimp, spawning occurs from February through October, during the spawning season, about more than one brood may be produced. During mating, males transfer a spermatophore to the female, which occurs within seven hours of molting, and the eggs are fertilized externally. The female attaches the eggs to her pleopods, where they remain until hatching 12 to 60 days after they are fertilized. It was easy to recognize

females, those carrying eggs, because the eggs are visible through the female's carapace. Females molt again after spawning (Anderson, 1985).

In a study of population characteristics of the grass shrimp *Palaemonetes pugio* population structure, mortality, fecundity, and size at sexual maturity from a lagoon system inlet in the southwestern Gulf of Mexico, the number of females was higher than for males in winter and spring, suggesting a reproductive strategy that increases the probability of the male finding a receptive female. The K (Bertalanffy growth coefficient) values were 0.48 for males and 0.43 for females. This could indicate a reduction of female energy investment in growth, directing it rather to reproduction. The highest mortality was encountered from April to September. The highest mortality was encountered from April to September. Female size at sexual maturity was estimated to be 2.41 cm TL, showing that ovarian development starts in winter and continues until early spring (Cházaro-Olvera, 2009).

1.7 Statement of Purpose

As the discussion above has indicated climate change may affect salinity and temperature in estuarine ecosystems and current acute toxicity testing in estuarine organisms is generally only done at standard temperature (20°C) and salinity (20psu) for most toxic chemicals, including contemporary use pesticides such as bifenthrin, flame retardants such as PBDE-47, and PPCPs such as triclosan, and ibuprofen. The grass, *P. pugio*, is an ecologically important estuarine invertebrate and has been used widely in acute toxicity testing. We propose to us adult *P. pugio* to assess potential impacts of higher temperature and salinities associated with global warming and sea level

rise/drought, respectively as manifestations of climate change. We will specifically assess whether current traditional acute toxicity testing at standard conditions (20°C and 20 psu) is providing effective risk assessment information with a changing climate by comparing acute toxicity at higher temperature (30°C) and salinity (35psu). In addition, we will test a mixture of the most toxic compounds to assess interactive effects under both standard and climate change conditions. Finally, we will test the effects of triclosan on the gut microbiome of the grass shrimp under standard conditions with a focus on vibrio bacteria antimicrobial resistance.

1.8 Objectives and Hypotheses

The overall objective of this study was to evaluate the effects of increasing temperature and salinity associated with climate change conditions on the ecotoxicology of selected CECs, including the contemporary use pesticide-bifenthrin, the antimicrobial agent-triclosan, the polybrominated flame retardant PBDE-47, and the anti-inflammatory agent -ibuprofen on adult estuarine grass shrimp, *Palaemonetes pugio*. Specific aims that were assessed include testing of the following hypotheses:

Hypothesis 1. HO: High salinity has no effect on selected CECs, including PBDE-47, ibuprofen, bifenthrin, triclosan, and a bifenthrin-triclosan mixture to grass shrimp, *P. pugio*.

1.HA: High salinity has effects on the selected CECs, including PBDE-47, ibuprofen bifenthrin, triclosan, and a bifenthrin-triclosan mixture to grass shrimp, *P. pugio*.

Hypothesis 2. **HO:** High temperature has no effect on the selected CECs, including PBDE-47, ibuprofen, bifenthrin, triclosan, and a bifenthrin-triclosan mixture to grass shrimp, *P. pugio*.

2. HA: High temperature has effects on the selected CECs, including PBDE-47, ibuprofen, bifenthrin, triclosan, and a bifenthrin-triclosan mixture to grass shrimp*, P. pugio*.

Hypothesis 3. HO: The combination of high salinity and temperature has no effect on the toxicology selected CECs, including PBDE-47, ibuprofen, bifenthrin, triclosan, and a bifenthrin-triclosan mixture to grass shrimp, *P. pugio*.

3.HA: The combination of high salinity and temperature has effects on the toxicology selected CECs, including PBDE-47, ibuprofen, bifenthrin, triclosan, and a bifenthrin-triclosan mixture to grass shrimp*, P. pugio*.

Hypothesis 4. HO: A mixture of triclosan and bifenthrin is not additively toxic to grass shrimp*, P. pugio* when exposed to the either high salinity, high temperature or the combination of high temperature and salinity.

HA: A mixture of triclosan and bifenthrin was additively toxic to grass shrimp*, P. pugio* when exposed to the either high salinity, high temperature or the combination of high temperature and salinity

Hypothesis 5: HO: The antimicrobial agent triclosan has no effect on the gut microbiome of the grass shrimp including no effect on vibrio bacteria and their antimicrobial resistance under standard conditions.

HA: The antimicrobial agent triclosan has a significant effect on the gut microbiome of the grass shrimp including no effect on vibrio bacteria and the antimicrobial resistance under standard conditions.

CHAPTER 2

MATERIALS AND METHODS

2.1 PBDE-47

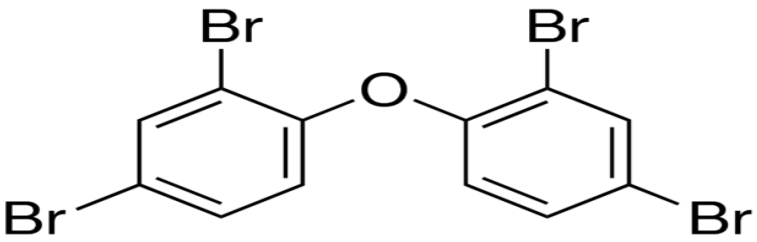

Figure 2.1 Chemical structure of the polybrominated diphenyl ether, (PBDE-47) (After Sigma Chemicals, 2019).

2.1.1 Collection and Maintenance

Adult grass shrimp *Palaemonetes pugio* were collected from the western branch of Leadenwah Creek, a tidal tributary of the North Edisto River estuary in South Carolina on Wadmalaw Island, SC just south of Charleston, SC. This collection site has been used by National Oceanic and Atmospheric Administration (Key et al., 2003), as a long-term collection site for grass shrimp used in lab toxicity tests. The land use in this area is rural agriculture with an average lot size of 12 acres and chemical analysis of sediments have indicated that contaminant levels are below sediment quality guidelines for metals, pesticides, PCBs and petroleum hydrocarbons. Seawater was collected from Cherry Point Landing, also considered free from contamination, at 25-35psu salinity and mixed with

Instant Ocean to develop 20psu water for acclimation and holding. Adult shrimp were acclimated in 10-gallon aquaria (60 X 30 X 30 cm) using a 14-h light: 12-h dark photoperiod. Grass shrimp were initially acclimated at 20 ˚C and 20 psu salinity. Grass shrimp used for toxicity tests to assess Climate Change temperature and salinity interactions were maintained at 30˚C and 35 psu for a minimum of 10 days prior to experiments. Water quality parameters were recorded every other day throughout the acclimation period, including water temperature (thermometer ˚C), salinity (American Optics Refractometer-psu), dissolved oxygen (YSI Oxygen Meter Model # Quatro Cable Assay, 4M - mg/L) and pH (pH meter). Shrimp were daily fed Tetramin® Fish Flakes during lab acclimation but were not fed during acute toxicity tests (Buikema et al., 1980).

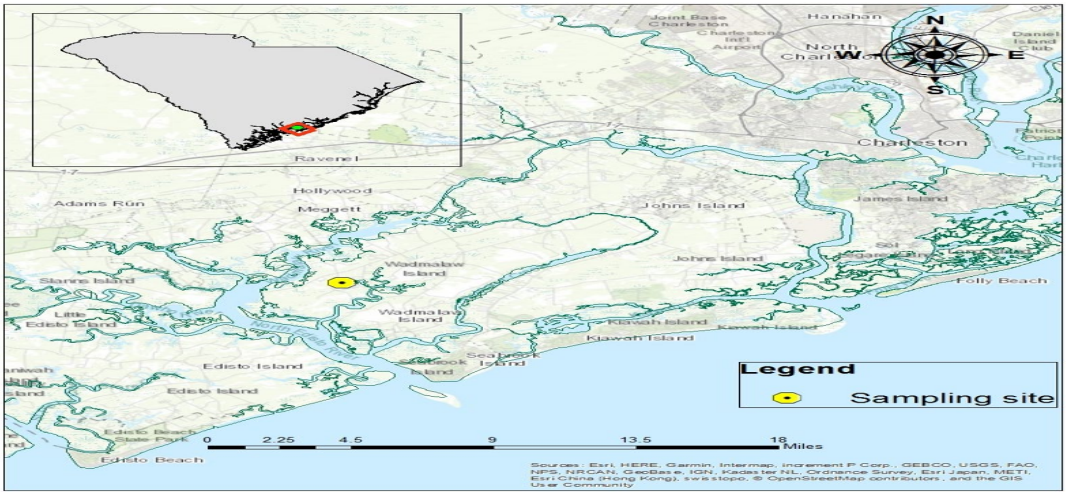

Figure 2.2 Map of collection site for grass shrimp located at the CTL site on the western branch of Leadenwah Creek on Wadmalaw Island, SC.

2.1.2 Acute Toxicity Tests PBDE-47

PBDE-47 high purity (≥97.0%) was weighed and mixed with pesticide grade acetone to produce a concentrated PBDE-47 stock solution of 12 mg/L, which was then

mixed with deionized water and acetone daily to a create a working stock. The working stock was then mixed with Instant Ocean seawater to deliver nominal PBDE-47 exposure concentrations of 100, 56, 32, 18, and 10 µg/L to each of three test chambers for each exposure concentration under different temperature and salinity conditions (20°C, 20 psu = Standard Conditions; 30°C, 35 psu = Climate Change Interaction Conditions). An acetone carrier (0.1% acetone) was added to each PBDE-47 exposure treatment as well as the controls in each test to assure adequate mixing of PBDE-47 into solution. All exposure chambers (1L glass beakers) were pre-cleaned using soap followed by a triple rinse in tap water followed by a triple rinse in deionized water with a final triple rinse in pesticide grade acetone. The final concentration of acetone carrier was kept at 0.1% in each PBDE-47 exposure treatment as well as the controls. Each beaker was wrapped in acetone-rinsed tinfoil to prevent and reduce evaporation (Konwick et al., 2005). All toxicity tests were conducted in a Revco® environmental chamber at 20 °C and a 14-h light: 10-h dark photoperiod while tests 30°C were run in a water bath. Synthetic seawater was used in each toxicity test and was prepared by adding Instant Ocean sea salt to the distilled water to provide 20 psu and 35 psu seawater. Each beaker (1-L) contained five animals with three replicates per PBDE-47 concentration and controls (n=15 shrimp/treatment) in each toxicity test. Water changes were made every 24 hours and supplemental aeration was not provided. Shrimp were not fed during the tests.

Aqueous static renewal 96h toxicity tests were conducted to determine the 24- 96-h LC_{50} (median lethal concentration) values for PBDE-47 at different temperatures and salinities. Before each daily media change, water quality parameters (temperature (°C, pH and salinity (psu)) were measured along with observations of survival. Dissolved oxygen

levels (mg/L) were measured in aerated seawater used for each toxicity tests. Cumulative mortality of the shrimp was recorded for each dose/replicate throughout each 96-h exposure. The criteria for death was either a lack of movement, an absence of movement when animals were gently probed with a glass rod or when the body color turned white or red. Dead animals were removed after each observation period, aeration was not provided during the test (Key et al., 2003).

2.1.3 Chemical analysis:

Brominated flame retardant 2,2′,4,4′-TetraBDE, 2,2′,4,4′-Tetrabromodiphenyl ether, PBDE 47, was purchased from Sigma as analytical grade (purity ≥97.0%). The stock solution was made in 100% pesticide grade acetone to produce nominal exposure concentrations used in each toxicity tests. The stock solution was analyzed chemically by Mass Spectrometry to quantify the levels of PBDE 47 which were reported in. This stock solution was analyzed by GC mass spectroscopy to quantify the levels of PBDE 47 which was within 185.1% the estimated nominal concentration (Table 3.2).

2.1.4 Statistical Analysis

Median lethal concentrations (LC50 values along with 95% confidence limits (CI)) were determined using the Probit method and the nominal concentrations to estimate the 24-96-h LC_{50} concentrations (Ellersieck and LaPoint, 1995), under both Standard and Climate Change exposure conditions. Differences in LC50 values at different time points between treatments were determined by Chi Square Analysis and only differences p values ≤ 0.05 were considered significantly different. In addition, the No Observable Effects Concentration (NOEC) and Lowest Observable Effects

Concentrations were calculated were calculated after 96h of exposure. The NOEC was the highest concentration tested that was not significantly ($p > 0.05$) different from the controls, whereas the LOEC was the lowest concentration tested that was significantly ($p \leq 0.05$) different from the controls as determined using ANOVA and Dunnett's test. Because toxicity data are usually not normally distributed, non-parametric tests (e.g. Kruskal Wallace and equivalent Dunnett tests (e.g. Student Newmans-Kuels and Tukeys) were used.

2.2 Ibuprofen

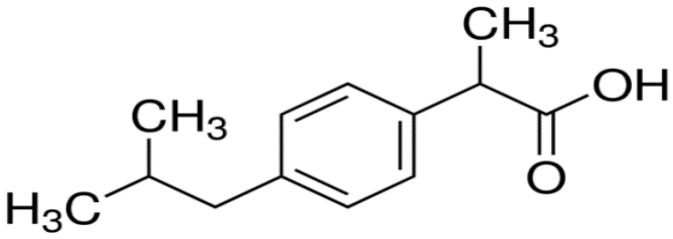

Figure 2.3 Chemical structure of the pharmaceutical, ibuprofen (After Sigma Chemicals, 2019).

2.2.1 Acute Toxicity Tests ibuprofen

High purity (≥98%) IBU was weighed and mixed with pesticide grade acetone and used to produce a concentrated IBU stock solution of 863.4 mg/L (0.864 mg/ml), which was then mixed with Instant Ocean seawater to deliver nominal IBU exposure concentrations of 297, 163, 90, 50 and 27.5 mg/L to each of three test chambers for each exposure concentration under different temperature and salinity conditions [e.g. 20 °C, 20 psu = Standard Conditions; high temperature effects from Climate Change (30 °C, 20 psu); high salinity effects from Climate Change and sea level rise (20 °C, 35 psu); and extreme Climate Change conditions of both high temperature and salinity (30 °C, 35

psu)]. The final concentration of acetone carrier was kept at 0.1% in each IBU exposure treatment as well as the controls.

All exposure chambers (1L glass beakers) were pre-cleaned using soap followed by a triple rinse in tap water followed by a triple rinse in deionized water with a final triple rinse in pesticide grade acetone. Each beaker was wrapped in acetone-rinsed tinfoil to prevent and reduce evaporation (Konwick et al., 2005). All toxicity tests were conducted in a Revco® environmental chamber at 20°C and a 14-h light: 10-h dark photoperiod for standard conditions while tests conducted at 30°C were run in a water bath. Synthetic sea water was used the dilution water in each toxicity test and was prepared by adding Instant Ocean sea salt were added to distilled water to provide 20 psu and 35 psu dilution water in each test. Each beaker (1-L) contained five animals with three replicates/ ibuprofen Concentration and controls (n=15 shrimp/treatment) in each toxicity test. Tests were run using static renewal methods with water changes were made every 24 hours. Shrimp were not fed during the tests.

Aqueous static renewal tests were conducted to determine 96h LC_{50} (median lethal concentration) values for IBU at different temperatures and salinities. Before each daily media change, water quality parameters (temperature, pH and salinity) were measured along with observations of survival. Dissolved oxygen levels were measured in dilution water prior to daily water changes and was always > 100% saturation. Cumulative mortality of the shrimp was recorded for each dose/replicate throughout each 96-h exposure. The criteria for death was either a lack of respiratory movement, an absence of movement when animals were gently probed with a glass rod or when the

body color turned white or red. Dead animals were removed after each observation period, aeration was not provided during the test (Key et al., 2003).

2.2.2 Chemical Analysis:

α-Methyl-4-(isobutyl) phenylacetic acid, (±)-2-(4-Isobutylphenyl) propanoic acid, ibuprofen was purchased from Sigma as analytical grade (purity ≥98%). The stock solution was made in 100% pesticide grade acetone to produce nominal exposure concentrations used in each toxicity tests. This stock solution was analyzed by GC mass spectroscopy to quantify the levels of IBU which was within 109% of the estimated nominal concentration (Table 3.4).

2.2.3 Statistical Analysis

Median lethal concentrations (LC50 values along with 95% confidence limits (CI) were determined using the Probit method and the nominal concentrations to estimate the 24-96-h LC_{50} concentrations (Ellersieck and LaPoint, 1995), under both Standard and Climate Change exposure Conditions. Differences in LC50 values at different time points between treatments were determined by Chi Square Analysis and only differences p values ≤ 0.05 were considered significantly different. In addition, the No Observable Effects Concentration (NOEC) and Lowest Observable Effects Concentrations were calculated were calculated after 96h of exposure. The NOEC was the highest concentration tested that was not significantly ($p > 0.05$) different from the controls, whereas the LOEC was the lowest concentration tested that was significantly ($p \leq 0.05$) different from the controls as determined using ANOVA and Dunnett's test. Because toxicity data are usually not normally distributed, non-parametric tests (e.g. Kruskal

Wallace and equivalent Dunnett tests (e.g. Student Newmans-Kuels and Tukeys) were used.

2.3 Bifenthrin

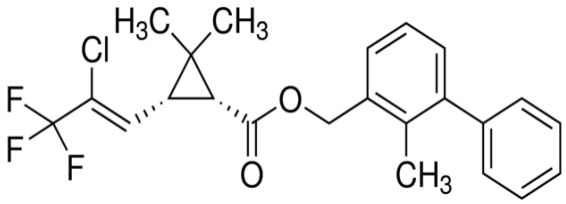

Figure 2.4 Chemical structure of the pyrethroid insecticide, bifenthrin (After Sigma Chemicals, 2019).

2.3.1 Acute Toxicity Tests bifenthrin

Bifenthrin high purity (98%) was weighed and mixed with pesticide grade acetone and used to produce a concentrated bifenthrin stock solution of 46 mg/L, which was then mixed with deionized water and acetone daily to a create a working stock. The working stock was then mixed with Instant Ocean seawater to deliver nominal bifenthrin exposure concentrations of 100, 56, 32, 18, and 10 ng/L to each of three test chambers for each exposure concentration (Standard Conditions(20 °C, 20 psu); increased temperature effects from Climate Change (30 °C, 20 psu); increased salinity effects from Climate Change and sea level rise (20 °C, 35 psu); and extreme Climate Change Conditions (30 °C, 35 psu)]. The final concentration of acetone carrier was kept at 0.1% in each bifenthrin exposure treatment as well as the controls. All exposure chambers (1L glass beakers) were pre-cleaned using soap followed by a triple rinse in tap water followed by a triple rinse in deionized water with a final triple rinse in pesticide grade acetone. Each beaker was wrapped in acetone-rinsed tinfoil to prevent and reduce evaporation

(Konwick et al., 2005). All toxicity tests were conducted in a Revco® environmental chamber at 20°C and a 14-h light: 10-h dark photoperiod while tests 30°C were run in a water bath. Synthetic seawater was used in each toxicity test and was prepared by adding Instant Ocean sea salt to the distilled water to provide 20 psu and 35 psu seawater. Each beaker (1-L) contained five animals with three replicates/ bifenthrin Concentration and controls (n=15 shrimp/treatment) in each toxicity test. Water changes were made every 24 hours and supplemental aeration was not provided. Shrimp were not fed during the tests.

Aqueous static renewal tests were conducted to determine 96-h LC_{50} (median lethal concentration) values for bifenthrin at different temperatures and salinities. Before each daily media change, water quality parameters (temperature, pH and salinity) were measured along with observations of survival. Cumulative mortality of the shrimp was recorded for each dose/replicate throughout each 96-h exposure. The criteria for death was either a lack of respiratory movement, an absence of movement when animals were gently probed with a glass rod or when the body color turned white or red. Dead animals were removed after each observation period, aeration was not provided during the test (Key et al., 2003).

2.3.2 Chemical Analysis technical grade

Bifenthrin: 3-[(1*Z*)-2-Chloro-3,3,3-trifluoro-1-propenyl]-2,2-dimethylcyclopropanecarboxylic acid (2-methylbiphenyl-3-yl) methyl ester, was purchased from sigma (purity 98%). The stock solution was made in 100% pesticide grade acetone to produce nominal exposure concentrations used in each toxicity tests.

This stock solution was analyzed by GC mass spectroscopy to quantify the levels of bifenthrin, which was within 65% of the estimated nominal concentration (Table 3.6).

2.3.3 Statistical Analysis

Median lethal concentrations (LC50 values along with 95% confidence limits (CI)) were determined using the Probit method and the nominal concentrations to estimate the 24-96-h LC_{50} concentrations (Ellersieck and LaPoint, 1995), under both Standard and Climate Change exposure conditions. Significant differences (p values < 0.05) in LC50 values at different time points between treatments were determined by Chi Square Analysis. In addition, the No Observable Effects Concentration (NOEC) and Lowest Observable Effects Concentrations were calculated after 96h of exposure. The NOEC was the highest concentration tested that was not significantly ($p > 0.05$) different from the controls, whereas the LOEC was the lowest concentration tested that was significantly ($p \leq 0.05$) different from the controls as determined using ANOVA and Dunnett's test. Because toxicity data are usually not normally distributed, non-parametric tests (e.g. Kruskal Wallace and equivalent Dunnett tests (e.g. Student Newmans-Kuels and Tukeys) were used.

2.4 Triclosan

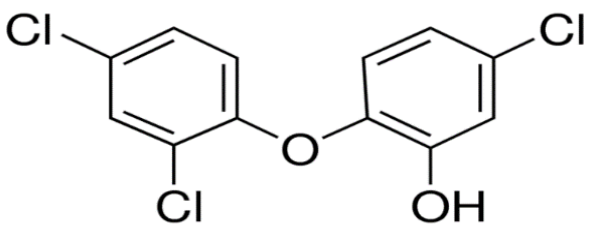

Figure 2.5 Chemical structure of the (PPCP) antimicrobial, triclosan (After Sigma Chemicals, 2019).

2.4.1 Acute Toxicity Tests Triclosan

Triclosan high purity (99.9%) was weighed and mixed with pesticide grade acetone and used to produce a concentrated triclosan stock solution of 1.435 mg/L. Daily this Concentrated Stock was mixed with deionized water and acetone to a create a Working Stock, which was then added to Instant Ocean and seawater to deliver nominal triclosan exposure concentrations of 1000, 800, 600, 400, 200 and 100 µg/L. For each of these triclosan concentration, a total of three test chambers were used for each exposure condition under different temperature and salinity regimes [Standard Conditions (20 °C, 20 psu); high temperature effects from Climate Change (30 °C, 20 psu); high salinity effects from Climate Change and sea level rise (20 °C, 35 psu); and extreme climate change conditions of high temperature , high salinity (30 °C, 35 psu). The final concentration of acetone carrier was kept at 0.1% in each triclosan exposure treatment as well as the. All exposure chambers (1L glass beakers) were pre-cleaned using soap followed by a triple rinse in tap water followed by a triple rinse in deionized water with a final triple rinse in pesticide grade acetone. Each beaker was wrapped in acetone-rinsed tinfoil to prevent and reduce evaporation (Konwick et al., 2005). All toxicity tests were conducted in a Revco® environmental chamber at 20 °C and a 14-h light: 10-h dark photoperiod while tests 30°C were run in a water bath. Synthetic seawater was used in each toxicity test and was prepared by adding Instant Ocean sea salt to the distilled water to provide 20 psu and 35 psu seawater. Each beaker (1-L) contained five animals with three replicates/ triclosan Concentration and controls (n=15 shrimp treatment) in each toxicity test. Water changes were made every 24 hours and supplemental aeration was not provided. Shrimp were not fed during the tests.

Aqueous static renewal tests were conducted to determine 96-h LC50 (median lethal concentration) values for triclosan at different temperatures and salinities. Before each daily media change, water quality parameters (temperature, pH and salinity) were measured along with observations of survival. Dissolved oxygen levels (mg/L) in aerated seawater used in each toxicity tests were always at or > saturation (e.g. 6.80 mg/L) prior to introduction into each aquarium. Cumulative mortality of the shrimp was recorded for each dose/replicate throughout each 96-h exposure. The criteria for death was either a lack of respiratory movement, an absence of movement when animals were gently probed with a glass rod or when the body color turned white or red. Dead animals were removed after each observation period, aeration was not provided during the test (Key et al., 2003).

2.4.2 Chemical analysis

Triclosan: 5-Chloro-2-(2,4-dichlorophenoxy) phenol, Irgasan, triclosan was purchased from sigma as analytical grade (purity 99.9%). The stock solution was made in 100% pesticide grade acetone to produce nominal exposure concentrations used in each toxicity tests. This stock solution was analyzed by GC mass spectroscopy to quantify the levels of triclosan, which was within 77% of the estimated nominal concentration (Table 3.8).

2.4.3 Statistical Analysis

Median lethal concentrations (LC50 values along with 95% confidence limits (CI)) were determined using the Probit method and the nominal concentrations to estimate the 24-96-h LC_{50} concentrations (Ellersieck and LaPoint, 1995), under both Standard and Climate Change exposure Conditions. Differences in LC_{50} values at

different time points between treatments were determined by Chi Square Analysis and only differences p values < 0.05 were considered significantly different. In addition, the No Observable Effects Concentration (NOEC) and Lowest Observable Effects Concentrations were calculated were calculated after 96h of exposure. The NOEC was the highest concentration tested that was not significantly ($p > 0.05$) different from the controls, whereas the LOEC was the lowest concentration tested that was significantly ($p \leq 0.05$) different from the controls as determined using ANOVA and Dunnett's test. Because toxicity data are usually not normally distributed, non-parametric tests (e.g. Kruskal Wallace and equivalent Dunnett tests (e.g. Student Newmans-Kuels and Tukeys) were used.

2.5 Bifenthrin – Triclosan Mixture

2.5.1 Acute Toxicity Tests mixture bifenthrin & triclosan

High purity (98%) standards of bifenthrin and triclosan were weighed and mixed with pesticide grade acetone to produce concentrated stock solutions (46 mg/L bifenthrin) and 1.1 mg/L) which were then mixed with deionized water and acetone daily to a create a working stock which was used in each toxicity test. This stock was then mixed with Instant Ocean seawater to deliver nominal bifenthrin and triclosan exposure concentrations for each toxicity test.

Initial toxicity tests were conducted with each individual compound (triclosan and bifenthrin) under Standard laboratory Conditions (20°C,20psu), high salinity (20°C, 35psu), high temperature (30°C,20psu) and Climate Change Conditions of both increased temperature and increased salinity (30°C,35psu) (Table 3.11). Mixture toxicity tests were then based upon this initial toxicity testing with each individual CEC under Standard and

Climate Change Conditions. Two mixture ratios of triclosan: bifenthrin were tested based on results of individual toxicity tests (Standard Conditions mixture ratio of triclosan: bifenthrin 7442:1 and exposure concentrations ranging from 145/0.012 (Tri/Bif) – 580/0.051 µg/L) and Climate Change Conditions mixture ratio of triclosan: bifenthrin = 7,273 and exposure concentrations ranging from 80/0.011 (Tri/Bif) – 320/0.043 µg/L). (Tables 3.12). Bifenthrin and triclosan exposure concentrations for each of the two mixtures were delivered to each of three test chambers for each exposure concentration under different temperature and salinity conditions [Standard Conditions (20°C,20 psu), high salinity (20°C, 35psu), high temperature (30°C, 20 psu), and Climate Change (30°C, 35 psu) Conditions]. An acetone carrier (0.1% Acetone) was added to each mixture of bifenthrin and triclosan exposure treatment as well as the controls in each test to assure adequate mixing of bifenthrin and triclosan into solution.

All exposure chambers (1L glass beakers) were pre-cleaned using soap followed by a triple rinse in tap water followed by a triple rinse in deionized water with a final triple rinse in pesticide grade acetone. Each beaker was wrapped in acetone-rinsed tinfoil to prevent and reduce evaporation (Konwick et al., 2005). All toxicity tests were conducted in a Revco® environmental chamber at 20°C and a 14-h light: 10-h dark photoperiod while tests 30°C were run in a water bath. Synthetic seawater was used in each toxicity test and was prepared by adding Instant Ocean sea salt to the distilled water to provide 20 psu and 35 psu seawater. Each beaker (1-L) contained five animals with three replicates/ mixture Concentration and controls (n=15 shrimp/treatment) in each toxicity test. Water changes were made every 24 hours and supplemental aeration was not provided. Shrimp were not fed during the tests.

Aqueous static renewal tests were conducted to determine 96h LC_{50} (median lethal concentration) values for mixture at different temperatures and salinities. Before each daily media change, water quality parameters (temperature, pH and salinity) were measured along with observations of survival. Cumulative mortality of the shrimp was recorded for each dose/replicate throughout each 96-h exposure. The criteria for death was either a lack of respiratory movement, an absence of movement when animals were gently probed with a glass rod or when the body color turned white or red. Dead animals were removed after each observation period, aeration was not provided during the test (Key et al., 2003).

The mixture toxicity tests were based on the protocols of (Marking,1985) and Pape-Lindstrom and Lydy (1997). All concentrations for triclosan and bifenthrin were derived from their individual LC_{50} value (Table 3.11). The five concentrations and control used in the mixture toxicity tests are listed in (Table 3.12). The highest exposure concentration for the two triclosan/ bifenthrin mixture test contained 100% of the triclosan 96 h LC50 (580 µg/L (Standard) and 320 µg/L (Climate Change) and 100% of the bifenthrin 96 h LC_{50} (0.051 (Standard) and 0.043 µg/L (Climate Change). Similarly, the lowest concentration for each mixture test contained 25% of the individual 96 h LC_{50s} for bifenthrin and triclosan (Table 3.11).

2.5.2 Chemical analysis

A. Bifenthrin: 3-[(1*Z*)-2-Chloro-3,3,3-trifluoro-1-propenyl]-2,2-dimethylcyclopropanecarboxylic acid (2-methylbiphenyl-3-yl) methyl ester, was purchased from sigma (purity 98%). The stock solution was made in 100% pesticide grade acetone to produce nominal exposure concentrations used in each toxicity tests.

This stock solution was analyzed by GC mass spectroscopy to quantify the levels of bifenthrin, which was within 65% of the estimated nominal concentration (Table 3.6).

B. Triclosan: 5-Chloro-2-(2,4-dichlorophenoxy) phenol, Irgasan, triclosan was purchased from Sigma as analytical grade (purity 99.9%). The stock solution was made in 100% pesticide grade acetone to produce nominal exposure concentrations used in each toxicity tests. This stock solution was analyzed by GC mass spectroscopy to quantify the levels of triclosan which was within 77% of the estimated nominal concentration (Table 3.8).

2.5.3 Statistical Analysis

Median lethal concentrations (LC_{50} values along with 95% confidence limits (CI)) were determined using the Probit method and the nominal concentrations to estimate the 24-96-h LC_{50} concentrations (Ellersieck and LaPoint, 1995), under both standard and climate change exposure conditions. Significant differences (p values < 0.05) in LC50 values at different time points between treatments were determined by Chi Square Analysis. In addition, the No Observable Effects Concentration (NOEC) and Lowest Observable Effects Concentrations were calculated were calculated after 96h of exposure. The NOEC was the highest concentration tested that was not significantly ($p > 0.05$) different from the controls, whereas the LOEC was the lowest concentration tested that was significantly ($p \leq 0.05$) different from the controls as determined using ANOVA and Dunnett's test. Because toxicity data are usually not normally distributed, non-parametric tests (e.g. Kruskal Wallace and equivalent Dunnett tests (e.g. Student Newmans-Kuels and Tukeys) were used.

The modified toxic unit approach to model joint toxicity (Marking, 1985) was used, primarily for its ease of use and understanding. In the toxic unit (TU) model, a value of 1 TU is assigned to the 50% effective concentration (LC50) value of contaminant. A sum of the TU contributed by each component describes the toxicity of a mixture as follows:

TU sum = Cw1/LC501 + Cw2/LC502 +……. Cwi/LC50i

where Cwi is the concentration of a chemical in a mixture and LC50i is the LC50 for the respective component chemicals of the mixture from 1 to i (McCarty et al.,1992). The empirically measured toxicity can then be compared to the expected toxicity (as predicted by the TU sum, which is generated using the LC50 values determined by tests of individual toxicants). When 50% mortality occurs at TU values lower than 1, the mixture is exhibiting greater than additive toxicity (synergism). Determination of less than additive toxicity (antagonism) is made when 50% mortality occurs at TU values greater than 1.

2.6 Triclosan Effects on the Antibiotic Resistance in Vibrio Bacteria

Aqueous static renewal 24-h toxicity tests were performed with triclosan. Chronic sublethal effects of TCS exposure on grass shrimp bacterial population were evaluated in two concentrations, which were the Maximum Exposure Concentration (MEC) and Minimum Inhibitory Concentration (MIC) reported for triclosan of 0.10 µg/L) and 300 µg/L, respectively. Toxicity tests were conducted in a Revco® environmental chamber at standard condition (20°C and 20psu). Each beaker (1L) contained six animals with three replicate triclosan concentrations and controls (n=18 shrimp treatment) in each toxicity test. Shrimp were not fed during the tests. After 24-h, 6 shrimp were pooled from each

beaker, rinsed in sterile phosphate buffered saline (1X PBS), pH 6, to remove external bacteria, and placed in 50 mL sterile conical tubes, weighed and then homogenized on ice in 30ml PBS using an electric tissue grinder for one minute. The tissue homogenates were used for assessing bacterial density as well as multiple antimicrobial resistance (MAR) testing. Changes in the grass shrimp bacterial densities were assessed using Tryptic Soy Agar (TSA, BD Difco) and CHROMagar Vibrio (DRG International) and Thiosulfate Citrate Bile Salts Sucrose (TCBS, BD Difco) which is selective for *Vibrio spp* (Uyaguari et al., 2009).

Serial dilutions (1:1, 1:10, and 1:100) were made from the shrimp homogenates and spread-plated in triplicate onto TSA plates, then incubated for 24-h at 37°C. After incubation, colonies on TSA plates were counted (a countable plate had between 20 and 100 colonies) to estimate the density of *Vibrio spp*. Replica plating technique were performed onto TCBS and CHROMagar Vibrio plates to isolates presumptive *Vibrio* from the total bacteria plates. The replica plates were incubated for 24h at 37°C. CHROMagar Vibrio was used which was a selective and differential media, where Vibrio species can be recognize based on the colony color. *V. parahaemolyticus* colonies were mauve colonies and *V. vulnificus*/*V. cholerae* colonies were ether blue or turquoise. Then, some colonies from TCBS and Chrome agar plates were streaked onto new CHROMagar plates and incubated for 24h at 37°C in shaker incubator to confirm the purity of each culture.

All presumptive *Vibrio spp*. isolates were preserved in TSB 2.5% NaCl and 25% (vol/vol) glycerol and stored at - 80°C for further analysis. Presumptive *Vibrio spp*. isolates were tested for multiple antibiotic resistance (MAR) following the method of

Kaspar *et al*. (1990). *Vibrio spp*. isolates were grown into 96 well plates containing tryptic soy broth with 2.5% NaCl supplemented on different antibiotics. Eleven individual antibiotics were tested at the MEC and MIC for each antibiotic with 2 replicate plates for each antibiotic: ampicillin (MEC = 10, MIC =30 mg/mL), amoxicillin (MEC =25, MIC = 75 mg/mL), ERY (MEC =15,MIC = 50 mg/mL), kanamycin A monosulfate (MEC = 25,MIC = 40 mg/mL), nalidixic acid (MEC =25,MIC = 50 mg/mL), neomycin (MEC = 30, MIC = 50 mg/mL), OTC hydrochloride (MEC = 30,MIC = 40 mg/mL), penicillin G (MEC = 75, MIC = 100 mg/mL), streptomycin sulfate (MEC = 16, MIC = 40 mg/mL), SMX (MEC = 0.0021 µg/mL, MIC = 50,500 mg/mL), ciprofloxacin (MEC = 5,MIC = 15 mg/ mL), and control plates without antibiotics (Kasper *et al*., 1990 ; Edge and Hill, 2005). The agar plates were incubated for 24 h at 37°C. After incubation, the growth results were recorded and classified as resistance or susceptible for each antibiotic.

CHAPTER 3

RESULTS

3.1 PBDE-47

Test conditions in each toxicity test are listed in Table 1. Under Standard Conditions temperature were 20°C, salinities 20 = psu, pH =7.70 and dissolved oxygen levels were ≤ 6.80 mg/L. Under Climate Change Conditions temperature were 30°C, salinities = 35 psu, pH = 8.10 and dissolved oxygen levels were ≤ 6.80 mg/L (Table 3.1). Measured concentrations of the PBDE-47 stock were measured at concentrations that were 85.1% of the nominal concentration (Table 3.2).

Grass shrimp, *Palaemonetes pugio,* exposed to PBDE-47 were found to be more sensitive at the higher temperature and salinity conditions than under standard testing conditions. The 96h LC50 results for Standard Conditions (20°C, 20 psu) resulted in a 96h LC50 of 201.48 ug /L (95% CI 184.15-218.80µg /L) compared to a 96-h LC50 of 31.30 µg /L (95% CI 28.69-33.92 µg /L) under high temperature, high salinity Climate Change Conditions (30°C, 35 psu) (Table 3.3). The combined exposure of high temperature and high salinity resulted in increased PBDE-47 toxicity to adult shrimp throughout the entire 96h of exposure compared to Standard Conditions (20°C,20 psu). PBDE-47 toxicity increased over time as evidenced by LC_{50}, NOEC, and LOEC values which all declined over time, indicating higher toxicity. After 96h of exposure under Standard Conditions the NOECs and LOECs were comparable under both treatment

conditions (< 9 µg/L). After 96h of exposure under Climate Change Conditions the NOECs and LOECs were comparable under both treatment conditions (9 µg/L).

Table 3.1 Water quality conditions measured in each toxicity test.

Water Quality Parameter	Value Measured
Temperature	20 or 30°C
Salinity	20 or 35psu
PH	7.7 or 8.1
Dissolved oxygen	5.6- 6.80 mg/L

Table 3.2 Nominal and measured concentrations of PBDE-47 observed in stock solutions used in each toxicity test.

Nominal Stock (mg/ml)	Measured Stock Concentration (ug/ml)	% of nominal
118.68 mg /ml	101 mg/L	85.1%

Table 3.3 The acute toxicity values for grass shrimp exposed to PBDE-47. The LC_{50} values for each test condition are based on observed percent mortality over time (24-96hours). The CI= 95% confidence interval. Significant ($p \leq 0.05$) differences in LC_{50} values between each temperature and salinity exposure condition associated with Climate Change and Standard Conditions at each individual exposure period (24, 48, 72 and 96h) are denoted by different letters ($^{a-d}$).Time points where LC_{50} values were significantly ($p \leq 0.05$) within the same treatment differed over time are noted by different numbers ($^{1-4}$).

Duration	Treatment	LC50(µg/L)	95% CI (µg/L)	p- value	NOEC (µg/l)	LOEC (µg/l)
24 h	PBDE-47 (20˚C,20psu)	>288 ^{a,1}	NC	NC		
	PBDE-47(30˚C,35psu)	131.99 ^{b,1}	120.79-143.19			
48 h	PBDE-47 (20˚C,20psu)	>288 ^{a,1}	NC	NC		
	PBDE-47 (30˚C,35psu)	131.39 ^{b,1}	119.15-143.62			
72 h	PBDE-47 (20˚C,20psu)	414.03 ^{a,1,*}	351.79-476.28	1.80		
	PBDE-47 (30˚C,35psu)	79.41 ^{b,2}	73.06-85.76			
96 h	PBDE-47 (20˚C,20psu)	201.48 ^{a,3}	184.15-218.80	1.61	< 9	9
	PBDE-47 (30˚C,35psu)	31.30 ^{b,3}	28.69-33.92		< 9	9

*= The 72h LC Value for PBDE-47 under standard conditions was predicted by the Probit model to > than the highest concentration tested.

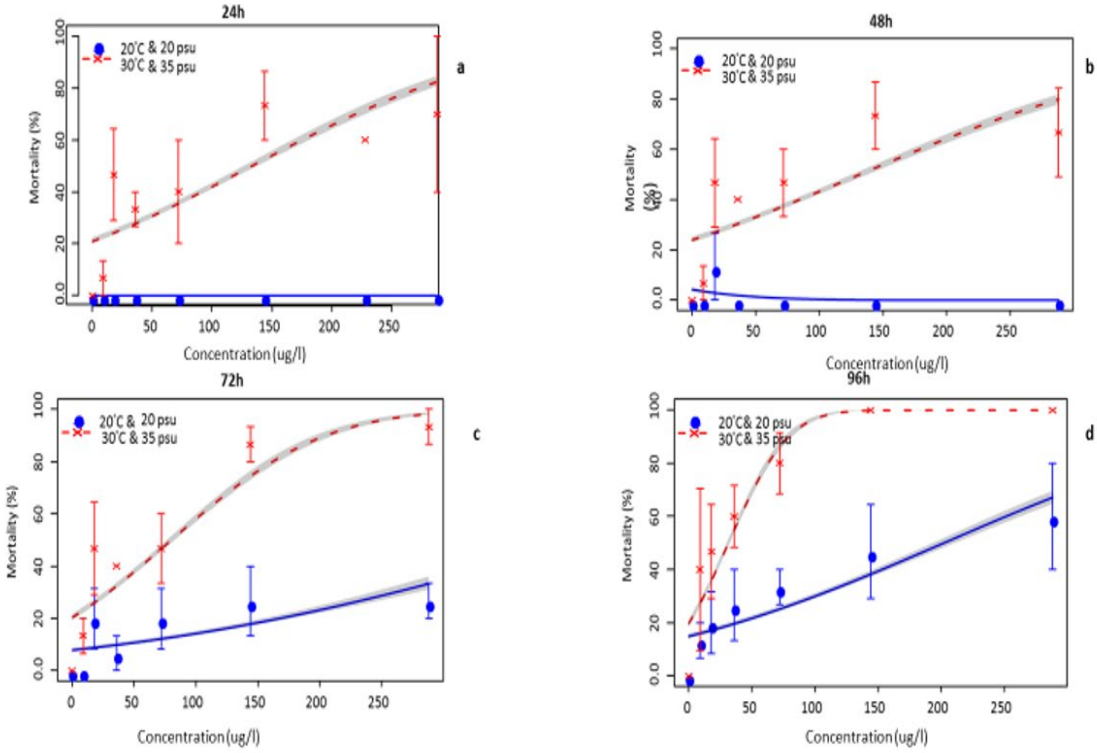

Figure 3.1.a.b.c.d Adult grass shrimp mortality in each PBDE-47 concentration (µg/L) after 24-96 hours of exposure under different conditions of Temperature (°C) & Salinity (psu).

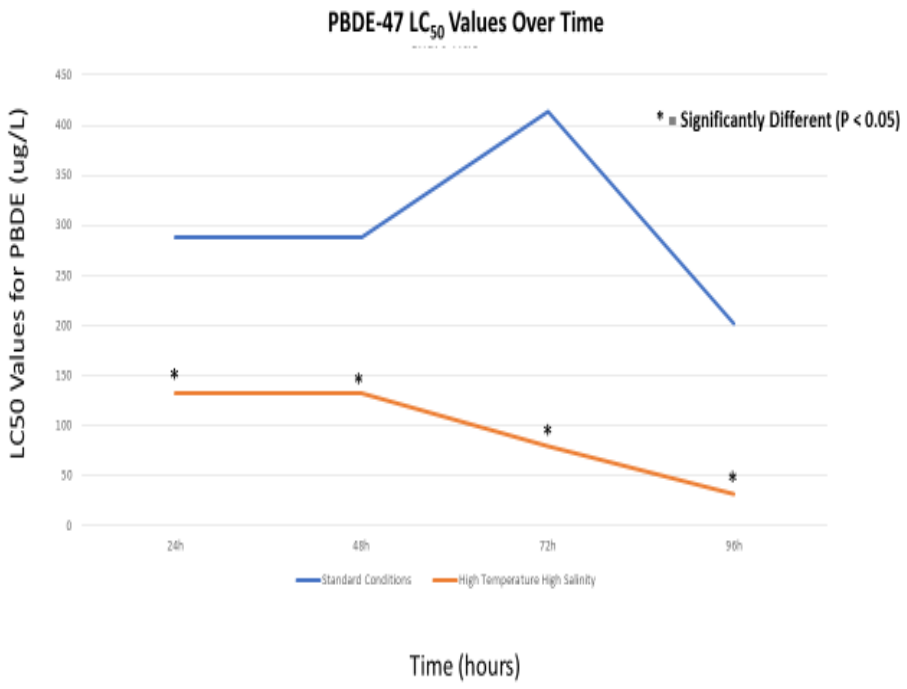

Figure 3.2 Comparison of different LC_{50} values curves for adult grass shrimp exposes to PBDE-47 under different temperature (˚C) & salinity (psu) conditions (Standard versus Climate Change Conditions).

3.2 Ibuprofen

Table 3.4 Nominal and measured concentrations of ibuprofen observed in stock solutions used in each toxicity test.

Nominal Stock (ug/ml)	Measured Stock Concentration (ug/ml)	% of nominal
0.846132 mg/ml	0. 9229 mg/L	109%

Grass shrimp, *Palaemonetes pugio,* exposed to IBU were generally found to be more sensitive to Climate Change exposure conditions of high exposure temperature, when compared to standard exposure conditions (Table 3.5). After 24h and 48h of exposure, there were significant ($P \leq 0.05$) differences in the toxicity of IBU at all different exposure combinations of temperature (20°C and 30°C) and salinity (20ºC, 35 psu) when compared to Standards Conditions (20°C, 20psu). Generally high salinity (20 °C, 35psu) and combined high temperature, high salinity (30°C, 35psu) conditions were more toxic than Standard Conditions (20°C,20psu). In addition, exposure only to high temperature (30°C, 20psu) resulted in increased IBU toxicity to adult grass shrimp when compared to combined high temperature and salinity (30 °C, 35psu) or just high salinity (20 °C, 35psu) as well as standard conditions (20°C, 20psu). After 72h of exposure, Standard Conditions (20°C, 20psu) were only more toxic than high salinity conditions (20 °C, 35psu) but was less toxic than both high temperature (30 °C, 20psu) and combined high temperature, high salinity conditions (30 °C, 35psu) which were significantly ($p \leq 0.05$) more toxic. After 96h of exposure, Standard Conditions (20 °C, 20psu) were significantly ($p \leq 0.05$) more toxic than high salinity (20 °C, 35psu), but was less toxic than both high temperature (30 °C, 20psu) and high temperature, high salinity conditions

(30 °C, 35psu). In addition, exposure to high temperature (30 °C, 20psu) resulted in increased IBU toxicity to adult grass shrimp when compared to combined high temperature and salinity (30 °C, 35psu), clearly indicating that increased temperature conditions associated with Climate Change significantly increased the toxicity of IBU. High salinity only increased the toxicity of IBU when accompanied by additional high temperature conditions.

The 96 h LC_{50} results for increased temperature conditions under Simulated Climate Change Conditions (30 °C,20 psu) was 32.69 mg/L (95% CI 31.11- 34.24 mg/L) was significantly more toxic than Standard Conditions (20 °C, 20psu) which had a significantly ($p \leq 0.05$) higher 96 h LC_{50} of 81.89 mg/L (95% CI 78.49-85.29 mg/L) (Table 3.5). Comparisons of Climate Change Conditions with only high salinity (20°C, 35psu) resulted in conditions that were less toxic than Standard Conditions after 24-96h and clearly indicated increased salinity *per se,* does not increase the toxicity of IBU. Conversely comparisons of Climate Change Conditions with only increased temperature (30°C, 20psu) resulted in conditions that were more toxic than the combined effects of high temperature and salinity (30°C, 35 psu) after 24-96h of exposure, with a 96h LC 50 value of 32.69 mg/L (CI= 31.11-34.24 mg/L) versus 61.68 mg/L (CL = 58.45-69.41 mg/L), respectively (Figures 3.3 a. b. c.d -3.4). This indicated that increased temperature was the major climate change variable that increased toxicity of IBU. High salinity only increased the toxicity of IBU under conditions of high temperature (30°C) and the interaction of increased temperature and salinity was less toxic than high temperature *per se.*

Time also played a significant role in IBU toxicity, as mortality significantly increased over time throughout the first 48h of exposures in all treatments regardless of temperature or the salinity regimes. For example, the 24h LC50 values ranged from 46.52-179.27 mg/L compared to a 48h LC50 values which ranged from 42.78-120.97 mg/L. Toxicity thereafter varied according to each treatment as toxicity under Standard Conditions (20 °C, 20psu) increased after 72h but remained the same after 96h of exposure. Toxicity in high salinity conditions (20 °C, 35psu) remained the same after both 72 and 96h of exposure while in high temperature conditions (30 °C, 20psu) they remained the same after 72h and then increased after 96h of exposure. The combination of high temperature and salinity (30 °C, 35psu) saw an increased in toxicity at both 72 and 96h of exposure. Increased temperature increased the toxicity of IBU by a factor of 2.51 after 96h of exposure (Table 3.5).

The No Observable Effect Concentration (NOEC) was <27.5 mg/L and the Lowest Observable Effect Concentration (LOEC) was 27.5 mg/L under high temperature conditions (30°C, 20 psu) after 24h and remained constant through the entire 96h of exposure. Higher temperature exposure increased the threshold for toxicity of IBU at these lower doses compared to Standard Conditions. The No Observable Effect Concentration (NOEC) was also 27.5 mg/L and the Lowest Observable Effect Concentration (LOEC) was also 50 mg/L under high temperature and high salinity conditions (30°C, 35 psu) after 96h of exposure. Higher temperature combined with high salinity exposure increased the threshold for toxicity of IBU at these lower doses compared to standard conditions Additional comparisons between high temperature conditions and high temperature combined with high salinity conditions indicated that the

additional stress of high salinity did not change the toxicity threshold for IBU and that thus higher temperature was the primary driver for the observed increased toxicity threshold. These differences in NOECs for IBU following Climate Change Conditions are significant, as these lower NOEC values would increase the scale of spatial impacts from use of this PPCP.

Table 3.5 Acute toxicity values for grass shrimp exposed to IBU at each time point and combination of temperature and salinity associated with climate change. Significant ($p \leq 0.05$) differences in LC_{50} values between each temperature and salinity exposure condition associated with Climate Change and Standard Conditions at each individual exposure period (24, 48, 72 and 96h) are denoted by different letters ($^{a-d}$).Time points where LC_{50} values were significantly ($p < 0.05$) different within each salinity and temperature exposure condition are noted by different numbers ($^{1-4}$).

Duration	Treatment	LC_{50}(mg/L)	95% CI (mg/L)	NOEC (mg/l)	LOEC (mg/l)
24 h	ibuprofen (20˚C, 20psu)	$179.27^{a,1}$	172.129-186.412		
	ibuprofen (20˚C, 35psu)	$143.42^{b,1}$	136.54-150.31		
	ibuprofen (30˚C, 20psu)	$46.52^{c,1}$	43.97-49.07		
	ibuprofen (30˚C, 35psu)	$140.85^{b,1}$	133.140-148.561		
48 h	ibuprofen (20˚C, 20psu)	$120.97^{a,2}$	115.326-126.620		
	ibuprofen (20˚C, 35psu)	$91.69^{a,b,2}$	NC		
	ibuprofen (30˚C, 20psu)	$42.78^{c,2}$	40.34-43.23		
	ibuprofen (30˚C, 35psu)	$93.60^{b,2}$	87.70-99.50		
72 h	ibuprofen (20˚C, 20psu)	$85.25^{a,3}$	81.70-88.81		
	ibuprofen (20˚C,35psu)	$92.83^{b,2}$	89.25-96.41		
	ibuprofen (30˚C, 20psu)	$40.95^{c,2}$	38.56-43.35		
	ibuprofen (30˚C, 35psu)	$70.94^{d,3}$	66.86-75.03		
96 h	ibuprofen (20˚C, 20psu)	$81.89^{a,3}$	78.49-85.29	27.5	50
	ibuprofen (20˚C, 35psu)	$92.83^{b,2}$	89.25-96.41	50	90
	ibuprofen (30˚C, 20psu)	$32.69^{c,3}$	31.11-34.24	27.5	50
	ibuprofen (30˚C, 35psu)	$61.68^{d,4}$	58.45-64.91	27.5	50

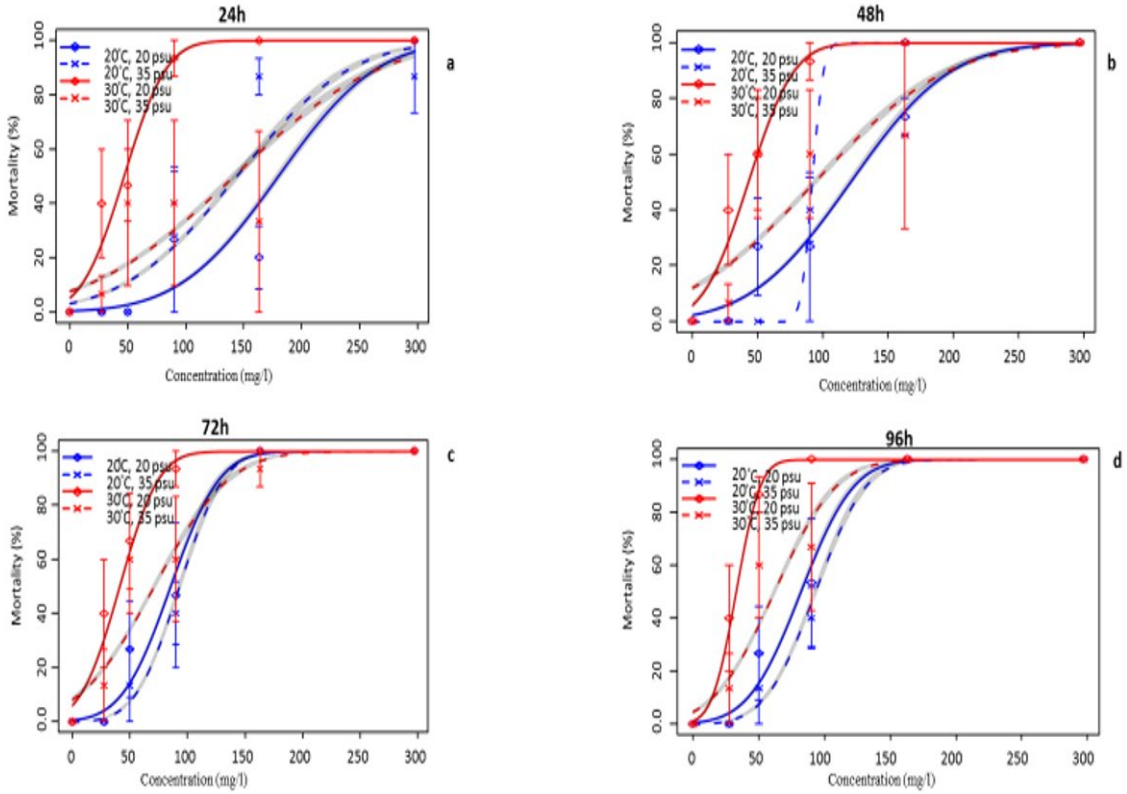

Figure 3.3 a. b. c.d Adult grass shrimp mortality in each ibuprofen concentration (mg/L) after 24- 96 hour of exposure under different conditions of temperature (°C) and salinity (psu).

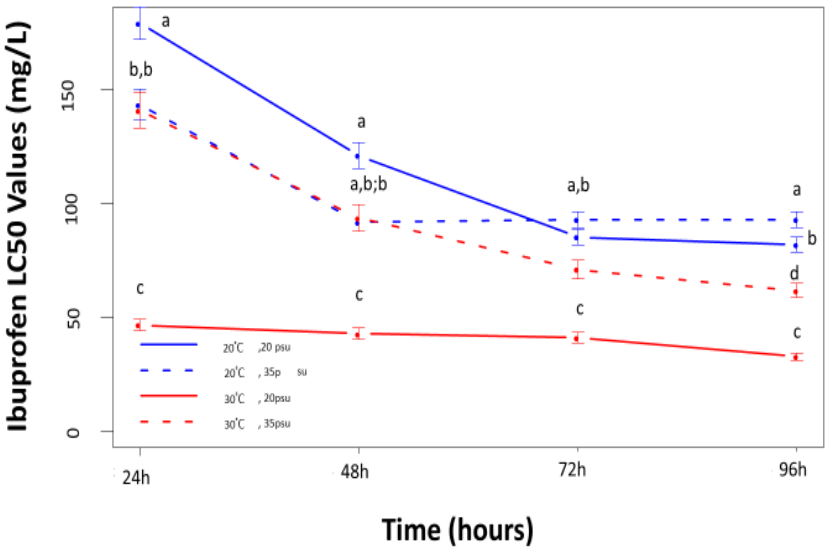

Figure 3.4 Comparison of different LC_{50} values (mg/L) for adult grass shrimp exposes to ibuprofen under different temperature (˚C) & Salinity (psu) conditions (standard versus different climate change conditions). LC50 values with different letters (^{a,b,c,d}) are significantly ($p < 0.05$) in comparison at each time point.

3.3 Bifenthrin

Test conditions in each toxicity test are listed in Table 1. Under Standard Conditions temperature were 20˚C, salinities 20 = psu, pH =7.70 and dissolved oxygen levels were ≤ 6.80 mg/L. Under Climate Change Conditions temperature were 30°C, salinities = 35 psu, pH = 8.10 and dissolved oxygen levels were 5.6- 6.80 mg/L. Measured Stock Concentrations of bifenthrin were measured at concentrations that were 66% of the nominal concentration (Table 3.6).

Grass shrimp, *Palaemonetes pugio,* exposed to bifenthrin were found to be more sensitive to Climate Change exposure conditions when compared to standard exposure conditions (Table 3.7; Figure 3.5 a.b.c.d -3.6). After 24h of exposure there were no differences in the toxicity of bifenthrin at all different exposure combinations of

temperature (20 and 30°C) and salinity (20 and 35 psu) when compared to Standards Conditions (20°C, 20psu). At 48h of exposure Standard Conditions (20°C, 20psu) were more toxic than all different exposure combinations of temperature (20 and 30°C) and salinity (20 and 35 psu). However, the combined exposure of increased temperature and increased salinity resulted in increased bifenthrin toxicity to adult shrimp at exposure periods of > 48-h (Figure 3.5 b). The 96 h LC_{50} results for Simulated Climate Change Conditions (30 °C and 35 psu) of 43.74 ng/L (95% CI 41.60- 45.87 ng/L) was significantly more toxic than Standard Conditions (20 °C, 20psu) which had a significantly ($p< 0.05$) higher 96 h LC_{50} of 51.13 ng/L (95% CI 48.96-53.29 ng/L) (Table 3.6). Comparisons of Climate Change Conditions with only increased salinity (20°C, 35psu) resulted in conditions that were less toxic than Standard Conditions after 48h and were not different after 96h of exposure. Comparisons of Climate Change Conditions with only increased temperature (30°C, 20psu) resulted in conditions that were more toxic than the combined effects of temperature and salinity (30°C, 35 psu) after 72 and 96h of exposure, with a 96h LC 50 value of 33.12 ng/L (CI= 31.71-34.53 ng/L) versus 43.74 ng/L (CL = 41.60-45.85 ng/L), respectively (Figure 3.5 c,d). This indicated that increased temperature was the major Climate Change variable that increased toxicity of bifenthrin. High salinity only increased the toxicity of bifenthrin under conditions of high temperature (30°C). Time also played a significant role in bifenthrin toxicity, as mortality significantly increased over time throughout the entire 96h of exposures in all treatments regardless of temperature or the salinity regimes. For example, 24h LC50 values ranged from 100-183 ng/L compared to 96h LC50 values which ranged from 33.12-53.47 ng/L. Thus, toxicity increased nearly a factor 2 over the 96h of exposure.

After 96h of exposure, the NOEC for Climate Change Conditions was lowered to 10ng/L compared to 18 ng/L under Standard Conditions, indicating only a 1.8-fold factor lowering of the concentration which had no effect. These differences in NOECs for bifenthrin following Climate Change Conditions are significant, as these lower NOEC values would increase the scale of spatial impacts from use of this pesticide. Both increased temperature and increased temperature and salinity conditions would result in lowered the 96h NOEC from 18 ng/L to 10 ng/L. Similar effects were also generally seen for LOECs under Climate Change Conditions.

Table 3.6 Nominal and measured concentrations of bifenthrin observed in the Concentrated Stock Solutions used in each toxicity test.

Nominal Stock (ug/ml)	Measured Stock Concentration(ug/ml)	% of nominal
46 ug/ml	30 ug /ml	65 %

Table 3.7 Acute toxicity values for grass shrimp exposed to bifenthrin at each time point and combination of temperature and salinity associated with Climate Change. Significant ($p < 0.05$) differences in LC_{50} values between each temperature and salinity exposure condition associated with Climate Change and Standard Conditions at each individual exposure period (24, 48, 72 and 96h) are denoted by different letters ($^{a-d}$).Time points where LC_{50} values were significantly ($p < 0.05$) different within each salinity and temperature exposure conditions are noted by different numbers ($^{1-4}$).

Duration	Treatment	LC_{50} (ng/L)	95% CI (ng/L)	NOEC (ng/L)	LOEC (ng/L)
24 h	bifenthrin (20 ˚C, 20psu)	$105.03^{a,1}$	NC		
	bifenthrin (20 ˚C, 35psu)	$105.03^{a,1}$	NC		
	bifenthrin (30 ˚C, 20psu)	$>100^{a,1}$	NC		
	bifenthrin (30 ˚C, 35psu)	$183.03^{a,1}$	149.12-216.95		
48 h	bifenthrin (20 ˚C, 20psu)	$73.96^{a,2}$	71.50-76.41		
	bifenthrin (20 ˚C, 35psu)	$98.44^{b,2}$	NC		
	bifenthrin (30 ˚C, 20psu)	$83.54^{c,2}$	540.55-1.43		
	bifenthrin (30 ˚C, 35psu)	$78.45^{d,2}$	74.41-82. 40		
72 h	bifenthrin (20 ˚C, 20psu)	$57.79^{a,3}$	55.46-60.12		
	bifenthrin (20 ˚C, 35psu)	$78.52^{b,3}$	76.14-80.89		
	bifenthrin (30 ˚C, 20psu)	$33.12^{c,3}$	31.71-34.53		
	bifenthrin (30 ˚C, 35psu)	$48.72^{d,3}$	46.29-51.14		
96 h	bifenthrin (20 ˚C, 20psu)	$51.13^{a,4}$	48.96-53.29	18	32
	bifenthrin (20 ˚C, 35psu)	$53.47^{a,4}$	51.53-55.41	18	32
	bifenthrin (30 ˚C, 20psu)	$33.12^{b,4}$	31.71-34.53	10	18
	bifenthrin (30 ˚C, 35psu)	$43.74^{c,4}$	41.60- 45.87	10	18

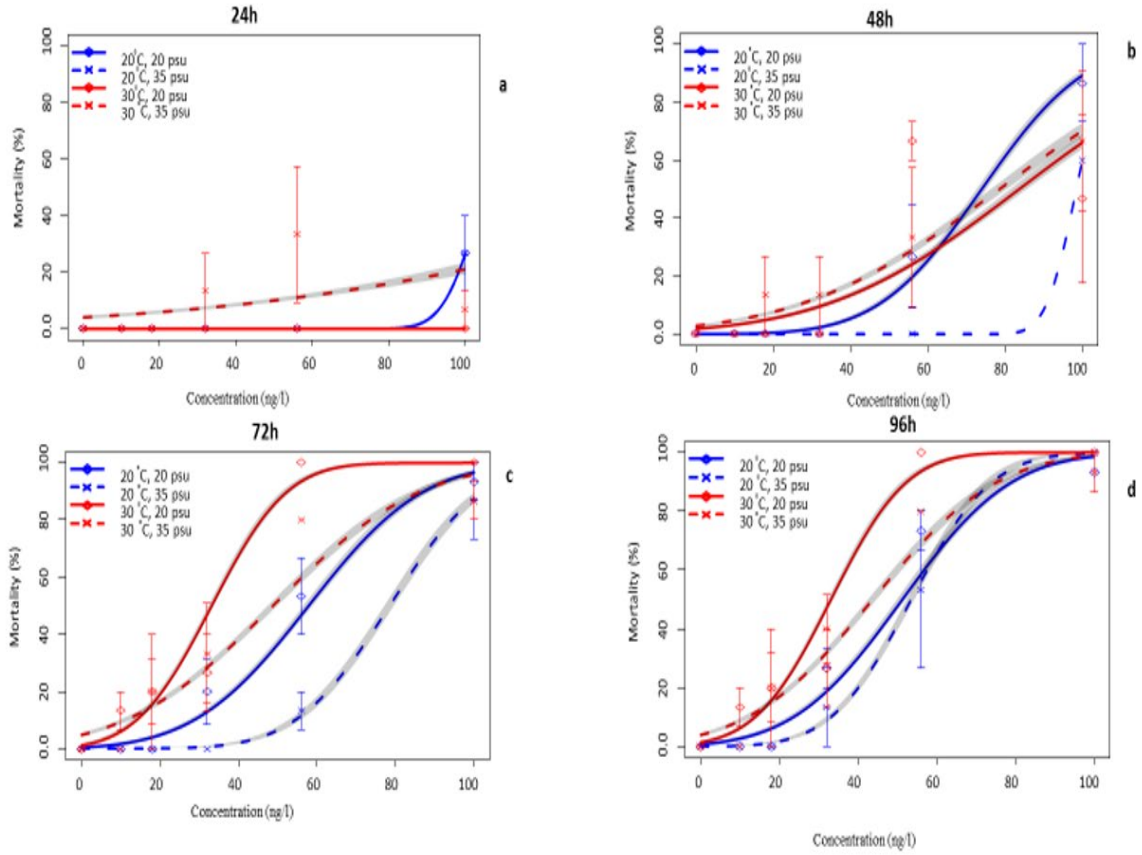

Figure 3.5 a.b.c.d Adult grass shrimp mortality in each bifenthrin concentration (ng/L) after 24-96 hours of exposure under different conditions of Temperature (°C) & Salinity (psu).

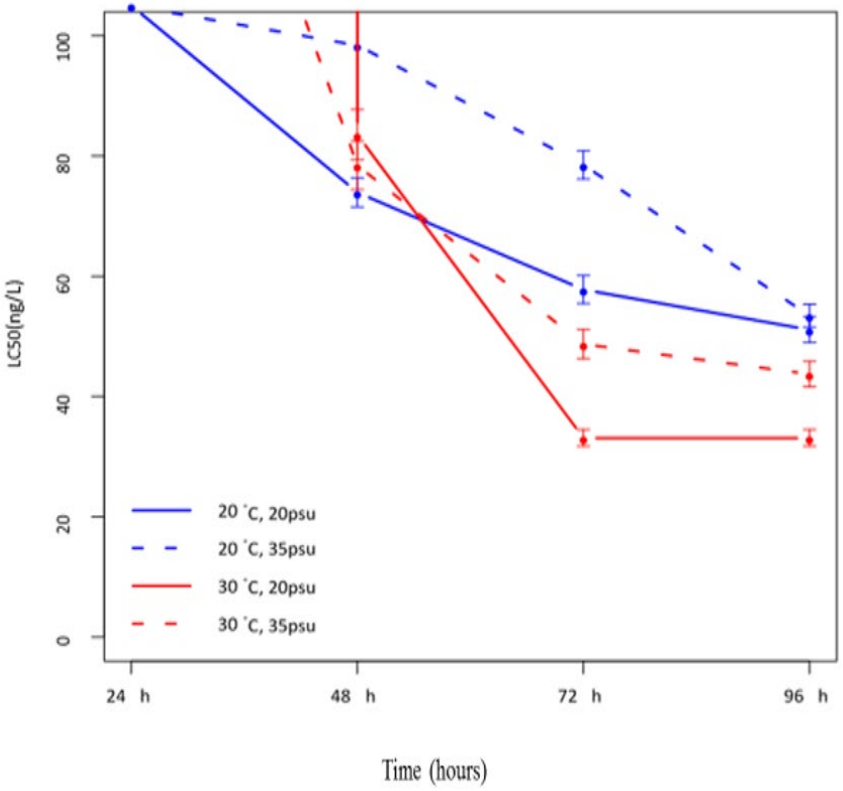

Figure 3.6 Comparison of different LC_{50} values curves for adult grass shrimp exposures to bifenthrin under different Temperature (˚C) & Salinity (psu) conditions (Standard versus Simulated Climate Change Conditions).

3.4 Triclosan

Grass shrimp, *Palaemonetes pugio*, exposed to triclosan were found to be more sensitive to the Climate Change exposure conditions temperature and salinity conditions of 30°C and 35psus, compared to standard conditions 20°C and 20psus as well as high temperature or high salinity conditions (Table 3.9). It has been shown in the first 24-h there was no mortality for both conditions in two lowest concentrations 100 and 200 µg/l. However, over the time the mortality began to increase in 600 µg/l in both conditions, but it was more significant for simulated high temperature, high salinity Climate Change conditions (30°C & 35psu) (Figure 3.7a). Results indicated that the LC_{50} for triclosan under Simulated Climate Change Conditions of high temperature, high salinity was more toxic, with a 96-h LC_{50} of 325 µg/L compared to an LC_{50} of 580 µg/L (CL = 560-600 ug/l) under Standard Conditions (Table 3.9). There were significantly lower 24-96-h LC50 value under Simulated Climate Change Conditions (30°C & 35psu), as the combination of increased temperature and salinity increased the toxicity of triclosan at all times (Figure 3.8). The 24-h to 96-h $LC5_0$ values determined for Standard Conditions (20°C, 20psu) for adult grass shrimp triclosan ranged from 1490 to 580 µg/L respectively. The 24-h to 96-h LC_{50} values determined for combined high salinity, high temperature conditions (30°C, 35psu) ranged from 730 to 320 µg/l respectively (Table 3.9). The 24-h to 96-h LC_{50} values determined for high salinity conditions (20°C, 35psu) was >1,000 µg/l while the 24-h to 96-h LC_{50} values determined for high temperature conditions (30°C, 20 psu) ranged from >1,000 -500 µg/L, respectively. The combined exposure of increased temperature and increased salinity resulted in increased triclosan toxicity for

adult grass shrimp compared to standard conditions (20°C, 20psu) and other Climate Change Conditions (30°C, 25psu and 20°C, 35 psu).

Table 3.8 Nominal and measured concentrations of triclosan observed in stock solutions used in each toxicity test.

Nominal Stock (µg/ml)	Measured Stock Concentration (ug/ml)	% of nominal
1.110 mg/ml	1.435 mg/L	130%

Table 3.9 Acute toxicity values for grass shrimp exposed to triclosan at each time point and combination of temperature and salinity associated with Climate Change. Significant ($p \leq 0.05$) differences in LC_{50} values between each temperature and salinity exposure condition associated with Climate Change and Standard Conditions at each individual exposure period (24, 48, 72 and 96h) are denoted by different letters ($^{a-d}$).Time points where LC_{50} values were significantly ($p \leq 0.05$) within the same treatment differed over time are noted by different numbers ($^{1-4}$).

Duration	Treatment	LC_{50}(µg/L)	95% CI (µg/L)	NOEC (µg/l)	LOEC (µg/l)
24 h	triclosan (20°C, 20psu)	$1490^{1,a}$	1330-1660		
	triclosan (20 °C,35 psu)	$>1,000^{1,b}$	NC		
	triclosan (30 °C,20 psu)	$> 1,000^{1,b}$	NC		
	triclosan (30°C, 35psu)	$730^{1,c}$	710-760		
48 h	triclosan (20°C, 20psu)	$970^{2,a}$	930-1000		
	triclosan (20 °C,35 psu)	$>1,000^{1,b}$	NC		
	triclosan (30 °C,20 psu)	$>1,000^{1,b}$	NC		
	triclosan (30°C ,35psu)	$550^{2,c}$	530-570		
72 h	triclosan (20°C ,20psu)	$710^{3,a}$	680-730		
	triclosan (20 °C,35 psu)	$>1,000^{1,b}$	NC		
	triclosan (30 °C,20 psu)	$>1,000^{1,b}$	NC		
	triclosan (30°C, 35psu)	$420^{3,c}$	409-438		
96 h	triclosan (20°C ,20psu)	$580^{4,a}$	562-607	180	320
	triclosan (20 °C, 35psu)	$>1,000^{1,b}$	NC	>1000	>1000
	triclosan (30 °C, 20psu)	$500^{2,a}$	479-520	180	320
	triclosan (30°C, 35psu)	$325^{4,c}$	NC	180	320

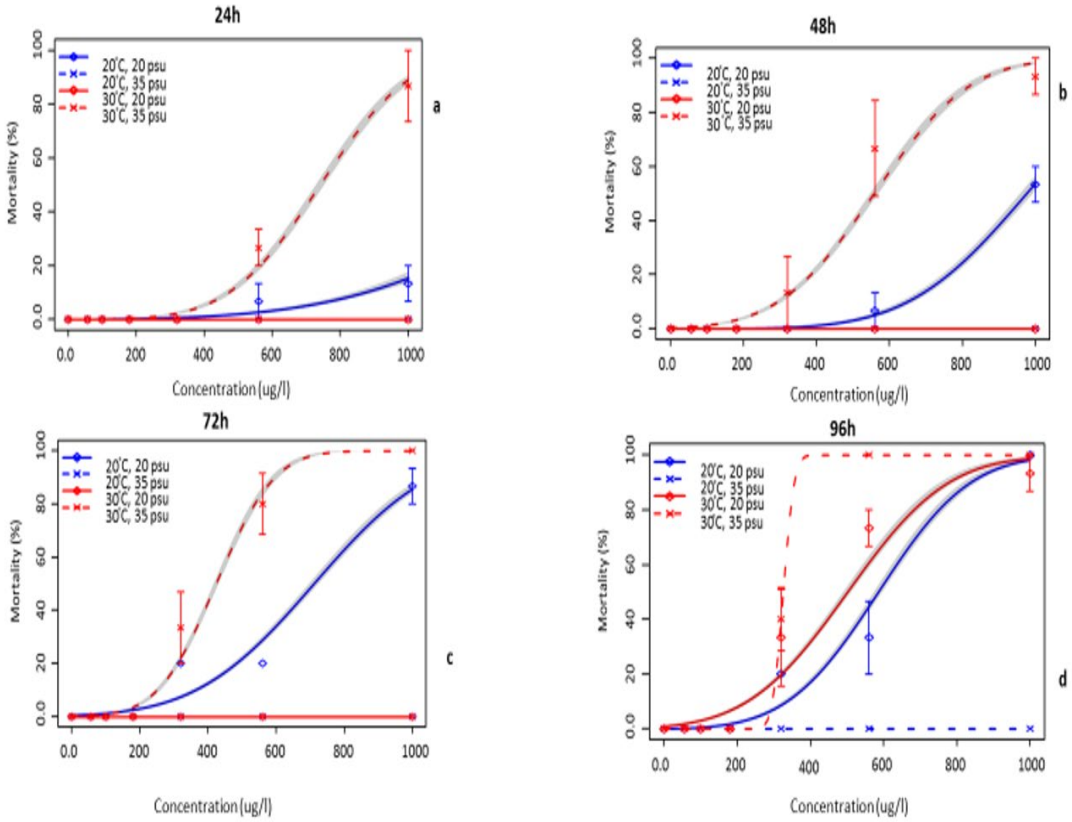

Figure 3.7 a. b. c.d Adult grass shrimp mortality in each triclosan concentration (µg/L) after 24- 96 hour of exposure under different conditions of temperature (˚C) and salinity (psu).

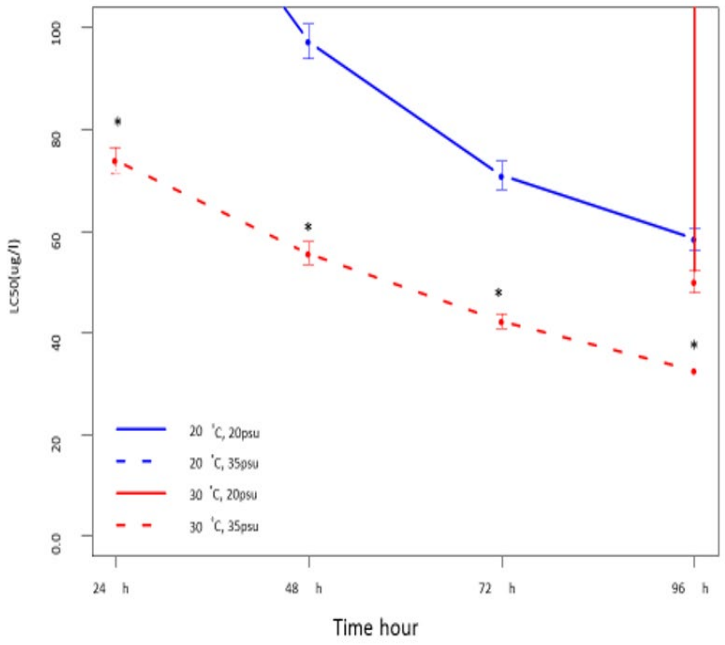

Figure 3.8 Comparison of different LC_{50} values curves for adult grass shrimp exposes to triclosan under different Temperature (°C) & Salinity (psu) conditions (Standard versus Simulate Climate Change Conditions). (*) Indicate significant lower 24-96-h LC_{50} value under Simulated Climate Change Conditions (30°C & 35psu).

3.5 Bifenthrin-Triclosan Mixture

3.1 Individual toxicity tests with triclosan and bifenthrin

Toxicity tests with each compound established acute LC50 and 95% CL values for each individual compound. Toxicity tests with bifenthrin indicated that both high temperature and the combination of high temperature and high salinity Climate Change conditions were significantly ($p \leq 0.05$) more toxic than Standard Conditions (Table 3.11). Standard Conditions (20°C, 20 psu) 96h LC50 values of 0.051 µg/L (95% CL = 0.049-0.053 µ g/L) were significantly ($P \leq 0.05$) less toxic than the high temperature, high salinity conditions (30°C, 35 psu) 96h LC 50 values of 0.043 µg/L (95% CL =

0.042-0.045 µ g/L). High temperature conditions were significantly ($p \leq 0.05$) more toxic with a 96h LC50 of 0.033 (95% CL = 0.032-0.035 µg/L) than both Standard Conditions and Climate Change Conditions of increased temperature and salinity. Bifenthrin exposures at high salinity conditions (20°C, 35 psu) were significantly ($p \leq 0.05$) less toxic than standard, increased temperature and Climate Change (high temperature and salinity) conditions (Table 3.11).

Triclosan results indicated that the LC_{50} for simulated Climate Change Conditions of high temperature and high salinity was significantly ($p \leq 0.05$) more toxic than high salinity, high temperature or standard conditions, with a 96 h LC_{50} of 320µg/L (95% CL = Not Calculatable) compared to an LC_{50} of 580µ g/L (95% CL = 562-607 µ g/L) under standard conditions, > 1,000µ g/L (95% CL = Not Calculatable) for high salinity and 500 ug/L (95% CL = 479-520 µ g/L) for high temperature conditions (Table 3.11). Only Climate Change Conditions of high temperature and high salinity increased the toxicity of Triclosan, with high salinity *per se* reducing its toxicity while high temperature had no effect on grass shrimp survival (Table 3.11).

3.2 Mixture Toxicity Tests

Two mixtures were tested with different ratios of triclosan: bifenthrin based on differences in toxicity for each individual compound under standard (Mixture 1 -Standard Condition Mixture) and Climate Change (Mixture 2 -Climate Change Condition Mixture) conditions for each individual compound (Tables 3.13-3.14).

Testing of the Standard Condition Mixture 1 indicated that mortality increased over time and that increased temperature and salinity Climate Change Conditions were

significantly ($p \leq 0.01$) more toxic than Standard Conditions at all time points tested (24, 48, 72 and 96 hours) (Table 3.13). A 96 h LC50 of 191.21 µg /L (95% CL = 183.44-198.99 µg/L) was observed for Climate Change Conditions (30°C,35psu) compared to a 96 h LC50 of 354.78 µg /L (95% CL =342.73-366.82 µg/L) under Standard Conditions (Tables 3.13-14, Figure 3.9).

Testing of the Climate Change Condition Mixture 2 indicated that mortality increased over time and that Climate Change Conditions were significantly ($p \leq 0.03$) more toxic than Standard Conditions at all time points tested (24, 48, 72 and 96 hours). A 96 h LC_{50} of 189.63 µg /L (95% CL =183.84-195.42 µg/L) was observed under Climate Change Conditions (30°C,35psu) compared to a 96 h LC_{50} of 306.93 µg /L (95% CL = 293.94-319.93µg /L) under Standard Conditions (Tables 3.14, Figure 3.9). Comparisons of results between Mixtures 1 and 2 (Table 3.15; Figure 3.9) indicated that there were significant ($p < 0.05$) differences between the 96h LC50 for Standard Condition Mixture 1 (96h LC50 of 354.78 µg/L (95% CL = 342.73-366.82 µg/L) versus Climate Change Conditions Mixture 2 (96h LC50 of 306.93 µg/L (95% CL = 293.94-319.93 µg/L) when tested under Standard Conditions (20°C,20 psu). Conversely there were no significant ($p > 0.05$) differences between the 96h LC50 for Standard Condition Mixture 1 (96h LC50 of 191.21 µg/L (95% CL = 183.44-198.99 µg/L) versus Climate Change Conditions Mixture 2 (96h LC50 of 189.64 µg/L (95% CL = 183.84-195.42 µg/L) when tested under Climate Change Conditions (30°C, 35 psu).

Assessment of the potential additivity of each bifenthrin and triclosan mixture indicated that the Standard Conditions Mixture 1, was less than additive with an Additive

Index of 1.32 while the Climate Change Mixture 2had an Additive Index of 0.99 which was slightly additive (Table 3.16, Figure 3.10).

Table 3.10 Nominal and measured concentrations of bifenthrin and triclosan observed in the Concentrated Stock Solutions used in each toxicity test.

Nominal Stock (ug/ml)	Measured Stock Concentration (ug/ml)	% of nominal
46 ug/ml	30 ug /ml	65 %
1.110 mg/ml	1.435 mg/L	130%

Table 3.11 The acute toxicity values for grass shrimp exposed to bifenthrin and triclosan individually. The LC_{50} values for each test condition are based on observed percent mortality over time (96 hours). The CI= 95% confidence interval. P-values are listed for comparison of mortality under Standards and Simulated Climate Change Condition at each time interval throughout the toxicity test, with P values of < 0.05 being significantly different (*).

Duration	Treatment	LC_{50}(µg/L)	95% CI (µg/L)	NOEC (µg/L)	LOEC (µg/L)
96 h	triclosan (20˚C 20psu)	580[a]	562-607	180	320
	triclosan (20 ˚C,35psu)	>1,000[b]	NC	>1000	>1000
	triclosan (30˚C,20 psu)	500[a]	479-520	180	320
	triclosan (30˚C 35psu)	320[c]	NC	180	320
96 h	bifenthrin (20˚C20psu)	0.051 [a]	0.049-0.053	0.018	0.032
	bifenthrin (20 ˚C,35psu)	0.053[a]	0.052-0.055	0.018	0.032
	bifenthrin (30˚C,20 psu)	0.033[b]	0.032-0.035	0.010	0.018
	bifenthrin (30˚C 35psu)	0.043[c]	0.042- 0.046	0.010	0.018

Table 3.12 Concentrations of triclosan and bifenthrin used in mixture toxicity tests. (A)Mixture 1 - Standard Conditions (20°C and 20 psu) for each individual compound, (B)Mixture 2 - Climate Change Conditions (30°C and 35 psu) for each individual compound.

A. Mixture 1-Standard Conditions Mixture Ratios (11,373:1) of triclosan: bifenthrin

% of LC_{50}	triclosan (µg/L)	bifenthrin (µg/L)
100	580	0.051
75	435	0.038
50	290	0.025
37.5	217.5	0.019
25	145	0.012

B. Mixture2-Climate Change Condition Mixture Ratios (7,442:1) of triclosan: bifenthrin

% of LC_{50}	triclosan (µg/L)	bifenthrin (µg/L)
100	320	0.043
75	240	0.032
50	160	0.022
37.5	120	0.016
25	80	0.011

Table 3.13 The results of acute toxicity tests for grass shrimp exposed to the Mixture 1 [Stand Conditions Mixture of triclosan (Tri) and bifenthrin (Bif)] for individual compounds under both standard (20˚C; 20psu) and Climate Change Conditions (30˚C;35psu). The LC_{50} values for each test condition are based on observed percent mortality over time (24-96hours). The CI= 95% confidence interval. Significant ($p \leq 0.01$) differences in LC_{50} values between each temperature and salinity exposure condition associated with Climate Change and Standard Conditions at each individual exposure period (24, 48, 72 and 96h) are denoted by different letters ($^{a-d}$).Time points where LC_{50} values were significantly ($p \leq 0.01$) within the same treatment that differed over time are noted by different numbers ($^{1-4}$).

Duration	Treatment	LC_{50}(µg/L)	95% CI (µg/L)	NOEC(µg/L)	LOEC(µg/L)
24 h	Tri&Bif(20˚20psu)	$1119.03^{a,1}$	904.31-1333.75	217.52	251.18
	Tri&Bif (30˚C35psu)	$293.71^{b,1}$	278.09-309.33	<145.01	<145.01
48 h	Tri&Bif (20˚C20psu)	$488.97^{a,2}$	469.80-508.15	145.01	177.60
	Tri&Bif (30˚C35psu)	$251.98^{b,2}$	238.51-265.452	< 145.01	<145.01
72 h	Tri&Bif (20˚C20psu)	$416.53^{a,3}$	402.84-430.23	145.01	177.60
	Tri&Bif (30˚C35psu)	$230.11^{b,3}$	218.57-241.64	<145.01	<145.01
96 h	Tri&Bif (20˚C20psu)	$354.78^{a,4}$	342.73-366.82	<145.01	<145.01
	Tri&Bif (30˚C35psu)	$191.21^{b,4}$	183.44-198.99	< 145.01	<145.01

Table 3.14 The results of acute toxicity tests for grass shrimp exposed to the Mixture 2 [Individual Climate Change Conditions for triclosan (Tri) and bifenthrin (Bif)] exposed to both standard (20˚C; 20psu) and Climate Change Conditions (30˚C;35psu). The LC_{50} values for each test condition are based on observed percent mortality over time (24-96hours). The CI= 95% confidence interval. Significant ($p \leq 0.03$) differences in LC_{50} values between each temperature and salinity exposure condition associated with Climate Change and Standard Conditions at each individual exposure period (24, 48, 72 and 96h) are denoted by different letters ($^{a\text{-}d}$). Time points where LC_{50} values were significantly ($p \leq 0.03$) within the same treatment that differed over time are noted by different numbers ($^{1\text{-}4}$).

Duration	Treatment	LC_{50}(µg/L)	95% CI (µg/L)	NOEC(µg/L)	LOEC(µg/L)
24 h	Tri&Bif (20˚C 20psu)	$491.81^{a,1}$	432.29-551.34	120.02	135.58
	Tri&Bif (30˚C 35psu)	$330.65^{b,1}$	311.76-349.54	<80.01	<80.01
48 h	Tri&Bif (20˚C 20psu)	$417.31^{a,2}$	382.99-451.64	120.02	135.58
	Tri&Bif (30˚C 35psu)	$290.73^{b,2}$	276.27-305.19	<80.01	<80.01
72 h	Tri&Bif (20˚C 20psu)	$361.45^{a,3}$	340.28-382.16	120.02	135.58
	Tri&Bif (30˚C 35psu)	$213.85^{b,3}$	206.69-221.01	<80.01	<80.01
96 h	Tri&Bif (20˚C 20psu)	$306.93^{a,4}$	293.94-319.93	80.01	97.99
	Tri&Bif (30˚C 35psu)	$189.63^{b,4}$	183.84-195.42	<80.01	<80.01

Table 3.15 The results of acute toxicity tests for grass shrimp exposed to both Standard Conditions Mixture 1 and the Climate Conditions Mixture 2 of triclosan (Tri) and bifenthrin (Bif) for individual compounds under both Standard (20°C; 20psu) and Climate Change Conditions (30°C;35psu). The LC_{50} values for each test condition are based on observed percent mortality over time (24-96hours). The CI= 95% confidence interval. Significant ($p \leq 0.05$) differences in LC_{50} values between each temperature and salinity exposure condition associated with climate change and standard conditions at 96h are denoted by different letters ($^{a-d}$).

Mixture Type	Exposure Conditions	96h LC50	95% CI (µg/L)	NOEC(µg/L)	LOEC(µg/L)
Standard	Tri&Bif (20°C 20psu)	354.78[a]	342.73-366.82	<145.01	<145.01
Condition	Tri&Bif (20°C 20psu)	306.93[b]	293.94-319.93	80.01	97.99
Climate Change	Tri&Bif (30°C 35psu)	191.21[a]	183.44-198.99	< 145.01	< 145.01
Conditions	Tri&Bif (30°C 35psu	189.63[a]	183.84-195.42	<80.01	<80.01

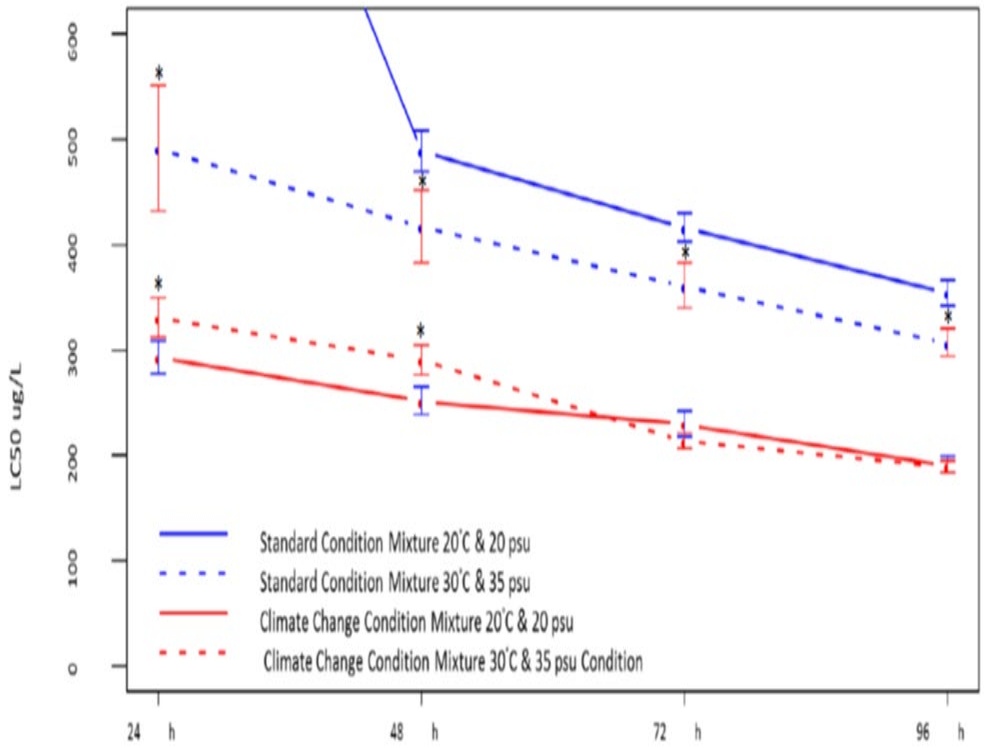

Figure 3.9 Comparison of different LC_{50} values curves for adult grass shrimp exposes to two different triclosan and bifenthrin mixtures (Standard Condition Mixture and Climate Change Condition Mixture) under different Temperature (˚C) & Salinity (psu) conditions (Standard versus Climate Change Conditions). Asterisks (*) denote differences between different salinity and temperature exposure conditions for each mixture.

Table 3.16 Comparison of joint toxicity of the triclosan/bifenthrin Standard Condition and Climate Change Mixtures Interaction Estimates of Additivity (S = Sum Additive Interactions

Triclosan and Bifenthrin Mixture Results

	Standard Conditions			Climate Change Conditions		
	Individual	Mixture		Individual	Mixture	
Compound	LC50	LC50	M/I	LC50	LC50	M/I
Triclosan	580 ug/L	305 ug/L	0.52	320 ug/L	188 ug/L	0.58
Bifenthrin	0.051 ug/L	0.041 ug/L	0.80	0.043ug/L	0.018 ug/L	0.41
Sum(S) of Additivity Interactions[a]			1.32			0.99

([a]S = $Triclosan_{mixture}/Triclosan_{individual}$ + $Bifenthrin_{mixture}/Bifenthrin_{individual}$;)
(When S >1= Less than Additive and When S<1 = Greater Than Additivity)

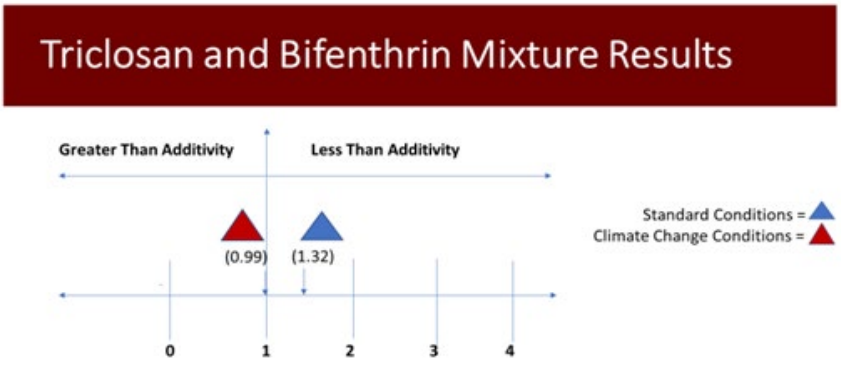

Figure 3.10 Graphical depiction of triclosan/bifenthrin mixture interactions from table 3.13-3.14. Note that The Climate Change Exposure Conditions enhanced the additivity of the additivity of the mixture to > additivity.

3.6 Triclosan Effects on the Antibiotic Resistance in Vibrio Bacteria

3.6.1 Total Vibrio Bacterial Density

The lower dose 0.1µg/l (MEC) of triclosan had a greater effect on Total Vibrio microbial growth and survival than the higher dose 300 µg/l (MIC) when compared to the control (Figure 3.11). Statistical analysis indicated both the control and high dose of triclosan was significantly different from the low triclosan dose ($P < 0.04$). There were no differences in Total Vibrio levels in comparisons between the control and the high triclosan dose after 24h ($p < 0.595$).

3.6.2 Bacteria Identification and Enumeration

The majority of Vibrio bacteria were *V. parahaemolyticus* (90%), *V. vulnificus* (2%) and *V. cholerae* (Figure 3.12). Statistical analysis of bacterial abundances for each identified Vibrio species that only the Maximum Exposure Concentration (MEC) of triclosan (0.10 µg/L) caused a significant reduction of bacterial abundance for *Vibrio parahaemolyticus* and *V. cholerae*.

For *V. parahaemolyticus*, the 0h control was significantly different from both the low and high triclosan dose ($p< 0.003$ and 0.02, respectively); however, the 24h control was only significantly different with low dose of triclosan. For *V, vulnificus*, the result indicated there were no difference between any triclosan treatments and control at any exposure time (0-24h). For *V. cholerae*, the result shows the 0h control was not significantly different from both the and high triclosan dose. The 24h control was a significantly ($p < 0.004$) different from the low triclosan dose (0.1µg/L). For unknown bacterial species both the 0 and 24h controls had significantly ($p < 0.05$) lower levels of bacteria than were measured in both the high and low triclosan doses.

3.6.3 The Vibrio bacteria

The Vibrio bacteria found in the microbiome of the grass shrimp following exposure to two doses of triclosan (MEC and MIC) were then tested for their antibiotic resistance for 11 different antibiotics and compared with the levels of antibiotic resistance found in control grass shrimp (Figures 3.14;3.15;3.16).

3.6.4 Within Treatment Comparisons

Within Treatment Comparisons (e.g. comparisons between the MEC and MIC for each antibiotic within Vibrio bacteria from the control, low dose (MEC) and high (MIC) dose of triclosan groups, respectively) only indicated altered antibiotic resistance with five antibiotics including Sulfamethoxazole (triclosan MIC), Neomycin (triclosan MEC and MIC), Erythromycin (triclosan MIC),Streptomycin (triclosan MIC), and Kanamycin (triclosan MEC and MIC) (Figure 3.14).

For Vibrio bacteria from controls there were no differences in the levels of antibiotic resistance observed for the MEC or MIC for each of the 11 antibiotics (Figure 19). For Vibrio bacteria from the low dose triclosan exposure group (MEC), differences in the levels of antibiotic resistance observed for the MEC or MIC were only observed for two of the 11 antibiotics (19%), including Neomycin and Kanamycin. For Vibrio bacteria from the high dose triclosan exposure group (MIC), differences in the levels of antibiotic resistance observed for the MEC or MIC for each antibiotic were only observed for five of the 11 antibiotics (46%), including Sulfamethoxazole, Neomycin, Erythromycin, Streptomycin and Kanamycin.

For Sulfamethoxazole, only the high dose of triclosan caused significant ($p < 0.05$) differences in the amounts of antibiotic resistance in Vibrio bacteria, as the Sulfamethoxazole Low and High MICs had significantly ($p \leq 0.05$) reduced levels of Vibrio bacteria when compared to the Neomycin MEC (Figure 3.14). There were no significant differences observed in Vibrio growth between the Sulfamethoxazole MEC and MIC exposure responses in bacteria from control grass shrimp. Similar responses were seen with Streptomycin as only bacteria from grass shrimp exposed to the high dose of triclosan caused significant ($p < 0.05$) differences in the amounts of antibiotic resistance in Vibrio bacteria, as the Streptomycin MICs had significantly ($p \leq 0.05$) reduced levels of Vibrio bacteria when compared to the Streptomycin MEC. There were no significant differences in antibiotic resistance between the Streptomycin MEC and MIC exposure response in bacteria from control grass shrimp (Figure 3.14).

For Neomycin, both the low and high doses of triclosan caused significant ($p < 0.05$) differences in the amounts of antibiotic resistance as the Neomycin, MIC significantly ($p \leq 0.05$) increased the levels of Vibrio bacteria while the Neomycin MEC significantly ($p \leq 0.05$) reduced levels of Vibrio bacteria. There were no significant differences between the Neomycin MEC and MIC response in bacteria from control grass shrimp (Figure 3.14).

Erythromycin and Kanamycin each had a somewhat similar effect as Neomycin, as low and high doses of Triclosan had an effect as the Erythromycin/ Kanamycin MIC significantly ($p \leq 0.05$) reduced the number of bacteria while the Erythromycin/ Kanamycin MEC significantly ($p < 0.05$) increased the levels of antibiotic resistance in Vibrio bacteria. There were also significant ($p < 0.05$) differences between the

Erythromycin MEC and MIC responses in bacteria from control grass shrimp, while there were no significant ($p \geq 0.05$) differences between the Kanamycin MEC and MIC responses in bacteria from control grass shrimp (Figure 3.14).

3.6.5 Between Group Comparisons

Between Group Comparisons (e.g. comparisons between the MEC and MIC for each antibiotic in Vibrio bacteria across the different treatments - control, low dose (MEC) and high (MIC) dose of triclosan groups, respectively) only indicated altered antibiotic resistance with seven antibiotics including Sulfamethoxazole (MIC), Erythromycin (MEC), Streptomycin (MEC and MIC), Amoxicillin (MEC and MIC), Ampicillin , (MEC and MIC) Kanamycin (MEC and MIC) and Penicillin (MEC and MIC) (Figure 3.15).

For Vibrio bacteria from controls, low dose (MEC) and high dose (MIC) triclosan exposed grass shrimp microbiome, which were then exposed to the MEC concentration of each of the 11 different antibiotics, there were only 5 antibiotics (46%) that altered growth and survival to generally significantly ($p < 0.05$) increase antibiotic resistance, including Streptomycin (increased antibiotic resistance), Amoxicillin, Ampicillin, Kanamycin and Penicillin (Figure 20). For Vibrio bacteria from controls, low dose (MEC) and high dose (MIC) triclosan exposed grass shrimp microbiome, which were then exposed to the MIC concentration of each of the 11 different antibiotics, there were only six antibiotics (55%) that altered growth and survival to either significantly ($p < 0.05$) increase antibiotic resistance with increasing triclosan dose (e.g. Amoxicillin and Penicillin); decrease antibiotic resistance with increasing triclosan dose (e.g. Sulfamethoxazole and Streptomycin) or to both increase antibiotic resistance at low dose

triclosan exposure and to decrease antibiotic resistance at high dose triclosan exposure (Ampicillin and Kanamycin) (Figure 3.15). This is clear evidence that Vibrio bacteria in the environment may react differently from Vibrio bacteria within humans, as the difference in dose may cause differing responses in terms of antibiotic resistance.

3.6.6 Multiple Antibiotic Resistance

Multiple antibiotic resistance isolates were observed as Vibrio bacteria in controls were resistant up to 8 different antibiotics while triclosan exposed Vibrio's were only resistant to 7 different antibiotics (Figure 3.16). Controls and the high triclosan exposure concentration had no Vibrio bacteria (0%) which were not antibiotic resistant while the low dose triclosan exposure group had 8.1% that had no antibiotic resistance.

The high doses of triclosan also had increased numbers of Vibrio isolates that were resistant to 6 or 7 different antibiotics (Figure 3.16). Only 31% of control isolate had resistance to > 6 different antibiotics compared to 41% of isolates at the low exposure dose of triclosan (MEC) and 74% of isolates at the high exposure dose of triclosan (MIC). Conversely 45% of control isolates and 46% of the low dose triclosan exposure group (MEC) were resistant to $\leq$ 3 antibiotics compared to only 5% of the high dose triclosan exposure group (MIC). This is clear evidence that increasing triclosan exposure will increase the amount of multiple antibiotic resistance in Vibrio bacteria.

Results of statistical regression analysis directly supports these results as using the negative binomial regression model indicated that grass shrimp exposed to triclosan had increased antibiotic resistance within the Vibrio bacteria of their microbiome. Antibiotic resistance was found in all treatments including the controls and both low and high dose

exposures of triclosan. In addition, statistical analysis indicated there was significantly ($p < 0.007$) more antibiotic resistance in the high triclosan exposure concentration (at the MIC) when compared to the controls. Statistical comparisons between the low and high triclosan doses were also significantly ($p < 0.001$) different with higher levels of antibiotic resistance in the higher (MIC) dose. At the low triclosan exposure concentrations there were no significant ($p > 0.58$) differences found when compared to controls.

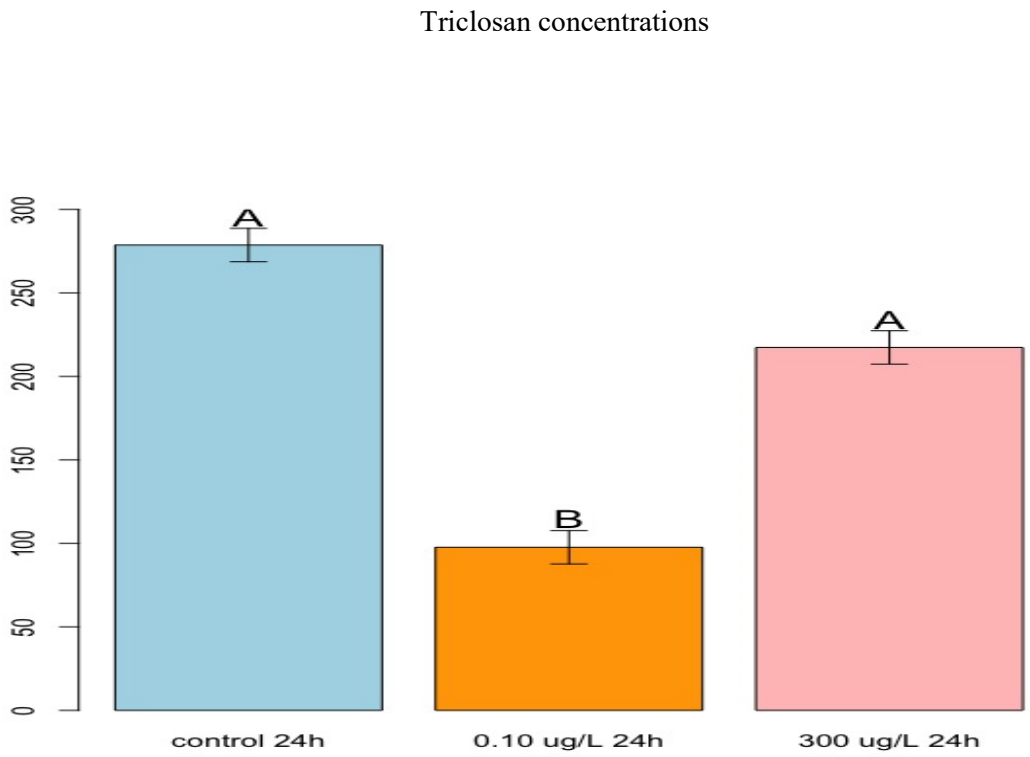

Figure 3.11 *Vibrio spp.* density in grass shrimp after 24 h exposure to triclosan treatments.

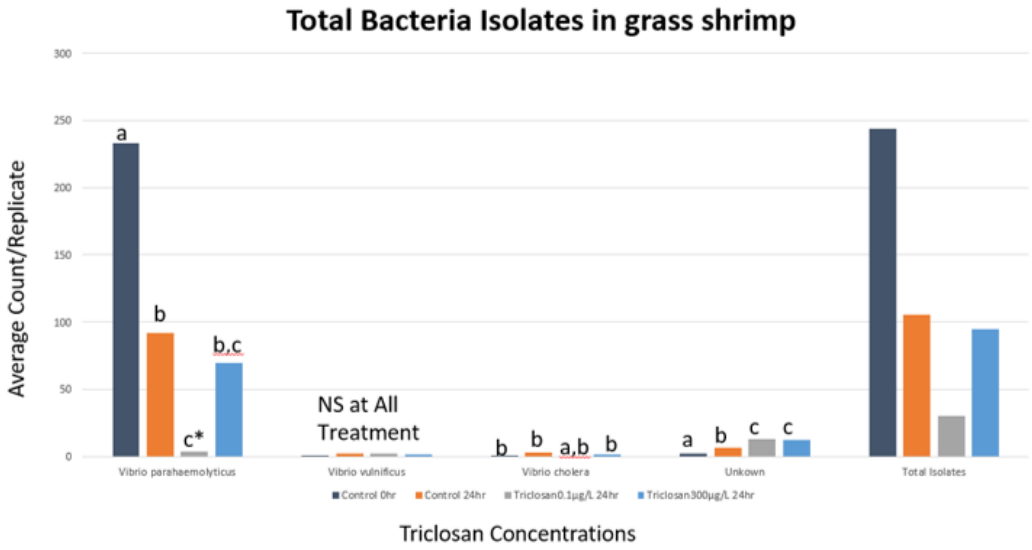

Figure 3.12 The distribution of different Vibrio bacterial species among the different isolates. (V.p. = *Vibrio parahaemolyticus*; V.v. = *Vibrio vulnificus*; V.c. = *Vibrio cholerae*) identified in the adult grass shrimp microbiome.

Figure 3. 13 Average Bacterial Counts/Replicate for bacterial species identified in the adult grass shrimp microbiome. Treatments with different letters (a, b, c) were significantly ($p \leq 0.05$) as indicated in the text.

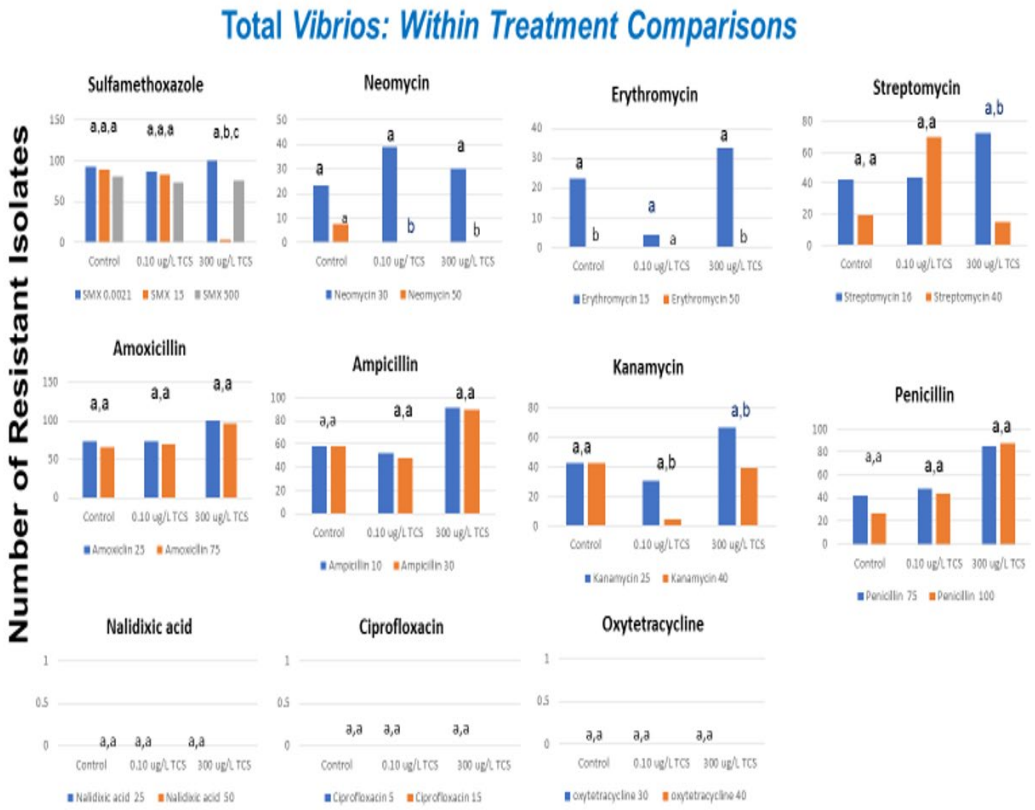

Figure 3.14 Number of resistant isolates from all *Vibrio spp*. within treatment comparisons. Treatments responded to 11 different antibiotics at multiple doses of each one to the grass shrimp after 24 h exposure to triclosan treatments.

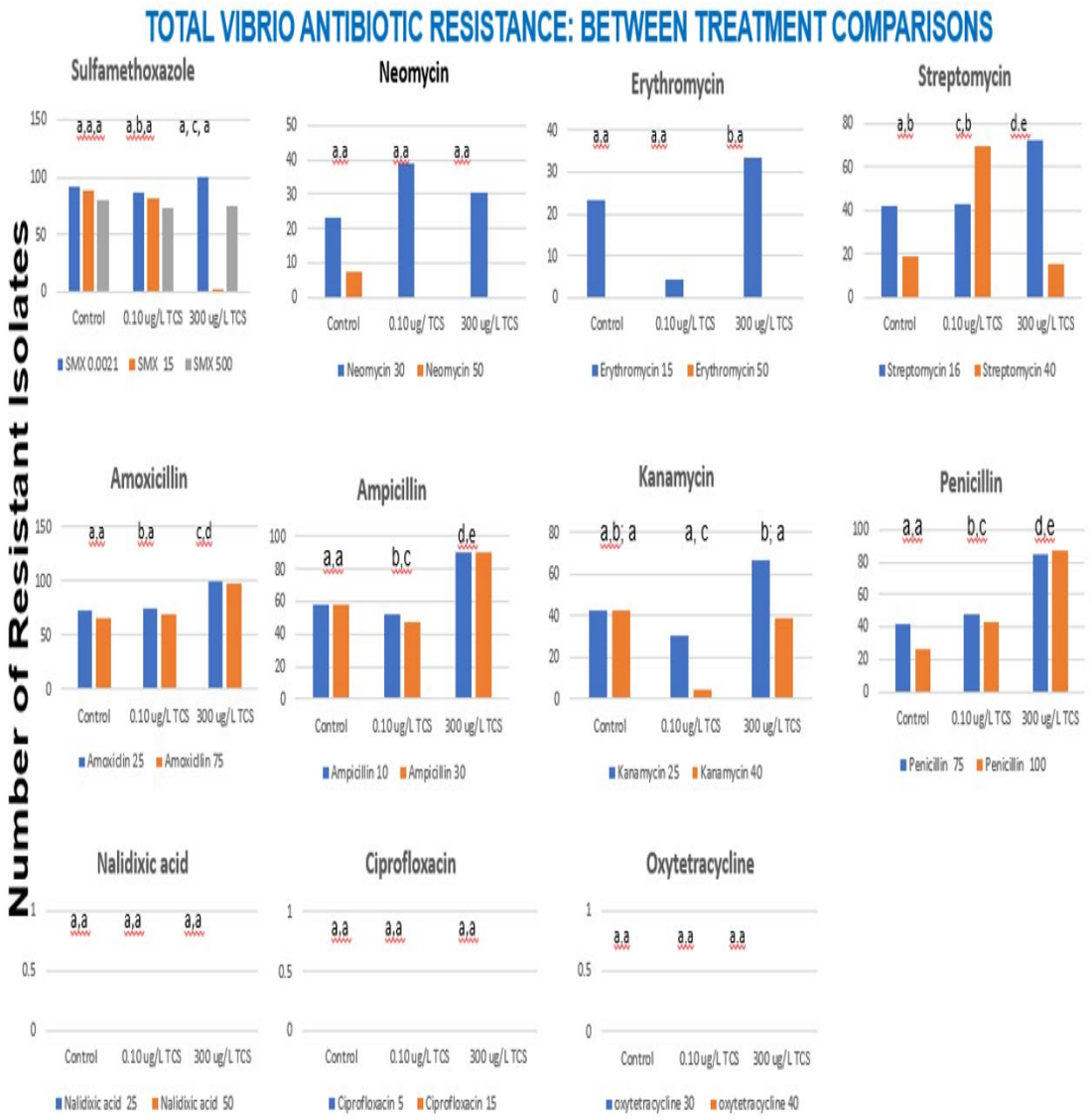

Figure 3.15 Number of resistant isolates from all *Vibrio spp*. between treatment comparisons. Treatments responded to 11 different antibiotics at multiple doses of each one to the grass shrimp after 24 h exposure to triclosan treatments.

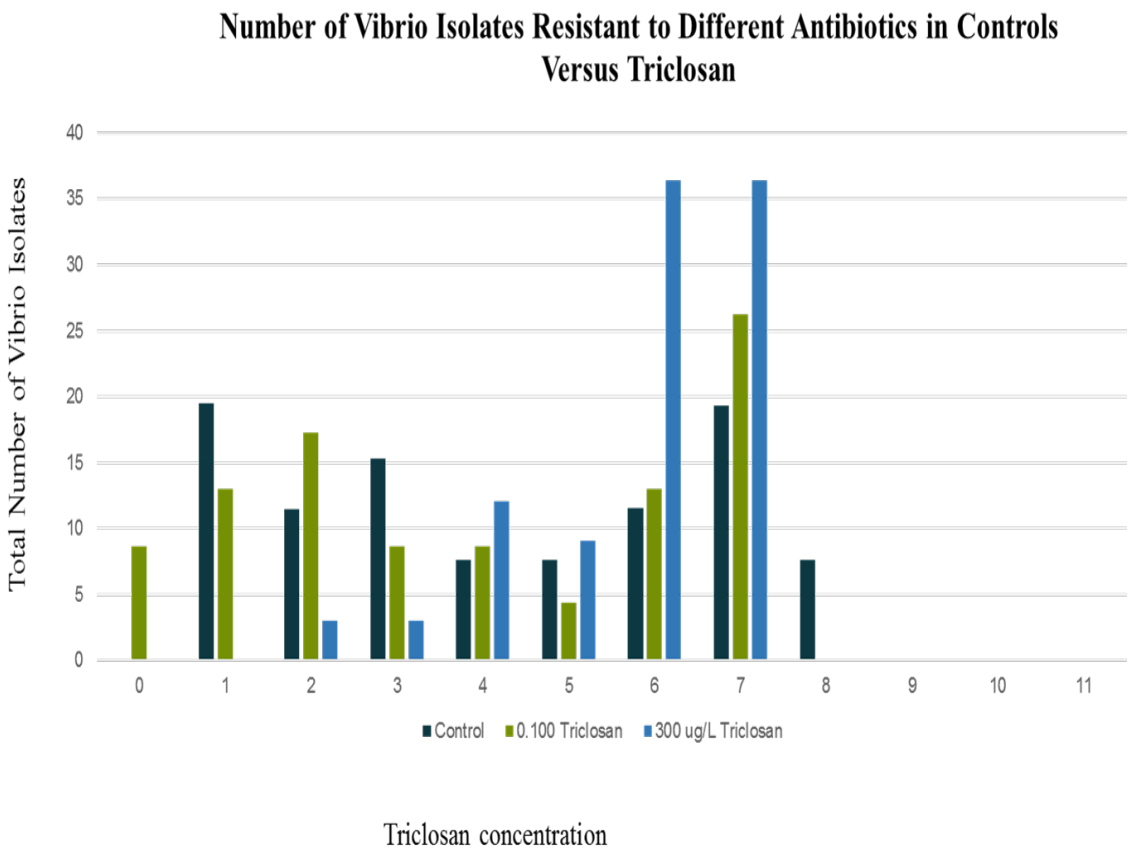

Figure 3.16 Number of vibrio *spp.* isolate resistance to different antibiotics in grass shrimp after 24-hour exposure to triclosan treatments.

CHAPTER 4

DISCUSSION

4.1 PBDE-47

PBDEs are the most important group of flame retardants, which contain a diversity of chemicals (WHO,1998). PBDEs have been shown to cause many adverse effects on aquatic life, wildlife and human health during last two decades (WHO,1988). PBDE-47 (2,2′,4,4′-tetrabromodiphenyl ether) is a major constituent of PentaPBDE, that were produced and used in North America to retard the flammability of polyurethane foam (Hale et al., 2003). PentaPBDEs have been detected in North America at levels up to 0.158 ng/L in surface waters, 2,290 ng/g in sewage sludge and 2100 pg/m^3 in outdoor air (Hale et al., 2003). Usually, PBDEs enter the environment by many routes such as manufacturing process, handling and recycling of treated products (Hale et al., 2003), and may be bioaccumulated by aquatic organisms. The concentrations of PBDEs detected in environmental water samples have been found to only be in pg/L amounts, while PBDE levels in river and marine sediments vary from ng/g to ug/g amounts (Watanabe and Sakai, 2003). Because of its high Kow PBDE-47 can be readily bioaccumulated by aquatic species. PBDEs were detected in sediment and caged mussels (*Elliptio complanata).* The sum of PBDE- 47, -99, -100, -153, -154 and -209 contributed between 92% and 96% of the total PBDE concentrations in all the mussel samples. Ratios of PBDE-47/99 and PBDE-100/99 and congener patterns in mussels and sediment were suggestive of the Penta formulation as the historical source (Richman et al.,2013). In

addition, PBDEs were detected in marine invertebrate muscle tissue of various crab, lobster and mussel species sampled along the French coast (Bodin et al., 2007). Some aquatic organism can bio-transform PBDE congeners including PBDE-47 like Spider crabs *(Maja brachydactyla*) (Bodin et al., 2007). Shrimp *Crangon* can also bioaccumulate PBDEs (Voorspoels et al., 2003). Hu et al. (2010) studied the concentrations of nine PBDE congeners including PBDE-47, in sixteen aquatic species collected from Baiyangdian Lake, North China. PBDE-47 was the predominant PBDE congener in most samples except for river snails and swan mussels. PBDEs had no impact on egg hatching rate of the marine copepod (crustacean) at any concentration tested (Wollenberger et al., 2005). Gandhi et al. (2017) studied the level of different polybrominated diphenyl ethers (PBDEs) in 18 fish species, PBDE-47 was the major congener in top predators. Once bioaccumulated, PBDE-47 may cause both acute and chronic toxicity to aquatic life.

There are limited studies on PBDE toxicity in other crustaceans. For example, Key et al. (2008) examined the effects of PBDE-47 at 25°C and 20psu, on adult and larval stages of the estuarine grass shrimp *P. pugio* and found 96h LC_{50} for larval shrimp of 23.60 µg/L (95% CI =14.51–38.37 µg/L). Adult shrimp were less sensitive with a much higher 96h LC50 of 78.07 µg/L (95% CI=65.1–93.63 µg/L). PBDE-47 was thus over three times more toxic to grass shrimp larvae than adult grass shrimp. There was 66.67% mortality in the highest concentration of 100.0 µg/L after 96h (Key et al.,2008). However, significant mortality was not observed in larval stages of the estuarine grass shrimp *P. pugio* until 72h after exposure. There was 87% mortality in the highest concentration of 100.0 µg/L after 96- h (Key et al., 2008). In the estuarine fish, *Fundulus heteroclitus*, an LC_{50} value could not be obtained for PBDE-47 as there was only 13.4%

mortality in the highest exposure of 0.1 mg/L (Key et al.,2009). Chiu et al. (2012) studied the toxic effects of PBDE-47 and PBDE-183 on a benthic oligochaete tubificid, *Monopylephorus limosus*. The LC_{50} values for PBDE-47 and PBDE-183 were 2,311 and 169 ng/g, respectively.

In this study, grass shrimp exposed to PBDE-47 were experienced greater mortality at the high temperature, high salinity conditions than under standard testing conditions. The 96h LC_{50} results for Standard Conditions (20˚C, 20 psu) resulted in a 96h LC50 of 201.48 µg/L (95% CI 184.15-218.80µg /L) compared to a 96-h LC50 of 31.30 µg/L (95% CI 28.69-33.92 µg /L) under Simulated Climate Change Conditions (30˚C & 35 psu) (Table 3.3). The toxicity under Climate Change Conditions was > 6.43 times more toxic than Standard Conditions. In evaluating our results and Key et al. (2008) there is a clear trend of increased toxicity with increasing exposure temperatures as LC50 values of 210.48 µg/L at 20˚C (this study), 78.07 µg/L at 25˚C and 31.30 µg/L at 30˚C in combination with higher salinity (this study). Given the fact that larval grass shrimp were > 3 times sensitive than adults (Key et al., 2008), effects of Climate Change Conditions may also affect larvae as well. There were also significant temporal differences in toxicity over time as climate change conditions of high temperature, high salinity led to immediate toxicity while toxicity was more gradual under Standard Conditions with significant mortality not occurring until 96h of exposure.

Many different aquatic organisms are highly sensitive to PBDEs such as PBDE-47. Crustaceans such as grass shrimp are highly sensitive to PBDE-47 as reported by Key et al (2008) for both adult and larval stages as well as in this study. Breitholtz and Wollenberger (2003) reported that adult benthic copepods, *Nitocra spinipes*, were less

sensitive than adult grass shrimp with only 42% mortality after 6 days exposure to concentrations as high as 130 µg/L PBDE-47; however just like the grass shrimp larval copepods stages were much more sensitive to lower exposure concentration (13-30 µg/L) than adults (Breitholtz& Wollenberger,2003). Fish embryo become more sensitive when exposed to PBDEs with respect to growth than in later stages (Chen et al., 2012). Exposure to PBDE-47 and PBDE-99 on embryo-larval stages of the marine flatfish turbot for 6 days, resulted in a higher toxicity of PBDE-47 compared to PBDE-99 (LC_{50} values for embryos and larvae, respectively,PBDE-47: 27.35 and 14.13 µg L−1; BDE-99: 38.28 and 29.64 µg L−1) (Mhadhbi et al.,2012). Significant larval mortality was not observed 72h after exposure to PBDEs on marine flatfish turbot, but at 96h, the mortality increased and reached statistical significance (Mhadhbi et al.,2012). The LC_{50} value was > 100 µg/L for the estuarine fish, *Fundulus heteroclitus* exposed to PBDE-47 at temperatures that ranged from 22.02 to 24.08°C and salinities that ranged from 19.62 to 21.39 ppt (Key et al.,2009). PBDEs caused a significant increase in embryo mortality for turbot (*Psetta maxima*), after just 48h. In addition, it caused a significant decrease in hatching success, malformations (embryos), *pericardial edema* and skeletal deformations (larvae) existed (Mhadhbi et al.,2012). Chen et al. (2012) indicated that 6-h post-fertilization zebrafish (*Danio rerio*) embryos, which were exposed to 1.25, 5, 20 µM concentrations of PBDE-47 had significantly affected spontaneous movement, decreased touch response and free-swimming speed, and altered larvae swimming behavior in response to light stimulation. Similarly, PBDEs impact on the development of marine copepod (*Acartia tonsa*) larvae which showed 50% inhibition when exposed to 13 µg/L BDE-47 for 5 days (Wollenberger et al., 2005). Key et al. (2009) found acetylcholinesterase (AChE) levels

on the estuarine fish, *Fundulus heteroclitus* were higher significantly at the PBDE-47 concentration of 12.5 ug/L compared with other compounds like simvastatin and irgarol. Fish have not been tested to see effects of increased temperature and salinity on PBDE-47 toxicity.

The time to mortality in aquatic species exposed to PBDEs shows that the compound generally is slow acting as mortality was not always immediate (Darnerud et al., 2001). The mortality was only about 33% after a 48-h exposure to 2000 µg/L PBDE-47 in marine copepod, *Acartia tonsa* (Wollenberger et al., 2005). Less than 50% mortality was observed in estuarine crustaceans like copepod *Nitocra spinipes* exposed to PBDE-47 (Breitholtz& Wollenberger,2003). Our results also support this finding as the onset of mortality was both temperature and salinity sensitive, as increased salinity and temperature caused the most rapid effects.

There are a few studied reported sublethal effects of PBDEs on crustaceans. Breitholtz and Wollenberger, (2003) studied the effects of three PBDEs on development, reproduction and population growth rate of the harpacticoid copepod *Nitocra spinipes*. Key et al. (2008) examined the effects of a polybrominated diphenyl ether (PBDE) compound, PBDE-47, on adult and larval stages of the estuarine grass shrimp *Palaemonetes pugio*, with assessed four sublethal physiological biomarkers glutathione (GSH), lipid peroxidation (LPx), cholesterol (CHL) and acetylcholinesterase (AChE).

Future studies of Climate Change interactions of temperature and salinity with PBDE-47 and other PBDEs should be conducted to examine more sublethal responses in aquatic species including fish and crustaceans. Our results clearly indicate that future climate

change conditions may enhance the toxicity of this particular PBDE, and it may similarly affect other PBDEs as well.

4.2 Ibuprofen

PPCPs are now one of the important emerging pollutants in the ecotoxicology field, because of their widespread use, persistence in the environment, and adverse effects on aquatic organisms (Henschel et al. 1997). IBU is generally considered one of the safest drugs used in medicine (Furey et al., 1992; DeArmond et al., 1995; and Kellenstein et al. 1999) and is on the World Health Organization's Model List of Essential Medicines (WHO, 2013). Ibuprofen tested at therapeutic doses extends life span of living organisms by 10-17% in yeast, nematodes and fruit flies and is thus considered an effective drug in humans a s a result as IBU use has reduced the occurrence of several chronic diseases (e.g. Alzheimer's, Parkinson's) (He et al., 2014). IBU is among the most consumed non-prescription medication worldwide (Mestre et al., 2007). Given its widespread use it is routinely found in raw sewerage and WWTP effluents and at concentrations ranging from ppt to ppb concentration range in the environment (Murdoch and Hay 2015). Additionally, disinfection of WWTP effluent with chlorine is inefficient (Westerhoff et al., 2005). IBU has a low solubility in water of 21 mg/L, a Log KOW of 3.97, a Bioconcentration Factor (BCF) of 3, and a half-life of 20 days (Bouissou-Schurtz et al., 2014). Chronic exposure of *Mytilus galloprovincialis* to IBU at an environmentally relevant concentration of 0.250 µg/L caused endocrine disruption and significant antioxidative metabolic stress including effects on superoxidase dismutase, catalase, glutathione reductase and phase II glutathione S-transferase after only 7 days of exposure (Gonzales-Rey and Bebianno 2012). Increased levels of lipid peroxidation and membrane

damage in the digestive gland of mussels were also observed (Gonzales-Rey and Bebianno 2012).

The acute toxicity of IBU has been shown in a number of aquatic invertebrates. Kim et al., (2009) studied acute toxicity of pharmaceutical and personal care products including IBU on freshwater crustacean *Thamnocephalus platyurus* and fish *Oryzias latipes*. The 24h median lethal concentration (LC_{50}) for *T. platyurus* was 19.59 mg/l, while the 96-hr LC50 values for the fish *O. latipes* was >100 mg/l. Other studies have reported IBU toxicity in invertebrate crustaceans such as *Daphnia magna* (0.5 to 50 ug/l) (Wang et al., 2016) and in vertebrates such as zebrafish (>10 μg L^{-1}) (David and Pancharatna, 2009) and Japanese medaka (1 to100 μg L^{-1}) (Flippin et al., 2007). Han et al. (2006) similarly reported a 48h LC50 value for IBU of 132.6 mg/L in *D. magna,* with a NOEC of 20 mg/L. Sung et al. (2014) studied effects of IBU to Green Neon Shrimp, *Neocaridina denticulate,* and reported 96 h LC_{50} values of 6.07 mg/L. The toxicity of ibuprofen in two crustacean species the copepod *Tisbe battagliai* and the shrimp *Atyaephyra desmarestii* resulted in 96h LC_{50} values of 49.7 mg/l for *T. battagliai* and 9.7 mg/l in *A. desmarestii* (Nieto et al, .2011).

Other studies have reported chronic sublethal effects of IBU exposure in aquatic species at lower exposure concentrations. De-Lange et al. (2006) found that ibuprofen concentrations of 0.001-0.100 μg/L decreased activity in the amphipod crustacean, *Gammarus pulex*. Wang et al. (2016) similarly investigated the influence of IBU at concentrations detected in the environment (0.5-50 μg/L) on expression of three genes involved in detoxification processes in *D. magna. Results indicated that effects on*

reproduction were observed including reductions in the total number of eggs, the total number of broods per female and body length.

Because of IBU toxicity in aquatic life, its occurrence, fate, risk and control in the aquatic environments have received great attention (Corcoran et al., 2010). Bouissou et al. (2014) conducted a risk assessment for PPCPs and found only five compounds had risk quotients (RQ = Lowest Concentration Causing Toxic Effect/Maximum Predicted Exposure Concentration) > 1 indicating potential for impacts on aquatic life including acetaminophen (RQ=1.6), IBU (RQ=600), diclofenac (RQ=15), oxazepam (RQ=2.1) and carbamazepine (RQ=3.2). Only IBU was identified as posing real environmental risk based on its actual maximum exposure concentration (MEC) (RQ=1.9). IBU was also listed on the Dirty Dozen List of CECs by the state of CA Expert Panel on CECs (Anderson et al., 2012). None of these previous assessments have included assessment of the effects of Climate Change on CECs such as IBU. Few previous studies link the effect of climate change (increased temperature and salinity interaction) with CECs (e.g. Delorenzo et al. 2009), particularly PPCPs such as IBU and especially in aquatic invertebrates such as the grass shrimp *Palaemonids pugio*.

In this study IBU was found to be more toxic at the higher temperature conditions under Simulated Climate Change Conditions (30°C & 20psu) compared to Standard Conditions (20°C, 20psu). The 96-h LC_{50} results for Standard Conditions resulted in a 96-h LC_{50} of 81.891 mg /L (95% CI 78.49-85.29 mg /L) compared to a 96-h LC_{50} of 32.69 mg /L (95% CI 31.11-34.24 mg/L) under high temperature Climate Change Conditions. Similarly, increased temperature and salinity in combination (30°C, 35 psu) was more toxic than Standard Conditions but was less toxic than high temperature conditions (30°C,

20 psu). High salinity Climate Change conditions were less toxic than Standard Conditions as well as high temperature conditions and combined high temperature, high salinity conditions. These findings indicate that increased temperature conditions associated with climate change will increase the acute toxicity of IBU.

Environmental stressors such as, temperature and salinity may produce additional stress to the organism. This can happen through deviation from the optimal conditions for an organism to function or by changing the physico-chemical behavior of contaminants within exposed organisms (Schiedek et al., 2007). IBU is a compound that was harmful to aquatic organisms at both temperatures tested (20°C and 30°C) in this study. High salinity did not increase acute toxicity but rather appeared to reduce toxicity, while high temperature increased IBU toxicity. Results in other species such as the shrimp *Atyaephyra desmarestii* exposed to IBU at 20°C and 25°C had 96h LC_{50} value and 95% confidence limits of 13.3 mg/L^{-1} (11.4–15.3 mg/L-^{1}) versus 10.1mg/L^{-1}(8.3–13.5 mg/L^{-1}) respectively (Nieto et al., 2016). Grass shrimp in this study were much more sensitive to effects from increased temperature stress. High temperature has also caused increased toxicity in other PPCPs such as Acetaminophen, Enrofloxacin and Chlortetracycline in *Daphnia magna* (Kim et al., 2010). With acetaminophen, toxicity increased 8.3-fold, from a 48 h EC_{50} of 39.7 mg/L at 15◦C to 4.8 mg/L at 25°C, using immobilization as the endpoint (Martins et al., 1013). Similarly, significant ($p \leq 0.002$) reductions in invertebrate biomass was highly correlated (rho = 0.810) with increasing concentrations of IBU and temperature was also the only other environmental parameter significantly related to invertebrate community abundance (Muñoz et al.,2009).

Field studies have shown that environmental concentrations of IBU in WWTP influent samples had higher concentrated concentrations of IBU metabolites compared to the parent compound. IBU metabolites may include carboxy ibuprofen and hydroxy ibuprofen, which were measured in both influent and effluent samples from a domestic WWTP (La Farre et al., 2008). This pharmaceutical has also been detected in the aquatic environments throughout the world. Corcoran et al. (2010) reported trace concentrations up to 0. 603 mg/L in raw wastewaters, up to 0.085 mg/L in treated effluents, and up to 0.005 mg/L in surface waters. Similarly other studies report surface waters IBU has been detected at the median concentration of 0.0098 mg/L (Canada) and ranges of 0.001–0.067 mg/L (Greece), < 0.015–0.414 mg/L (Korea), 0.005–0.280 mg/L (Taiwan), ND–0.008 ug/L (France), and ND–1.417 mg/L (China) (Almeida et al. 2013; Luo et al. 2013). Luo et al. (2014) reported the average concentration of IBU in groundwater for Europe is 0.00003 mg/L, with the maximal concentration of 0.00395 mg/L. In Europe, environmental concentrations of 0.00009 mg/L of IBU were measured in the Rhine at Main, Germany) (Ternes, 1998), 0.00237 mg/L in the Tyne Estuary in England (Thomas and Hilton, 2004) and to 0.0027 mg/L in the Catalonia region of Spain (Ferrer et al., 2001). Higher concentrations of IBU (0.300–3.000 mg/l) have been found in the effluent discharges from WWTPs and hospitals (Chen, 2008). In Germany, IBU was detected (3.350 mg/L) in sewage, lower concentrations of 0.010–0.500 mg/L in river water, and even lower concentrations of 0.000001-0.000006 mg/L were measured in drinking water (Stumpf et al., 1996). IBU was found in groundwater at much lower concentrations of 0.0002-0.0006 ug/L (Rabiet et al., 2006). Similarly, Ferrando-Clement et al. (2012) reported maximum IBU and related metabolites concentration ranging from 0.01374-

0.094 mg/L in WWTP influent versus concentration of 0.0019-0.0107 mg/L in in WWTP effluent. Total IBU concentrations for the parent compound and 5 different metabolites approached 0.0199 mg/L in WWTP effluent. Concentrations of 0.0039 mg/L were detected in aquatic environments (Ferrando-Clement et al. 2012).

The maximum IBU concentrations measured in the above studies was 3.9 mg/L in effluent and maximum surface water concentrations of 0.50 and 1.417mg/L in Europe and Asia, respectively. These concentration of IBU are well below concentrations causing acute toxicity in this study (e.g. NOEC < 27.5 mg/L). The Margin of Safety (NOEC/MEC) for these maximum concentrations measured in effluent is 55.8 and ranged from 7.1-19.8 in surface waters. When an uncertainty factor is applied of 10 is applied to these Margins of Safety estimates are 0.71 for effluent and 1.98 -5.58 in surface waters. However, streams in some regions of the U.S. and other parts of the world are > 90% effluent, particularly during extended dry weather periods such as drought such as in California (Anderson et al., 2012). Increased drought conditions in the future may possibly result in higher concentrations of PPCPs such as IBU in these effluent dominated streams that will begin to approach levels of concern under climate change conditions. In addition, the increased temperature conditions that may also co-occur under Climate Change Conditions would result in increased toxicity of IBU and possibly other PPCPs. These findings clearly underscore the importance of these Climate Change interactions with ibuprofen and suggest that future studies should address chronic toxicity Climate Change interactions such as effects on growth, reproduction and development in crustaceans.

4.3 Bifenthrin

Pyrethroids insecticides have been detected in a variety of environmental samples, including surface waters and sediments, but measured concentrations are typically not available for marine and estuarine waters (He et al., 2008). Pyrethroids are highly toxic to aquatic life when compared to other pesticides compounds, especially invertebrates. Evaluation of sensitive marine and estuarine species is essential for the development of toxicity testing and risk-assessment protocols. Estuarine crustaceans are particularly sensitive to pyrethroid exposure at low levels that may be encountered in the environment (DeLorenzo et al., 2014). Two estuarine crustacean species, *Americamysis bahia* (mysid shrimp) and *P. pugio* (grass shrimp), were tested with several commonly used pyrethroid compounds and adult and larval grass shrimp *P. pugio* were more sensitive than the mysids to all the pyrethroids tested, with acute toxicity observed in the low ng/L concentrations for some pyrethroids (DeLorenzo et al. 2014). Similarly, DeLorenzo and Fulton (2012) found that permethrin was the most toxic of several pesticides to marine and estuarine crustaceans, as diuron and chlorothalonil were 100-fold less toxic than permethrin. The toxicity of insecticide etofenprox was assessed, by exposing three grass shrimp life stages - adults, larvae, and embryos with resulting 96 h LC_{50} of 1260 ng/L for adults (DeLorenzo & De Leon, 2010). Duration of exposure also significantly affected insecticide toxicity, with lower toxicity values determined after 96 h versus 24 h of exposure (Key et al., 2005; DeLorenzo et al., 2006; Harper et al. 2008).

Bifenthrin was also reported by the State of California Expert Panel on Contaminants of Emerging Concern as one of three contemporary use pesticides posing significant ecological risks to aquatic life (Muraya et al., 2014). These findings clearly

indicate the importance of regulating the use of pyrethroid compounds within coastal zone environments. (DeLorenzo et al. 2014). Bifenthrin was the most toxic of four pyrethroids tested (bifenthrin, cyfluthrin, lambda cyhalothrin and tralomethrin) to *Ceriodaphnia dubia* (96 h LC_{50} = 70 ng/L) (Mokry and Hoagland 1990). The 96 h LC_{50} for the amphipod *Hyalella azteca* was 10 ng/L bifenthrin and sublethal effects on growth inhibition were observed at 6 ng/L (Anderson et al., 2015). Harper et al. (2008) similarly found that bifenthrin was super toxic to grass shrimp, *P. pugio*, with 96 h LC_{50} for adults of 20 ng/L (95% CI = 15-25 ng/L) and 13 ng/L (95% CI = 0 11–16 ng/L) for larval stages (20 ppt seawater at 25°C).

In mesocosm experiments, 24-h and 96 h caged grass shrimp LC_{50} values were 61 and 51 ng/L, respectively, as bifenthrin was found to be toxic to adult grass shrimp in salt marsh conditions at environmentally relevant exposure levels (Pennington et al., 2013). Results from this laboratory study are very similar to the Pennington et al. (2013) mesocosm study, with a 96 h LC_{50} value of 51.13 ng/L (95% CI 48.96-53.29 ng/L) determined for grass shrimp under standard conditions of 20°C and 20 psu.

Grass shrimp (*P. pugio)* were found to be more sensitive to bifenthrin exposure at 35 °C and 35psu, simulating future Climate Change Conditions, compared to Standard Conditions (20 °C and 20 psu) traditionally used for pesticide registration testing. These results were surprising since most studies have shown that pyrethroids have inverse temperature coefficients (EPA, 2010). However, several studies have found that pesticides were more toxic at the higher temperature simulating climate change than under standard testing conditions, due possibly in part to an increased metabolic rate at higher temperature, in increased water movement across the gills and increased pesticide

uptake as a result (DeLorenzo et al., 2009; 2014). For example, changes in temperature and salinity representing Climate Change altered the sensitivity of grass shrimp to the pyrethroid insecticide permethrin (DeLorenzo et al., 2012). Response to the changes in temperature and salinity will be different among aquatic organisms and different pesticides. Other classes of pesticides have also exerted higher toxicity under simulated Climate Change Conditions as chlorothalonil toxicity increased in grass shrimp exposed at 35 $^{\circ}$C as compared to those exposed at 25°C in both larval and adult life stages (DeLorenzo et al., 2012). Chlorothalonil toxicity also increased with increased salinity as compared to Standard Conditions but was significant ($p=0.026$) only after 96-h of exposure (DeLorenzo, & Fulton, 2012).

Our results not only indicated that combined increased temperature and salinity conditions associated with Climate Change generally increased the toxicity of bifenthrin as 96h LC_{50} values were significantly ($P \leq 0.05$) reduced under conditions of both increased temperature and increased temperature and salinity associated with Climate Change. Climate models clearly indicate increasing global temperatures and increasing sea level rise which will lead to more coastal flooding and increased salinities (IPPC, 2007, 2018; Union for Concerned Scientists, 2014). Our results indicate that the onset of toxic effects (LOEC and NOEC) levels were significantly affected, with 96 h NOECs and LOECs of 18 and 13.4 ng/L for Standard Conditions versus 96-h NOECs and LOECs of < 10 ng/L under Climate Change Conditions. These findings have direct implication for future pesticide risk assessments as EPA has recently conducted Pesticide Advisory Panels to address future climate change conditions (EPA, 2010). Recommendations form the EPA Panel included the inclusion of climate modeling and data on temperature and

sea level rise (salinity) into future pesticide risk assessments. Several studies have reported that increased temperatures may affect pesticide toxicity, for example organophosphates tend to be more toxic to beetles under warmer conditions (e.g., 30-32°C) (Grafius 1986, Turnbull & Harris 1986). Pyrethroids often manifest greater toxicity to arthropods at cooler ambient temperatures, typically around 15-16°C (Harris & Kinoshita 1977, Harris & Turnbull 1978, DeVries & Georghiou 1979, Hirano 1979, Sparks et a1. 1983, Grafius 1986, Turnbull & Harris 1986). Sparks et al. (1982) found that fenvalerate and deltamethrin had a negative temperature coefficient (i.e., more toxic at lower temperatures) when applied to the cabbage looper, *Trichoplusia ni* (Hubner), but had a neutral or positive relationship when applied to the fall armyworm, *Spodoptera frugiperda* (J. E. Smith), or the tobacco budworm, *Heliothis virescens* (F.). Thus, time and concentration as well as temperature and salinity may play crucial roles for pesticide risk assessment under future climate conditions. Estuarine organisms are exposed to a wide range of temperature and salinity; thus, it is important to examine the interaction between temperature, salinity, and pesticide toxicity (DeLorenzo et al., 2009), particularly with Climate Change and sea level rise exposure scenarios in future pesticide risk assessments**.**

4.4 Triclosan

Pharmaceuticals and personal care products (PPCPs) may include human veterinary medicines, human prescription drugs, biologics, diagnostic agents, fragrances, sunscreen agents, and other compounds. These compounds may enter aquatic environment as complex mixtures via a number of routes but primarily by both untreated and treated sewage (Daughton & Ternes, 1999). Pharmaceuticals have a specific mode of

action and often may persist within the body (Kolpin et al. 2002) and have been identified as potentially toxic chemicals in many aquatic environments ((Laura Martín-Díaz et al., 2009). Triclosan is an antimicrobial compound used in many personal care products and it is considered an environmental pollutant because of its widespread use and persistence in aquatic ecosystems (Kolpin et al., 2002; Fent et al., 2006; DeLorenzo, 2016). Hedgepeth et al. (2012) measured triclosan in surface waters of Charleston Harbor, SC which averaged 39.4 ng/L. DeLorenzo et al. (2008) also similarly measured triclosan in estuarine surface waters of SC including Charleston Harbor with a maximum concentration of 1 ng/L. Maximum surface water concentrations measured in the state of CA (Expert Panel on CECs) was 1,500 ng/L in ocean outfalls for WWTPs in southern California (Anderson et al. 2012).

DeLorenzo et al. (2008) assessed triclosan toxicity to three life stages of the grass shrimp *Palaemonetes pugio. P. pugio* larvae were more sensitive to triclosan than adult shrimp or embryos (Table 4.1) Acute aqueous toxicity values (96-h LC_{50}) were 305 µg/L for adult shrimp, 154 µg/L for larvae, and 651 µg/L for embryos. The presence of sediment decreased triclosan toxicity in adult shrimp (24-h LC_{50s} were 620 µg/L with sediment, and 482 µg/L without sediment. Comparisons of triclosan toxicity among different aquatic species indicated that the 24-h aqueous adult grass shrimp LC_{50} (452 µg/L) was lower than values in other studies for freshwater fish (e.g., the fathead minnow, *Pimephales promelas*, 24-h LC_{50} 5,360 µg /L and bluegill sunfish, *Lepomis macrochirus*, 24-h LC50 5,440 µg/L) (Orvos et al., 2002). Larvae of the amphibian *Acris crepitans blanchardii* have demonstrated sensitivity to TCS with a 96-h LC_{50} value of 367 µg/L during early development (Palenske et al., 2010). The 96-h aqueous median

triclosan lethal concentration (LC_{50}) for *Americamysis bahia* (mysid) and *Ampelisca abdita* (amphipod) were 74 and 73 mg/L, respectively (Perron et al., 20 12).

Table 4.1 Adult triclosan 24-96-h median lethal concentration (LC_{50}) values (µg/L) for various freshwater and estuarine crustaceans.

Species	Life Stage-Test	Toxicity value[1]	References
Palaemonetes pugio	Adult-aqueous	96-h LC_{50} = 305	DeLorenzo et al. 2008
	Adult-aqueous	24-h LC50 = 482	DeLorenzo et al. 2008
	Adult-sediments	24-h LC50 = 620	DeLorenzo et al. 2008
	Larvae-aqueous	96-h LC_{50} = 15	DeLorenzo et al. 2008
	Embryo-aqueous	96-h LC_{50} = 154	DeLorenzo et al. 2008
Americamysis bahia	*Mysid Shrimp*	48-h LC_{50} = 74.3	Perron et al. 2012
Ampelisca abdita	Amphipod	96-h LC_{50} = 73.4	Perron et al. 2012
Thamnocephalus platyurus	Artemia	24-h LC_{50} = 470	Kim et al. 2009
Oncorhynchus mykiss	Rainbow trout	96-h LC_{50} = 390	CIBA 1999
Pimephales promelas	Fathead minnow	96-h LC_{50} = 260	Orvos et al. 2002
Lepomis macrochirus	Bluegill sunfish	96-h LC_{50} = 370	Orvos et al. 2002

[1] = LC50 Values reported as ug/L in aqeous exposures and ug/kg dry in sediments

Results of this study have indicated that grass shrimp *Palaemonetes pugio* exposed to triclosan were found to be more sensitive under Climate Change exposure conditions of increased temperature and salinity conditions (30°C and 35psu) compared to standard conditions (20°C and 20psu). The LC_{50} for triclosan under Simulated Climate Change Conditions was 325 µg/L which was significantly ($P < 0.004$) lower than the 96-h LC_{50} of 580 µg/L (CL = 560-600 µg/l) under Standard Conditions (Table 3.9). Generally,

toxicity increased under all exposure conditions throughout the 96h of exposure and significant differences in treatment were observed at all exposure times.

Analysis of differences between high temperature and/or high salinity effects versus combined high temperature, high salinity conditions indicated that co-exposure to both temperature and salinity was more toxic. This suggest that triclosan in combination with increased temperature and salinity may have more interactive effects than just increased temperature or increased salinity alone. Increased salinity significantly reduced the toxicity of triclosan compared to standard conditions while increased temperature alone was not significantly different from standard conditions. However, increased temperature and salinity was significantly ($P< 0.05$) different from Standard Conditions at all exposure times (24-96h).

The effects of (PPCPs) on aquatic organisms could be much lower, and although animals are not obviously harmed by a contaminant. In addition, in natural ecosystems environmental impacts may often result from chronic low sublethal concentration exposures that cause metabolic stress that may lead to reduced fitness and health as well as mortality (Jensen and Bro-Rasmussen, 1992). It has been noted that some antibiotics such as Enrofloxacin and Chlortetracycline, are significantly toxic to aquatic life and many species may have increased toxicity with increasing water temperature, such as in *Daphnia magna* (Kim et al., 2010). Chronic, low level exposure to antibiotics and anti-microbial agents such as triclosan are extremely problematic as they may often lead to increased antibiotic resistance to aquatic microbes such as Vibrio bacteria that may adversely affect aquatic species such as shrimp and oysters (Huq et al., 1983; Vernberg et al., 1997) and pose a health hazard to humans. The additional stress caused by climate

change such as increased temperature, altered salinity, and decreased pH (e.g. increased acidity) on the toxicity of PPCPs is not well known. Furthermore, changes in these variables usually associated with Climate Change may change the transport, transfer, deposition and fate of contaminants (Macdonald et al., 2005) and resulting affect the toxicity of different classes of chemicals (Marquis et al., 2010). The ability of some species to adapt, which may lead to significant changes in the structure, function and services of ecosystems (Schiedek et al., 2007). This study has shown the combination of increased temperature and increased salinity that may result from climate change increased the toxicity of triclosan when compared to optimal growth conditions for grass shrimp.

Algae generally seem to be the most vulnerable taxon when exposed to triclosan with a 96-h EC50 value of 1.4 µg/L for *Scenedesmus subspicatus* based on reduced biomass (Orvos et al., 2002). Other studies with triclosan in fish have indicated that Increased exposure duration did not appear to increase the toxicity of triclosan in chronic exposures (e.g. *Danio rerio* - 9-day LC50 5,220 µ g/L) or *Oryzias latipes* -14-day LC50 5,400 µg/L) (Tatarazako et al., 2004). Elodie et al., (2017) studied effect of triclosan on early life stages of zebrafish up to 7 days post fertilization and found that Zebrafish exposed to 2, 20, 50 or 100 µg/L of triclosan had lower average mortality than was observed in control fish after 24 hours of exposure. Significant toxicity was only observed at the highest tested concentration. Similarly, Orvos et al., (2002) determined toxicity values for triclosan in fathead minnows, bluegill sunfish, and *Daphnia magna* of 260 µg /L (96-h LC50), 370 µg/L (96-h LC_{50}), and 390 µg/L (48-h EC_{50}), respectively. Exposure of the mussel, *M. galloprovincialis* to 300 ng/L of triclosan for 28-days resulted

in significant uptake of triclosan (Gatidou et al., 2010). The triclosan chronic LC50 values were 0.4 and 0.2 mg/L for10-day in the midge *Chironomus tentans* and the freshwater amphipod *Hyalella azteca*, respectively (Dussault et al., 2008).

Triclosan can accumulate and persist in aquatic ecosystems and my bioaccumulate in both vertebrate and invertebrate species (Orvos et al. 2002; Fair et al., 2009). While triclosan at measured environmental concentration may be acutely nontoxic to mammals there are different studies show that triclosan may affect other trophic levels in aquatic ecosystems, particularly affecting the microbial loop community at the base of most aquatic food webs. Microbes and algae may be most affected as EC_{50} values for algal species ranging from 0.7µg/L to 62.5µg/L (Orvos et al., 2002) and Microtox IC50 values of 150 ug/L have been reported in *Vibrio fischeri* (Scott et al., 2016).

Triclosan effects on the immune system of bivalves were assessed. For instance, different concentrations of TCS (2–100 mg/L) show that triclosan significantly reduced cell viability in the oyster *Crassostrea virginica* at the two highest tested concentrations (50–100 mg/L) (Lund et al., 2005). Triclosan can reduce the production of reactive oxygen intermediates (ROI) in hemocytes in various doses of triclosan from 2 to 10 mg/L for 4-h at 4°C in the oyster *Crassostrea virginica* (Chu et al. 2008). Triclosan modes of action (resistance) or toxicity have been demonstrated in some aquatic organisms but are not yet fully understood. Different evidence suggests that this personal care product exerts toxicity through effects on the thyroid axis, causing disruption and oxidative stress induction, leading to growth and developmental impairment as well as behavioral and reproduction effects (Oliveira et al., 2009; Schnitzler et al., 2016). Triclosan may also play a role in antibiotic resistance in microbes within the environment and cause toxicity

in aquatic organisms, like other antibiotics (DeLorenzo et al., 2016). It was also the only antibiotic/antimicrobial agent that posed a significant environmental risk based upon findings from the state of California Expert Panel on CECs, which listed it as one of 12 Dirty Dozen CECs and suggested triclosan monitoring should be required in future environmental assessments (Anderson et al., 2012).

Environmental concentrations of triclosan have been measured throughout the environment including freshwater in lakes, rivers and streams with concentrations ranging from 1.4–40,000 ng/L versus concentrations of <0.001–1500 ng/L in marine waters (SCCS, 2010; Dhillon et al., 2015). Similarly, sediment concentrations in freshwater lakes, rivers and streams ranged from <100–53,000 µg/kg dry weight (dw) versus 0.02–35 µg/kg dw in marine sediments (SCCS, 2010, Dhillon et al., 2015). Highest environmental concentrations have been measured in wastewater influent (20–86,161 ng/L), wastewater effluent (23–5370 ng/L), WWTP biosolids (20–133,000 µg/kg dw), and WWTP activated/digested sludge (580–15,600 µg/kg dw) (SCCS, 2010; Dhillon et al. 2015). Thus, greatest exposure to triclosan have generally been found downstream of WWTPs. The US Geological Survey detected triclosan in 57.6% of streams and rivers sampled in a nation-wide survey but at concentrations < 2,700 ng/L (Koplin et al. 2002). While the increased acute toxicity observed with triclosan in this study in grass shrimp under Climate Change Conditions occurred at concentrations (LC_{50} = 325 µg/L and NOEC =240 µg/L) well above maximum environmental exposure levels (2.700 µg/L), chronic effects on aquatic species at lower environmental concentrations remain a significant concern, particularly effects on microbial antibiotic resistance and toxic effects on algae. Orvos et al. (2002) reported a 96-h growth rate EC50 for triclosan

of 2.8 µg/L for the freshwater algae *Scenedesmus subspicatus*. Matozzo et al., (2012) studied effects of triclosan (TCS) on the immune parameters of the clam *Ruditapes philippinarum* were investigated after a 7-day exposure to sublethal triclosan concentrations (300, 600, and 900 ng/L). Similarly, grass shrimp and other crustaceans are known to house vibrio bacteria as a major microbial fauna in their gut microbiome (Uyagauri et al. 2008, DeLorenzo et al. 2016) and other antibiotics such as oxytetracycline have been shown to significantly increase the antibiotic resistance of these gut microbiome species within the grass shrimp. The effects of future Climate Change scenarios such as increased temperature and salinity changes may enhance this type of antibiotic resistance and may pose increased hazards to aquatic life and human use of estuarine ecosystems (Scott et al., 2016).

4.5 Bifenthrin-Triclosan Mixture

Aquatic organisms are exposed to a wide range of toxicants in the environment, and water quality criteria are derived from single compounds only, but disregard the effects for synergism, antagonism and additively (Altenburger et al., 1990; Drescher and Boedeke,1995). The interactivity of chemicals produces a toxic response on the aquatic environment, because water bodies are almost always contaminated with multiple toxicants (Faust et al., 1993). It has been found that estuarine waters analyzed for pesticides contain a mixture of pesticides (Scott et al., 1999; Key et al., 2003b). Due to widely uses and chemical properties, pesticides probably have ability to contaminate estuarine areas. Therefore, estuarine organisms, such as grass shrimp (*Palaemonetes pugio*), may be exposed to a pesticide mix instead of just individual pesticides. The toxicity of most compounds is generally studied under Standard Conditions for optimal

temperature and salinity conditions for the species being tested. Very few studies have examined both individual compound toxicity under the more rigorous conditions of high temperature and high salinity that may result from Climate Change. For grass shrimp Standard Conditions are typically 20°C and 20 psu. In this study, we further tested the effects of high temperature (30°C), high salinity (35 psu) and the combination of high temperature and high salinity (30°C, 35 psu) to Simulate Climate Change Conditions and how this may affect the toxicity of the contemporary use pesticide – bifenthrin and the pharmaceutical and personal care product – triclosan, individually and in mixture.

Numerous studies have evaluated the toxicity of pesticide mixtures. Key et al, (2007) examined the toxicity of three pesticides, (atrazine, fipronil and imidacloprid) singly and in mixture, to grass shrimp *(P. pugio*) larvae. In mixtures, fipronil plus atrazine and imidacloprid plus atrazine had no change in toxicity compared to fipronil and imidacloprid tested singly. However, exposure of grass shrimp (*Palaemonetes pugio*) larvae to the mixture of fipronil plus atrazine and imidacloprid plus atrazine had no change in toxicity compared to fipronil and imidacloprid tested singly. Similarly, a fipronil/ imidacloprid mixture did not show greater than additive toxicity, but when atrazine was added to the fipronil/imidacloprid mixture, greater than additive toxicity occurred.

Exposure to joint toxicity is mixture of two organophosphorus pesticides diazinon and chlorpyrifos to *Ceriodaphnia dubia* through 48- to 96-h exposure. The 48-h LC_{50} values ranged between 0.26 and 0.58 mg/L for diazinon and between 0.058 and 0.079 mg/L for chlorpyrifos. The 96-h values were approximately 65% of their respective 48-h

values. The LC_{50s} for the mixtures ranged between 0.89 and 1.46 TUs, with an average of 1.13 Tus (Bailey et al., 1997).

A study examining the toxicity of three mixture of the pesticide's atrazine, metolachlor, and fipronil, using 96 h survival bioassays with *Hyalella Azteca* were conducted ((Lizotte et al.,2009).). Significant mortality occurred in *H. azteca* with 96-h survival pesticide mixture effects concentrations ranging from 10.214–11.997, 5.822–6.658, 0.650–0.817, and 0.030–0.048 µg L-1 for atrazine, metolachlor, fipronil, and fipronil-sulfone, respectively. Significant differences ($p \leq 0.001$) were also detected among treatment classes for several the OP-herbicide mixtures (Lizotte et al.,2009). Both single toxicant and binary mixture bioassays were examined on nine commonly detected different pesticides chemical classes including organophosphate (OP) insecticides as well as triazine, triazinone, and substituted urea herbicides on the aquatic midge *Chironomus tentans* (Lydy & Austin, 2004). The toxicity of two of the organophosphorus pesticides diazinon and chlorpyrifos in mixture were additively toxicity to *C. dubia* (Bailey et al.,1997). The LC_{50s} for the mixtures for the 48- and 72-h exposure periods ranged from 0.79 to 1.14 TUs, with an average of 0.98 TUs. These values with unity suggest that the two pesticides were additive with respect to acute toxicity. Results for these two samples could not be calculated for the 24-h interval because less than 50% mortality occurred in the highest concentration of chlorpyrifos tested (Bailey et al.,1997).

Two of herbicides (atrazine and cyanazine) and an organophosphate insecticide (chlorpyrifos) toxicity has been estimated on fourth-instar larvae of the aquatic midge, *Chironomus tentans* (Jin-Clark et al.,2002). The mortality was 10% of midges in 48-h acute toxicity bioassays. exposure to 0.25 mg/L of chlorpyrifos. There was no significant

acute toxicity to *C. tentans*, when exposed to high concentrations (up to 1,000 mg/L) of atrazine or cyanazine alone. However, exposure to of atrazine and cyanazine caused significant synergistic effects on the toxicity of chlorpyrifos when midges were exposed to mixtures of atrazine or cyanazine (10, 100, 1,000 mg/L) with chlorpyrifos (0.25 mg/L) (Jin-Clark et al.,2002). The toxicity of chlorpyrifos was enhanced by 1.8- and 2.2-fold by atrazine and cyanazine, respectively, at the 50% effective concentration levels, at fixed concentrations (200 mg/L) of the herbicides (-Jin-Clark et al.,2002). Acute toxicity tests 96-h using *Chironomus tentans*, atrazine was also found to produce synergistic (greater than additive) toxicity in a binary mixture with methyl-parathion. Results suggest that the response addition model does not always accurately predict mixture toxicity for chemicals with differing modes of action (Pape-Lindstrom & Lydy, 1997).

Individual compound and mixture toxicity tests of antifouling biocides used in boat paints were analyzed with a battery of toxicity bioassays to evaluate the toxic effects of these compounds on *Vibrio fischeri*, *Daphnia magna*, and *Selenastrum capricornutum* (Fernández-Alba et al., 2002). They studied the toxicity of irgarol, individually and in binary mixtures with three other pesticides (the fungicide chlorothalonil, and the herbicides atrazine and 2,4-D), to the marine phytoplankton species *Dunaliella tertiolecta*. Irgarol in mixture with chlorothalonil exhibited synergistic toxicity to *D. tertiolecta*, with the mixture being approximately 1.5 times more toxic than the individual compounds. Irgarol and atrazine, both triazine herbicides, were additive in mixture. The toxicity threshold of 2,4-D was much greater than typical environmental levels and would not be expected to influencw irgarol toxicity.

Certain pesticide applications in the coastal zone may increase the toxicological risk to resident phytoplankton populations including sublethal effects. For example, chlorothalonil and 2,4-D exposure led to negative population growth rates, indicating cell death (DeLorenzo& Serrano, 2006). DeLorenzo& Serrano, (2006) found that the mixture of three pesticides irgarol, atrazine, and chlorothalonil have negative affect on *D. tertiolecta* population growth rate at environmentally plausible concentrations.

Irgarol in mixture with chlorothalonil exhibited synergistic toxicity to *D. tertiolecta*, with the mixture being approximately 1.5 times more toxic than the individual compounds. Irgarol and atrazine, both triazine herbicides, were also additively toxic in mixture exposures; however, the threshold for toxicity of 2,4-D was much greater than typical environmental levels and would not be expected to influence irgarol toxicity (DeLorenzo & Serrano 2006).

While these numerous studies describe above have assessed the toxicity of contemporary use pesticide mixtures, very few studies have tested for pesticide mixture effects with other CECs such as PPCPs. In addition, even fewer studies have evaluated these potential mixture effects und Climate Change Conditions. Results from this study show that Climate Change Condition generally were more toxic compared to standard conditions for both bifenthrin and triclosan both individually and in mixture. Bifenthrin was found to be more sensitive to the climate change conditions of increased temperature and salinity, with a 96-hour LC_{50} of 53.47 ng/L (95% CI 51.53-55.41 ng/L) at 20°C, 20 psu compared to a significantly ($p < 0.001$) lower 96-hour LC_{50} of 43.74 ng/L (95% CI = 41.60- 45.87 ng/L) at 30°C, 20 psu. High temperature conditions were significantly ($p \leq 0.05$) more toxic with a 96h LC50 of 0.033 (95% CL = 0.032-0.035 µg/L) than both

Standard Conditions and Climate Change Conditions of increased temperature and salinity. Bifenthrin exposures at high salinity conditions (20°C, 35 psu) were significantly ($p \leq 0.05$) less toxic than standard, increased temperature and climate change (high temperature and salinity) conditions. In mixture exposures, bifenthrin and triclosan were more toxic under Climate Change Conditions of increased temperature and salinity. Both mixture ratios of triclosan: bifenthrin (Mixture 1 – based on individual toxicity tests under standard conditions and Mixture 2 – based on individual toxicity tests under Climate Change Conditions) were more toxic under conditions of increased temperature and salinity conditions.

Little is known about the fate of pharmaceutical compounds in the aquatic environment. Low concentrations of these bioactive compounds may have significant effects on aquatic organisms (Halling-Sørensen et al., 1998). Normally, aquatic organisms are exposed to complex mixtures of pharmaceuticals at low concentrations (Kolpin et al., 2002; Calamari et al., 2003). Many of pharmaceutical compounds like diclofenac DCF can be categorized as a toxic compound whereas carbamazepine CBZ and IBU are compounds that are harmful to aquatic organisms at both temperatures (Trombini et al., 2002). There are two characteristics that may make the joint toxic effect of a pharmaceutical mixtures a major issue for hazard and risk assessment. The ecotoxicity of a pharmaceutical mixture is typically higher than the effects of each individual component and such a mixture can have a considerable ecotoxicity, even if all individual pharmaceuticals are present only in low concentrations that do not provoke significant toxic effects if acting singly on the exposed organisms (Backhaus, 2014).

Results of this study have indicated that grass shrimp *Palaemonetes pugio* individually exposed to triclosan were found to be more sensitive under Climate Change exposure conditions of increased temperature and salinity conditions (30°C and 35psu) compared to standard conditions (20°C and 20psu). Toxicity tests with triclosan individually, indicated that the LC_{50} for Simulated Climate Change Conditions of high temperature and high salinity was significantly ($p \leq 0.05$) more toxic than high salinity, high temperature or Standard Conditions, with a 96 h LC_{50} of 320µg/L (95% CL = Not Calculatable) compared to an LC_{50} of 580µ g/L (95% CL = 560-600 µg/L) under Standard Conditions, > 1,000µ g/L (95% CL = Not Calculatable) for high salinity and 500 ug/L (95% CL = 479-520 µg/L) for high temperature conditions. Only Climate Change Conditions of high temperature and high salinity increased the toxicity of triclosan, with high salinity *per se* reducing its toxicity while high temperature *per se* had no effect on grass shrimp survival.

The presence of these pharmaceuticals in the environment may be used as indicator to predict aquatic ecosystem effects. For example, mixture of fluoxetine, ibuprofen and ciprofloxacin in experimental freshwater communities were studied (Richards et al., 2004). They evaluated the aquatic toxicity of individual pharmaceuticals and mixtures with *Daphnia magna*, a common freshwater zooplankton, for acute (6-day) effects. Exposure to a single pharmaceutical in the 1–100 µg/l range yielded no apparent effects on the normal life processes of *D. magna*. A mixture of fluoxetine (36 µg/l) and clofibric acid (100 µg/l) caused significant mortality; the same fluoxetine concentration mixed with 10 µg/l clofibric acid resulted in significant deformities, including malformed carapaces and swimming setae. Aquatic toxicity of pharmaceutical mixtures can be

unpredictable, and complex compared to individual pharmaceutical effects and timing and duration of pharmaceutical exposure influence aquatic toxicity (Flaherty and Dodson,2005). A mixture of seven common pharmaceutical agents (acetaminophen, diclofenac, gemfibrozil, ibuprofen, naproxen, salicylic acid, and triclosan) was tested for their effects on the freshwater amphipod *Hyalella azteca* over three generations. The concentration of each chemical (100 ng/L) was representative of the upper range observed for these substances in Canadian fresh waters, except in the immediate vicinity of effluent discharges (Borgmann et al., 2007).

Pharmaceutical compounds, such as acetaminophen, enrofloxacin and chlortetracycline, may significantly increase their toxicity to *Daphnia magna* as water temperature increases (Kim et al., 2010). Significant mixture exposure may be observed in areas closer to industrial and wastewater treatment plant effluent discharges where mixtures of pharmaceuticals and other chemicals are more likely to occur; however, in this study significant mixture effects of the seven pharmaceutical tested were not observed. These chemicals do not, therefore, appear to be substances of major concern for Hyalella in most Canadian fresh waters (Borgmann et al., 2007). Acute exposure to triclosan (1, 10, 100 µg/l) yielded no significant effects on survivorship, morphology, ephippium production, fecundity, or sex ratio. Chronic exposure to 10 µg/l triclosan significantly increased sex ratio of the first brood only (Flaherty et al., 2005). Acute exposure to erythromycin (10 and 100 µg/l), lincomycin (1 and 10 µg/l), sulfamethoxazole (10 and 100 µg/l), or trimethoprim (10 µg/l) yielded no detectable effects on survivorship and morphology of adults or neonates, ephippium production, fecundity, or sex ratio of Daphnia (Flaherty et al., 2005).

Acute exposure to a pharmaceutical mixture of 36 µg/l fluoxetine and 100 µg/l clofibric acid, concentrations that yielded no apparent effects when tested individually, caused significant mortality. On average, 62.5% of Daphnia exposed to this pharmaceutical mixture died by day 6, compared to a 10% mortality rate among control Daphnia. Acute exposure to fluoxetine (36 µg/l) and a lower concentration of clofibric acid (10 µg/l) resulted in the presence of morphological abnormalities in an average of 19% of Daphnia (Fisher combined $P < 0.001$ (Flaherty and Dodson,2005). Pharmaceuticals compounds have negative effects on aquatic animals. A microcosm study with a mixture of ibuprofen, fluoxetine and ciprofloxacin showed significant mortality in fish and macrophyte, and increased abundance and decreased diversity in both phytoplankton and zooplankton communities after chronic exposure (renewal of concentrations every 48 h for 5 weeks) to concentrations between 60 and 100g/L of the individua pharmaceuticals (Richards et al., 2004).

Assessment of the effect of three pharmaceuticals, the antidepressant fluoxetine, the analgesic ibuprofen (IBU) and the anti-epileptic carbamazepine, and one cationic surfactant, cetyltrimethylammonium bromide (CTAB), on the activity of the benthic invertebrate *Gammarus pulex* (Crustacea: Amphipoda: Gammaridae) was conducted (De Lange et al., 2006). Exposure to low concentrations (10–100 ng/L) of fluoxetine and ibuprofen resulted in a significant decrease in activity, whereas the activity of *G. pulex* at higher concentrations (1µg/L-1 mg/L) was not different from controls (De Lange et al., 2006). The results obtained in the comparison between measured and predicted mortality for the diclofenac (DF)+ ibuprofen (IBU) mixture at different temperatures found that at 20°C lower mortality was observed than that predicted by the Concentration addition

(CA) model, whereas at 25°C the observed mortality is higher than that predicted at the lowest exposure concentrations tested. In the higher concentrations tested the effect observed in the mixture seems to fit the CA model. At 25°C the observed mortality values do not fit either of the models at the lowest concentrations, although a trend towards the CA model is observed at the highest concentrations. Similarly, for diclofenac (DF) + carbamazepine (CBZ), at low concentrations the observed data does not fit any model at 20°C. However, at 25°C the curve seems to fit the independent action (IA) model better. For ternary mixtures, a different behavior for mortality is observed with respect to the curves described by both models. At 20°C the experimental curve shifts more towards the CA prediction at low concentrations, whereas at high concentrations, the curve is closer to that predicted by IA. At the increased temperature (25°C), at both low and high concentrations, the CA model is the one that better predicted the combined effects of the mixture (Nieto et al., 2016).

Studies of the lethal and sub-lethal toxicity of three pharmaceutical compounds, Diclofenac (DF), Ibuprofen (IBU) and Carbamazepine (CBZ) on the shrimp, *Atyaephyra desmarestii,* both individually and in mixture under two temperature 20°C and25°C were conducted. LC_{50}(96-h) values were obtained individually at 20°C and 25°C for each compound and mixture. At 25°C, mortalities in binary and ternary mixtures were higher than at 20°C (Nieto et al., 2016).

Mixture toxicity of the compounds could be accurately predicted using simple predictions additivity for each compound (Cleuvers, 2004). While acute exposure to 10 µg/l erythromycin, triclosan, or trimethoprim individually yielded no apparent effects on Daphnia development or reproduction, an antibiotic mixture of these three

pharmaceuticals (total antibiotic concentration 30 µg/l) elicited a significant decrease in sex ratio (Flaherty and Dodson,2005). Acute exposure to 1 and 10 ug/l each of erythromycin, triclosan, trimethoprim, lincomycin, and sulfamethoxazole (total antibiotic concentrations of 5 and 50 µg/l, respectively) did not significantly alter Daphnia sex ratio (Flaherty and Dodson,2005). Exposure to 100 µg/l of each antibiotic (total antibiotic concentration of 500 µg/l) significantly increased the number of males produced. Because of advances in analytical techniques, there has been an increased awareness of the widespread distribution of low concentrations of pharmaceutical compounds in the aquatic environment (Daughton and Ternes, 1999; Kolpin et al., 2002; Metcalfe et al., 2003). While these above studies have assessed the toxicity of PPCP mixtures under standard conditions, very few studies have assessed effects under increased temperature and increased salinity conditions resulting from climate change.

Mixture exposures of triclosan and bifenthrin were more toxic under Climate Change Conditions than standard conditions. The LC_{50} for this mixture under Simulated Climate Change Conditions of high temperature, high salinity was more toxic, with a 96h LC_{50} of 325 µg/L compared to an LC_{50} of 580 µg/L under Standard Conditions. Two mixture of triclosan and bifenthrin were tested in this study with differing ratios of triclosan: bifenthrin based upon individual tests under standard conditions (Mixture 1) and climate change conditions (Mixture 2). Each of these mixtures were then tested under Standard and Climate Change Conditions. Results indicated that Mixture 2 (based upon toxicity for each individual chemical under Climate Change Conditions) was more toxic than Mixture 1 ((based upon toxicity for each individual chemical under Standard Conditions) under both Standard and Climate Change Conditions. The triclosan and

bifenthrin Mixture 2 based upon Climate Change Conditions approached levels that were more than additively toxic (Additive Index = 1.00) with an Additive Index of 0.99 versus 1.32 for the Standard Condition Mixture. This underscores the importance of future toxicity test design for mixtures under Climate Change conditions. Should mixture ratios for each chemical be based upon individual toxicity tests under Standard Conditions of based upon Climate Change Conditions. Our results clearly indicated that the Mixture 2 ratio of triclosan: bifenthrin was more toxic than the mixture ratios of triclosan: bifenthrin used in Mixture 1. Future mixture studies assessing Climate Change effects should consider these findings when selecting mixture ratios to test, as our results clearly showed that mixture ratios based upon individual toxicity test done under Climate Cchange Conditions enhanced the additive toxicity of these two compounds.

4. 6. Triclosan Effects on the Antibiotic Resistance in Vibrio Bacteria

Global Climate Change plays a big role in increasing and spreading Vibrio worldwide by the variations in the weather that introduce warmer waters into colder regions and alter the salinity profile along the longitudinal axis of coastal rivers (Baker-Austin et al. 2016). Climate Change may thus affect both physical and chemical factors such as, temperature, sunlight, pH, salinity and organic matter, and O2 content, which may change drastically, both temporarily and spatially, and influence viability, abundance and ecology of bacteria (Murphree & Tamplin ,1995). Increasing the sea surface temperatures may help the habitat and geographical range of pathogens to expand (Austin et al., 2013; Vezzulli et al., 2016).

Triclosan is one of the most common antimicrobial compounds found in PPCPs entering surface waters in the U.S. (Arpin-Pont et al., 2016). TCS can damage the cell by causing the cell contents to physically leak out of the membrane, in a (0.1–0.3 w/v%). This TCS mechanism of action is wide spectrum, showing activity against many microorganisms such as gram-positive and gram-negative bacteria, some fungi, and to some extent, mycobacteria (Saleh et al., 2010). Minimum inhibitory concentrations of TCS for different bacterial strains range between 10 and 3,000 µg/L (Bhargava and Leonard 1996). Therefore, it could have an effect on the number of beneficial intestinal bacterial strains (probiotic), that help with survival and growth of aquatic animals and reduce disease outbreaks (Zhang et al,.2009). Lately, probiotics have been used in the aquaculture field to control the spread of disease, enhance aquatic species immune response, and improve water quality (Zhang et al,.2009). Oxytetracycline is one of the few antibiotics approved for shellfish aquaculture of shrimp. Grass shrimp exposed to the higher than 250 mg/L OTC caused significant mortality in intestinal bacteria. This had a significant effect on digestive tract bacteria survival at or above the level of the acute no-observable-effect concentrations for grass shrimp (Uyaguari e et al,.2009). Exposure to OTC showed a significant positive growth for *Vibrio alginolyticus*, while over time, another bacterial species abundance declined (Uyaguair et al,.2009). However, lower concentration of OTC (1, 16, and 32 mg/L) did not show significant bacterial mortality (Uyaguari et al,.2009). There were no differences in total bacterial population densities for shrimp exposed to lower concentration of 1, 16, and 32 mg/L OTC and control (p. 0.05) (Uyaguari et al,.2009).

Vibrio species have been reported to be the dominant species in the digestive tracts of grass shrimp (Uyaguari et al., 2009). The most abundant species after OTC exposure were *V. alginolyticus* (26.1%), *A. hydrophila* (19%), *V. vulnificus* (13.8%), *V. cholerae* (5.6%), and *V. parahaemolyticus* (3.7%), with Vibrios accounting for more than 68% of all isolates (Uyaguari et al,.2009). Time plays an important role to reduce *vibrio spp.* growth during increased exposure to OTC concentrations. For example, *V. vulnificus, V. parahaemolyticus, V. cholerae*, and Vibrio spp. concentrations changed compared to the control. However, another Vibrio, *V. alginolyticus*, dramatically increased compared to the control (Uyaguari et al,.2009).

Vibrio bacteria are naturally widespread, living in certain coastal waters. These bacteria are detected in tissues and/or organs of many marine species such as, algae and animals, e.g., abalones, bivalves, corals, fish, shrimp, sponges, squid, and zooplankton (Thompson & Swings, 2004). The genus Vibrio includes more than 30 species, and many are pathogenic to humans and/or have been associated with food-borne diseases (Chakraborty et al., 1997). Vibrio bacteria are widely distributed in all environments throughout the world; especially in warmer waters, notably when temperatures rise above 17˚C and, depending on the species, tolerate a range of salinities (Wright et al., 1996). There are three main Vibrio spp.: *Vibrio cholera, V. vulnificus*, and *V. parahaemolyticus*, which are considered foodborne pathogens that cause illness through raw seafood consumption as well as wound infections. These three strains are an important pathway for pathogens to both humans and marine organisms (CDC, 2016). *Vibrio parahaemolyticus* is an important foodborne pathogen for humans. It transfers to human by consumption of raw or undercooked seafood, mainly shellfish, with many symptoms

such as acute gastroenteritis characterized by diarrhea, headache, vomiting, nausea, and abdominal cramps (Su & Liu, 2007). *V. parahaemolyticus* has been found in freshly caught shrimp. Vibrio can be detected in all life stages of culture shrimp (Cann et al., 1981).

Results from this study indicated that *Vibrio spp*. abundance in the lower dose of 0.1µg/l (MEC) dose of triclosan was significantly reduced compared to controls and higher doses of 300 µg/l (MIC) compared to the control. Delorenzo et al (2016) similarly reported that grass shrimp exposed to TCS had significantly increased *Vibrio spp*. density which was 351% higher within grass shrimp exposed to 0.33 mg/L TCS when compared to controls. Vibrio spp. density were reduced by 78% and 81% with grass shrimp exposure to 30 mg/L and 60 mg/L Sulfamethoxazole (SMX), respectively (DeLorenzo et al.,2016). Similarly, a mixture of triclosan (TCS)/SMX and a mixture of Oxytetracycline (OTC)/Erythromycin (ERY)/SMX reduced the Vibrio spp. density by 80% and 94%, respectively (DeLorenzo et al.,2016). Further analysis of the V*ibrio spp*. results shows that after exposure to TCS, the majority of the population was composed of *Vibrio parahaemolyticus* compared with another *Vibrio spp.*

Analysis of antibiotic resistance in Vibrio bacteria from the grass shrimp microbiome to was found in all treatments including both triclosan and the controls. Generally, the exposure of grass shrimp to antimicrobial TCS caused an increase in multiple antibiotic resistance in the shrimp-associated Vibrio bacteria. Statistical analysis indicated there was significantly was greater antibiotic resistance in the high triclosan dose (MIC) when compared to controls. The lower triclosan dose (MEC) was not significantly different from controls but was significantly different from the high dose of

triclosan (MIC). Higher doses of triclosan (MIC) also increased the number of isolates resistant to 6 or 7 different antibiotics and a greater proportion of Vibrio bacteria had increased multiple antibiotic resistance (> 6 antibiotics) in both the low (MEC) and high (MIC doses when compared to controls. Only 31% of control isolate had resistance to > 6 different antibiotics compared to 41% of isolates at the low exposure dose of triclosan (MEC) and 74% of isolates at the high exposure dose of triclosan (MIC). Conversely 45% of control isolates and 46% of the low dose triclosan exposure group (MEC) were resistant to $\leq$ 3 antibiotics compared to only 5% of the high dose triclosan exposure group (MIC). This is clear evidence that increasing triclosan exposure will increase the amount of multiple antibiotic resistance in Vibrio bacteria. Vibrio bacteria have been shown to be highly sensitive to practically all antimicrobials (Jones & Oliver, 2009). Other studies have shown similar effects as both SMX and TCS have a significant increase in resistance as compared to the control (DeLorenzo et al.,2016). Other studies with grass shrimp isolates exposed to 1, 16, and 32 mg/L OTC showed an average resistant value of 13% after 24h; however, after 48h of exposure the values were 6.67% for 16 mg/L and O% from the 1 mg/L exposure concentration (Uyaguari et al,.2009). At higher OTC concentrations of 32 mg/L after 96h of exposure, bacterial resistance was significantly ($p < 0.0056$) higher than the lower OTC exposures (1 and 16 mg/L) and controls (Uyaguari et al,.2009).

Antibiotic concentrations range in the soil or water environment from about a few nanograms to hundreds of nanograms per liter or kg of soil, which are detected in areas with high levels of anthropogenic inputs such as hospital and waste water treatment plant effluents as well as confined feeding animal operations in livestock and aquaculture

operations (Verlicchi & Zambello,2015). As a result, extreme use of antibiotics throughout time by human agriculture and aquaculture systems, antimicrobial resistance has emerged in many bacterial genera (Cabello, 2006). In a study of antimicrobial sensitivity, it was determined that in 100 strains of Vibrio isolated from the *Litopenaeus vannamei* shrimp, there was increased antimicrobial resistance. This antimicrobial resistance was confirmed in all *Vibrio spp*. including *V. parahaemolyticus* and *V. cholerae* (Albuquerque Costa et al,.2015).

Differences in MEC and MIC exposures with triclosan were very evident in this study and future studies should similarly use both MEC and MIC concentrations to fully assess the ecotoxicological hazards and risks. For Vibrio bacteria from controls, low dose (MEC) and high dose (MIC) triclosan exposed grass shrimp microbiome, which were then exposed to the MEC concentration of each of the 11 different antibiotics, there were only five antibiotics (46%) that altered growth and survival, increasing antibiotic resistance (Ampicillin, Amoxicillin, Kanamycin and Penicillin, Streptomycin) compared to six antibiotics (Ampicillin, Amoxicillin, Kanamycin and Penicillin, Streptomycin and Sulfamethoxazole) for the MIC concentration of each of the 11 antibiotics.

Of these six antibiotics there were different responses including significantly ($p < 0.05$) increasing antibiotic resistance with increasing triclosan dose (e.g. Amoxicillin and Penicillin); decreasing antibiotic resistance with increasing triclosan dose (e.g. Sulfamethoxazole and Streptomycin) or to both increase antibiotic resistance at low dose triclosan exposure and to decrease antibiotic resistance at high dose triclosan exposure (Ampicillin and Kanamycin). This is clear evidence that Vibrio bacteria in the environment may react differently from Vibrio bacteria within humans, as the difference

in dose may cause differing responses in terms of antibiotic resistance. Future studies with other antibiotics should more fully address these differences between MEC and MIC responses among other classes of bacteria and under climate change conditions of increased temperature.

CHAPTER 5

CONCLUSION

Increasing temperatures and salinities are direct impacts associated with Climate Change and increased sea level rise associated with it. These environmental changes will affect the ecotoxicology of chemical contaminants found in coastal ecosystems and will affect the health and well-being of aquatic organisms and in turn may increase public health threats. Results of this study have clearly shown that increased temperature and or the combination of increased temperature and salinity will significantly increase the toxicity of selected CECs in the grass shrimp, *P. pugio.* The specific conclusions form these studies (Table 5.1) have indicated that:

5.1 PBDE-47

1. The combination of high temperature and salinity altered the toxicity of the brominated flame retardant, PBDE-47, increasing the toxicity of this compound by a factor of 6.4 in the estuarine adult grass shrimp, *Palaemonetes pugio* compared to Standard Conditions.

2.In addition, Climate Change Conditions of high temperature, high salinity altered the onset of mortality, resulting in a much more rapid onset of significant mortality within the first 24 hours of exposure that was not observed until 96h of exposure under standard conditions (20°C, 20psu).

3. High salinity *per se*, did not significantly increase the acute toxicity of PBDE-47.

4.This alteration of the toxicity of PBDE-47 caused by high temperature, high salinity conditions associated with climate change should be further tested with other life history stages of invertebrates and with other PBDE isomers to more fully examine how climate change may affect this class of contaminant.

5.2 Ibuprofen

4. The combination of increased temperature and salinity altered the toxicity of ibuprofen (IBU) with increased temperature enhancing the toxicity of ibuprofen by a factor of 2.5 to the estuarine adult grass shrimp, *Palaemonetes pugio*, under Simulated Climate Change Conditions (30°C & 20 psu) when compared to Standard Conditions (20°C &20psu).

5. In addition, exposure to high temperature *per se* (30°C, 20psu), resulted in increased IBU toxicity to adult grass shrimp when compared to combined increased temperature and salinity conditions (30°C, 35psu), clearly indicating that high temperature conditions associated with climate change significantly increased the toxicity of IBU. Thus, the interaction of increased temperature and salinity was less toxic than high temperature *per se.*

6. High salinity only significantly increased the toxicity of IBU when co-occurring with increased temperature and decreased its toxicity at lower temperatures under Standard Conditions. Thus, high salinity conditions were less toxic than high temperature *per se*.

5.3 Bifenthrin

7. Climate Change combinations of increased temperature and salinity conditions (30°C & 35 psu) enhanced the toxicity of the pyrethroid insecticide bifenthrin by a factor of 1.5 to adult grass shrimp, *P. pugio*, compared to Standard Conditions (20°C & 20 psu).

8. Increased temperature (30°C) *per se*, enhanced the toxicity of bifenthrin at both 20 and 35 psu salinities while high salinity (35 psu) conditions *per se* significantly decreased the toxicity of bifenthrin.

9. Climate Change effects associated with increased fossil fuel combustion and increased CO_2 emissions have the potential to increase both global temperatures, alter precipitation patterns, and increase the rate of sea level rise resulting in higher temperatures and salinities in coastal areas, which may increase the toxicity of chemicals such as bifenthrin. Future risk assessment should include testing of other classes of high use pesticides under Simulated Climate Change Conditions to fully protect coastal ecosystem health.

5.4 Triclosan

10. The combination of high temperature and high salinity altered the toxicity of antimicrobial triclosan enhancing the toxicology of triclosan by a factor of 1.8 to the estuarine adult grass shrimp, *Palaemonetes pugio*, under Simulated Climate Change Conditions (30°C,35 psu) when compared to standard conditions (20°C &20psu).

11. High salinity alone significantly reduced the toxicity of triclosan compared to standard conditions while high temperature alone was not significantly different from standard conditions. Both high salinity conditions and high temperature conditions were significantly different from combined high temperature, high salinity conditions.

12. This increased toxicity under Climate Change Conditions suggests that triclosan may hay have the potential to also enhance sublethal effects in grass shrimp as well.

13. Future studies should address sublethal effects such as growth, reproduction and other heath indicators (e.g. changes in the microbiome) under Climate Change Conditions of

increased temperature and salinity to fully understand Climate Change interaction with this chemical.

5.5 Triclosan-Bifenthrin Mixture

14. Both mixture of triclosan: bifenthrin exposure in combination of high temperature and high salinity altered the toxicity of antimicrobial triclosan enhancing the toxicology of the two different mixtures by a factor of 1.6-1.9 to the estuarine adult grass shrimp, *Palaemonetes pugio*, under simulated Climate Change Conditions (30°C,35 psu) when compared to standard conditions (20°C &20psu).

15. High salinity alone significantly reduced the toxicity of both triclosan: bifenthrin mixtures compared to standard conditions while toxicity in the high temperature alone was not significantly different from standard conditions. Both high salinity conditions and high temperature conditions were significantly different from combined high temperature, high salinity conditions.

16. The triclosan: bifenthrin Mixture 2 (Mixture ratios were based on Climate Change Condition toxicity tests results) was significantly more toxic than triclosan: bifenthrin Mixture 1 (Mixture ratios were based on standard change condition toxicity tests results).

17. Mixture 1 (Standard Condition Ratios) was less than additively toxic (Additive Index of 1.32) while Mixture 2 (Climate Change Condition Ratios) was additively toxic (Additive Index of 0.99). able 5.1. Summary of Climate Change effects on the ecotoxicology of selected CECs.

Table 5.1 Summary of Acute Toxicity Tests with CECs under Standard and Climate Change Conditions.

Compound	Temperature (T)	Salinity (S)	Combined T-S	Enhancement
PBDE-47	?	?	+	6.4 (T-S)
Ibuprofen	++	-	+	2.5 (T)
Bifenthrin	++	-	+	1.5 (T)
Triclosan	0	-	+	1.8 (T-S)
Mixture 1 SC[a]	0	-	+	1.9 (T-S)
Mixture 2 CC[b]	0	-	+	1.6 (T-S)

++ = Most Toxic Interaction; + = Increased Toxicity vs SC; - = Decreased Toxicity with SC; and 0 = No Difference in Toxicity with SC

[a] = Mixture 1 (SC) was less than Additively Toxic; [b] = Mixture 2 (CC) was slightly > than Additively Toxic; [c] = Climate Toxicity Enhancement Factor = 96h LC50 Standard Conditions/Climate Change.

5.6 Triclosan Effects on the Grass Shrimp Microbiome

Results of microbiome assessments of grass shrimp exposed to triclosan at the MEC and MIC concentration (Table 5.2) and then tested under both Standard Conditions found that:

18. Triclosan exposure at the MEC significantly reduced the number of total Vibrio and *V. parahaemolyticus* bacteria in the grass shrimp microbiome (Table 5.2).

19. *V. parahaemolyticus* bacteria was the dominant bacteria in the grass shrimp microbiome, accounting for 90% of the microbes.

20. Surprisingly only the MEC exposure had an effect on the gut microbiome while the at the higher MIC concentration there was no effect on survival (Table 5.2).

Table 5.2 Summary of effects triclosan on Vibrio bacterial levels on the grass shrimp microbiome.

Microbial Metric	Control	MEC Triclosan (LOW)	MIC Triclosan (HIGH)
Total Vibrios	0	-	0
V. parahaem (90%)	0	-	0
V. vulnificus	0	0	0
V. Cholerae	0	0	0

0 = Control Conditions or Not Different from the Control

- = Significantly reduced from the Control

MEC = Maximum Exposure Concentration

MIC = Minimum Inhibitory Concentration

5.7 Assessment of Vibrio Bacteria Exposure to 11 Different Antibiotics

The microbiome of grass shrimp exposed to triclosan at the MEC and MIC exposure concentrations which were then tested for multiple antibiotic resistance (MABR) found that:

21. There were significant differences in MABR observed in Vibrio bacterial comparisons between controls and each triclosan exposure treatment (MEC and MIC).

22. MABR in Vibrio bacteria in both the MEC and MIC triclosan exposures increased with increasing triclosan dose

23. Within Treatment Comparisons of Vibrio bacteria exposed to each of the 11 antibiotics at the MEC and MIC found only 4.3% of the exposures had significant

differences in MABR in controls versus 8.6% and 30.4% in the low (MEC) and high (MIC) doses of triclosan of triclosan exposures (Table 5.3).

24. Within Treatment Comparisons of Vibrio bacteria exposed to each of the 11 antibiotics at the MEC and MIC also found that there was increased resistant to an increasing number of antibiotics in comparisons between in controls (1 antibiotic) versus the low (MEC = 2 antibiotics) and high (MIC = 5 antibiotics) doses of triclosan exposures.

25. Between Treatment Comparisons of Vibrio bacteria exposed to each of the 11 antibiotics at the MEC and MIC found only 0% of the exposures had significant differences in MABR in controls versus 8.6% and 21.7% in the low (MEC) and high (MIC) doses of triclosan of triclosan exposures.

26. Between Treatment Comparisons of Vibrio bacteria exposed to each of the 11 antibiotics at the MEC and MIC also found that there was increased resistant to an increasing number of antibiotics in comparisons between in controls (0 antibiotic) versus the low (MEC = 2 antibiotics) and high (MIC = 5 antibiotics) doses of triclosan exposures.

27. Between Treatment Comparisons of Vibrio bacteria exposed to each of the 11 antibiotics at the MEC and MIC further found 52% of the exposure comparisons had significant differences in MABR in low (MEC) versus high (MIC) doses of triclosan of triclosan exposures and there was resistance to 7 different antibiotics (Table 5.3).

Table 5.3 Comparisons of MABR in Vibrio bacteria from the grass shrimp microbiome in controls and MEC and MIC triclosan exposed grass shrimp assessed exposed to 11 different antibiotics.

Microbial Metric	Control	MEC Triclosan (LOW)	MIC Triclosan (HIGH)
Within Treatment MABR Comparisons	0 (4.3% ABR) (1 Antibiotic)	+ (8.6% ABR) (2 Antibiotics)	++ (30.4% ABR) (5 Antibiotics)
Between Treatment MABR Comparisons	0	+ (8.6% ABR) (2 Antibiotics)	++ (21.7%) (6 Antibiotics)
		++ (52%) (7 Antibiotics)	

0 = Control Responses or Similar to Controls

+ = Significantly Increased

++ = Very Significantly Increased

28. MABR increased with increasing Triclosan dose and increased antibiotic dose, as highest levels of antibiotic resistance were found in the MIC versus the MEC and Controls.

29. Only 31% of control isolate had resistance to > 6 different antibiotics compared to 41% of isolates at the low exposure dose of triclosan (MEC) and 74% of isolates at the high exposure dose of triclosan (MIC).

30. Conversely 45% of control isolates and 46% of the low dose triclosan exposure group (MEC) were resistant to $\leq$ 3 antibiotics compared to only 5% of the high dose triclosan exposure group (MIC).

31. This is clear evidence that Vibrio bacteria in the environment at MEC exposure levels may react differently from Vibrio bacteria within humans which will be exposed to much higher doses at the MIC. These difference in dose may cause differing responses in terms of antibiotic resistance.

32. Future studies with other antibiotics should more fully address these differences between MEC and MIC responses among other classes of bacteria and under climate change conditions of increased temperature.

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