Acute Toxicity and Sub-Lethal Effects of Non-Point Source Pollutants on Invertebrates

by

Jocelyn Ann Romano

University Program in Integrated Toxicology
Nicholas School of the Environment and Earth Sciences
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Celia Bonaventura

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the University Program in
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ABSTRACT

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Abstract

Non-point source pollution is not generated from any single source, rather can arise from a mixture of agricultural, residential, and industrial activities. As a result of these activities millions of tons of chemicals enter into aquatic environments annually with the potential to disrupt the fragile ecosystems existing within. Common anthropogenic compounds most frequently seen in estuarine environments include pesticides, antifoulants, polycyclic aromatic hydrocarbons (PAH), and industrial solvents.

This dissertation examines the acute toxicity and sub-lethal effects of diuron, CuPT, B(a)P, and styrene in the mud snail, *Ilyanassa obsoleta*, the American oyster, *Crassostrea virginica*, the sea urchin, *Lytechinus variegatus*, and/or the barnacle, *Amphibalanus* (= *Balanus*) *amphitrite*. In addition, the general effects of non-point source pollution within the Rachel Carson Estuarine Research Reserve (RCERR) were examined at six sites in order to gain a better understanding of the current health of this unique habitat.

Of the four compounds tested, only the industrial solvent, styrene, resulted in an LC_{50} (1341 μg L^{-1}, *I. obsoleta*) that was within the range of currently reported environmental levels. Diuron and CuPT did not elicit mortality at environmentally relevant concentrations, but did significantly reduce fecundity in *I. obsoleta* and *C. virginica* and fertilization success and larval development in *L. variegatus*. The only

notable sub-lethal effect elicited by the PAH, benzo(a)pyrene, was a significant decrease in egg capsule production by *I. obsoleta* following exposure to concentrations as low as 50 μg L^{-1}.

Within the RCERR, animals from Sites 4, 5, and 6 were observed to have significant differences with respect to fecundity, condition index, and/or ECOD activity when compared to conspecific organisms from control Site 1. This is most likely a consequence of their proximity to anthropogenic sources. Large variation in mortality (15-98.9%) was observed when families of *A. amphitrite* from a single population where exposed to CuPT.

It is often difficult to extrapolate data from laboratory findings into natural populations. Frequently the organisms used under laboratory conditions are genetically very similar, while field population can vary with anthropogenic exposure. Caution must be taken when developing protocols for risk assessment to ensure that actual environmental conditions are being represented.

Dedication

For Leona, Betty, Daniel, and Joseph

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List of Abbreviations

ANOVA	Analysis of Variance
B(a)P	Benzo(a)pyrene
BPH	Benzo(a)pyrene hydroxylase
CYP450	Cytochrome P450
CuPT	Copper pyrithione
DDT	Dichloro-diphenyl-trichloroethane
Diuron	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DMSO	Dimethylsulfoxide
DUML	Duke University Marine Laboratory, Beaufort, NC
ECOD	Ethoxycoumarin-O-deethylase
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act of 1972
FSW	Filtered Seawater
IMO	International Maritime Organization
LC_{50}	Lethal Concentration to kill 50% of test animals
NCDENR	North Carolina Department of Environment and Natural Resources
NCDMF	North Carolina Division of Marine Fisheries
NCERRS	North Carolina Estuarine Research Reserve System
NERRS	National Estuarine Research Reserve System

NPSP	Non-Point Source Pollution
PAH	Polycyclic Aromatic Hydrocarbon
RCERR	Rachel Carson Estuarine Research Reserve
TBT	Tributyl Tin
USEPA	United States Environmental Protection Agency
VOM	Volatile Organic Molecules
ZPT	Zinc Pyrithione

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Chapter 1

Introduction:

Non-Point Source Pollution in Estuaries

1.1 Background

The United States Environmental Protection Agency (USEPA) defines non-point source pollution (NPSP) as "pollution coming from many diffuse sources" (USEPA, 1994). This type of pollution is not generated from any single source; rather can arise from a mixture of agricultural, residential, and industrial activities. NPSP is typically caused by precipitation moving over or through terrestrial areas resulting in the transport of chemical compounds to fresh and saltwater systems (Carpenter et al, 1998; EPA, 1994). NPSP can also include aquatic based pollution stemming from commercial and recreational boating activity (Flory and Alber, 2005).

An estimated 140 million tons of fertilizer and several million tons of pesticides enter the environment each year as a result of agricultural practices (Food and Agricultural Organization of the United Nations, 2006). In addition, accidental spills and intentional disposal of other pollutants also contribute to NPSP. Jackson et al (2001) reported nearly a half million tons of oil and gasoline components entering the environment through accidents in the year 2001. As a result of these accidental and intentional practices, hundreds of thousands of tons of synthetic chemicals, nutrients and various anthropogenic compounds find their way into lakes, rivers, streams and ultimately oceans each year (Schwarzenbach et al, 2006), but not without first filtering through transitory environments such as estuaries.

Estuaries can be described as partly enclosed tidal inlets where fresh and salt water mix (Little, 2000). They are places of extraordinary biological importance and home to an immense diversity of plants and animals. Estuarine environments are critical ecosystems that serve as feeding grounds for migratory waterfowl, nurseries for juvenile fish and invertebrate larvae, and provide shelter for many types of benthic organisms including ecologically important molluscs, crustaceans, and echinoderms.

Estuaries are not only ecologically important but provide income and commercial livelihoods to hundreds of thousands of people worldwide. In 2004, the North Carolina Department of Environment and Natural Resources (NCDMF, 2007) estimated that 34 million pounds of blue crab were harvested resulting in a dockside value of $23 million. In 2005 an estimated 71,398 bushels of oysters worth nearly $1.7 million were harvested (NCDMF statistics, accessed 16 December 2006). In addition, millions of pounds of Atlantic croaker, summer flounder, and other recreational sport fish are harvested within the Pamlico and Albemarle (NC) sounds and estuaries annually (NCDENR, 2007).

With so much ecological and commercial value placed on estuarine environments, it is unfortunate that they are all too often the 'end of line' or 'dumping grounds' for many anthropogenic compounds. For decades estuarine environments such as salt marshes and mangrove swamps, have been viewed as unsightly and nasty environments. So much so that in the early 1900's the British newspaper, *The Punch*,

published an article titled "Have you ever been in a mangroove swamp, my dear – Nature at its most revolting" (Little, 2000).

Fortunately attitudes have changed since then. In an effort to protect these resources new government legislation mandates water quality monitoring and requires environmental impact studies for areas of commercial and residential development. These legislative efforts were also instrumental in the creation of the National Estuarine Research Reserve System (NERRS). The NERRS represents 27 different biogeographical areas of the United States (U.S.) set aside where long term research, monitoring, and protection of resources have been implemented. One of these sites exists within the North Carolina Estuarine Research Reserve System (NCERRS) and was named after a pioneer in environmental protection and monitoring, Rachel Carson.

1.2 North Carolina Rachel Carson Estuarine Research Reserve

The North Carolina Rachel Carson Estuarine Research Reserves (RCERR) represents an ideal location for monitoring the impact of coastal development and the input of anthropogenic compounds on estuarine organisms. The site includes a number of islands covering more than 2500 acres within the midst of one of the state's fastest growing areas. The islands and estuarine waters at the site are strongly influenced by the Newport River, the North River Channel, the Beaufort Inlet and the continually changing Back Sound conditions. The twice-daily tides, a low variation in salinity over the western section, and the topography of the entire site creates a diverse and

productive estuarine ecosystem. Habitats found within the site include tidal flats, flooded salt marshes, ocean beach, sub-tidal soft bottoms, hard surfaces, and dredge spoil areas.

Over the past few decades the area surrounding the reserve has become the target of residential and industrial development. Numerous housing developments, recreational boating facilities, industry, and agricultural areas have all contributed to the influx of pollutants into this reserve. The estuary's own sediment may also serve as a continual source of contaminants due to years of pollutants partitioning into this environmental compartment.

Some recent advances have been made in an effort to clean up the aquatic environment, mainly by controlling pollution from point sources such as industries and sewage treatment plants. Unfortunately, non-point source pollution remains one of the largest causes of water quality problems. NPSP is the main reason that approximately 40 percent of rivers, lakes, and estuaries are not clean enough to allow basic uses such as fishing or swimming (New York Sea Grant, 2001). Industrial development, land reclamation, and waste disposal sites are currently the most common uses for estuarine environments and remain the major sources of anthropogenic compounds.

The chronic input of contaminants into the RCERR and other estuarine environments has the potential to upset the balance of the ecosystem by altering the reproduction and development of ecologically vital organisms. Various anthropogenic

compounds are known to exist within estuarine environments. Most commonly seen are antifoulants, pesticides, industrial solvents, and any number of polyaromatic hydrocarbons (PAHs). In this dissertation the effects of non-point source contamination on six sites within and four sites outside the RCERR were examined. These sites were chosen based on their proximity to anthropogenic sources of pollution (Figure 1.1). Each site will be discussed in detail throughout the chapters of this dissertation. In addition, the reproductive and developmentally specific effect of four different compounds representing the major types of anthropogenic inputs (i.e. pesticides, antifoulants, petroleum by-products and industrial solvents) will be examined.

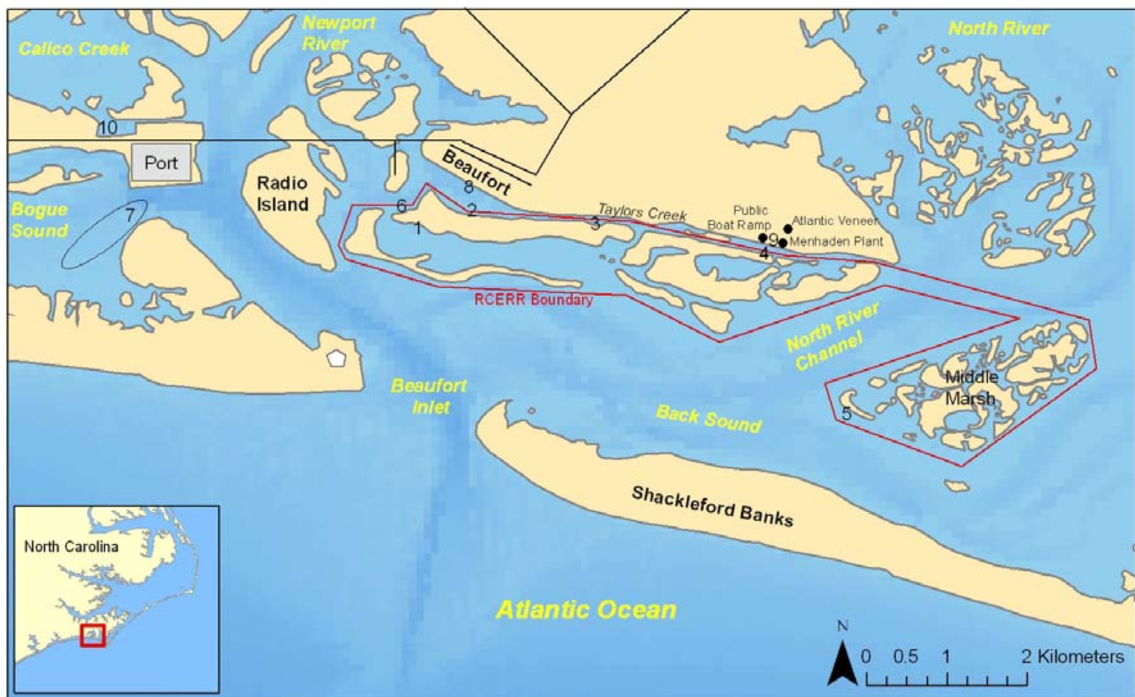


Figure 1.1 The Rachel Carson Estuarine Research Reserve (RCERR) and the 10 sites sampled for this dissertation.

1.3 Anthropogenic Compounds

Anthropogenic compounds are chemicals that are man made and enter into the environment through human activity. Along with bacteria and nutrients from livestock and pet waste, sediment from improperly managed construction sites and excess fertilizers used in agriculture and residential practices, anthropogenic compounds such as pesticides, petroleum by-products, and industrial solvent make up the majority of NPSP. The three major sources of these man-made compounds are agriculture, domestic and urban runoff, and industry (Schwarzenbach et al, 2006). The impact from a compound entering an environment is not easily estimated and many variables need to be considered when evaluating potential toxicity. Toxicity is heavily weighted on a number of factors but most importantly whether or not the compound is persistent and mobile in the environment.

Tributyltin (TBT) is a classic example of a chemical that was originally hailed for its long standing persistence and effectiveness as an antifouling agent. The characteristics that made TBT economically invaluable to the antifouling market (high effectiveness, environmental stability, and low water solubility) were exactly the same characteristics that made it detrimental to organisms in the environment. TBT and other organotins found their niche in the antifouling market in the late 1960s and early 1970s. However, within a decade, reports surfaced associating the use of organotins with decreased spatfall and unnatural shell thickening in the oyster, *Crassostrea gigas* (Alzieu

et al, 1986; Alzieu, 2000). During this same time period work in the United Kingdom linked TBT to endocrine disruption in the marine gastropod, *Nucella lapillus*. Bryan et al, (1986, 1987) reported the masculinization of females (imposex) and widespread decline in population numbers due to exposure to TBT. Although the USEPA, through the 'Organotin Antifouling Paint Control Act' of 1988, regulates the use of TBT in paints and sets standards for the amount of biocides that can leach from the paint into water, this compound is still used at unregulated volumes in many countries and serves as a continued source of non-point source pollution.

Beside gastropod deformities (Gibbs et al, 1987; Bauer et al, 1995), other consequences of NPSP include shell malformations and steroid metabolic variations in bivalves (Morcillo et al, 1998) reduced reproductive capacity in crustaceans (McKenney et al, 1998; Oberdörster and McClellan-Green, 2000), altered developmental patterns in echinoderms (Chapter 4, this dissertation), and depressed immune system and physiological function in both vertebrates and invertebrates (Nakata et al, 2002; Oyama et al, 2003). This variety of effects is alarming in view of the ecological importance of both vertebrate and invertebrate species within estuarine ecosystems.

The following studies examine the effects of four representative compounds from several major classes of commonly found environmental pollutants. These pollutants include pesticides such as the herbicide diuron, antifoulants such as the biocide, copper pyrithione (CuPT), PAHs such as benzo (a) pyrene (B(a)P), and industrial solvents such

as styrene. All four of these compounds were examined for their potential to cause mortality and elicit sub-lethal effects on reproduction, fecundity, and/or larval development in four model invertebrate species typically found in North Carolina estuarine waters.

1.3.1 Pesticides

The USEPA defines a pesticide as ‘any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest’ (USEPA, 2007). Pesticide usage is a critical concern in many coastal areas, where inputs from agriculture and urban areas impact the surrounding estuaries and marshes. Unfortunately pesticide detection is not a routine test in most standard water quality assessments. As a result, the type and amount of pesticides in many coastal waters are unknown and are estimated based on potential use and proximity to agricultural and residential areas. The RCERR is one such place where pesticide contaminants in sediments and water samples are unknown. We chose the commonly used herbicide and antifouling additive, diuron as our representative NPSP pesticide.

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea (Figure 1.2)) has been found in marine environments (Thomas et al, 2001) and surface waters (Moncada, 2004; Green and Young, 2006) at concentrations from the low μg L^{-1} to mg L^{-1} range. This compound belongs to the *N*-phenylurea class of chemicals and was originally approved for use by the USEPA in 1967. Diuron inhibits photosynthesis by reversibly inhibiting

photosynthetic electron flow to the plastoquinone in the photosystem II complex (PSII) (Hayes, 1978; Ware, 1978). This inhibition is caused by blocking the electron transport chain just after the primary electron acceptor Q_A (for a review of PSII herbicides see Oettmeier, 1992; Jones and Kerswell, 2003).

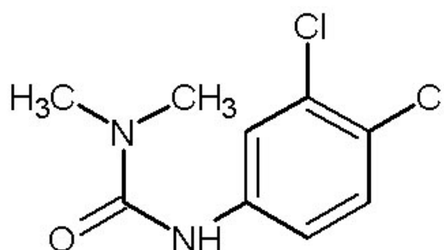


Figure 1.2 Diuron chemical structure.

The USEPA estimated that 9 to 10 million pounds of diuron were used in agricultural practices in 2003. In addition, 2-3 million pounds were used in 'right of way' management along roads, railways, and fences (USEPA, 2003). It is not known how much diuron is used through application of antifouling coatings on recreational water crafts in the United States. However, Boxall et al (2000) reported that up to 10 tons (20 thousand pounds) of diuron were used on leisure craft in the United Kingdom (U.K.) during 2000. Recent reports have placed diuron concentrations at ranges between 6.74 $\mu g\ L^{-1}$ in coastal waters of the U.K. (Thomas et al, 2001) up to 1.3 mg L^{-1} in U.S. surface waters (Moncada, 2004; Green and Young 2006). These levels have raised concerns because diuron inhibits photosynthesis in aquatic environments at concentrations as low

as 0.3 μg L^{-1} (Jones and Kerswell, 2003). The most likely source of these environmental levels of diuron are a result of agricultural runoff and leaching from antifouling coatings.

Unfortunately little is known regarding the effects of diuron on organisms inhabiting contaminated aquatic environments. A few studies exist that report LC_{50} (48 hour) values of diuron ranging from 4.3 mg L^{-1} to 42 mg L^{-1} in fish, and from 1 mg L^{-1} to 2.5 mg L^{-1} for aquatic invertebrates (Okamura et al, 2002). In addition, diuron has also been reported to disrupt mitosis in early development of the Pacific oyster, *Crassostrea gigas* at realistic environmental concentrations. Based on this evidence, classification of diuron has been deemed moderately toxic to fish and aquatic invertebrates.

1.3.2 Antifoulants

Antifoulants are compounds used to combat fouling organisms such as barnacles, tunicates, algae, and bacterial films often found on submerged surfaces (i.e. boat hulls, docks, pilings, etc). Fouling is a major problem because it decreases ship performance and costs the shipping industry millions of dollars each year in dry dock fees, loss of trade, and increased fuel consumption. Controlling fouling has strategic, economic and environmental consequences (Johnson and Miller, 2003; Johnson and Gonzalez, 2004). In the past, various broad spectrum biocides such as arsenic, organomercury, organochlorides, and organotins have been used to manage fouling organisms. Within the past few decades these broad spectrum biocides were determined

to have unacceptable environmental impacts, the worst of which were local and regional population (mostly invertebrate) extinctions (Gibbs et al, 1991; Stewart et al, 1992). As a result antifouling companies have started adding organic biocides (such as broad spectrum pesticides) to coating formulations to increase effectiveness while at the same time reducing metal concentrations (Voulvoulis et al, 1999).

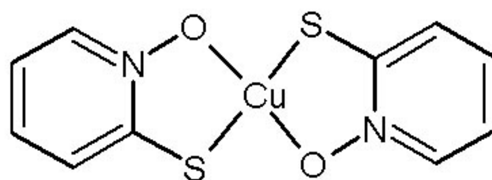


Figure 1.3 Copper pyrithione (CuPT) chemical structure.

Copper pyrithione (Figure 1.3) (in addition to diuron) is one such broad spectrum biocide that is presently being added to existing coatings in an attempt to lower overall copper concentrations in antifouling paints (Voulvoulis et al, 1999). Copper pyrithione (2-mercaptopyridine N-oxide copper salt) (CuPT) is a microbial biocide that was introduced into the market as an antifoulant in 1996 (Arch Chemicals; Maraldo and Dahllöf, 2004). Mackie et al (2004) reported total pyrithiones (including sodium-, zinc-, and copper conjugates) within U.K. waters exceeding 0.3μg L^{-1}. Concentrations of these chemicals are expected to increase with the eventual phase-out of other antifoulants such as the organotins. For these reasons the biocide additive, CuPT was chosen as the NPSP antifouling representative examined in this study.

While limited information exists on CuPT, zinc pyrithione (ZPT) (a sister compound to CuPT) has been in use much longer and may provided some insight into CuPT's mode of toxicity. ZPT has been used as an anti- fungal, bacterial, and algal compound since the 1960s (Turley et al, 2005). It complexes immediately with Cu^{2+} ions after dissociation in marine environments to form the related transchelate CuPT (Mackie et al., 2004). Both CuPT and ZPT are lipophilic compounds with half-lives ranging between 15 min to 30 days (Turley et al., 2005; Maraldo and Dahllöf, 2004), depending upon environmental conditions. The fastest degradation rates of ZPT and CuPT occurred in the presence of light or sandy and aerobic sediments (Turley et al, 2005). While absolute certainty of the mode of action of CuPT is still unclear, Ermolayeva and Sanders (1995) suggest that pyrithiones act by interfering with the activity of the primary proton pump, H^+-ATPase, resulting in the disruption of cell membranes. It is assumed that CuPT functions through a similar process.

1.3.3 Polycyclic Aromatic Hydrocarbons

In addition to pesticides and antifoulants, aquatic environments are also plagued with by-products from petroleum based activities. Polycyclic aromatic hydrocarbons (PAHs) are a large class of compounds that are comprised of two or more fused aromatic rings. Natural sources of PAHs include forest fires and volcanic eruption (Wcislo, 1998), however most PAHs result from the burning of fossil fuels or from incomplete combustion. PAHs are most often lipophilic, have low water solubility, sorb

to organic particles, and accumulate in aquatic sediments, which together make them a potentially dangerous class of chemicals (Meyer and Quinn, 1973). There are more than 100 different types of PAHs (Agency for Toxic Substance and Disease Registry, 1995), 16 of which are designated "compounds of interest" by the USEPA (Miles and Delfino, 1999). PAHs vary in their chemical characteristics and in their capacity to elicit biological effects. In aquatic environments PAHs become rapidly associated with particles in the water and are deposited in sediments, where they are consumed by benthic organisms (McElroy et al, 1989). It is thought that the majority of damage from PAH exposure results from oxidative stress which can influence detoxification pathways and cause production of genetic abnormalities (Livingstone et al, 1990; Grundy et al, 1996; Ericson and Balk, 2000; McElroy et al, 2000).

One of the most common PAHs in aquatic environments is benzo(a)pyrene (B(a)P). B(a)P (Figure 1.4) is a 5-ring polycyclic aromatic hydrocarbon (PAH). It has been reported at levels exceeding 50 $\mu g\ Kg^{-1}$ in highly polluted areas of the U.S, England, Finland, Lithuania, and Russia (Madany et al, 1994; Kirso et al, 2002; Hylland, 2006).

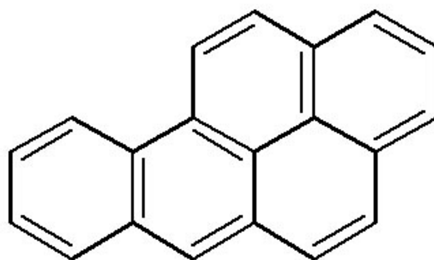


Figure 1.4 Benzo(a)pyrene (B(a)P) chemical structure.

While extensive studies have been done on aquatic vertebrates, relatively little data exist on *in vivo* B(a)P and metabolism of other PAHs in invertebrates (James, 1989; Livingstone, 1990, 1991). Molluscs and crustaceans comprise two of the most heavily impacted classes of invertebrates within estuarine environments (Elder and Dresler, 1988; Grundy et al, 1996; Carman et al, 1997; Wootton et al, 2003). Molluscs and crustaceans exposed to PAH compounds, like B(a)P, experience sub-lethal effects that include changes in molting, growth, behavior, the induction of enzymatic activities such as ethoxycoumarin-O-deethylase (ECOD) (Oberdörster et al, 1999; 2000) and severe immunological alterations such as inhibition of phagocytosis and damage to lysosomes (Grundy et al, 1996; Wootton et al, 2003; Auffret et al, 2004). These physiological changes are thought to result from the production of polar biochemically reactive electrophilic species which interact with nucleic acids and proteins (Stegeman and Lech, 1991; Stegeman et al, 1992; Xue and Warshawsky, 2005).

With so much recreational activity within and surrounding the RCERR it is inevitable that levels of PAHs will continue to rise within the Reserve. A potential problem exists in these compounds' affinity for high organic based sediments (Schwarzenbach et al, 1992). Organisms living in these muddy environments are exposed daily to chronic levels of B(a)P and other PAHs and are forced to adapt to their presence. A major concern addressed in this dissertation is how B(a)P affects reproduction and development of the benthic organisms living in these estuarine environments.

1.3.4 Industrial Solvents

Organic solvents have been widely used in various industrial practices. Their main application includes formulation of products, paint thinning, and cleaning of materials to remove contaminants (Siva, 1990; Callahan and Green, 1995). During these applications, solvent emission and waste solvent generation frequently occurs. Organic solvents are known to cause adverse health effects in both human and wildlife (Burrell, 1993). Non-point source pollution from organic solvents is typically divided into either volatile organic molecules (VOMs; anthropogenic compounds that are emitted into the atmosphere) or terrestrial pollutants that enter into ground water, sediments, and marine environments.

Styrene (Figure 1.5) is routinely used in the production of polystyrene, latex, and resins and is typically released into the environment during production, storage,

transport, use or through disposal practices (Gibbs and Mulligan, 1997). Styrene has been ranked 23rd out of all chemicals in terms of total amount released and is listed among the top ten carcinogenic VOMs (Gibbs and Mulligan, 1997). Styrene has an estimated worldwide production capacity of over 16 million tons (Miller et al, 1994) and was reported in some U.S. oil refinery wastewater effluents at concentrations over 30 µg (Pelish et al, 2003). In other parts of the world, styrene has been reported in wastewater effluent at concentrations exceeding 5.0 mg L^{-1} (Gomez et al, 2001).

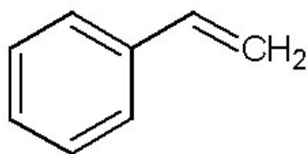


Figure 1.5 Styrene chemical structure.

In mammals, styrene is distributed among the liver, pancreas, kidneys and brain and can accumulate within adipose tissue (Bond, 1989). While several studies on the effects of styrene exposure in mammals have been performed, a limited number of investigations have been conducted with aquatic vertebrates and even fewer with aquatic invertebrates. Reports on the toxicity of styrene to aquatic invertebrates are scarce and usually limited to acute studies under open and static conditions. The results

from these assays are quite variable due to poor solubility and the high volatility of styrene (Cushman et al, 1997; Gibbs and Mulligan, 1997).

Typically industries using industrial solvents are located on rivers or coastlines that allow for easier transportation of manufactured goods. Unfortunately these rivers and coastlines often border estuarine environments with little monitoring of industrial effluents. According to the USEPA Toxics Release Inventory Program (March 22, 2007) industries in and around Beaufort, NC released a total of 113,859 pounds of styrene in 2005, thus, styrene was chosen as a representative industrial compound for this study.

1.4 Laboratory and Field Experiments

Estuaries are critical habitats that are among some of the most productive environments in the world. Yet, they also rank among the most anthropogenically contaminated, mostly as a result of non-point source pollution. Assessing these types of environments can be extremely difficult due to the numerous variables encountered and the degree of chemical mixtures in the environment. A majority of studies examining the effects of anthropogenic compounds on organisms are done under laboratory settings where such variables as concentration, exposure time, and environmental factors can be controlled. Unfortunately examining organisms in the field is not as simple or as organized. One concern discussed in this dissertation is the variation in toxicity of compounds among populations. With field organisms being exposed to variable

environmental conditions and input of anthropogenic compounds, determining the exact effect a chemical compound has in nature often proves difficult.

Registering chemical compounds with the USEPA requires a battery of EPA-mandated assays including acute, sub-chronic, and chronic toxicity tests. Regulations governing compounds such as pesticides, antifoulants, petroleum products, and industrial solvents are typically based on laboratory toxicity assays that use organisms with little genetic variability (Forbes and Depledge, 1992). A major concern with these assays is that because laboratory populations are far removed from natural populations the regulatory assays may not accurately predict natural impacts. Animals in the field are exposed to a range of anthropogenic compounds that could induce natural variations in response. These variations may include altered physiological, metabolic, and detoxification pathways both among and within populations of organisms (Barata et al, 2002; Virgilio et al, 2005; Piola and Johnston, 2006).

Another major problem is that ecotoxicological assessments often neglect a potential pollutants' impact on representatives of the invertebrate phyla (Depledge and Billinghurst, 1999; Rittschof and McClellan-Green, 2005). This is alarming in view of the ecological importance of invertebrate systems in the global environment and their sensitivity to many toxic compounds. Since invertebrates are key components of all ecosystems, the threat of negative reproductive and developmental effects should be fully evaluated to ensure environmental protection. The major focus of this dissertation

was to investigate acute toxicity and sub-lethal effects of non-point source pollutants on invertebrates. Four model organisms were used in this dissertation to investigate mortality and sub-lethal effects including fecundity, fertilization success, and/or larval development. The model species used in these studies included the mud snail, *Ilyanassa obsoleta,* the American oyster, *Crassostrea virginica,* the variegated sea urchin, *Lytechinus variegatus,* and the barnacle, *Amphibalanus amphitrite*.

1.4.1 Ilyanassa obsoleta

The mud snail, *Ilyanassa obsoleta*, (Say) is a common, soft sediment, intertidal inhabitant found along the east coast of the United States. Mud snails tend to aggregate in tens of thousands (Straw and Rittschof, 2004) and are reproductively active from January through April (Oberdörster and McClellan-Green, 2000; Rittschof et al, 2002; Straw and Rittschof, 2004; Oberdörster et al, 2005). They feed predominately on microorganisms that grow in and on the surfaces of sediments, oysters, and on decaying matter such as fish and crabs (Britton and Morton, 1994; Giannotti and McGlathery, 2001).

Mud snails have been used extensively in organotin related studies (Oberdörster and McClellan-Green, 2000; Rittschof et al, 2002; Straw and Rittschof, 2004; Oberdörster et al, 2005) and have been shown to exhibit imposex induction (a condition characterized by the development and superimposition of male accessory sex organs (Jenner, 1979; Gibbs et al, 1987,1991; Oehlmann et al 1996)), altered cytochrome P450 metabolism

(Oberdörster et al, 1998), alterations in neuropeptide hormone expression (Oberdörster and McClellan-Green, 2000), and behavioral castration (disruption of sexual behavior) following anthropogenic exposure (Straw and Rittschof, 2004). *I. obsoleta* was considered an excellent model organism, because of its rich literature base in previous studies, ease of collection, and overall ecological importance in mud flat environments.

1.4.2 Crassostrea virginica

The American oyster, *Crassostrea virginica*, (Gmelin) is of both great economic and ecological value. Oysters have been cultivated for more then 2000 years, with the current U.S. harvest estimated at over 30 million pounds; 75% of which is comprised of *C. virginica* (Southern Regional Aquaculture Center Publication, 2001). They can be found attached to hard substrates such as rocks or other shells. Their flattened, distorted shells are variable in size and can grow to 15 cm in length (Calvo et al, 2000). Oysters are typically found in estuaries including embayments with low salinities and in shallow water of tidal to subtidal depth. If uncrowned and healthy, this bivalve can live upwards of 20 years, but more typically have life expectancy in the 5–7 year range (Kennedy, 1996).

The reproductive cycle begins with spawning, which is usually triggered by water temperature above 25° C (Kennedy, 1996). After fertilization in the open water, cell division proceeds rapidly and within hours, fertilized eggs develop into microscopic larvae. Settling occurs when the larva cements itself onto a hard substrate and

metamorphoses into a tiny oyster termed a spat. Sexual maturity can occur within 4 months in warmer waters, but has usually been found to take between 1–3 years in colder waters such as those surrounding the RCERR (Kennedy, 1996).

C. virginica is often used as a sentinel species due to its ability to bioaccumulate pollutants from the environment. Healthy oyster populations increase estuarine water clarity by filtering out suspended particles (Gerritsen et al, 1994; Brumbaugh et al, 2000). This bivalve mollusk was chosen as a model because of its invaluable ecological and commercial importance and for the fact that it comprises a major constituent of the RCERR.

1.4.3 Lytechinus variegatus

The urchin *Lytechinus variegatus* (Lamarck) occurs in shallow water from North Carolina to Brazil, and throughout the Caribbean and the Gulf of Mexico (Hill and Lawrence, 2003; M. Wise, *personal correspondence*). The species is usually thought of as inhabiting seagrass beds, but is more typically found on rock, oyster substrate, and hard bottoms within and surround the Reserve (Watts et al, 2001). *L. variegatus* is a broadcast spawner, which has reproductive cycles that correspond with changes in a variety of environmental factors including temperature, salinity, photoperiod, and food availability (Beddingfield and McClintock, 1998).

One reason *L. variegatus* was chosen as a model organism was because gametes can be obtained in the laboratory with the use of KCl and fertilization and larval

development are easily observed. The eggs and early embryos of *L. variegatus* are transparent which makes visualizing cleavage and development relatively straightforward (Ettensohn et al, 2004). In addition, the early development of sea urchin embryos is highly synchronous, i.e., when a batch of eggs is fertilized, all of the resulting embryos typically develop on the same time course (McCarthy and Young, 2002). This synchronous development makes biochemical and molecular studies of early embryos possible, and it also allows for detection of a number of disturbances in development by anthropogenic compounds. The sea urchin has been widely used as a test species in developmental toxicity studies (Heslinga, 1976; Bellas et al, 2005, Rosen et al, 2005; Wong and Wessel, 2005)

1.4.4 Amphibalanus amphitrite

Amphibalanus (= *Balanus*, Clare and Rittschof, 1989) *amphitrite* (Pitombo, 2004) is a small sessile barnacle that can be found throughout the world in the mid-intertidal to shallow subtidal zones (Zullo, 1979) mainly as a result of ballast water introduction. In the wild, barnacles can be found on a number of different substrates including oysters, salt marsh cordgrass, rocks, and seawalls (Holm et al, 2000). These barnacles are hermaphrodites, but cross-fertilization occurs in dense populations. In such cases, males deposit sperm directly into the mantle cavity of adjacent functional females via the penis (Ruppert and Barnes, 1996). Fertilized eggs are brooded in the mantle cavity and produce free-swimming planktonic larvae (Rittschof et al, 2003).

Attachment and metamorphosis of cypris larvae are extremely sensitive to a range of environmental and anthropogenic factors (Holm et al, 2000). *A. amphitrite* typically live 120 days under laboratory conditions (Rittschof et al, 2003) and can produce up to 10,000 eggs per brood and up to 24 broods per year (Costlow and Bookhout, 1956; El-Komi and Kajihara, 1991) under natural conditions. Because of this organism's sensitivity to anthropogenic factors and its role as a target species of antifoulants, it is an excellent test organism to understand how copper pyrithione impacts mortality and variations of toxicity within populations and families.

1.5 Objectives

The objectives of this study were (1) to determine the effects of four representative compounds (diuron, copper pyrithione, benzo(a)pyrene, and styrene) on acute toxicity, fecundity, and ECOD activity in the mud snail, *Ilyanassa obsoleta* and then compare these results with these same organisms at each of the six sites within the RCERR (Chapter 2); (2) to determine the effects of four representative compounds on acute toxicity, condition index, fecundity (field sites only), and ECOD activity in the oyster, *Crassostrea virginica* and then compare our results with animals from RCERR (Chapter 3); (3) to examine the effects of diuron and copper pyrithione on mortality, fertilization, and larval development in the sea urchin, *Lytechinus variegatus* (Chapter 4); (4) to examine the variation in toxicity of copper pyrithione among populations and families of the barnacle, *Amphibalanus amphitrite* (Chapter 5). Lastly Chapter 6

summaries the results and conclusions for this study and presents a brief examination of invertebrate health within the RCERR.

Chapter 2

The effects of diuron, copper pyrithione, benzo (a) pyrene, and styrene exposure on acute toxicity, reproduction, and ECOD activity in the mud snail, *Ilyanassa obsoleta*: A laboratory and field survey in the Rachel Carson Estuarine Research Reserve, Beaufort, NC

2.1 Introduction

Pesticides, antifoulants, petroleum by-products, and industrial solvents are common pollutants found in estuarine waters. Exposure to these compounds can result in mortality as well as sub-lethal impacts. Imposex induction (Gibbs et al, 1987), developmental and metabolic changes (Morcillo et al, 1998), and reproductive and behavioral alterations (Oberdörster and McClellan-Green, 2000; Oberdörster and Cheek, 2001; Straw and Rittschof, 2004) are just a few examples of the chronic effects observed in marine invertebrates following exposure to anthropogenic compounds. The concentration of these type of chemicals that are required to cause mortality are chemical- and species-specific but are generally several orders of magnitude greater than levels required to elicit chronic effects. Exposure of animals through accidental spills or illegal dumping of toxic material often results in acute toxicity however, it is the slow bioaccumulation and bioconcentration of anthropogenic compounds that go unseen, that are the real cause for concern.

The rationale for this study was to examine acute and sub-lethal effects of typical non-point source pollution (NPSP) in the model organism *Ilyanassa obsoleta*. Diuron, copper pyrithione, benzo(a)pyrene, and styrene were chosen as representative compounds of pesticides, antifoulants, petroleum by-products, and industrial solvents typically found in estuarine environments. Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) belongs to the *N*-phenylurea class of chemicals and has been one of the

major herbicides used since the 1950s. It has recently been added to marine antifouling coatings. It inhibits photosynthesis in plants and algae by reversibly inhibiting electron flow to the plastoquinone in the photosystem II (PSII) complex, resulting in the blockage of the electron transport chain (Oettmeier, 1992; Jones and Kerswell, 2003). Diuron has been reported at concentrations ranging from a few μg L^{-1} to over 1 mg L^{-1} in some United States (U.S.) surface waters (Moncada, 2004; Green and Young, 2006) and has been reported to inhibit photosynthesis in aquatic environments at concentrations as low as 0.3 μg L^{-1} (Jones and Kerswell). The majority of the diuron entering the aquatic environment results from agricultural and residential runoff (Moncada, 2004; Green and Young, 2006).

Copper pyrithione (2-mercaptopyridine N-oxide copper salt) (CuPT) is a microbial biocide that was introduced into the market as an antifoulant in 1996 (Arch Chemicals; Maraldo and Dahllöf, 2004) and is presently added to existing coatings in an attempt to lower overall copper concentrations (Voulvoulis et al, 1999). Little is known regarding its current environmental concentrations or its mode of toxicity however; Mackie et al (2004) reported total pyrithiones (including sodium-, zinc-, and copper conjugates) exceeding 0.3 μg L^{-1} within United Kingdom (U.K.) waters. In the aquatic environment CuPT is formed through the transchelation of its sister compound zinc pyrithione (ZPT), which is also used in some antifouling coatings (Mackie et al, 2004; Turley, 2005). CuPT is lipophilic with a half-live ranging between 15 min to 30 days

(Turley et al, 2005; Maraldo and Dahllöf, 2004), depending on environmental conditions. The fastest degradation rates of CuPT occur in the presence of light or aerobic sandy sediments through photolysis and hydrolysis, respectively (Turley et al, 2005). While the mode of action of CuPT is still unclear, Ermolayeva and Sanders (1995) suggest that pyrithiones act by interfering with the activity of the primary proton pump, H^+-ATPase. This is hypothesized to result from the collapse of cell membrane pores leading to ion gradient destabilization and ultimately cell apoptosis (Bragadin et al, 2003). It is assumed that CuPT operates by similar mechanisms.

Benzo(a)pyrene (B(a)P) is a commonly occurring polycyclic aromatic hydrocarbon (PAH) found in marine environments. In nature its' main sources include forest fires and volcanic eruption (Wcislo, 1998) but it is typically introduced into the marine environment as a result of incomplete combustion and industrial activities (Hylland, 2006). B(a)P is listed by the U.S. Environmental Protection Agency (USEPA) as a possible carcinogen (USEPA, 1996; 1999) and has been found at concentrations greater than 50 $\mu g\ Kg^{-1}$ in highly polluted areas of the U.S., England, Finland, Lithuania, and Russia (Kirso et al , 2002; Saltiene et al, 2002). The main problem associated with B(a)P and its metabolites is their ability to interact with nucleic acids and proteins (Stegeman and Lech, 1991; Stegman et al, 1992; Xue and Warshwsky, 2005) resulting in genetic and biochemical alterations.

Styrene is a volatile organic molecule (VOM) found in motor vehicle exhaust, solvents, and other industrial practices (Gibbs and Mulligan, 1997). It is ranked 23rd in terms of total amount released into U.S. sediments and is routinely listed among the top carcinogenic VOMs (U.S. Toxic Release Inventory, 1997). According to USEPA Toxic Chemical Release Inventory, total styrene released into U.S. land and water bodies from 1987-1993 totaled over 2 millions pounds. Some global concentrations have even been reported at levels exceeding 5.0 mg L^{-1} (Gomez et al, 2001). In mammals, tissue distribution of styrene occurs within the liver, pancreas, kidneys and brain and can accumulate within fatty tissue (Bond, 1989). While several studies on the effects of styrene exposure in mammals have been reported, limited studies have been preformed using aquatic vertebrates and even fewer in aquatic invertebrates.

Anthropogenic compounds such as pesticides, antifoulants, petroleum by-products, and industrial solvents enter into rivers, lakes and estuaries (Schwarzenbach et al, 2006) daily. Organisms living in these environments will either die or invoke coping strategies to deal with such toxicant related stress. Coping strategies often include such detoxification mechanisms as activation of the cytochrome P450 system, glutathione induction, metallothionein production, sequestration of metals, and reallocation of energy supplies (Notten et al, 2006; Moraga et al, 2005).

It is known that animals in stressful conditions often reallocate energy away from secondary biological activities such as reproduction (i.e. egg capsule production) to

mechanisms necessary for survival (Notten et al, 2006; Moraga et al, 2005). One such mechanism is the cytochrome P450 detoxification system (CYP450). These phase I enzymes are responsible for biologically transforming both endogenous and exogenous compounds such as polycyclic aromatic hydrocarbons (PAHs), pesticides, and other xenobiotics (Livingstone, 1985, 1989, 1997; Sarkar et al, 2006). Typical reactions associated with CYP450 include oxidation, reduction and hydrolysis (Stegeman and Hahn, 1994; DiGiulio et al, 1995; Lanis and Yu, 1999). These reactions can be quantified by the measurement of specific enzyme activities. One such enzyme that has historically been used to estimate PAH exposure is ethoxycoumarin-O-deethylase (ECOD) (Goksøyr and Förlin, 1992).

In this study mortality, egg capsule production, and ECOD activity were assessed in the mud snail, *Ilyanassa obsoleta* after exposure to four representative classes of anthropogenic compounds (diuron, CuPT, B(a)P, and styrene). These same endpoints were assessed in mud snails from six sites within the Rachel Carson Estuarine Research Reserve (RCERR) Beaufort, NC. The area surrounding the reserve has recently undergone an increase in residential development and recreational activity. The chronic input of contaminants from these sources into the estuarine environment has the potential to upset the balance of the existing ecosystem. The purpose of this study was to provide insight into the effects of non-point source pollution in the ecologically important model organism *I. obsoleta.*

2.2 Methods and Materials

2.2.1 Study Sites

Six sites surrounding the RCERR were chosen for their proximity to various industrial, residential, and coastal sources of pollution (Table 2.1, Figure 2.1). Site 1 has no notable levels of PAHs, PCBs, or heavy metals, has been used as a control site in previous studies (Oberdörster and McClellan-Green, 1998, 2000; Straw and Rittschof, 2004; Oberdörster et al, 2005; McClellan-Green et al, 2006), and is considered AA quality shellfish water by the North Carolina Department of Environment and Natural Resources (NCDENR). Site 2 is located along the Carrot Island side of Taylors Creek, across from the Beaufort, NC waterfront and is approximately 100 m from a public marina and other residential and private docks. Site 3 is located on the same tidal creek as Site 2 but receives wastewater discharge from a tertiary sewage treatment pipe approximately 200 m across the creek. Site 4 is the final site located on the Carrot Island side of Taylors Creek and is regularly exposed to persistent and residual contaminants from an active boat launch, commercial fishing vessels, and a nearby wood veneer production facility. Site 5 is located along the North River Channel and was chosen in order to evaluate anthropogenic input resulting from agricultural and recreational activities in the North River. Site 6 is situated on the western end of the RCERR across from Radio and Pivers Islands. It receives tidal flows from the North and Newport River estuary and is an active waterway for boats entering the Beaufort Inlet.

Table 2.1 **Mud snail collection site coordinates and descriptions**

Site	Latitude	Longitude	Description
1	34° 42.38 N	76° 40.10 W	Reference site in Carrot Island embayment
2	34° 42.74 N	76° 39.58 W	West end of Beaufort waterfront, marine, moorings
3	34° 42.64 N	76° 38.62 W	Midway along Taylors Creek near 3° sewage pipe
4	34° 42.50 N	76° 37.96 W	Across Taylors Creek from industry and boat ramp
5	34° 41.67 N	76° 37.26 W	Along North River Channel
6	34° 42.81 N	76° 40.23 W	Across Gallants Channel from DUML

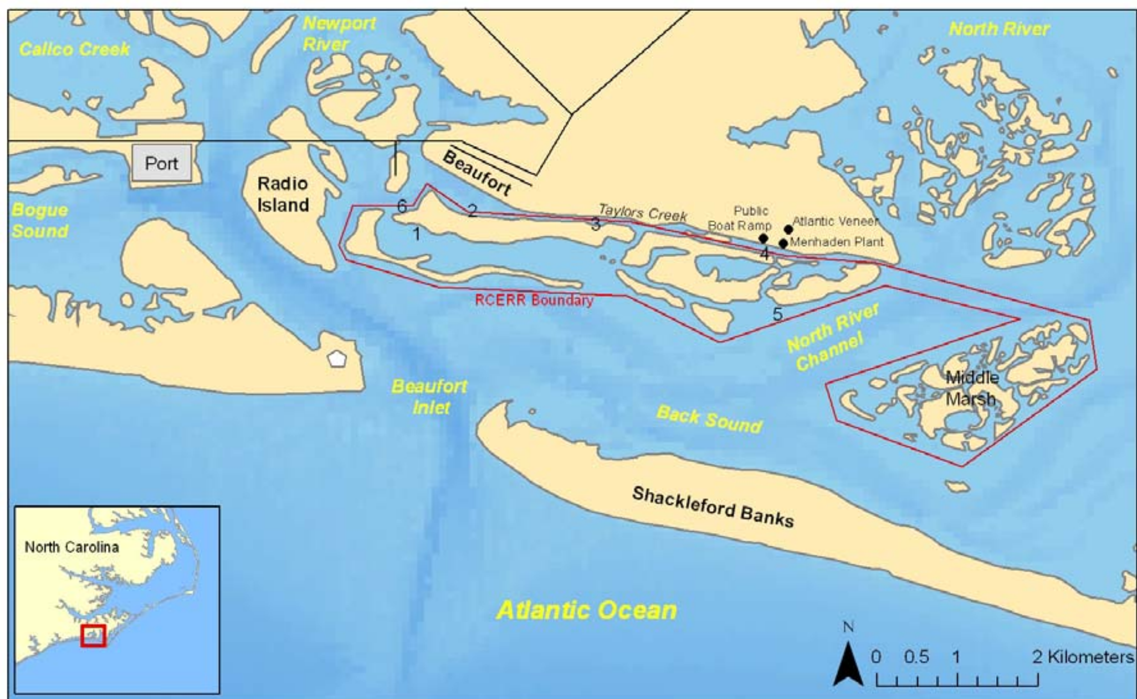


Figure 2.1 Map of the Rachel Carson Estuarine Research Reserve (RCERR), Beaufort, NC with mud snail collection sites indicated by site number.

2.2.2 Experimental Solutions

Diuron (Sigma-Aldrich, St. Louis, MO) stock solutions were prepared in acetone at a concentration of 10 g L^{-1} following Jones and Kerswell (2003) and Chesworth et al. (2004). CuPT (Arch Chemicals, Norwood, CT) stock solutions were prepared in dimethylsulfoxide (DMSO) at a concentration of 2 g L^{-1} following Kobayashi and Okamura (2002). B(a)P(Sigma-Aldrich, St. Louis, MO) was prepared in warm acetone at a concentration of 1 g L^{-1} following McClellan-Green (*personal correspondence*). Styrene (Fisher Scientific Company, Fair Lawn, NJ) stock solution was prepared in ethanol at a concentration of 10 g L^{-1} following Ohtani et al (2001). Stock solutions were kept at room temperature in dark bottles to avoid photolysis for no longer then 1 week. Working solutions were made immediately before use by dilution in filtered seawater (FSW) to appropriate concentrations.

2.2.3 Diuron, CuPT, B(a)P, and Styrene Acute Toxicity – LC_{50}

Approximately 500 *I. obsoleta* snails were collected from Site 1 during the first week of November 2005. Snails were maintained at the Duke University Marine Laboratory (DUML) on a 14L:10D cycle in continuously flowing single pass sand-filtered seawater (30–35 salinity, approx. 4°C) and allowed to graze on algae growing on the tanks for 48 hours prior to use.

Acute toxicity assays were 96 h in duration with daily mortality checks and water and treatment renewal. Snails (all greater than 1 cm in length) were housed in

groups of twenty in 1 L acid-washed glass jars and allowed to acclimate to a 14L:10D light cycle (seawater at 20°C, 35 ppt) for 24 hr before the start of the experiment. All controls, (FSW, acetone, DMSO, and ethanol), were tested in triplicate at the highest concentration of their respective compounds. Three replicates of 25, 50, 75, 100, and 1000 μg L^{-1} diuron and CuPT; 10, 50, 100, 500 μg L^{-1} B(a)P and 50, 500, 1000, and 5000 μg L^{-1} styrene were tested and analyzed for acute toxicity. LC_{50}, the concentration at which mortality occurs in 50% of snails, was estimated using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA).

2.2.4 Compound-Specific Egg Capsule Production and ECOD Activity Assays

Approximately 500 *I. obsoleta* snails were collected from Site 1 during February 2006 and kept at DUML under the same conditions as described in section *2.2.3* for 48 hours prior to use. The test concentrations for assays were based on preliminary findings, acute toxicity measurements, and environmentally relevant concentrations reported in the literature.

Compound-Specific Egg Capsule Production Assay

Groups of twenty *I. obsoleta* were placed in 1 L acid-washed glass jars and exposed to 750 mL of FSW, acetone, DMSO, and ethanol controls at the highest concentration used in their respective compounds. In addition, snails were exposed to 750 mL of 100 and 1000 μg L^{-1} diuron; 3.21 and 49.7 μg L^{-1} CuPT; 10, 50, and 100 μg L^{-1}

B(a)P and 50, 500, 1000, and 5000 μg L^{-1} styrene. Both control and treated snails were exposed for 30 days with daily renewal of compound and mortality checks. Each assay was performed in triplicate. Snails grazed on biofilms produced in the glass jars and were fed fish every seven days following Oberdörster et al (1998). At the end of the 30 day exposure period, snails were dissected and sexed (Oberdörster et al., 1998). The number of females and egg capsules per jar was recorded. Statistical analysis was preformed to compare controls to treatments with the use of a One Way Analysis of Variance and a Holm-Sidak post-hoc test (SigmaPlot, SYSTATSoftware Inc. San Jose, CA).

Compound-Specific ECOD Activity Assay

Groups of twenty *I. obsoleta* were placed in 1 L acid-washed glass jars and exposed to 750 mL of FSW and respective solvent controls. In addition snails were exposed to 750 mL of 100 μg L^{-1} diuron; 10 μg L^{-1} CuPT; 50 μg L^{-1} B(a)P and 100 μg L^{-1} styrene for 96 h with daily water and treatment renewal. Each exposure was preformed in triplicate.

After 96 h snails were dissected and gonad-digestive gland removed. Gonad-digestive glands from five snails were combined per sample, rinsed in 50 mM Tris (pH 7.4), 1.15% KCl, then blotted and weighed to the nearest 0.1 gram. Tissue was homogenized in 50mM Tris (pH 7.4), 1.15% KCl at a 1:3 (v/v) ratio and centrifuged at

9,000 x g for 10 minutes. Supernatant was stored at -80°C until analyzed. The protein content of all samples was determined using the Bradford method (Bradford, 1976).

Solutions consisting of 1 mL of 2 mM 7-ethoxycoumarin in Tris-HCl (pH 7.6), 400 µL of 50 mM Tris-HCl (pH 7.6), and 100 µL of sample, were added to glass tubes and pre-warmed 5 minutes in a shaking water bath at 30°C. At the end of the pre-incubation, 25 µL of 5 mM NADPH was added to each tube and incubated at 30°C for 20 minutes. The reaction was stopped by plunging tubes in an ice bath and deproteinated by adding 1 mL of cold 5% (w/v) zinc sulphate. Samples were centrifuged at 2000 x g for 15 minutes at 4°C. One mL of deproteinated supernatant was transferred to clean glass tube containing 2 mL of 0.5 M glycine-NaOH buffer (pH 10.5) (Lake, 1987). ECOD activity was measured following Livingstone (1991) (pmol hydroxycoumarin min^{-1} mg^{-1} protein) using a Perkin-Elmer (Norwalk, CT) Model LS50B luminescence spectrophotometer at excitation and emission wavelengths of 380 and 456 nm, respectively. Statistical analysis was performed using paired T-tests comparing each treatment with its respective control (SigmaPlot, SYSTATSoftware Inc. San Jose, CA.)

2.2.5 Site-Specific Egg Capsule Production and ECOD Activity Assays

Approximately 100 to 150 *I. obsoleta* were collected from each of the six sites during the last week of January 2006 to coincide with the normal snail breeding period

(Oberdörster and McClellan-Green, 2000). Snails were maintained under the same conditions as section *2.2.3* for 48 hours prior to use.

Site-Specific Egg Capsule Production Assay

Groups of twenty *I. obsoleta* were placed in 1 L acid-washed glass jars and exposed to FSW for 30 days. Mortality assessment and water renewal was preformed daily as described in *2.2.4*. At the end of the 30 day period, snails were dissected and sexed (Oberdörster et al, 1998). The number of females and egg capsules per jar were counted and recorded. The average numbers of egg capsules per female were compared to Site 1 using a paired T-test for sites 2, 3, 5, and 6 and a Mann-Whitney Rank Sum Test for Site 4 (failed normality test) (SigmaPlot, SYSTATSoftware Inc. San Jose, CA.).

Site-Specific ECOD Activity Assay

Twenty snails from each of the six sites in the RCERR were collected and immediately dissected. Groups of five snails per sample were rinsed in 50mM Tris (pH 7.4), 1.15% KCl, blotted and weighed to the nearest 0.1 gram. Protein concentration and ECOD activity were determined as described in section *2.2.4*. Statistical analyses were performed comparing Site 1 (reference site) to each of the other 5 sites using paired T-tests (SigmaPlot, SYSTATSoftware Inc. San Jose, CA.).

2.3 Results

2.3.1 Diuron, CuPT, B(a)P, and Styrene Acute Toxicity – LC_{50}

No mortality was observed in *I. obsoleta* exposed to FSW or any of the solvent controls. Exposure to CuPT caused mortality at concentrations as low as 25 μg L^{-1} and exhibited a LC_{50} of 63.2 μg L^{-1} (95% C.I. 50.3–79.3 μg L^{-1}; Figure 2.2). Styrene produced mortality in exposed snails at concentrations as low as 50 μg L^{-1} and resulted in a LC_{50} of 1341 μg L^{-1} (95% C.I. 1020.0–1765.0 μg L^{-1}). No LC_{50} could be estimated for diuron or B(a)P. B(a)P exposures did produce a lowest observable effective concentration (LOEC) at 10 μg L^{-1} and a 20% mortality at 500 μg L^{-1}.

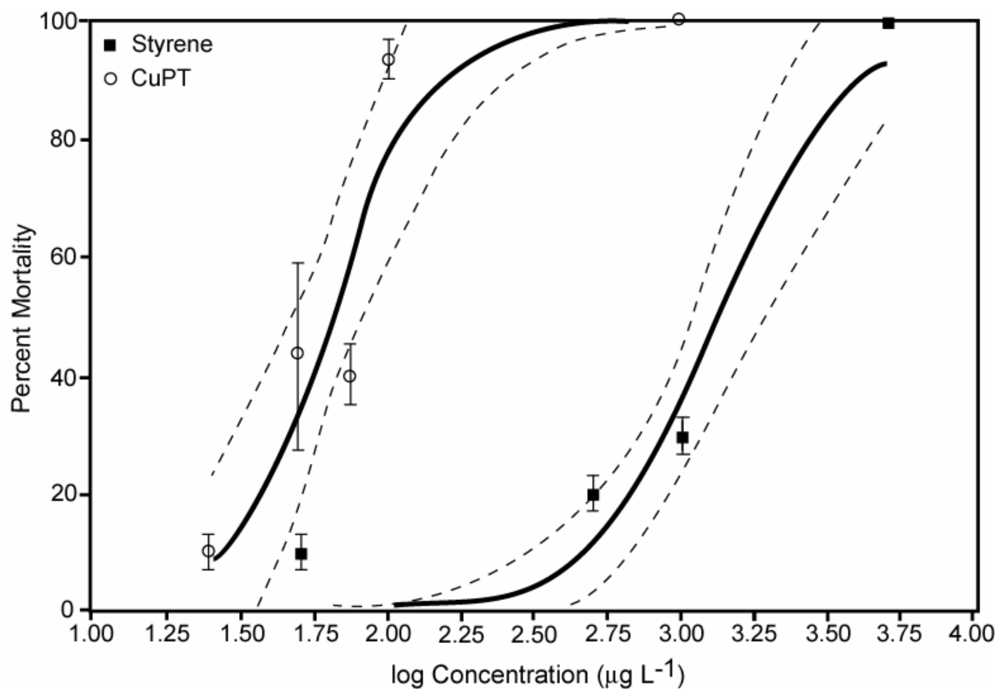


Figure 2.2 Mortality in *Ilyanassa obsoleta* after 96 h exposure to CuPT (○) and styrene (■). Dashed lines indicate 95% confidence interval.

2.3.2 Compound-Specific Egg Capsule Production Assay

The average number of egg capsules laid per female for FSW was 73.2 (±3.2; Standard Error of the Mean). This was not significantly different from the acetone, DMSO, or ethanol controls, thus all controls were combined to increase statistical power. Diuron significantly ($p<0.05$) decreased egg capsule production in both the 100 and 1000 $\mu g\ L^{-1}$ treatments with egg capsule laid per female values of 43.3 (±3.3) and 53.8 (±6.4), respectively (Figure 2.3a). Likewise snails exposed to 3.21 and 49.7 $\mu g\ L^{-1}$ CuPT produced significantly fewer egg capsules with an average of 54.5 (±4.6) and 0 (±0) egg capsules laid per female, respectively ($p<0.05$) (Figure 2.3b). The 50 and 100 $\mu g\ L^{-1}$ concentrations of B(a)P differed significantly from the control with average egg capsules laid per female of 55.3 (±6.8) and 54.2 (±1.8), respectively ($p<0.05$) (Figure 2.3c). All styrene treatments (50, 500, 1000, and 5000 $\mu g\ L^{-1}$) produced significantly fewer ($p<0.05$) number of egg capsules per female (50.46 (±4.9), 34.39 (±2.7), 23.92 (±3.0), and 10.9 (±5.7), respectively) than the control (Figure 2.3d). Thus, exposure of mud snails to each of the test compounds significantly reduced the number of egg capsules laid.

2.3.3 Compound-Specific ECOD Activity Assay

The average ECOD activity in snails exposed to FSW was 0.003 (±0.003) pmol hydroxycoumarin $min^{-1}\ mg^{-1}$ protein. This was significantly lower then the acetone control of 0.013 (±0.001) pmol hydroxycoumarin $min^{-1}\ mg^{-1}$ protein), but was not different than either the DMSO or ethanol controls. ECOD activity measured in *I.*

obsoleta exposed to diuron, CuPT, or styrene was not significantly different from FSW or their respective solvent controls (acetone, CUPT, and ethanol). Exposure of snails to 50 μg L^{-1} B(a)P had significantly decreased ($p<0.05$) ECOD activity (0.004 (±0.003) pmol hydroxycoumarin min^{-1} mg^{-1} protein) when compared to the acetone control (Figure 2.4).

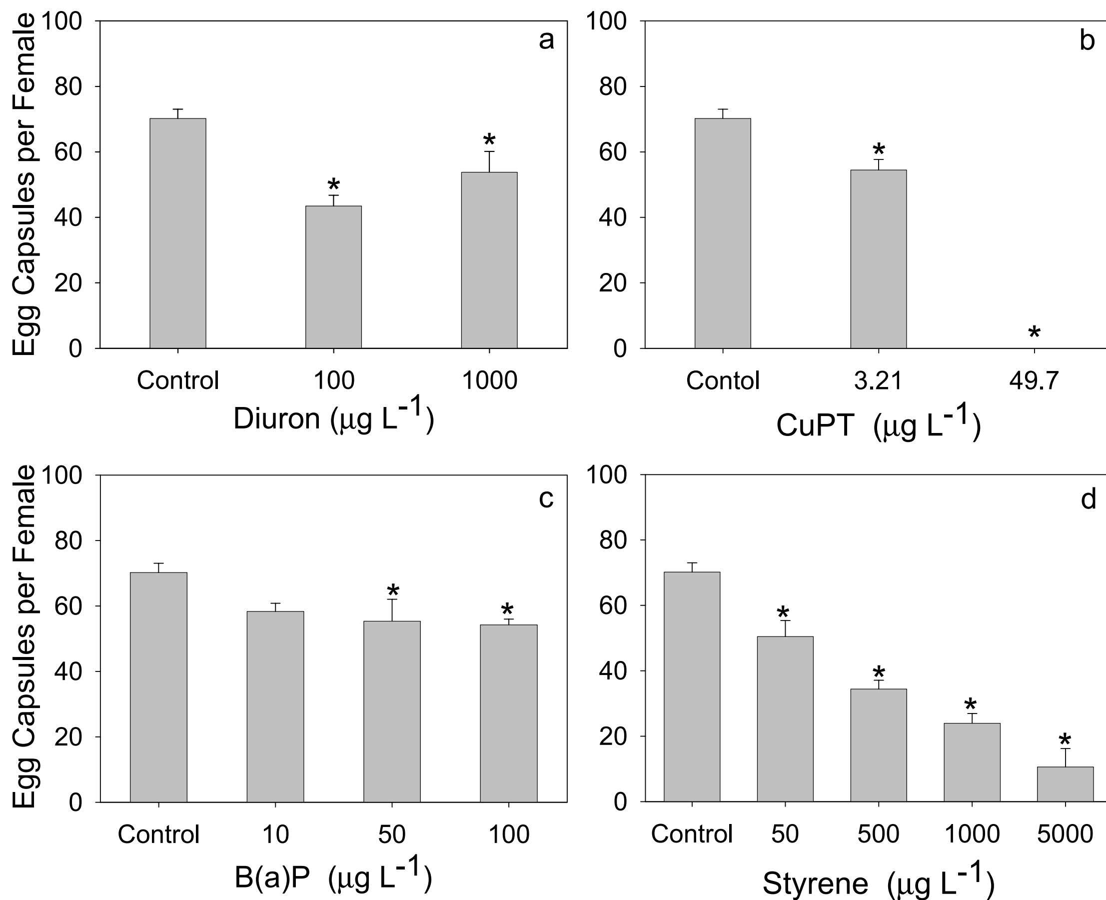


Figure 2.3 Average number of egg capsules per female *Ilyanassa obsoleta* after 30 d exposure. Treatment included (a) diuron and acetone solvent control, (b) CuPT and DMSO solvent control, (c) B(a)P and acetone solvent control, and (d) styrene and ethanol solvent control. Error bars are standard error.*p<0.05.

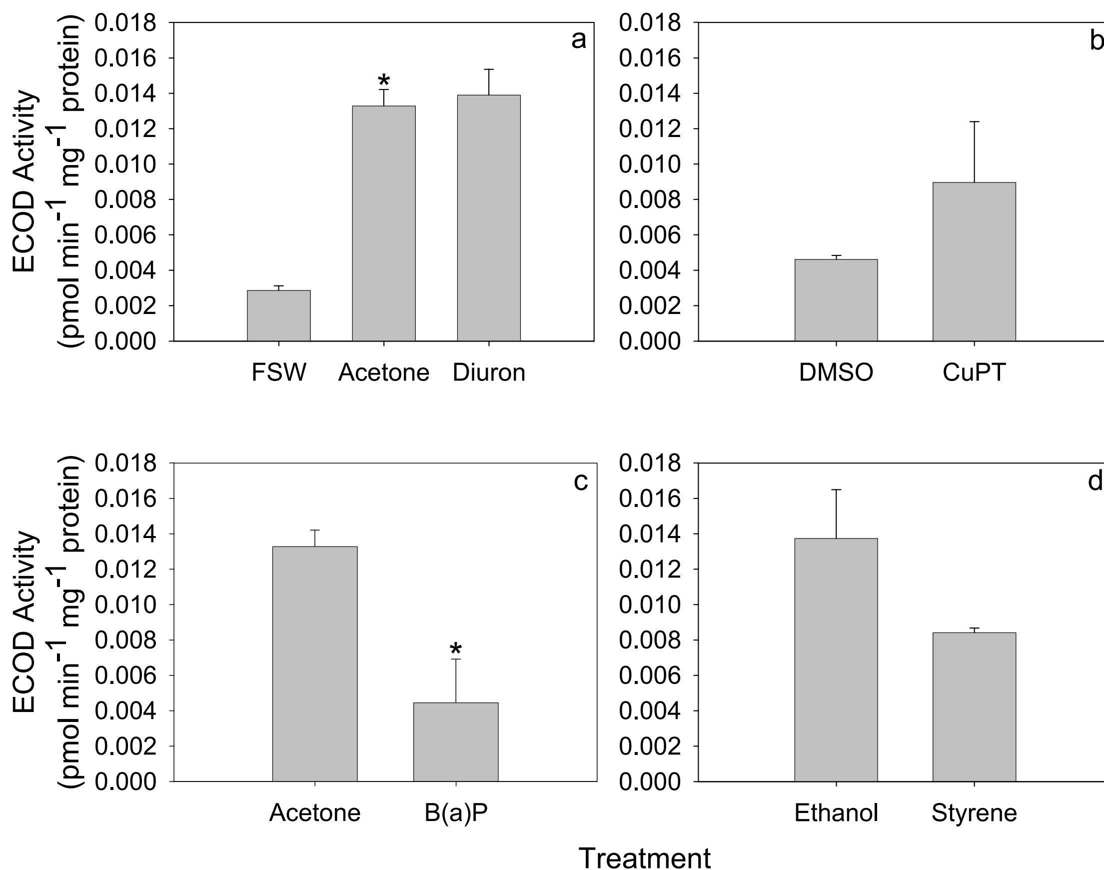


Figure 2.4 ECOD activity in *Ilyanassa obsoleta* after 96 h exposure. Exposures include (a) FSW, acetone solvent and diuron control (asterisk indicates significance solely between FSW and acetone solvent control), (b) DMSO solvent control and CuPT, (c) acetone solvent control and B(a)P, and (d) ethanol solvent control and styrene. Error bars are standard error. *p<0.05

2.3.4 Site Specific Egg Capsule Production Assay

The average number of egg capsules laid per female ranged from 0.55 to 60.45 between the six sites (Figure 2.5). Egg capsules laid per female averaged 55.5 (±3.0) at Site 1 (Figure 2.5). This was significantly higher than averages at sites 3, 4, and 6. Site 6

average 35.7 (±4.1; p=0.05) egg capsules laid per female. Site 4 had the lowest number of egg capsules laid per females, averaging 0.55 (±0.21; p=0.05). Site 3 fell in between the number of egg capsules laid per female at Site 4 and 6, with egg capsules laid per female averaging 4.7 (±0.9; p=0.001). Site 2 averaged 50.68 egg capsules laid per female which was similar to Site 1. Site 5 had the highest number of egg capsules laid per female with a value of 60.45 (±5.57) but this level was not significantly different from Site 1.

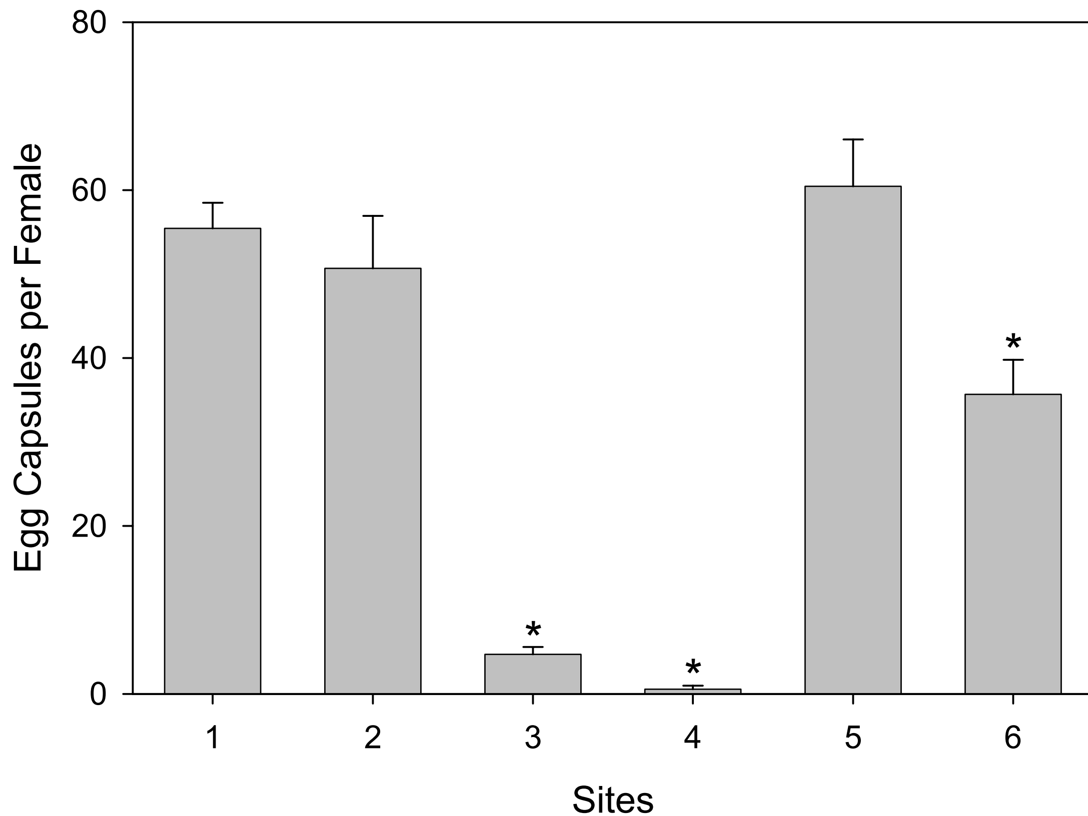


Figure 2.5 Average number of egg capsule per female *Ilyanassa obsoleta* at sites 1–6. Error bars are standard error. *p<0.05 compared to control site 1.

2.3.5 Site Specific ECOD Activity Assay

ECOD activity in snails collected from Site 1 was 0.015 (±0.005) pmol hydroxycoumarin min^{-1} mg^{-1} protein). This level of activity was higher, although not significantly, than Sites 2, 4, 5, or 6 (Figure 2.6). Site 3 snails exhibited approximately the same level of activity as Site 1. Snails from all sites were highly variable with respect to ECOD activity, resulting in large standards of deviation.

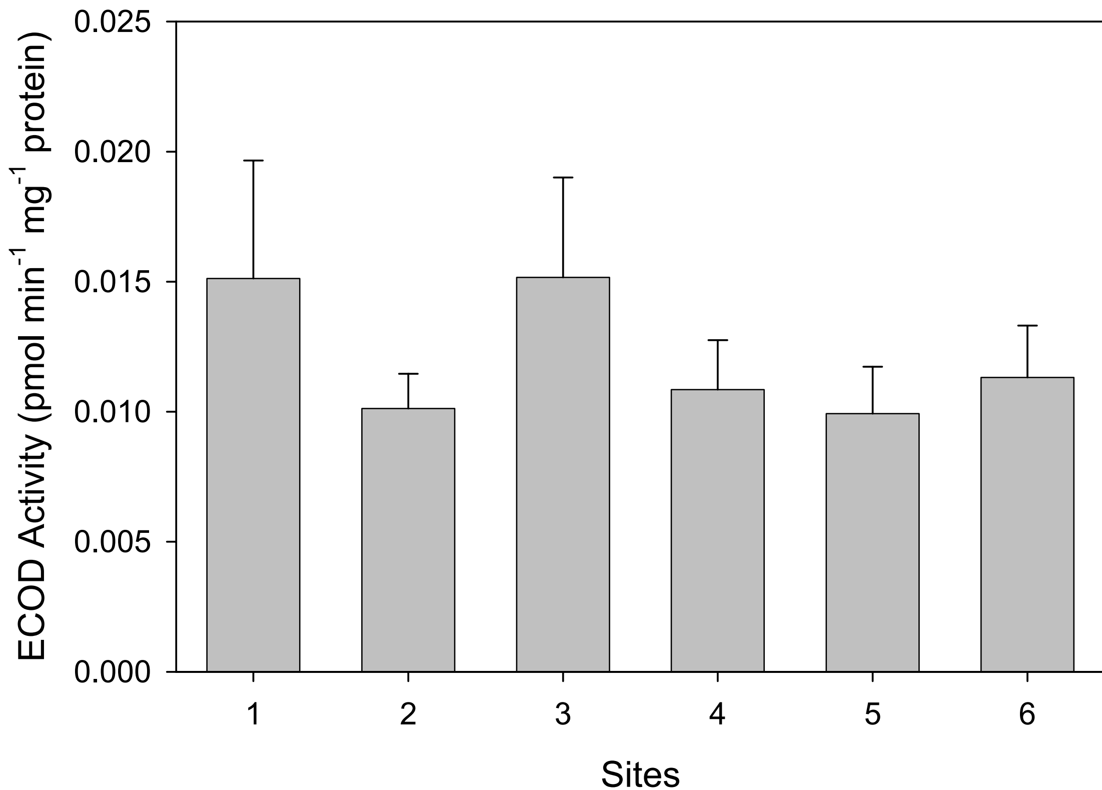


Figure 2.6 ECOD activity in *Ilyanassa obsoleta* at sites 1–6. Error bars are standard error.

2.4 Discussion

2.4.1 Diuron, CuPT, B(a)P, and Styrene Acute Toxicity – LC_{50}

LC_{50}s represent the lethal concentration of a toxicant that is required to cause mortality in 50% of exposed organisms. Concentrations causing mortality in *I. obsoleta* are within the range of reported values for other marine organisms (Table 2.2). In this study only CuPT and styrene were able to elicit such acute effects, 63.2 μg L^{-1} and 1341.0 μg L^{-1}, respectively. B(a)P causes mortality in only 20% of snails exposed at the highest concentration of 500 μg L^{-1}. Diuron was unable to produce mortality even at the highest concentration of 1000 μg L^{-1}. Realistically, the concentrations of diuron, CuPT, and B(a)P required to elicit mortality in *I. obsoleta* in this study are not environmentally relevant. However, the LC_{50} for styrene (1341 μg L^{-1}) is well below some present environmental levels. Concentrations in various Argentinean wastewater effluents have been reported as high as 5 mg L^{-1} (Gomez et al, 2001). In addition, styrene was one to ten orders of magnitude more toxic to mud snails than other organisms such as the fish, *Pimephalus promelas* and earthworm, *Eisenia foetida* (Cushman et al, 1997). The reason some organisms are more susceptible to toxicants than others is not fully understood at this time, but may be explained by variations in metabolic processes, physiology, or detoxification pathways (Davis and Hidu, 1969; Lytle and Lytle, 1990; Au et al, 1999; Lannig et al, 2006).

Table 2.2 Toxicity data of diuron, copper pyrithione (CuPT), benzo(a)pyrene (B(a)P, and styrene.

Compound	Organism	LC_{50}	Reference
Diuron	*Mercenaria mercenaria* (clam)	5 mg L^{-1}	Davis and Hidu, 1969
	Americamysis bahia (shrimp)	1.1 mg L^{-1}	U.S. EPA, 2000
	Daphnia magna (water flea)	0.4 mg L^{-1}	Shcherban, 1972
	Pterconarcys californicus (stone fly)	3.6 mg L^{-1}	Sanders and Cope, 1968
	Aedes aegypti (mosquito)	1.2 mg L^{-1}	Nebeker and Schytema, 1998
	Chironomus tentans (midge)	3.3 mg L^{-1}	Knapek and Lakota, 1974
	Lytechinus variegatus (echinoderm)	7.14 mg L^{-1}	Chapter 4, this dissertation
CuPT	*Heptacarpus futilirostris* (shrimp)	2.5 µg L^{-1}	Mochida et al, 2006
	Pagrus major (fish)	9.3 µg L^{-1}	Mochida et al, 2006
	Aquatic fish	4.3–43.6 µg L^{-1}	Yamada and Kauno, 2002*
	Pinmephales promelas (fish)	4.3 µg L^{-1}	NRAAVC, 2001
	Oncorhynchus mykiss (fish)	7.6 µg L^{-1}	Okamura et al., 2002
	Lytechinus variegatus (echinoderm)	14.04 µg L^{-1}	Chapter 4, this dissertation
B(a)P	*Daphnia pulex* (water flea)	5 µg L^{-1}	Trucco et al, 1983
	xenopus laevis (frog embryo)	13.4 mg L^{-1}	Propst et al, 1997
	Solea solea (fish)	5 mg L^{-1}	Au et al, 1999
	Gammarus duebeni (amphipod)	11 mg L^{-1}	Lawrence and Poulter, 1998
Stryene	*Pimephales promelas* (fish)	10 mg L^{-1}	Cushman et al, 1997
	Hyalella azteca (amphipod)	9.5 mg L^{-1}	Cushman et al, 1997
	Eisenia fostida (earthworm)	120 mg Kg^{-1}	Cushman et al, 1997

* orginal article in Japanese, abstract in english

2.4.2 Compound-Specific Egg Capsule Production Assay

Little is know about the endocrine or other physiological effects of diuron in aquatic invertebrates (Nebeker and Schuytema, 1998). *I. obsoleta* exposed to both 100 and

1000 μg L^{-1} diuron produced significantly ($p<0.05$) lower numbers of egg capsules per females then control animals. One possible explanation is that diuron may be interfering with endocrine function resulting in decreased fertilization or fecundity. Nebeker and Schuytema (1998) reported decreased survival and number of young produced in *Daphnia pulex* exposed to diuron at concentrations of 7.7 mg L^{-1} and in the fathead minnow, *Pimephales promelas* at concentrations of 31.2 mg L^{-1}. These concentrations are 1 to 2 orders of magnitude higher than that which produced decrease egg capsule production in *I. obsoleta.* Such low sub-lethal effects were also reported by Romano et al (in preparation) where exposure of sea urchins, *Lytechinus variegatus* to 0.1μg L^{-1} diuron significantly impacted sperm function. In addition, Christian and Tate (1982) reported that another urea pesticide, fluometuron, also decreased fecundity in the flatworm, *Fasciola hepatica*, at realistic environmental concentrations.

One other possible explanation may be related to the decrease in photosynthetic food available to *I. obsoleta* during the period of egg capsule production. Diuron has been observed to inhibit photosynthesis at concentrations as low as 0.3 μg L^{-1} (Jones and Kerswell, 2003). It was expected in addition to fish meat every seven days, snails would also graze on algae and other photosynthetic growth within the glass jars. The removal of this added food source in combination with the toxicant itself may have been enough to nutritionally limit snails causing them to reallocate energy from fecundity mechanisms into starvation and detoxification coping strategies. Similar energy

reallocation in times of stress has been observed in marine organisms such as the mysid shrimp, *Neomysis integer* following tributyltin exposure (Verslycke et al, 2003).

Snails that were exposed to 3.21 and 49.7 μg L^{-1} CuPT were also observed to have significantly decreased egg capsule production. This may have also resulted from some interference in endocrine function or physiological mechanism. At this time the mode of toxicity of CuPT is thought to include cell membrane disruption resulting in altered ATP synthesis (Chandler and Segel, 1978; Dinning et al, 1998; Bragadin et al, 2003). In CuPT exposed snails, decreased ATP supplies may interfere with either egg capsule production directly or in the physical release of egg capsules from the oviduct. Typically eggs are released from the ovary and fertilized in the oviduct (Sullivan and Maugel, 1984). They are then transferred to the albumen gland and embedded within fibrous material which is ultimately passed through the oviduct and out into the mantle (Sullivan and Maugel, 1984). It is possible that decreased ATP production affects one or more steps of egg capsule production and/or extrusion. Unfortunately ATP levels and/or synthesis were not measured as part of this study. Histological analysis may help identify any causative abnormal morphology in the egg capsule gland and/or oviduct resulting from CuPT exposure.

Styrene significantly reduced egg capsule production at concentrations of 50, 500, 1000, and 5000 μg L^{-1}. Styrene is metabolized by cytochrome P450 isozymes (CYP2E1 and CYP2F2 in mammals) to styrene oxide (Linhart et al, 2000) which in turn has been

reported to cause glutathione depletion (Hynes et al, 1999). Information regarding the toxicity of styrene to aquatic organisms is very minimal in the literature and mainly limited to acute studies (Gibbs and Mulligan, 1997). The cause of decreased egg capsule production following styrene exposure in snails in not known, but similar reductions in fertility have been reported in *Ceriodaphnia dubia* at styrene dimer and trimer concentrations as low as 0.04 µg L^{-1} (Tatarazako et al, 2002). Further histological analysis examining the egg capsule gland and oviduct could provide insight into this problem.

2.4.3 Compound-Specific ECOD Activity Assay

The cytochrome P450 (CYP450) system is an integral part of the detoxification process and is typically associated with phase I type reactions (Landis and Yu, 1999) such as oxidation, reduction, and hydrolysis (Stegeman and Hahn, 1994; Di Giulio et al, 1995). Benzo(a)pyrene is a classical PAH inducer of cytochrome P450 activity (specifically CYP4501A) in many species and has historically been quantified by ECOD activity (Goksøyr and Förlin, 1992). In the present study, none of the test compounds significantly increased ECOD activity. The one surprising observation was that the acetone control ($p<0.05$) and to some extent the ethanol control (although not significantly) induced ECOD activity in snails when compared to FSW (Figure 2.4).

Acetone and ethanol are metabolized by some of the same enzyme systems and can both induce expression of cytochrome P4502E1 (Lieber and Decarli, 1972; Brady et al, 1988; Sohda et al, 1993; Forkert et al, 1994; Bondoc et al, 1999). Brady et al (1988) and

Forkert et al (1994) reported that acetone significantly increased both the microsomal protein content and the activity of cytochrome P4502E1 in rat liver 18 h after a single dose of 15 nmol kg^{-1} body weight and in mouse liver 24 h after exposure to a single oral dose of 5 mL kg^{-1}. Ethanol has also been found to induce microsomal protein content and P4502E1 activity in rat liver (Lieber and Decarli, 1972, Lieber, 1999).

The use of ECOD activity to assess CYP2E1 induction has been demonstrated in mammals for nearly a decade (Wronska et al 1997; Gonzalez and Tarloff, 2004) and recently in invertebrates by Downs et al (2001) through the cross reactivity of mammalian CYP2E1 antibodies in *I. obsoleta*. Since acetone and ethanol can induce CYP2E1 induction and CYP2E1 can be quantified by ECOD activity, the increased ECOD activities seen in this study could be attributed to the acetone and ethanol exposures. A repeat of this study should be preformed to verify this supposition.

In snail exposure to B(a)P, ECOD activity was significantly ($p<0.05$) less than activity measure in the solvent control, acetone. This was unexpected since B(a)P has long been known to induce ECOD activity in higher order species (Stegeman and Hahn, 1994; Padros and Pelletier, 2000). One explanation may be the short duration of the exposure. B(a)P is considered a chronic toxicant and acute toxicity in not normally seen at environmentally relevant concentrations. Grinwis et al (2000) reported similar findings in the European flounder, *Platichthys flesus*. B(a)P was found not to induce CYP1A at exposure concentrations of 28 μg L^{-1} nor with intraperitoneal administration at

a dose of 25 mg Kg^{-1} body wt during a short-term. The reason for the lack of ECOD induction in compound exposed *I. obsoleta* may be related to the short duration of the experiment. Alternatively it is possible that invertebrates such as the mud snail do not respond to classical aryl hydrocarbon receptor (AhR) ligands in the same manner as higher organisms. This could be due to altered receptor mechanisms or composition of cytochrome P450 isozymes. The induction of P4501A activity has been reported to be very low (~2X background) in both gastropod and bivalve molluscs, particularly during active breeding periods (Livingstone and Farrar 1985; Hamers et al, 2004). Although the reason behind the seasonal differences it is not known, it could explain the decreases seen in our study since snails were collected in the middle of their breeding season (February).

2.4.4 Site-Specific Egg Capsule Production Assay

Decreased reproductive potential was also observed at some of the six RCERR sites. Sites 3, 4, and 6 were 11, 100, and 1.5 fold (respectively) lower with respect to egg capsule production per female than animals from reference Site 1. Site 3 is approximately 200 m from a tertiary-treated sewage discharge pipe. Sewage treatment effluents are known to contain pharmaceuticals, personal hygiene by-products and industrial chemicals (Daughton and Ternes, 1999; Kummerer, 2001).These type of compounds can have a wide range of effects on organisms living in proximity their discharge and in many cases produced endocrine disrupting effects (Kime, 1999;

Sumpter, 1998). In a recent study, Cœurdassier et al (2005) exposed snails, *Lymnaea palustris*, to wastewater effluent for four weeks resulting in decreased egg production per female, eggs per cluster, and egg clusters per snail. The decrease in egg capsules laid per female observed in this study may be due to a similar process involving the proximity to wastewater effluent. A study directly exposing *I. obsoleta* to the effluent discharge would test this hypothesis.

Both Sites 4 and 6 experience heavy boat traffic. Site 4 is located across from an active boat launching facility as well as docks for commercial fishing vessels. Snails from Site 4 exhibited the lowest number of egg capsules per female, over 100 fold lower than animals from the reference site. Site 6 is located on the western end of the RCERR adjacent to Radio and Pivers Islands and the Beaufort Inlet and produced approximately half the number of egg capsule as snails from Site 1. A study by Romano et al (manuscript in preparation) reported higher concentrations of PAH (pyrene, benzo(a)pyrene, and naphthalene) in the vicinity of Site 4 than Site 1 (unfortunately sediment from Site 6 was not tested). If snails are being stressed by these and other contaminants such as copper, TBT, and antifouling compounds then they (similarly to the diuron, CuPT, styrene compound assays) could also be experiencing endocrine system alterations or possibly reallocating energy supply from reproduction to survival and detoxification (Servia et al, 2006; Heiden et al, 2005).

It is interesting to note that in another study by Romano et al (manuscript in preparation), imposex levels at Site 1 were reported to be 0%, while a site directly across from site 4 reported an imposex rate of over 90%. The observed increased in imposex levels in snails from the area adjacent Site 4 may explain the dramatically lower number of egg capsules laid per female. It is possible that endocrine disrupting compound (EDC) contaminants entering the waterway across the creek are also deposited at Site 4. Unfortunately the imposex levels at Site 4 were not measured. Oehlmann et al (2000) and McClellan-Green (2005) have both reported significant decreases in fecundity and egg capsule production after exposure to nanomolar concentrations of triphenyltin and tributyltin, respectively. Unfortunately imposex levels were also not recorded at Sites 3 or 6. If snails were negatively impacted by organotin exposure, it is also possible their fecundity was impacted.

2.3.5 Site Specific ECOD Activity Assay

There was no statistically significant inhibition or induction of ECOD activity at any of the six sites sampled within the RCERR. This may be due to the time of year the animals were collected. Livingston and Farrar (1985) reported seasonal changes in benzo(a)pyrene hydroxylase activity (BPH) in the mussel, *Mytilus edulis* with BPH activity being low with the onset of spawning. They reported increases in BPH activity during the non-reproductive period. The *I. obsoleta* breeding season begins around mid-January and progresses through the end of April (Oberdörster et al, 1998, 2005;

Oberdörster and McClellan-Green, 2000). Since snails were obtained in January, it is possible that instead of increasing enzymatic activity to detoxify PAHs, snails ovodeposit these toxicants within the eggs. Other studies have reported toxic compounds being ovodeposited in eggs (Stewart et al, 1997; Dimitriou et al, 2003). In a study by Goldberg et al (2004) TBT was found in egg capsules of the snail *Adelomelon brasiliana* at concentrations ranging between 0.264 and 1.86 μg per egg.

To conclude, estuarine environments are unique and delicate ecosystems that serve as spawning grounds, nurseries, and living environments for numerous organisms and aquatic life. Unfortunately they are all too often the 'end of line' for pesticides, antifoulants, petroleum by-products, and industrial solvents. While these compounds can elicit acute responses at high concentrations, they typically cause far more silent problems such as endocrine disruption and reproductive damage. Although some of the concentrations used in the acute toxicity assays were not environmentally relevant (at present), the concentrations that resulted in decreased egg capsule production per female were relevant. The exact mechanism causing the decrease in fecundity in diuron, CuPT, B(a)P, and styrene exposed *I. obsoleta,* is not known at this time. Other than PAHs, the presence of these compounds within the RCERR is only hypothesized based on human influences. What is known is that some anthropogenic compound or mixtures of compounds are having negative impacts on fecundity in *I. obsoleta* within the Rachel

Carson Research Reserve. Further assessments must be made to evaluate the causes of such problems and to monitor the Reserve's continued health.

Chapter 3

The effects of diuron, copper pyrithione, benzo (a) pyrene, and styrene exposure on acute toxicity, condition index, fecundity, and ECOD activity in the American oyster, *Crassostrea virginica*: A laboratory and field survey in the Rachel Carson Estuarine Research Reserve, Beaufort, NC.

3.1 Introduction

The Rachel Carson Estuarine Research Reserve (RCERR) is home to a wide variety of marine invertebrates. One of its inhabitants is the American oyster, *Crassostrea virginica* (Gmelin, 1791). The oyster is a sessile bivalve mollusc of great ecological and economic importance. It is often used as a sentinel species due to its ability to bioaccumulate pollutants from the environment (Paez-Osuna et al, 2002). Healthy oyster populations increase estuarine water clarity by filtering out suspended particles (Gerritsen et al, 1994; Brumbaugh et al, 2000) and often serve as the only natural hard substrates for other marine animals in these soft-bottomed environments providing (Soniat et al, 2004; Woods et al, 2005).

Recently oysters have been the target of parasites, disease, and anthropogenic contaminates in the Chesapeake Bay, the estuaries and bays of North Carolina, and in other coastal Atlantic waters (Ewart and Ford, 1993; Smith et al, 2005; Encino et al, 2006). As result, the oyster industry has suffered dramatically over the past two decades. In 1983, more than 724,000 pounds of oyster meat was harvested with an estimated value of $1.02 million. This is compared to just 260,000 pounds in 2003, with an estimated value of $1.12 million according to the N.C. Division of Marine Fisheries (NCDMF). The purpose of this study was to assess the role of non-point source contaminants on oyster decline, by evaluating the effects of representative contaminants on metabolic and

physiological parameters. We also addressed current populations of *C. virginica* within the Rachel Carson Estuarine Research Reserve (RCERR) in Beaufort, NC in an effort to determine whether they are affected by non-point source contaminants. Acute and sub-lethal chronic effects were examined in *C. virginica* after exposure to four representative anthropogenic compounds (diuron, copper pyrithione, benzo(a)pyrene, and styrene) and at six sites within the Rachel Carson Estuarine Research Reserve (RCERR). These compounds were chosen to represent types of pesticides, biocides, petroleum by-products, and industrial solvents typically found in estuarine environments.

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea is urea herbicide that has been used in agriculture since the 1950s and has recently been added to some antifouling coatings. It inhibits photosynthesis by interfering with electron flow to the plastoquinone in photosystem II (PSII) complex, resulting in the blockage of the electron transport chain (Oettmeier, 1992; Jones and Kerswell, 2003). Diuron has been reported in some United States (U.S.) surface waters at concentrations ranging from a few $\mu g\ L^{-1}$ to over 1 $mg\ L^{-1}$ (Moncada, 2004; Green and Young, 2006) and has been found to inhibit photosynthesis in aquatic environments at concentrations as low as 0.3 $\mu g\ L^{-1}$ (Jones and Kerwell, 2003). The majority of the diuron entering the aquatic environment is a result of agricultural and residential runoff (Moncada, 2004; Green and Young, 2006).

Copper pyrithione (2-mercaptopyridine N-oxide copper salt) (CuPT) is a broad spectrum biocide that was introduced into the antifouling market in 1996 (Arch

Chemicals; Maraldo and Dahllöf, 2004). It is presently added to many existing coatings in an attempt to lower overall copper concentrations (Voulvoulis et al, 1999). Little is know regarding its mode of toxicity or its current environmental concentration however Mackie et al (2004) reported total pyrithiones (including sodium-, zinc-, and copper conjugates) within United Kingdom (U.K.) waters exceeding 0.3 μg L^{-1}. In the aquatic environment other pyrithiones (sodium and zinc) transchelate (Mackie et al., 2004; Turley et al, 2005) to CuPT with half-lives ranging between 15 min to 30 days (Turley et al., 2005; Maraldo and Dahllöf, 2004), depending upon environemntal conditions. The fastest degradation rates of CuPT occur in the presence of light or sediments (Turley et al, 2005). While absolute certainty of the mode of action of CuPT is unclear, Ermolayeva and Sanders (1995) suggest that pyrithiones in general act by interfering with the activity of the primary proton pump, H^{+}-ATPase. This is hypothesized to result from the collapse of cell membrane pores which lead to ion gradient destabilization and ultimately cell apoptosis (Bragadin et al, 2003). It is assumed that CuPT operates by a similar mechanism.

Benzo(a)pyrene (B(a)P) is a frequently occurring polycyclic aromatic hydrocarbon (PAH) found in aquatic environments. B(a)P is naturally produced as a result of forest fires and volcanic eruption (Wcislo, 1998) but is typically introduced into the marine environment as a result of combustion and industrial activities (Hylland, 2006). B(a)P is listed by the U.S. Environmental Protection Agency as a possible

carcinogen (USEPA 1996, 2002) and has been found at concentrations greater than 50 μg Kg^{-1} in highly polluted areas of the U.S., England, Finland, Lithuania, and Russia (Kirso et al , 2002; Saltiene et al, 2002). B(a)P toxicity results from its (and its metabolites) ability to interact with nucleic acids and proteins (Stegeman and Lech, 1991; Stegman et al, 1992; Xue and Warshwsky, 2005) resulting in biochemical and genetic alterations

Styrene is a volatile organic molecule (VOM) found in solvents, motor vehicle exhaust, and from other industrial activities (Gibbs and Mulligan, 1997) It is one of the top carcinogenic compounds released into the environment (U.S. Toxic Release Inventory, 1997) and has been reported in some South American wastewater effluents at concentrations exceeding 5.0 mg L^{-1} (Gomez et al, 2001). Numerous studies have shown that styrene is deposited in the liver, pancreas, kidneys and brain of mammals and can accumulate to significant levels lipid rich tissues (Bond, 1989). While several studies on the effects of styrene exposure in mammals and other vertebrates have been reported, few have been performed in aquatic invertebrates.

In this study mortality, condition index, egg production, and ECOD activity were assessed in the American oyster, *Crassostrea virginica* after exposure to four representative classes of compounds and at six sites within the RCERR. An organism's ability to deal with non-point source contaminants is critical to survival in most polluted environments.

Condition index is one method employed to evaluate how oysters and other bivalves are affected by their environment (Van Dolah et al, 1992; Rheault and Rice, 1996) and has historically been used to assess oyster growth with respect to anthropogenic contaminants (Lawrence and Scott, 1982; Lauenstein and O'Conner, 1988; Lytle and Lytle, 1990; Yevich and Zaroogian, 1990). The literature is replete with different methods for calculating condition index, but it more often includes a ratio involving wet and dry weights, together with other physical parameters such as length, width, or volume. This study employs the condition index as described by Abbe and Sanders (1988), which compares dry tissue weight to the interior volume of oysters. Changes in growth can be attributed to physiological changes experienced by oysters in terms of carbohydrate, protein, lipid, and mineral content as well as parasitism and the effects of anthropogenic compounds (Austin et al, 1993; Cheung and Tse, 1993; Marin-Mezquita et al, 1997).

The P450 system is an integral part of the detoxification process and is typically associated with reactions such as oxidation, reduction, and hydrolysis (Stegeman and Hahn, 1994; DiGiulio et al, 1995; Landis and Yu, 1999). Ethoxycoumarin-O-deethylase (ECOD) has been widely used to assess substrate specificity of cytochrome P450 in mammals and some invertebrates (Goksøyr and Förlin, 1992). The major catalysts for ECOD activity in mammals are P450 isoyzmes 1A1, 1A2, 2B, and 2E1 which are induced by a range of anthropogenic compounds (Yamazaki et al, 1996).

The area surrounding the reserve has recently undergone an increase in residential development and anthropogenic activity. The persistent input of pollution from these sources into the environment has the potential to upset the existing ecosystem. The purpose of this study was to provide insight into the effects of non-point source pollution in the ecologically and economically important model organism *C. virginica.*

3.2 Methods and Materials

3.2.1 Study Sites

Site 1 (Figure 3.1) is located within the southwest portion of the RCERR and has been used as a reference site in several pervious studies (Oberdörster et al, 1998, 2005; Oberdörster and McClellan-Green, 2000; Straw and Rittschof, 2004; McClellan-Green et al, 2006). Site 2 is located on the Carrot Island side of Taylors Creek, a tidal creek, and is approximately 200 m across from a public marina and numerous residential and public docks. Site 3 is situated to the east of Site 2 along the Carrot Island side of Taylors Creek and is approximately 100 m from a tertiary treated wastewater drainage pipe. Site 4 is even further east along the Carrot Island side of Taylors Creek and is regularly exposed to persistent and residual petroleum by-products from an active boat launch, a dock for commercial fishing vessels, and a nearby wood veneer production facility. Site 5 is located along the North River Channel and was chosen in order to evaluate

anthropogenic input resulting from agricultural and recreational activities in the North River. Site 6 is situated on the western end of the RCERR across from Radio and Pivers Islands. It receives tidal flows from the North River and Newport River estuary and is an active waterway for boats entering the Beaufort Inlet.

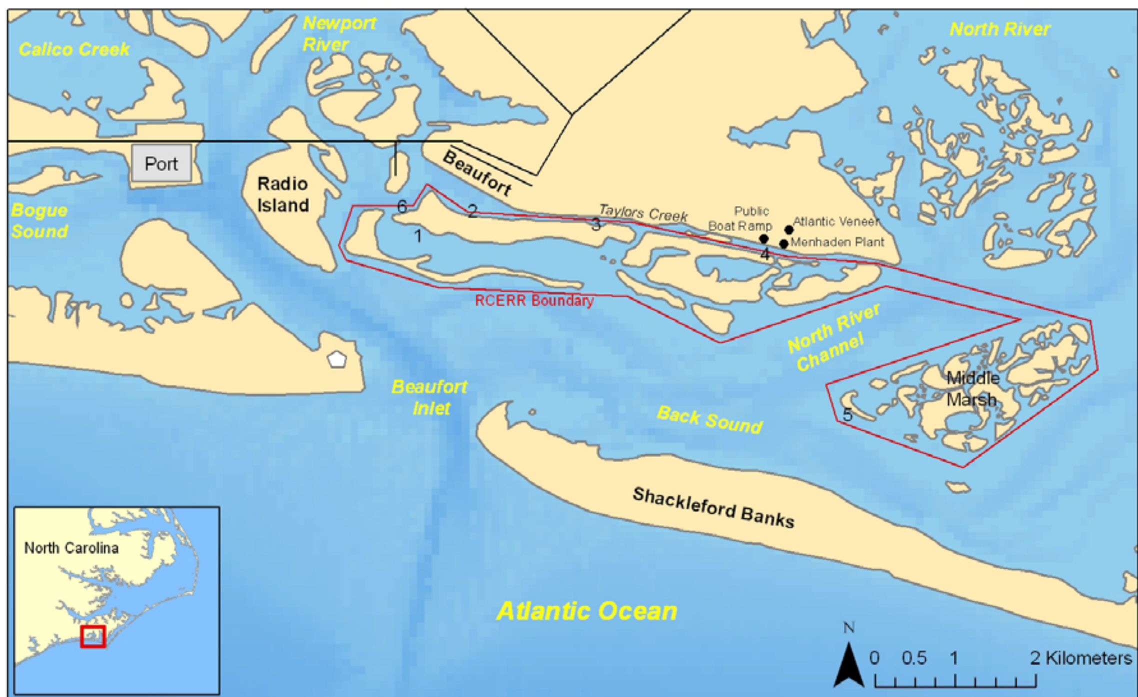


Figure 3.1 Map of the Rachel Carson Estuarine Research Reserve (RCERR), Beaufort, NC with oyster collection sites indicated by site number.

3.2.2 Experimental Solutions

Stock solutions of diuron (Sigma-Aldrich, St. Louis, MO) were prepared in acetone at a concentration of 10 g L^{-1} following Jones and Kerswell (2003) and Chesworth et al (2004). Stock solutions of CuPT (Arch Chemicals, Norwood, CT) were prepared in

dimethylsulfoxide (DMSO) at a concentration of 2 g L^{-1} following Kobayashi and Okamura (2002). B(a)P(Sigma-Aldrich, St. Louis, MO) stock solution was prepared following P. McClellan-Green, *personal correspondence*, in warm acetone at a concentration of 1 g L^{-1}. Stock solutions of styrene (Fisher Scientific Company, Fair Lawn, NJ) were prepared following Ohtani et al (2001) in ethanol at a concentration of 10 g L^{-1}. All stock solutions were kept in amber bottles or wrapped in aluminum foil at room temperature for no longer then 1 week to avoid photolysis. Working solutions were made directly before use by diluting stock solutions in filtered seawater (FSW) to appropriate concentrations.

3.2.3 Diuron, CuPT, B(a)P, and Styrene Acute Toxicity – LC_{50}

Approximately 80–120 oysters were collected from Site 1 during the months April, June, and July 2005. Oysters were maintained at the Duke University Marine Laboratory (DUML) on a 14L:10D cycle in continuously flowing single pass sand-filtered seawater (30–35 salinity, approx. 4°C) for 48 hours prior to 96 h mortality assays.

Ninety six hour acute toxicity assays were conducted with water renewal and mortality checked daily. Oysters (all over 7 cm in length) were housed in groups of 10–12 in 54 L glass aquaria and allowed to acclimate to a 14L:10D light cycle, temperature of 18–20°C, and a salinity of 35 ppt for 24 hr before the start of each experiment. Each compound was tested at different time periods. FSW and solvent controls were tested at the highest concentration of their respective compounds in each treatment (diuron/

acetone, CuPT/DMSO, B(a)P/acetone, styrene/ethanol). Compound treatments consisted of the following: 1, 5, 10, and 50 mg L^{-1} diuron; 5, 10, 50, 100 μg L^{-1} CuPT; 10, 50, 500 μg L^{-1} B(a)P; and 50, 500, 5000 μg L^{-1} styrene.

Animals were deemed dead if a gap between the two valves was evident following removal from the tanks indicating that the abductor muscle was no longer functioning. Desiccation and odor were also used to assess mortality. Acute toxicity or the lethal concentration required to cause mortality in 50 % of animals was estimated using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA).

3.2.4 Compound-Specific Condition Index and ECOD Activity Assays

Approximately 80–120 oysters were collected from Site 1 in November 2006. Oysters were maintained on a 14L:10D cycle under the same condition as in section *3.2.3* for 48 hours prior to treatment. Following exposure, the oysters were assessed for compound specific condition index and ECOD activity. The concentrations used in these assays were determined from preliminary findings and acute toxicity measurements and were deemed to be environmentally relevant based on reports in the literature.

Compound-Specific Condition Index Assay

Groups of 3 *C. virginica* were placed in large acid-washed glass finger bowls and exposed to FSW, DMSO, acetone, and ethanol controls at the highest concentration of their respective compounds. Treatment exposures were conducted in triplicate at concentrations in 1L volumes of 1000 μg L^{-1} diuron; 10 μg L^{-1} CuPT; 50 μg L^{-1} B(a)P; and

2000 μg L^{-1} styrene. All assays were carried out for 96 h with daily water renewal and mortality checks. At the end of the 96 h exposure period, whole wet weight, body wet weight, and empty shell wet weight were recorded. Oysters were then dried for 48 h at 85°C and whole dry weight, body dry weight, and empty shell dry weight were recorded. Condition index was determined following Abbe and Sanders (1988).

Condition Index = (dry total oyster weight (g)/dry shell cavity volume) x 100

Dry Shell Cavity Volume = whole weight – empty shell weight

The condition index values were compared between controls and their respective treatments using paired T-tests (SigmaPlot, SYSTATSoftware Inc. San Jose, CA).

Compound-Specific ECOD Activity Assay

Groups of 3 *C. virginica* were placed in large acid-washed glass finger bowls and exposed to the same solvent controls and compound treatments as in section *3.2.4* for 96 h with daily water renewal and mortality checks. Oysters were dissected and digestive gland removed. Digestive glands from individual oysters were then rinsed in 50 mM Tris (pH 7.4), 1.15% KCl, blotted and weighed to the nearest 0.1 g. Tissue was then homogenized in 50 mM Tris (pH 7.4) and 1.15% KCl at a 1:3 (v/v) ratio and centrifuged at 9,000 x g for 10 minutes. Supernatant was stored at -80°C until assayed. The protein content of all samples was determined using the Bradford method (Bradford, 1976).

Solutions consisting of 1 mL of 2 mM 7-Ethoxycoumarin in Tris-HCl (pH 7.6), 400 μL of 50 mM Tris-HCl (pH 7.6), and 100 μL of sample, were added to glass tubes and pre-warmed 5 minutes in a shaking water bath at 30°C. At the end of the incubation,

25 μL of 5 mM NADPH was added to each tube and incubated at 30°C for 20 minutes. The reaction was stopped by plunging tubes into an ice bath and deproteinated by adding 1 mL of cold 5% (w/v) zinc sulphate. Samples were centrifuged at 2000 x g for 15 minutes at 4°C (Lake, 1987). One mL of deproteinated supernatant was transferred to clean glass tube containing 2 mL of 0.5 M glycine-NaOH buffer (pH 10.5). ECOD activity was measured following Livingstone (1991) (pmol hydroxycoumarin min^{-1} mg^{-1} protein) using a Perkin-Elmer (Norwalk, CT) Model LS50B luminescence spectrophotometer at 380/456 and excitation and emission, respectively. Statistical analysis was compared between controls and their respective treatments using paired T-tests for CuPT, B(a)P, and a Mann-Whitney Rank of Sums for diuron (failed normality test) (Sigma Plot, SYSTAT Software Inc. San Jose, CA).

3.2.5 Site-Specific Condition Index, Eggs per Female, and ECOD Activity Assays

Approximately 60 oysters were collected from each of the six sampling sites during June 2006. Once collected, oysters were returned and maintained under the same acclimation conditions as the acute and exposure assays.

Site-Specific Condition Index Assay

Similar testing conditions and procedures used in the compound-specific assays (section 3.*2.4*) for condition index were employed for the site-specific assay. Five oysters from each site were opened and whole wet weight, body wet weight, and empty shell

wet weight were recorded. Oysters were then dried for 48 h at 85°C and whole dry weight, body dry weight, and empty shell dry weight were recorded. Condition index was determined following Abbe and Sanders (1988). Statistics were preformed on condition index values per site using a Kruskal-Wallis One-Way Analysis of Variance on Ranks (SigmaPlot, SYSTAT Software, Inc. San Jose, CA).

Site-Specific Eggs per Female Assay

Five oysters from each of the six sites were immediately dissected following collection. A drop of gonad from each oyster was placed on a slide and sex determined using a 20X Wild Heerbrugg dissecting microscope. The presence of eggs indicated the oyster was female and gravid.

Once females were identified, the gonad was sliced/scraped downward into a small finger bowl and the tissue diced into small pieces. Water was used to dislodge eggs from the gonad and the material decanted into a 250 mL beaker. This was repeated four times to ensure all eggs were removed. The egg and tissue mixture was then filtered through a 75 μm sieve and eggs collected on a 20 μm sieve. Eggs were suspended in 200 mL FSW. Five 10 μL aliquots were placed on a single slide and the number of eggs counted. Aliquots were averaged for each oyster. Statistics were preformed using paired T-tests to compare the average number of eggs per female at Site 1 with the remaining sites (SigmaPlot, SYSTATSoftware Inc. San Jose, CA).

Site-Specific ECOD Activity Assay

Similarly to section *3.2.5*, five oysters from each of the six sites were immediately dissected following collection. Individual oysters were rinsed in 50 mM Tris (pH 7.4), 1.15% KCl, blotted and weighed to the nearest 0.1 g. The protocols described in section *2.4.2* were used here to determine protein concentration and ECOD activity. Statistical analysis was preformed using SigmaPlot (SYSTAT Software Inc. San Jose, CA). ECOD activity from oysters at Site 1 were compared to those from Sites 2, 3, 4, 5 and 6 using paired T-tests.

3.3 Results

3.3.1 Diuron, CUPT, B(a)P, and Styrene Acute Toxicity – LC_{50}

No mortality was observed in *C. virginica* exposed to FSW or any of the solvent controls. Diuron had a lowest observable effective concentration (LOEC) of 5 mg L^{-1} and produced a LC_{50} of 10.85 mg L^{-1} (95% C.I. 7.49–15.72 mg L^{-1}; Figure 3.2). CuPT produced a LOEC in oysters of 10 μg $L^{-1}$and resulted in a LC_{50} of 74.53 μg L^{-1}(95% C.I. 57.22–97.1 μg L^{-1}; Figure 3.2). A LC_{50} in *C. virginica* exposed to B(a)P or styrene could not be determined. This is likely due to the solubility limits of B(a)P and the volatility of styrene in the aerated tanks.

3.3.2 Compound-Specific Condition Index Assay

The average condition index value for *C. virginica* exposed solely to FSW was 97.1 (Standard Error of the Means ± 1.5) (Figure 3.3). This was equivalent to the acetone,

DMSO, and ethanol controls. There were significant differences between oysters exposed to 1000 μg L^{-1} diuron and those exposed to the acetone control (C.I. = 86.8 (± 1.8; p=0.006)). Oysters exposed to 10 μg L^{-1} CuPT were significantly different from the DMSO control (C.I = 82.4 (±1.2; p=0.002). Styrene (2000 μg L^{-1}) exposed oysters also had a significantly lower condition index (C.I. = 84.8 (± 3.1; p=0.033) when compared to their ethanol control. No significant difference in condition index could be determined between 50 μg L^{-1} B(a)P, (C.I. = 85.4 (± 4.7)) and its acetone control.

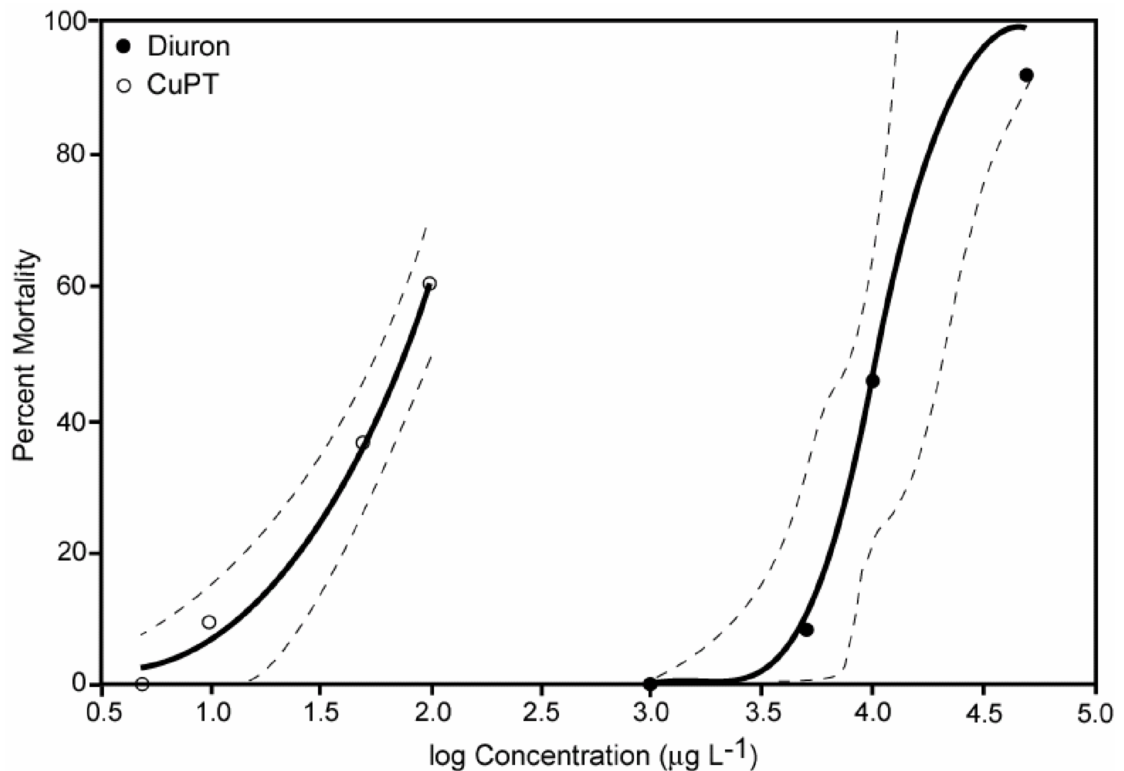


Figure 3.2 Mortality in *Crassostrea virginica* after 96 h exposure to diuron (●) and CuPT (○). Dashed lines indicate 95% confidence intervals.

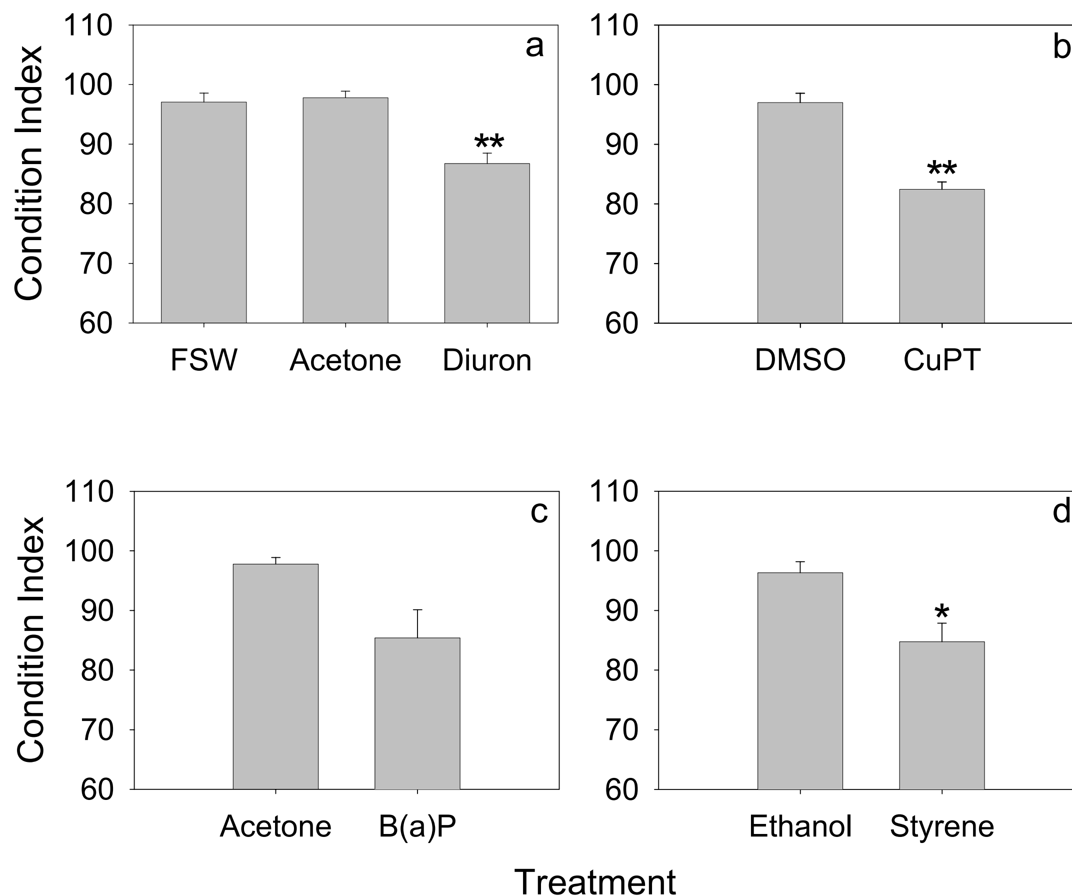


Figure 3.3 Average condition index value for *Crassostrea virginica* after 96 h exposure to (a) FSW, acetone control, and diuron, (b) DMSO control and CuPT, (c) acetone control and B(a)P, and (d) ethanol control and styrene. Error bars are standard error. *p<0.05, **p<0.005.

3.3.3 Compound-Specific ECOD Activity Assay

The average ECOD activities in oysters exposed to FSW was 0.007 (± 0.0037) pmol hydroxycoumarin min^{-1} mg^{-1} protein (Figure 3.4). This was significantly lower ($p<0.05$) than for oysters exposed to the solvent control, acetone (0.032 (± 0.0033) pmol

hydroxycoumarin min^{-1} mg^{-1} protein). Neither DMSO nor ethanol was found to be significantly different than FSW. ECOD activity was decreased, although not significantly, by all four compounds when compared to their individual solvent controls (Figure 3.4).

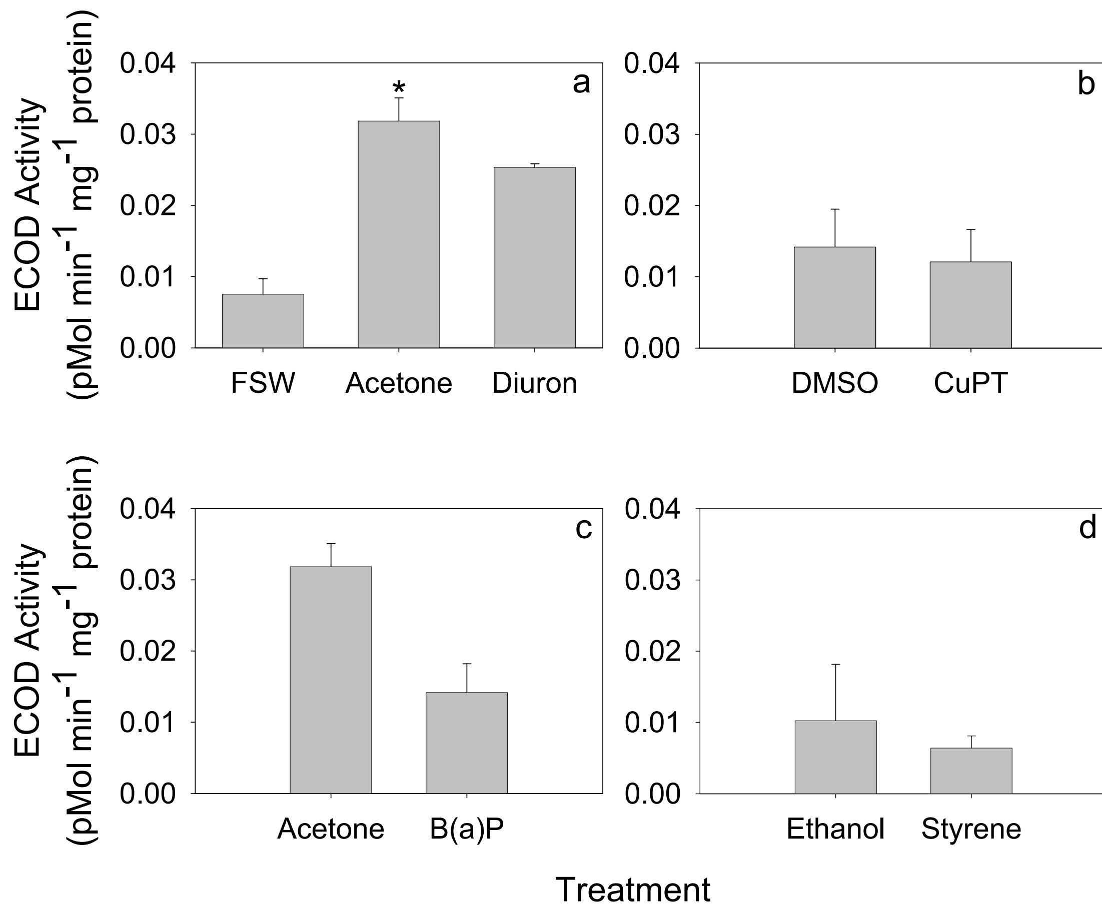


Figure 3.4 ECOD activity in *Crassostrea virginica* after 96 h exposure to (a) FSW, acetone solvent and diuron control, (b) DMSO solvent control and CuPT, (c) acetone solvent control and B(a)P, and (d) ethanol solvent control and styrene. Error bars are standard error.* $p<0.05$ between FSW and Acetone.

3.3.4 Site Specific Condition Index Assay

The average condition index ranged from 85.76 to 99.86 between the six sites (Figure 3.5). Condition index averaged 99.86 (±0.6) at control Site 1. This was significantly ($p<0.001$) different from condition index values from sites 4, 5, and 6 having condition index values of 84.6 (±2.3), 85.61 (±3.3), 85.76 (±2.0), respectively.

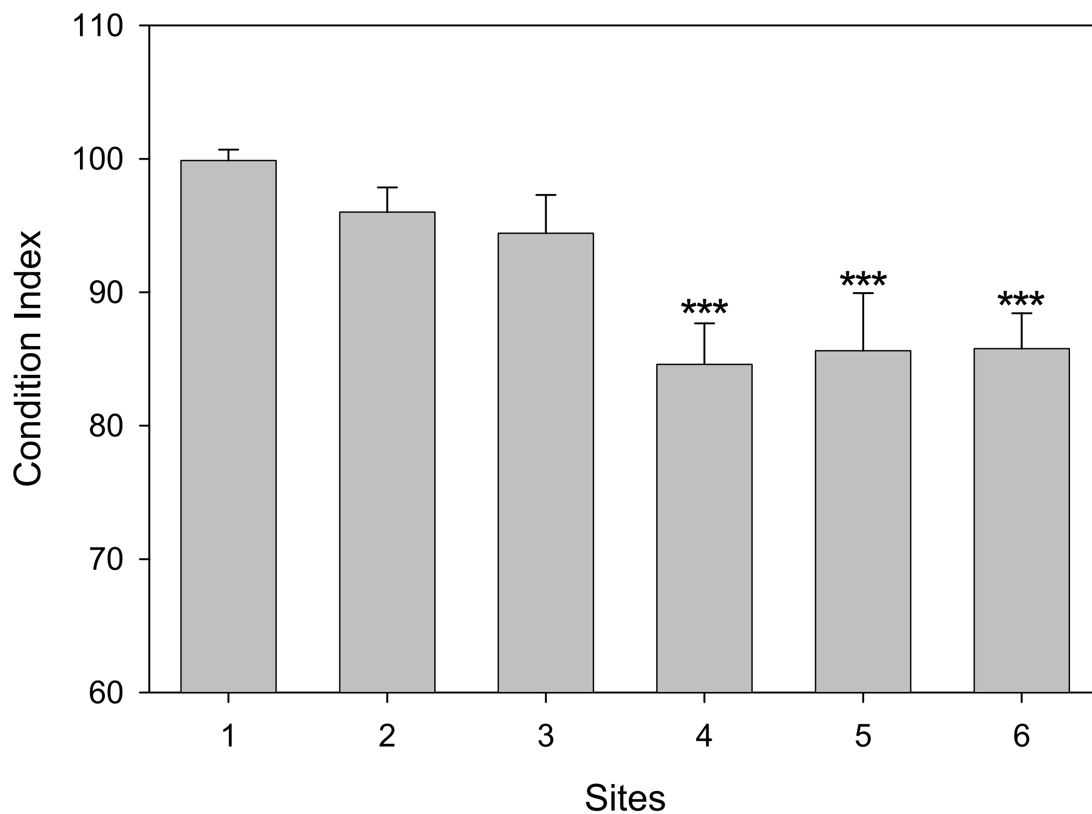


Figure 3.5 Average condition index value for *Crassostrea virginica* from sites 1–6. Error bars are standard error. ***p<0.001.

3.3.5 Site-Specific Eggs per Female Assay

The average number of eggs per gram of female oysters from Sites 1–6 range from 5.2×10^4 to 3.2×10^5 (Figure 3.6). Site 3 had the lowest number of eggs per gram with an average of 5.2×10^4 eggs per gram ($\pm 8.2 \times 10^3$), while site 2 had the highest eggs per gram averaging 3.2×10^5 ($\pm 9.1 \times 10^4$). Sites 4, 5 and 6 resulted in average eggs per gram of 6.3×10^4 ($\pm 2.8 \times 10^4$), 2.3×10^5 ($\pm 4.5 \times 10^5$), and 3.1×10^5 ($\pm 3.6 \times 10^4$) respectively. Only Site 6 was found to be significantly different from Site 1 (6.6×10^4 ($\pm 1.3 \times 10^4$)) ($p<0.05$).

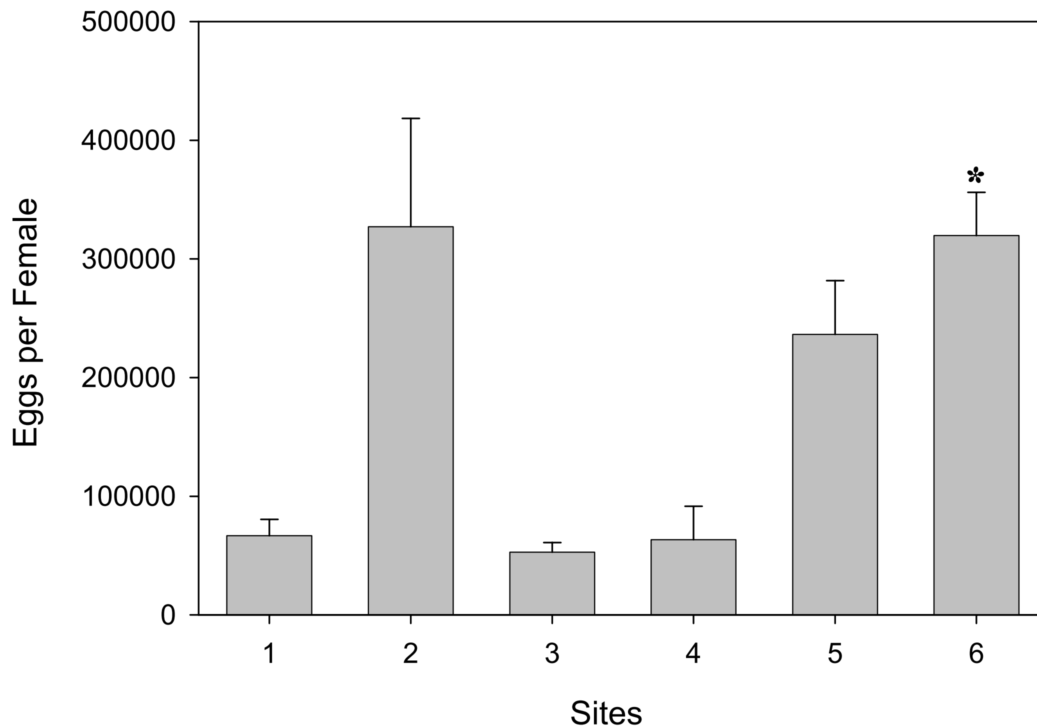


Figure 3.6 Average number of egg capsules per *Crassostrea virginica* at sites 1–6. Error bars are standard error. *p<0.05.

3.3.6 Site-Specific ECOD Activity Assay

ECOD activity in oysters collected from Sites 1–6 surrounding the RCERR were found to have enzyme activities ranging from levels of 0.017 (± 0.002) to 0.062 (± 0.002) pmol hydroxycoumarin min^{-1} mg^{-1} protein, (Figure 3.7) respectively. Only Site 6 (0.062 (± 0.002) pmol hydroxycoumarin min^{-1} mg^{-1} protein) was found to be significantly different ($p<0.001$) from the control Site 1 (0.017 (± 0.002) pmol hydroxycoumarin min^{-1} mg^{-1} protein).

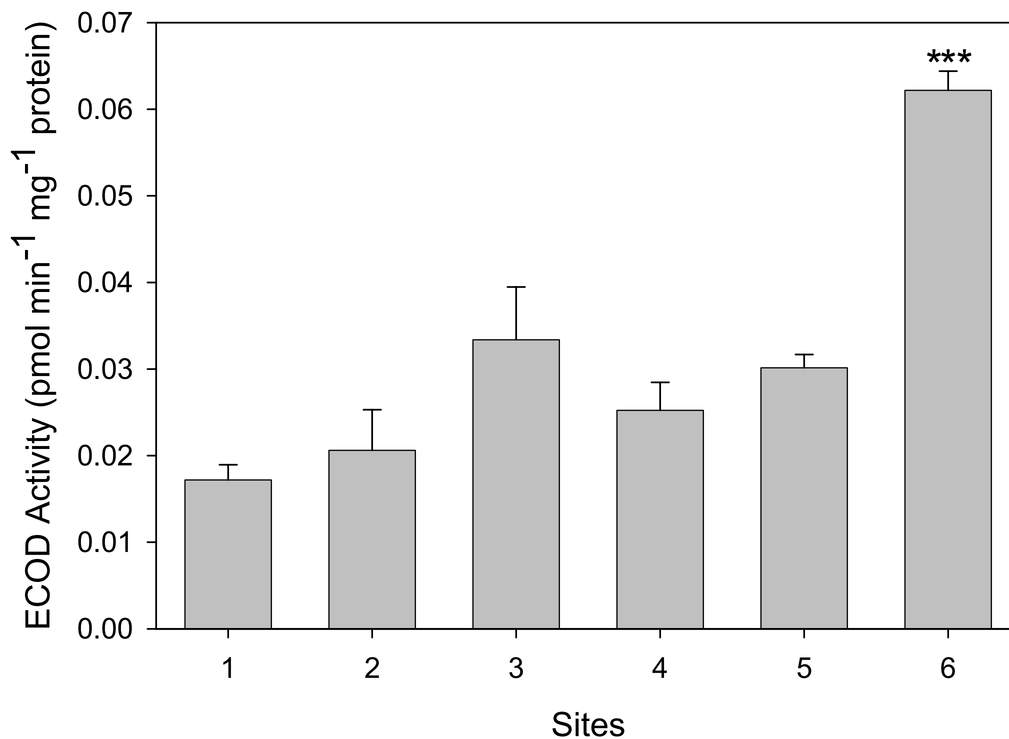


Figure 3.7 ECOD activity in *Crassostrea virginica* at sites 1–6. Error bars are standard error. ***p<0.001.

3.4 Discussion

3.4.1 Diuron, CUPT, B(a)P, and Styrene Acute Toxicity – LC_{50}

In the present experiment diuron and CuPT caused mortality in *C. virginica* at concentrations that are not environmentally realistic. Moncada (2004) as well as Green and Young (2006) reported concentrations of diuron in surface waters ranging from 2–30 $\mu g\ L^{-1}$. Total pyrithiones have only been found up to 100 nM in some U.K. rivers and harbors (Mackie et al, 2004). These are both 1000x lower than levels necessary to achieve mortality in *C. virginica*. B(a)P and styrene did not produce mortality even at their highest respective concentrations.

Although concentrations of diuron and CuPT required to cause mortality in oyster are not environmentally realistic, it is interesting to note that CuPT causes mortality at concentrations two orders of magnitude lower than diuron. Similar results were reported for other model species (Table 3.1). Mochida et al (2006) and NRAAVC (2001) reported LC_{50} values for CuPT at concentrations three orders of magnitude below those of diruon in the shrimp, *Heptacarpus futilirostris* and in fish, *Pinmephales promelas.*

Table 3.1 Toxicity data of diuron, copper pyrithione (CuPT), benzo(a)pyrene (B(a)P), and styrene.

Compound	Organism	LC_{50}	Reference
Diuron	*Mercenaria mercenaria* (clam)	5 mg L^{-1}	Davis and Hidu, 1969
	Americamysis bahia (shrimp)	1.1 mg L^{-1}	U.S. EPA, 2000
	Daphnia magna (water flea)	0.4 mg L^{-1}	Shcherban, 1972
	Pterconarcys californicus (stone fly)	3.6 mg L^{-1}	Sanders and Cope, 1968
	Aedes aegypti (mosquito)	1.2 mg L^{-1}	Nebeker and Schytema, 1998
	Chironomus tentans (midge)	3.3 mg L^{-1}	Knapek and Lakota, 1974
	Lytechinus variegatus (echinoderm)	7.14 mg L^{-1}	Chapter 4, this dissertation
CuPT	*Heptacarpus futilirostris* (shrimp)	2.5 μg L^{-1}	Mochida et al, 2006
	Pagrus major (fish)	9.3 μg L^{-1}	Mochida et al, 2006
	Aquatic fish	4.3–43.6 μg L^{-1}	Yamada and Kauno, 2002*
	Pinmephales promelas (fish)	4.3 μg L^{-1}	NRAAVC, 2001
	Oncorhynchus mykiss (fish)	7.6 μg L^{-1}	Okamura et al., 2002
	Lytechinus variegatus (echinoderm)	14.04 μg L^{-1}	Chapter 4, this dissertation
B(a)P	*Daphnia pulex* (water flea)	5 μg L^{-1}	Trucco et al, 1983
	xenopus laevis (frog embryo)	13.4 mg L^{-1}	Propst et al, 1997
	Solea solea (fish)	5 mg L^{-1}	Au et al, 1999
	Gammarus duebeni (amphipod)	11 mg L^{-1}	Lawrence and Poulter, 1998
Stryene	*Pimephales promelas* (fish)	10 mg L^{-1}	Cushman et al, 1997
	Hyalella azteca (amphipod)	9.5 mg L^{-1}	Cushman et al, 1997
	Eisenia fostida (earthworm)	120 mg Kg^{-1}	Cushman et al, 1997

* orginal article in Japanese, abstract in english

One possible explanation for this differential in diuron and CuPT toxicity may be their respective modes of action. Diuron is a photosystem II inhibitor (Moncada, 2004) and acts by interfering in the electron transport chain of photosynthetic organisms

(Oettmeier, 1992; Lansen et al, 1993; Jones and Kerswell, 2003). The mode of action for CuPT is thought to be disruption of the cell membrane resulting in altered ATP synthesis (Chandler and Segel, 1978; Dinning et al, 1998 Bragadin et al, 2003). One would expect CuPT to have greater consequences in non-photosynthetic organisms than diuron because it produces mortality at concentrations three orders of magnitude lower. The real and underlying problem with diuron is that it can inhibit photosynthesis at concentrations as low as 0.3 $\mu g\ L^{-1}$ (Jones and Kerswell, 2003). This could theoretically threaten the photosynthetic food sources that oysters and other marine organisms rely upon, potentially causing indirect mortality.

3.4.2. Compound-Specific Condition Index Assay

Condition index is an important tool in accessing bivalve health. It typically compares dry/wet tissue weight to interior bivalve volume (Abbe and Sanders, 1988) and is used as a tool for monitoring environmental pollutants (Lawrence and Scott, 1982; Scott and Lawrence, 1982; Rosas et al, 1983). Oysters have been observed to suffer changes in their lipid, mineral and carbohydrate content in response to some anthropogenic compounds (Austin et al, 1993). A perfect condition index score using Abbe and Sanders (1988) method of assessment is 100. *C. virginica* exposed to FSW resulted in a condition index value of 97.1. This was not significantly different from any of the solvent controls. Condition index values observed in diuron, CuPT, and styrene were all significantly less then their respective controls. Condition indexes of oysters

exposed to B(a)P were not observed to be significantly different from their acetone control. One reason for the lack of difference between B(a)P and acetone may be that the oysters were exposed to the treatments for only 96 h. B(a)P is well known carcinogen with the ability to produce DNA adducts over chronic time periods (Garman et al, 1997; Ericson et al, 1998; Reichert et al, 1998; Ericson et al, 1999). The duration of B(a)P exposure may not have been long enough to produce significant changes in weight. Because we wanted to examine the acute affects of compound exposure on condition index and not other variables such as algal food (especially because of the photosynthetic inhibitory effect of diuron) the assays were only run 96 h. The effects of chemical exposures on condition index are typically monitored 20 to 30 days (Lannig et al, 2006) with feedings occurring 2 to 3 time per week. The significant difference found between diuron, CuPT, and styrene and their respective acetone, DMSO, and ethanol controls are unclear at this time, but may be due to utilization of lipid, and carbohydrate stores as a result of starvation (Davis and Hidu, 1969; Lytle and Lytle, 1990; Au et al, 1999; Lannig et al, 2006).

3.4.3 Compound-Specific ECOD Activity Assay

The cytochrome P450 (CYP450) system is an integral part of the detoxification process and is typically associated with phase I type reactions (Landis and Yu, 1999) such as oxidation, reduction, and hydrolysis (Stegeman and Hahn, 1994; Di Giulio et al, 1995). Benzo(a)pyrene is a classical PAH inducer of cytochrome P450 activity

(specifically CYP4501A) in many species and has historically been quantified by ECOD activity (Goksøyr and Förlin, 1992). In the present study, none of the test compounds significantly increased ECOD activity. The one surprising observation was that the acetone control ($p<0.05$) and to some extent the ethanol control (although not significantly) induced ECOD activity in oysters when compared to FSW (Figure 3.4).

Acetone and ethanol are metabolized by similar enzyme systems and can both induce expression of cytochrome P4502E1 (Lieber and Decarli, 1972; Brady et al, 1988; Sohda et al, 1993; Forkert et al, 1994; Bondoc et al, 1999). Brady et al (1988) and Forkert et al (1994) reported that acetone significantly increased both the microsomal protein content and the activity of cytochrome P4502E1 in rat liver 18 h after a single dose of 15 nmol kg^{-1} body weight and in mouse liver 24 h after exposure to a single oral dose of 5 mL kg^{-1}. Induction of microsomal protein content and P4502E1 activity in rat liver has also been reported to be induced by ethanol (Lieber and Decarli, 1972, Lieber, 1999).

The use of ECOD activity to assess CYP2E1 induction has been demonstrated in mammals (Wronska-Nofer et al 1997; Gonzalez and Tarloff, 2004) and invertebrates (Downs et al, 2001). Since acetone and ethanol can induce CYP2E1 induction and CYP2E1 can be quantified by ECOD activity, the increased ECOD activities seen in this study could be caused by the acetone and ethanol exposures. A repeat of this study should be preformed to verify this hypothesis.

In oysters exposure to B(a)P, ECOD activity was less (but not significantly) than activity measure in the solvent control, acetone. This was unexpected since B(a)P has long been known to induce ECOD activity in higher order species (Stegeman and Hahn, 1994; Padros and Pelletier, 2000). One explanation may be the short duration of the exposure. B(a)P is considered a chronic toxicant with acute toxicity not normally observed at environmentally relevant concentrations. Similar reports of B(a)P related decreases in ECOD activity have been reported by Grinwis et al (2000) in the European flounder, *Platichthys flesus* exposed to B(a)P at concentrations as high as 28 $\mu g\ L^{-1}$

Another possible explanation for the lack of ECOD induction seen in *C. virginica* may be attributed to the fact that oysters do not respond to classical aryl hydrocarbon receptor (AhR) ligands in the same manner as higher organisms. This could be due to altered receptor mechanisms or composition of cytochrome P450 isozymes. The induction of P4501A activity has historically been very low (~2X background) in both gastropod and bivalve mollusks (Hamers et al, 2004), particularly during active breeding periods (Livingstone and Farrar, 1985). Since oysters were collected for the ECOD assays in November, it is possible that seasonal differences contributed to the overall lower ECOD activity.

3.4.4 Site Specific Condition Index Assay

Site specific condition index values helped estimate the health of organisms at particular sites within and around the RCERR. Comparisons of condition index values

were then made among all sites for an overall assessment of oyster health in the RCERR. Condition index values ranged between 85 to over 99. Control Site 1 had the highest condition index value followed closely by Sites 2 and 3, indicating that these organisms were in good health. Site 4, 5, and 6 all had condition index values that were significantly lower than Sites 1 and 2. Site 4 experiences heavy boat traffic and is across (100 m) from an active public boat ramp, docks, and industrial development. Site 5 is located at the mouth of the North River and receives pollutants from residential and agricultural sources up stream. Site 6 is also an area of high boat traffic located within the Beaufort Inlet, which serves as the main thoroughfare to Beaufort Harbor. Romano (2007, chapter 2) reported lower egg capsule production in female *Ilyanassa obsoleta* from Site 4, thus, it is not surprising that oysters from this same site may be stressed. It is possible that disease, fewer nutrients, and/or contaminant exposure in this area is causing oysters to reallocate energy from carbohydrate and lipid production to supplying energy for detoxification and survival (Servia et al, 2006).

3.4.5 Site-Specific Eggs per Female Assay

As a result of the condition index finding from Sites 1–6, we decided to investigate whether oysters with lower condition indexes also had lower numbers of eggs per gram tissue (female). Female oysters that are of harvestable size, 7 cm or greater, can release as many as 100 millions eggs during a reproductive season spanning from early to late spring and again from early to late fall (Williams, 2001).

Oysters from Site 6 were found to have significantly more eggs per gram tissue (female) than oysters from Site 1. At this time we are not sure how to interpret this data. The most likely cause for differences in egg concentration are water temperature and nutrition. Because Site 1 is shallower and does not experience the strong tidal currents of Site 6, it is possible that the temperatures at Site 1 reached the ideal spawning temperatures, between 15.5 and 20°C (Williams, 2001), prior to sampling, therefore oysters from this site were already depleted. Collection of oysters at these two sites may have occurred after and before spawning, respectively. It is also possible that animals from Site 1, while healthier, are nutritionally limited with respect to egg production. Site 1 is a low lying mud flat with few inputs while Site 6 experiences a strong tidal flow with nutrient input from up river sources.

3.4.6 Site-Specific ECOD Activity Assay

The cytochrome P450 system is responsible for metabolizing many different types of substrates including fatty acids, prostaglandins, xenobiotics, and steroid hormones (Nebert et al, 1989). While some substrates can inhibit CYP450 activity such as tributyltin (Fent, 1996; Klaassen et al, 1996), PAHs tend to induce their activity (Livingstone, 1991). Monitoring ECOD activity is one method to assess PAH induced CYP450 expression. In this study ECOD activity in *C. virginica* was significantly higher at Site 6 than at control Site 1. One possible explanation might be the proximity of Site 6 to the Beaufort Inlet. During the spring, summer, and early fall months Beaufort Inlet

receives heavy boat and recreational activity. Long term exposure to compounds such as PAHs and petroleum by-products are known to induce ECOD activity. Ismert et al (2002) exposed the snail, *Helix aspersa* to PAH-saturated conditions for 4 weeks. They recorded significant induction of ECOD activity. Induction of ECOD activity has also been seen with 3-methylchoanthrene in the aquatic invertebrates *Mytillus edulis* and *Littorina littorea* (Livingstone et al, 1988). Further studies including other benthic invertebrates and soil PAH assays should be preformed to verify this hypothesis.

To conclude, the concentrations of diuron and CuPT required to cause mortality in *C. virginica* are several orders of magnitude higher than presently reported environmental levels. It is interesting to note, however, that CuPT causes mortality at concentrations two orders of magnitude lower than diuron. One could easily expect CuPT to have more consequences in non-photosynthetic organisms than diuron. CuPT's mode of toxicity (Ermolayeva and Sanders, 1995) could potentially shut down all physiological activities within an organism that rely on ATP production. Diuron does not appear to be especially toxic to *C. virginica* however as diuron use arises or if concentrations reach or exceed levels that inhibit photosynthesis, this could pose problems. Diuron could then result in indirect mortality due to food limitability.

At the present time we have not determined the exact anthropogenic contaminants with the RCERR. But is does appear that something is affecting the overall health of oysters at Sites 4, 5, and 6. Many other assessments such as water quality and

sediment analysis assays need to be preformed before any concrete cause and effect relationship can be made.

Chapter 4

Effects of diuron and copper pyrithione on adults, gametes and embryos of the sea urchin, *Lytechinus variegatus.*

4.1 Introduction

Fouling of submerged surfaces by living and non-living substances is inevitable. Fouling decreases ship performance and costs the shipping industry millions of dollars each year in dry dock fees, loss of trade, and increased fuel consumption. Controlling fouling is both strategically and economically important, but can have grave environmental consequences (Johnson and Miller, 2003; Johnson and Gonzalez, 2004). In the last 50 years, broad spectrum metal biocides in polymer coatings have been used to manage fouling. Many of these biocides (especially the organotins) have unacceptable impacts, the worst of which had been the local and regional extinctions of certain species (Gibbs et al, 1991; Stewart et al, 1992). Besides the obvious acute toxicity, sub-lethal impacts of these compounds have been observed and include morphological abnormalities (Alzieu et al, 1991; Morcillo et al, 1998), sexual abnormalities (Jenner, 1979; Gibbs et al, 1987, 1991; Bauer et al, 1995), and reduced or altered reproductive capacity including behavioral castration (Medesani et al, 2004; Oberdörster and Cheek, 2001; Oberdörster et al, 1998, Straw and Rittschof 2004). In marine mammals, there has also been evidence of immune disorders related to organotin uptake (Nakata et al, 2002).

Because of the myriad number of impacts, organotin antifoulants were the subject of an International Maritime Organization (IMO, a component of the United Nations, 2004) Convention in which coating companies voluntarily withdrew organotins

from the global commercial market (D. Rittschof, *personal correspondence*). After the withdrawal of organotin based antifoulants, coating companies depended heavily on the use of copper oxide in their paint formulations. This unfortunately led to increased copper concentrations in waters with heavy boating activity and resulted in violations of the Clean Water Act of 1972 (Rosen et al, 2004). As a consequence, copper biocide coatings have been banned in several countries including Sweden and Denmark and restricted in several U.S. states (Rosen et al, 2004).

In response to new requirements for the reduction of copper leaching from antifoulants, companies have begun adding organic biocides to their coating formulations. These biocides work to reduce copper release rates and increase the overall effectiveness of the coating. Diuron and copper pyrithione (CuPT) are two biocides currently added to antifoulant coatings. Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a phenylurea herbicide that has been used in agricultural applications since the 1950s. It is now employed as a biocide in antifouling coatings throughout the United Kingdom (U.K.), France, Japan, and the United States. (Boxall et al, 2000; Thomas et al, 2000; Okamura et al, 2003). Diuron inhibits photosynthesis at concentrations as low as 0.3 $\mu g\ L^{-1}$ (Jones and Kerwell, 2003) by interfering with the plastoquinone in the photosystem II complex (Pruess and Hall, 1995; Jones and Kerswell, 2003) of plants and algae. Concentrations of diuron in surface waters have been found at concentrations

ranging from a few μg L^{-1} to over 1 mg L^{-1} in some U.S. surface waters, mainly as a result of agricultural runoff (Moncada, 2004; Green and Young, 2006).

Copper pyrithione (2-mercaptopyridine N-oxide copper salt) (CuPT) is a microbial biocide that was introduced into the market as an antifoulant in 1996 (Arch Chemicals; Maraldo and Dahllöf, 2004). Mackie et al (2004) have reported total pyrithiones (including sodium-, zinc-, and copper conjugates) within UK waters at levels exceeding 0.3 μg L^{-1}. While limited information exists on CuPT, zinc pyrithione (ZPT) (a sister compound to CuPT) has been in use much longer and may provided some insight into CuPT's mode of toxicity. ZPT has been used as an anti- fungal, bacterial, and algal compound since the 1960s (Turley et al, 2005) and complexes with Cu^{2+} ions after dissociation in marine environments to form CuPT (Mackie et al, 2004). Both CuPT and ZPT are lipophilic compounds with half-lives ranging between 15 min to 30 days (Turley et al, 2005; Maraldo and Dahllöf, 2004), depending on environmental conditions. The fastest degradation rates of these pyrithiones occurred in the presence of light or aerobic sediments (Turley et al, 2000). While absolute certainty of the mode of action of CuPT is unclear, Ermolayeva and Sanders (1995) suggest that pyrithiones act by interfering with the activity of the primary proton pump, H^{+}-ATPase. This is hypothesized to result from the collapse of cell membrane pores which lead to ion gradient destabilization and ultimately cell apoptosis (Bragadin et al, 2003). It is assumed that CuPT operates by a similar mechanism.

The aim of this project was to investigate both lethal and sub-lethal effects of environmentally relevant concentrations of diuron and CuPT on the sea urchin *Lytechinus variegatus*. *L. variegatus* has been used extensively in marine pollution bioassays (Kobayashi, 1980; Kobayashi and Okamura, 2004) and appears to be a well characterized and sensitive species (Ramachandran et al, 1997; Yarolslavtseva and Sergeeva, 2002; Geraci et al, 2004). In this study, acute toxicity in adult *L. variegatus*, sub-lethal fertilization effects on exposed adults and gametes, and the impact of short term exposures on development were examined.

4.2 Materials and Methods

4.2.1 Study site and preparation

Adult variegated urchins, *Lytechinus variegatus* (Lamarck), were collected during the summer months of 2004 and 2005 from Bogue Sound, NC (see Figure 4.1), and purchased from Sea Life Inc. of Tavernier, FL during winter months. *L. variegatus* from these sources have been used in studies of normal embryonic development for over two decades (Wessel et al, 1998; Gross and McClay, 2001; Gross et al, 2003). Urchins were maintained at the Duke University Marine Laboratory (DUML) for up to one week on a 14L:10D cycle in continuously flowing single pass sand-filtered seawater (30–35 salinity, 25°C) and allowed to graze on oyster rubble and algae growing on the tanks until used in experiments.

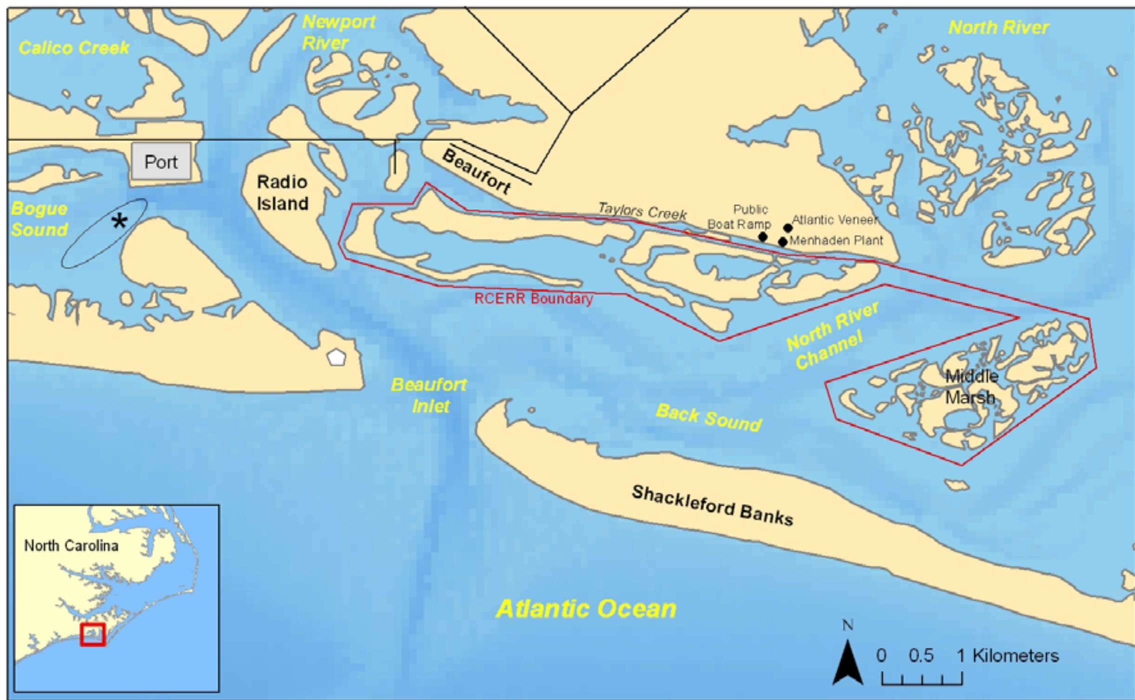


Figure 4.1 Beaufort, NC area sampling site. *Lytechinus variegatus* were collected by dredge in Bogue Sound indicated by the star and surrounding circle.

4.2.2 Experimental Solutions

Stock solutions of diuron (Sigma-Aldrich, St. Louis, MO) were prepared in acetone at a concentration of 10 g L^{-1} following Chesworth et al (2004) and Jones and Kerswell (2003). CuPT (Arch Chemicals, Norwood, CT) stock solutions were prepared in dimethylsulfoxide (DMSO) at a concentration of 2 g L^{-1} following Kobayashi and Okamura (2002). Stock and working solutions were kept in dark bottles at room temperature to avoid photolysis for no longer than one week. Working solutions were made immediately before use by diluting stock solutions in filtered seawater (FSW). All

working solutions of diuron contained 0.0001% (v/v) acetone and those for CuPT contained 0.00005% (v/v) DMSO.

4.2.3 Acute Toxicity

Acute toxicity assays were 96 hr in duration with daily static renewal of treatments and mortality checks. Urchins (all roughly 7 cm test diameter) were housed individually in 1 L acid-washed glass jars and allowed to acclimate to a 14L:10D light cycle (20°C and 35 ppt) for 48 hr before the start of the experiment. Data were pooled from three replicate assays for each biocide. In each iteration approximately 70 urchins were assayed with five or 15 replicate urchins per treatment. A filtered seawater (FSW) control, solvent control and several biocide concentrations were employed for each assay. Diuron was tested at concentrations between 0.5 and 200 mg L^{-1} while CuPT was tested between 0.1 and 200 μg L^{-1}. The data for each test was pooled, log10 transformed, and modeled as four-parameter sinusoidal does-response curves using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA).

4.2.4 Gamete Toxicity and Percent Fertilization following Adult Exposure to Biocides

Adult *L. variegatus* were exposed to 6.53 mg L^{-1} diuron, 7.09 μg L^{-1} CuPT, or controls for 96 h with daily treatment renewal. Concentrations were chosen as a result of preliminary studies. The same acclimation, testing conditions, and procedures described

for the acute toxicity assays were used. At the end of the exposure period urchins were induced to spawn with an injection of 1 mL of 0.55 M KCl. For each treatment, eggs from five females were pooled and sperm from five males were pooled. Eggs were collected by placing spawning females aboral side down in individual 250 mL beakers of FSW. Eggs were allowed to settle by gravity then washed by decanting and resuspending in FSW three times. Spawning males were placed oral side up on a tray of ice and "dry" sperm was collected by pipetting directly from the gonopores. The sperm was kept on ice until used. A sperm suspension was made by diluting 25 μL of pooled dry sperm into 5 mL of FSW (Ettensohn et al, 2004).

All possible pairwise crosses of gametes from FSW control, diuron-treated, and CuPT-treated adults were carried out (9 total crosses, see Table 4.1). All crosses are referenced by a code that indicates the exposure of the female parents followed by the exposure of the male parents, such as "diuron-CuPT" for a cross of a diuron-treated female with a CuPT-treated male. Fertilization crosses were made by mixing 10 μL of egg suspension with 5 μL of sperm suspension. Crosses (5 replicates) were carried out in a polystyrene 96-well microplate containing 200 μL of FSW (Ettensohn et al, 2004) with an egg:sperm ratio of approximately 1:4000. Fertilization success was scored by counting eggs with and without a fertilization envelope 30 minutes after the mixing of sperm and egg using a 40x magnification on a Leitz-Wetzlar scope.

Percent Fertilization after Gamete Exposure to Biocides

Eight control female and eight control male *L. variegatus*, selected at random, were induced to spawn following the same procedure as in 4.2.4 (*Gamete Toxicity and Percent Fertilization following Adult Exposure to Biocides*). After the final seawater wash, eggs were resuspended (10% (v/v)) in 0.1, 1, 10, or 1000 μg L^{-1} diuron, 0.1, 1, 10 or 100 μg L^{-1} CuPT, acetone, DMSO, or FSW for 30 minutes. Alternatively, 25 μL of dry sperm was suspended in 5 mL of identical treatments. Crosses of gametes exposed to identical treatments were made (i.e. 10 μg L^{-1} CuPT eggs crossed with 10 μg L^{-1} CuPT sperm). Fertilization crosses were made by mixing 10μL of egg suspension with 5 μL of sperm suspension (1:4000 egg:sperm dilution (Ettensohn et al, 2004)) in five wells of a polystyrene 96-well microplate ultimately containing 200 μL of test compound. Fertilization success was scored by counting eggs with and without a fertilization envelope 30 minutes after mixing of egg and sperm.

4.2.5 Embryo Development

The impacts of diuron and CuPT exposure on embryo development in *L. variegatus* were tested by exposing FSW fertilized eggs and embryos to biocides in 30 minute intervals at 18°C. Seven 30 minute exposure intervals (from t= 0 to t= 240 min) followed by two 120 min exposure intervals (from t= 240 min to t= 480 min) were conducted. The two longer exposure periods were included because development in FSW up to the hatched blastula stage was slower at 18°C than in previous reports where higher temperatures were used (Ettensohn et al, 2004).

Eggs from five FSW females and sperm from five FSW males were collected, pooled, and crossed as described above to produce a 105 ml reservoir of fertilized eggs. From this reservoir, aliquots of 20–40 fertilized eggs were taken and added to wells of a 96-well microplate containing 200 μL of 0.1, 1, 10, or 1000 μg L^{-1} diuron, 0.1, 1, 10 or 100 μg L^{-1} CuPT, acetone control, DMSO control, or FSW alone. Four replicate aliquots per treatment per time interval were used. The fertilized eggs remaining in the reservoir were allowed to develop into embryos at room temperature with gentle aeration. A new set of aliquots (20-40) embryos were taken from this reservoir every 30 minutes and added to new microplate wells containing 200 μL of treatment solution. At the end of each 30 minute time period embryos were preserved in their current stage of development by adding a few drops of buffered formalin. [Note: Formalin preservation did not induce abnormal development (unpublished observations)]. The cell stage and any abnormal development were recorded for all eggs in each replicate for each exposure interval.

Mean cell number (the total number of cells per embryo divided by the total number of embryos) was calculated for embryonic stages from the 1 cell to 64 cell stage but not for hatched blastulas. FSW embryos reached the hatched blastula stage during the last exposure period (t= 360–480 min). For this period, we used the proportion of embryos reaching the hatched blastula stage as our test statistic.

4.2.5 Statistics

The percentages of successful fertilization after adult exposure were arcsin-square root transformed to meet assumptions of normality and equal variance and tested with a one-way analysis of variance (ANOVA) followed by Holm-Sidak post-hoc tests. Fertilization rates of exposed gametes and percentages of embryos reaching the hatched blastula stage after 480 minutes were similarly tested but without transformation.

One-way ANOVAs followed by Holm-Sidak post-hoc tests were performed on mean cell numbers and the percentages of embryos undergoing abnormal development between the treatments for any particular time period, and separately for diuron and CuPT. Data for both mean cell number and abnormal development did not always meet the assumptions of equal variance and normality even after being arcsin-square root transformed. Since more data fit the assumptions after transformation than before, parametric one-way ANOVAs were performed using the transformed data despite the assumption violations.

When both solvents and FSW controls were tested, the solvent treatments and not the FSW treatments were used as controls in statistical analyses. All data points are presented in graphs as mean with error bars representing standard errors of the mean. All ANOVAs were performed using SigmaStat (SYSTAT Software, Inc. San Jose, CA).

4.3 Results

4.3.1 Acute Toxicity

Mortality was concentration-dependent in the acute toxicity assays, (Figure 4.2). The 96 h LC_{50} (95% CI) for adult *L. variegatus* exposed to diuron was 7.14 mg L^{-1} (4.9–10.3 mg L^{-1}). The 96 h LC_{50} (95% CI) for adult *L. variegatus* exposed to CuPT was 14.04 μg L^{-1} (8.0–24.6 μg L^{-1}).

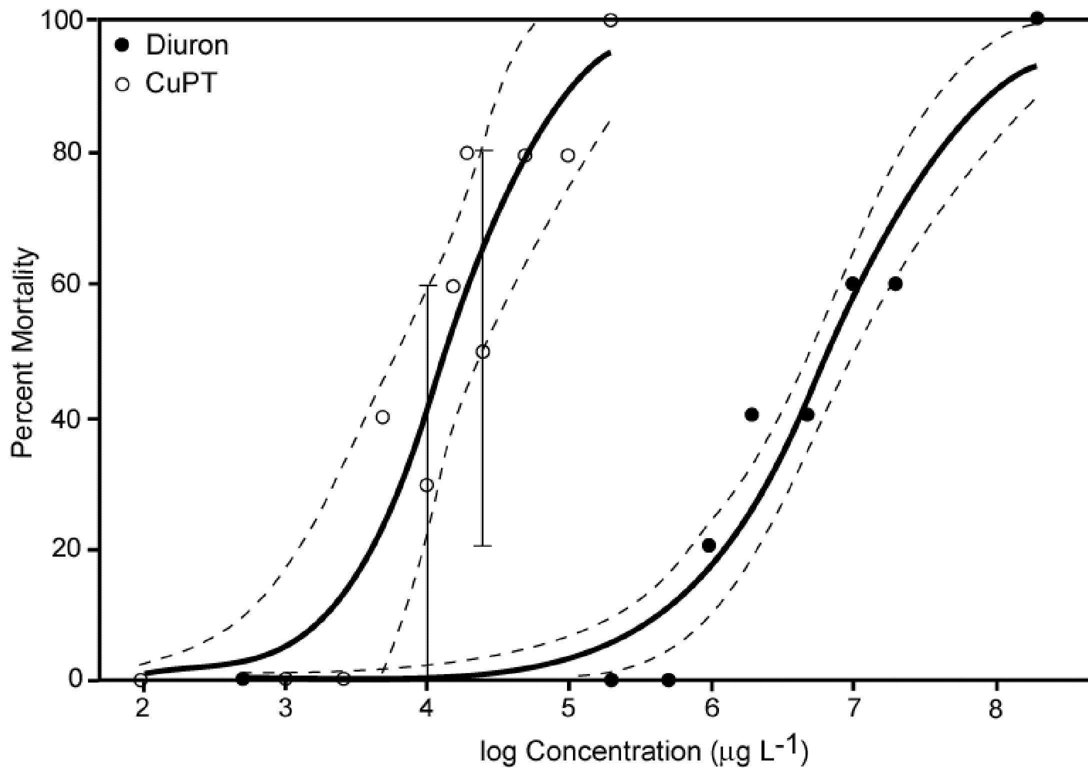


Figure 4.2 Mortality in adult *Lytechinus variegatus* after 96 h exposure to diuron (●) and CuPT (○). Dash lines represent 95% confidence interval.

4.3.2 Gamete Toxicity and Percent Fertilization following Exposure of Adults to Biocides

Nine crosses were made with gametes from adults exposed to three different treatments (FSW, 6.53 mg L^{-1} diuron, and 7.09 mg L^{-1} CuPT). The FSW-FSW cross had a 98.64% fertilization success level and was used as the control cross to which all other crosses were compared (see Table 4.1). Treatment of adults with either biocide significantly decreased fertilization success, except for those observed in the FSW-CuPT and diuron-FSW crosses. The lowest three percentages of successful fertilization (75.2%, 76.12%, and 84.3% ($p < 0.01$, Holm-Sidak post-hoc)) occurred when adult males were exposed to diuron. All crosses made with CuPT exposed females showed significantly reduced percentages of successful fertilization (all $p < 0.01$, Holm-Sidak post-hoc) and ranged from 89.26% when crossed with FSW males to 76.12% when crossed with diuron males.

Percent Fertilization after Gamete Exposure to Biocides

When gametes from FSW parents were exposed after spawning, crosses of gametes in the presence of FSW, acetone, or DMSO resulted in fertilization success levels of 96.3%, 96.6%, and 97.1% respectively (Figure 4.3). The three lowest concentrations of diuron exposed gametes resulted in significantly reduced ($p<0.001$, Holm-Sidak post-hoc) fertilization when compared to their acetone control. The highest diuron treatment, 1000 μg L^{-1}, showed no significant effect on fertilization. Gametes from FSW parents that

were exposed to all concentrations tested, from 0.1 to 100 µg L^{-1}, resulted in significantly reduced (all p<0.001, Holm-Sidak post-hoc) percentages of fertilization.

Table 4.1
Fertilization success of gametes from adult male and female *L. variegatus* exposed to 6.53 mg L^{-1} diuron or 7.09 mg L^{-1} copper pyrithione (CuPT) or FSW (control)

Exposure		
Female	**Male**	**% Fertilization**[a]
Control	Control	98.64 ± 1.31
Control	Diuron	75.20 ± 6.01**
Control	CuPT	93.48 ± 7.71
Diuron	Control	94.78 ± 4.39
Diuron	Diuron	84.38 ± 7.68**
Diuron	CuPT	91.00 ± 3.73**
CuPT	Control	89.26 ± 2.85**
CuPT	Diuron	76.12 ± 16.67**
CuPT	CuPT	85.96 ± 7.45**

[a]plus or minus standard deviation

**p<0.01 Holm-Sidak post-hoc tests after ANOVA

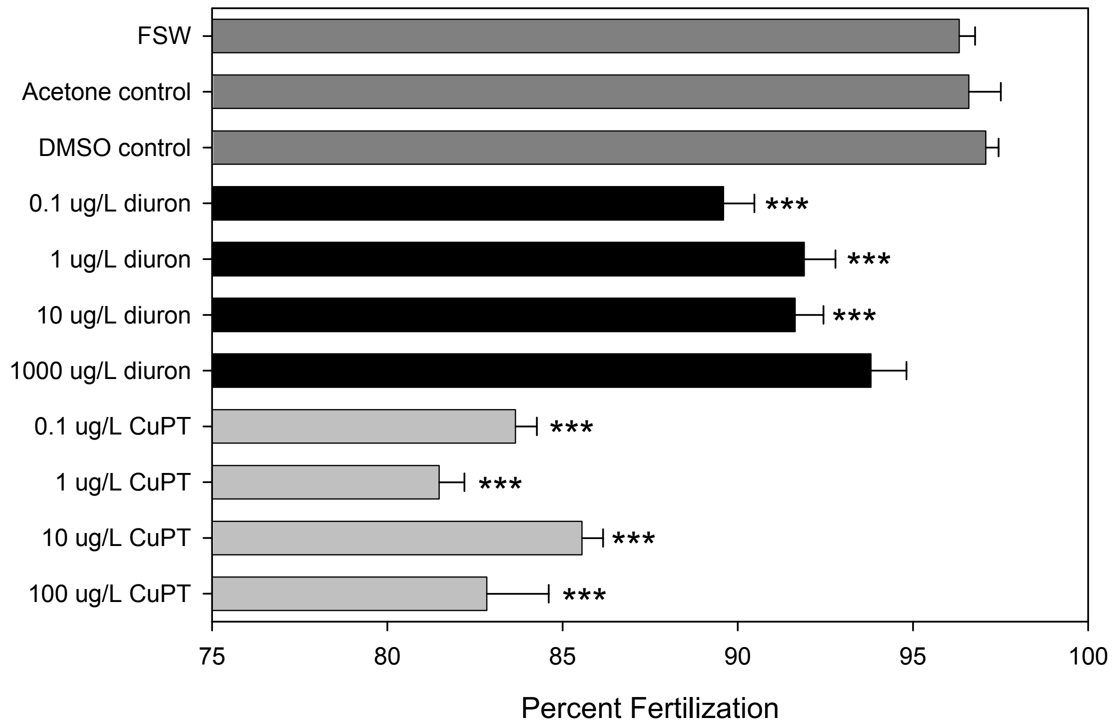


Figure 4.3 Fertilization success of gametes exposed to diuron, copper pyrithione, and controls. Sperm and eggs were exposed to the same treatments in each cross.

4.3.3 Embryo Development after Gamete Exposure to Biocides

Embryos from a FSW-FSW cross were exposed to biocides in 30 minute exposure periods. Development was delayed in embryos exposed to diuron starting at the 60–90 min. exposure period. Significantly fewer embryos advanced to the next developmental stage following exposure in all four diuron treatments (Figure 4.4a). Beginning with the 60–90 min. period and later, exposure to the highest diuron treatment, 1000 µg L^{-1} resulted in mean cell numbers that were significantly lower than each acetone control

(all $p<0.05$, Holm-Sidak post-hoc; Figure 4.4a). In addition, all four diuron treatments showed significantly reduced mean cell numbers during the 60–90, 180–210, and 210–240 min. exposure periods (all $p<0.05$, Holm-Sidak post-hoc). The control FSW embryos were in the 2 cell stage in the former exposure period and the 16 cell stage during the latter two periods (please refer to photos in Figure 4.6 for control FSW cell stages). These three exposure periods were the only intervals in which the lowest two diuron treatments, 0.1 and 1 $\mu g\ L^{-1}$, elicited a significant effect.

During the two hour exposure periods from 360–480 min., 89.7% of FSW embryos and 93.3% of acetone embryos reached the hatched blastula stage (Figure 4.4b). All diuron treatments significantly lowered the percentages of embryos developing from the 64 cell stage to the hatched blastula stage (all $p<0.001$, Holm-Sidak post-hoc). The lowest 0.1 $\mu g\ L^{-1}$ diuron treatment was the only diuron treatment to show any appreciable (77.6%) development into the hatched blastula stage. The 1, 10, and 1000 $\mu g\ L^{-1}$ diuron exposures resulted in 0%, 1%, and 0% development to the hatched blastula, respectively.

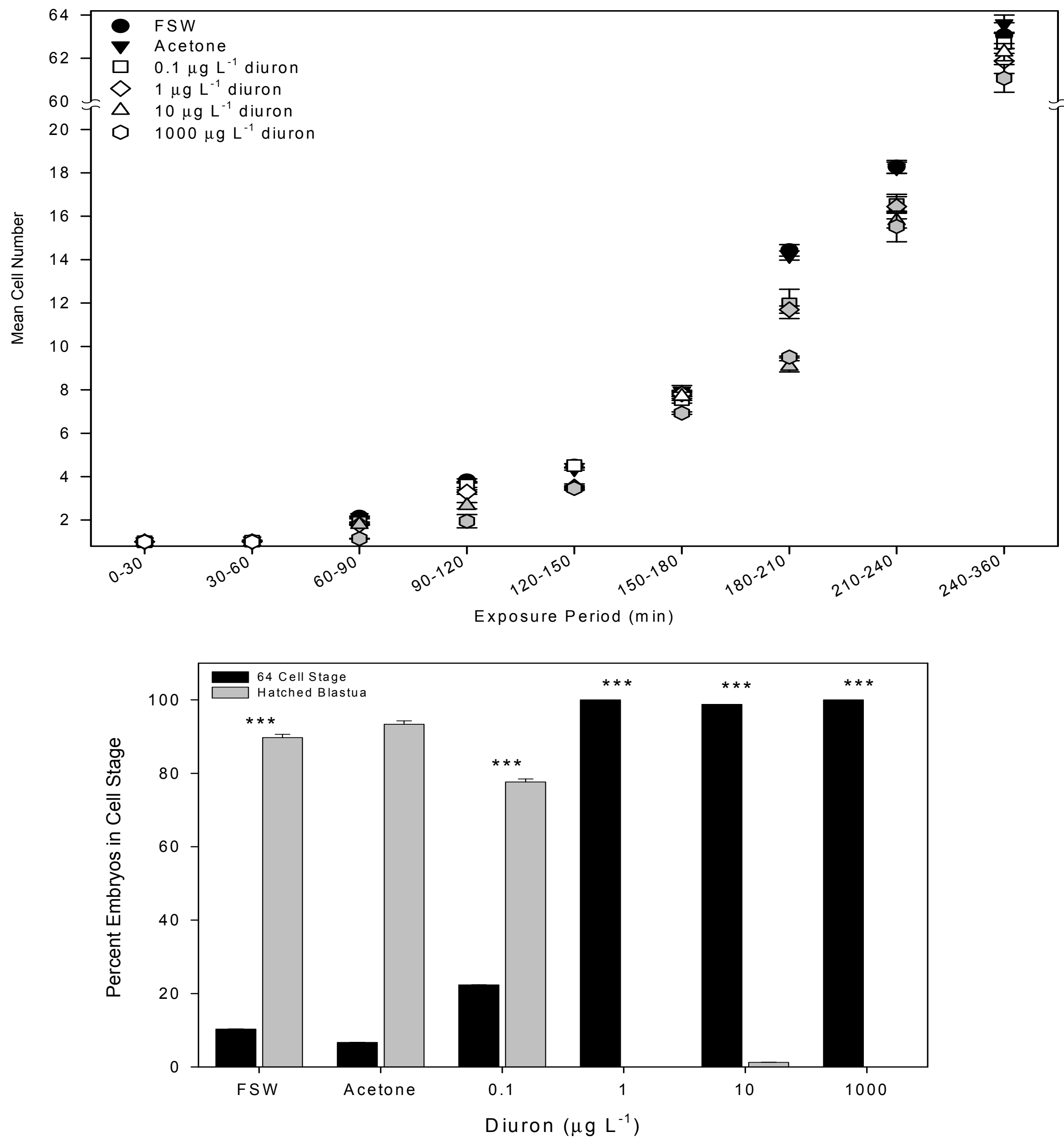
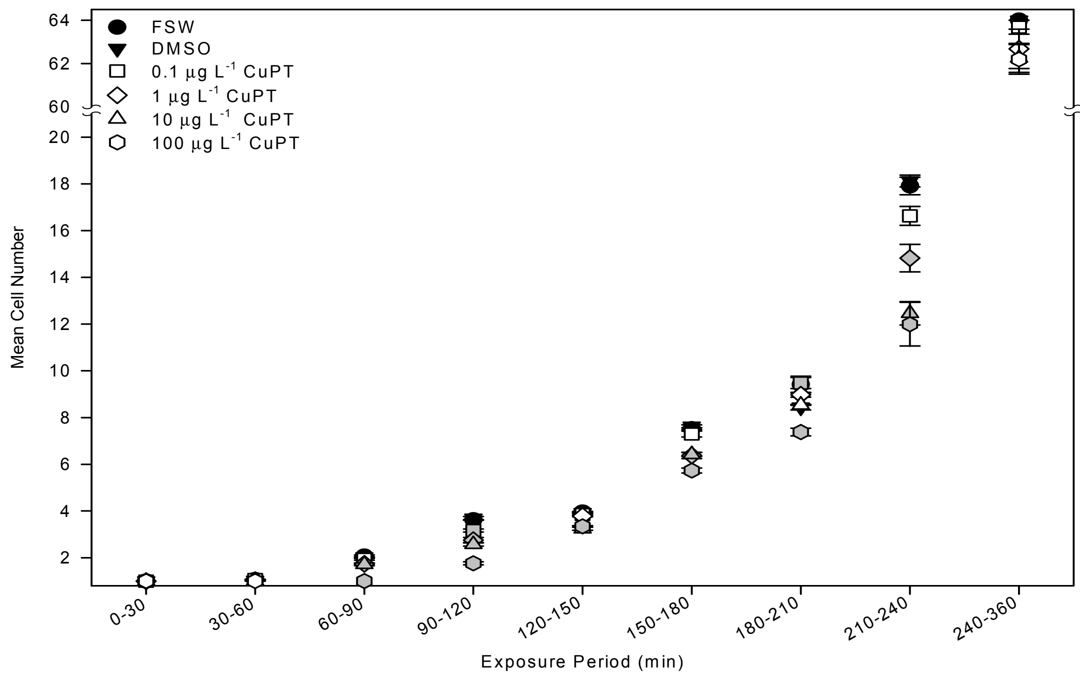


Figure 4.4 Embryo development during diuron exposure. (a) Mean cell number of embryos exposed to diuron during 30 min exposure periods (t = 0–360 min). Black symbols, control treatments; gray symbols, significantly different from acetone; open symbols, not significantly different. (b) Percentage of embryos exposed to diuron in the 64 cells stage and the hatched blastula stage at the end of the 360–480 min exposure period. ***p<0.001.

Embryos exposed to CuPT during development (Figure 4.5a) also developed more slowly than DMSO controls. Mean cell numbers were significantly reduced for CuPT treated embryos after the 60–90 min. exposure period in which the control FSW cells were in the 2 cell stage (please refer to photos in Figure 4.7). All CuPT treatments showed significantly reduced mean cell numbers during the 90–120 min. period (control FSW embryo was in the 4 cell stage) ($p<0.05$, Holm-Sidak post-hoc).

In the 360–480 min. exposure period control treatments of FSW and DMSO resulted in 98.3% and 96.4% of embryos reaching the hatched blastula stage. All CuPT treatments significantly reduced the percentage of embryos developing from the 64 cell stage to the hatched blastula stage during the 360–480 min. exposure period (Figure 4.5b; $p<0.001$, Holm-Sidak post-hoc). The lowest two CuPT treatments, 0.1 and 1 $\mu g\ L^{-1}$, were the only exposures in which embryos progressed to the hatched blastula stage (8.3% and 2.4%, respectively).



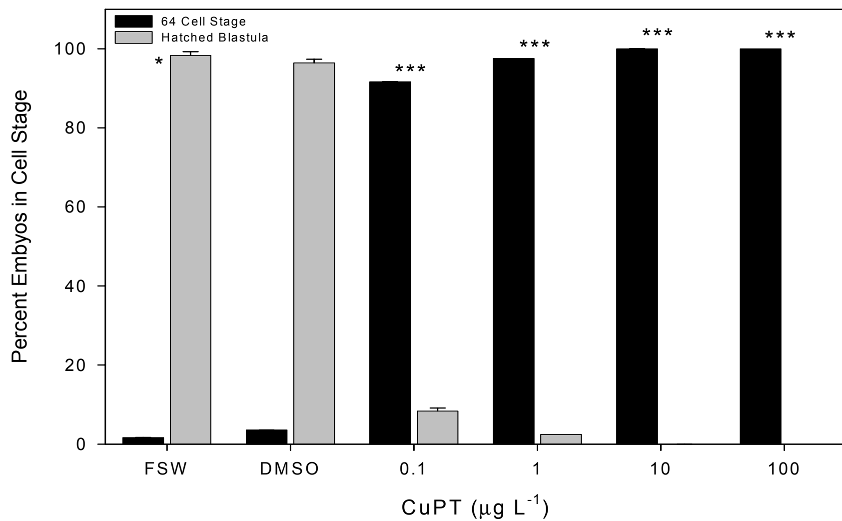


Figure 4.5 Embryo development during CuPT exposure. (a) Mean cell number of embryos exposed to CuPT during 30 min exposure periods (t = 0–360 min). Black symbols, control treatments; gray symbols, significantly different from DMSO; open symbols, not significantly different. (b) Percentage of embryos exposed to CuPT in the 64 cells stage and the hatched blastula stage at the end of the 360–480 min exposure period. *p<0.05, ***p<0.001

4.3.4 Abnormal Embryo Development

The percentages of embryos exposed to FSW or acetone that developed abnormally ranged between 0 and 3.4% (Figure 4.6). In contrast, there was a direct relationship between diuron concentration and the percentage of embryos exhibiting abnormal development, reaching a maximum of 19.5% by the hatched blastula stage in embryos exposed to 1000 μg L^{-1}. The lowest diuron treatment, 0.1 μg L^{-1}, did not significantly alter development however exposure to 1 μg L^{-1} diuron resulted in significantly higher percentages of abnormal embryos during the 150–180 ($p<0.01$, Holm-Sidak post-hoc) and 360–480 ($p<0.05$, Holm-Sidak post-hoc) min. exposure periods. Exposure of embryos to 10 μg L^{-1} diuron resulted in the production of significantly more abnormalities than control solvent ($p<0.01$, Holm-Sidak post-hoc) during four separate exposure periods. Higher percentages (all $p<0.05$, Holm-Sidak post-hoc) of abnormal development also occurred with the highest diuron treatment of 1000 μg L^{-1} during all exposure periods except the 60–90 and 120–150 min. time intervals. During these two periods, when control FSW cells were in the 2 cell and 4 cell stages respectively, none of the treatments produced an elevated rate of abnormal development. In addition, only exposure to 1 mg L^{-1} during the 90–120 interval resulted in the development of abnormal embryos.

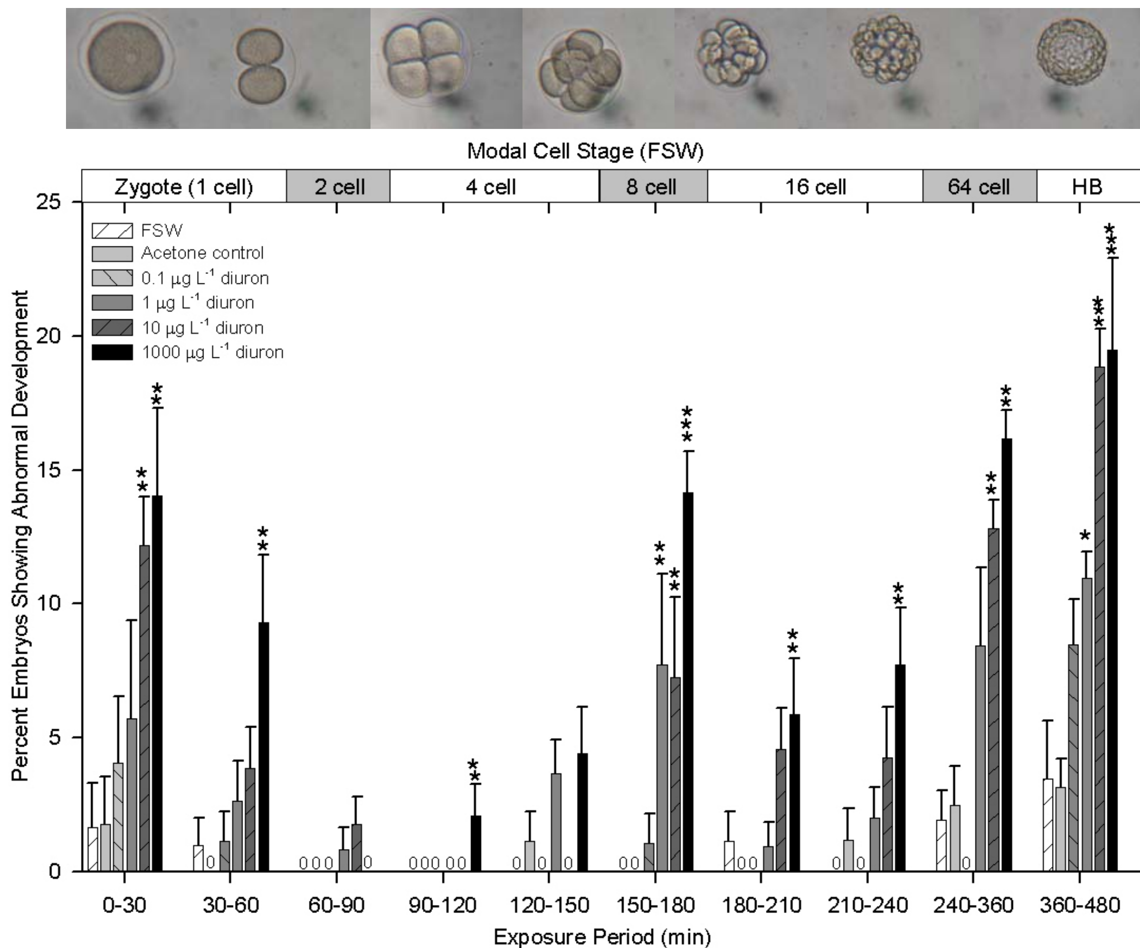


Figure 4.6 Abnormal development in embryos exposed to diuron, acetone, and FSW. The modal cell stage of FSW embryos and the end of each exposure period can be seen across the top of the figure. *p<0.05; **p<0.01; ***p<0.001

As with diuron, exposure of urchin embryos to CuPT showed a direct relationship between chemical concentrations and percentage of developmental abnormalities (Figure 4.7). These abnormalities occurred at levels of 0 to 3.1% for control exposures up to 21.3% for 64-cell stage embryos exposed at the highest, 100 μg L^{-1}, CuPT treatment. Percentages of abnormal development were significantly elevated during only one

exposure period using 1 μg L^{-1}, during four periods using 10 μg L^{-1}, and eight out of ten periods using 100 μg L^{-1} CuPT (Figure 4.7).

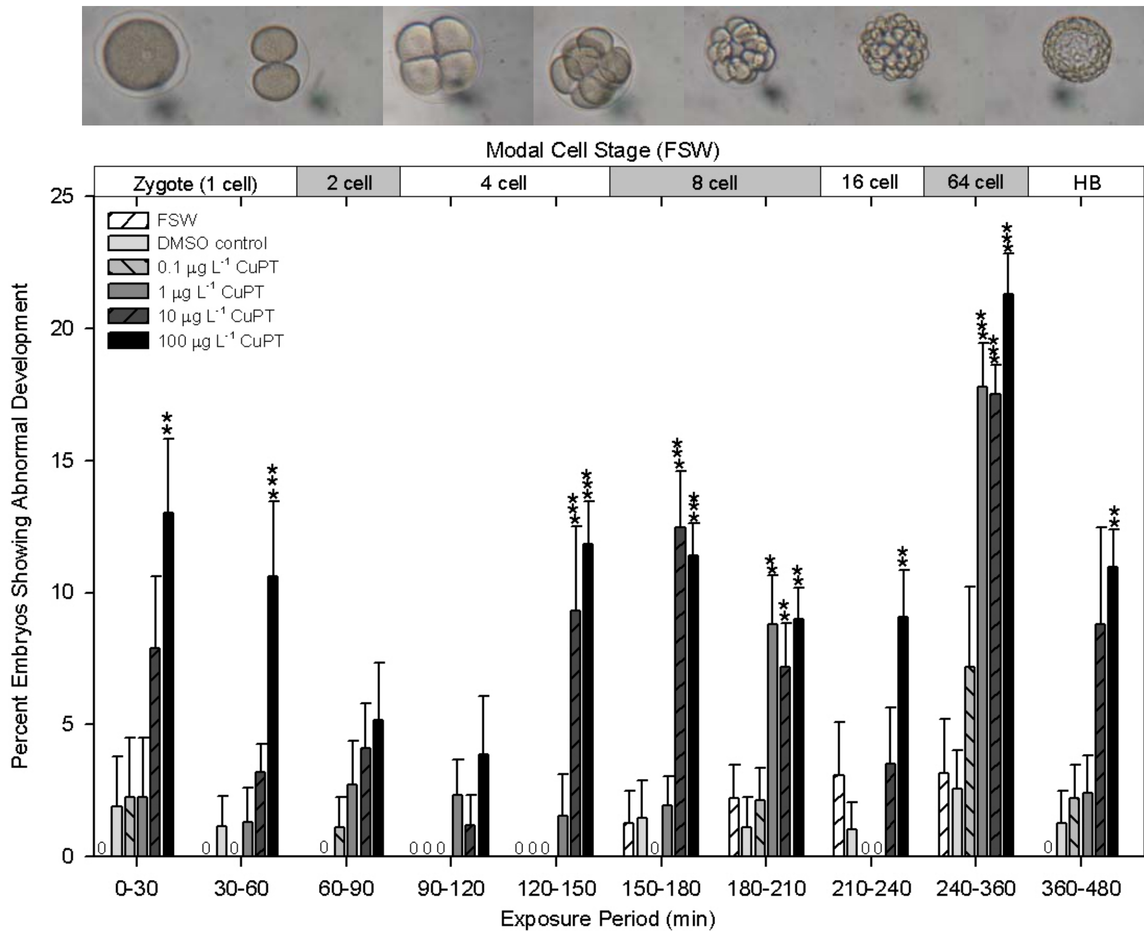


Figure 4.7 Abnormal development in embryos exposed to CuPT, DMSO, and FSW. The modal cell stage of FSW embryos and the end of each exposure period can be seen across the top of the figure. *p<0.05; **p<0.01; ***p<0.001.

There were no significant differences in abnormal development during the 60–90 and 90–120 min. periods in which the control cell stages in FSW were 2 and 4 cell, respectively (Figure 4.7). These were the only two time periods without any significant

differences following CuPT exposure. Urchin embryos exposed 240–360 min. after fertilization appear to be especially vulnerable to abnormal development when exposed to CuPT. The three highest treatments produced significantly elevated percentages of abnormally developed embryos at levels between 17.5 and 23.3% during this time interval. Control embryos in the 240–360 min exposure period were mostly in the 64 cell stage.

4.4 Discussion

In this study adult mortality, percent fertilization, and embryonic development in the non-target species, *Lytechinus variegatus* were examined following exposure to the biocides, diuron and CuPT. Our objectives were: (1) to determine the concentrations which would elicit 50% mortality of adults (LC_{50}); (2) observe the effect that adult exposure (at LC_{50} concentrations) would have on gamete fertilization; (3) determine the effects that pre-exposure of gametes (egg and sperm) would have on fertilization; and (4) assess timing of exposure on embryonic development and metamorphosis.

4.4.1 Diuron

Diuron caused mortality in adult *L. variegatus* at 1 mg L^{-1} with an LC_{50} of 7.14 mg L^{-1} (Figure 4.1). This LC_{50} is well within range of those reported for other aquatic invertebrates (Table 4.2) such as opossum shrimp, *Americamysis bahia* (1.1 mg L^{-1}; EPA

Pesticide Database, 2000), clams, *Mercenaria, mercenaria* (5 mg L^{-1}; Davis and Hidu, 1969), and water fleas, *Daphnia magna* (0.4 mg L^{-1}; Shcherban, 1972). It is also similar to values reported for terrestrial invertebrates such as mosquitos, *Aedes aegypti* (1.2 mg L^{-1}; Knapek and Lakota, 1974), stoneflies, *Pterconarcys califoricus* (3.6 mg L^{-1}; Sanders and Cope, 1968), and the midge, *Chironomus tentans* (3.3 mg L^{-1}; Nebeker and Schytema, 1998). Overall, diuron exhibits low acute toxicity in *L. variegatus* and other invertebrates.

Table 4.2
Recent toxicity data of diuron, copper pyrithione (CuPT), and zinc pyrithione (ZPT) in aquatic species.

Compound	Organism	LC_{50}	Reference
Diuron	*Mercenaria mercenaria* (clam)	5 mg L^{-1}	Davis and Hidu, 1969
	Americamysis bahia (shrimp)	1.1 mg L^{-1}	U.S. EPA, 2000
	Daphnia magna (water flea)	0.4 mg L^{-1}	Shcherban, 1972
	Aedes aegypti (mosquito)	1.2 mg L^{-1}	Knapek and Lakota, 1974
	Pterconarcys califoricus (stonefly)	3.6 mg L^{-1}	Sanders and Cope, 1968
	Chironomus tentans (midge)	3.3 mg L^{-1}	Nebeker and Schuytema, 1998
	Oncorhynchus mykiss (fish)	74 mg ai L^{-1}	Okamura et al, 2002
CuPT	*Heptacarpus futilirostris* (shrimp)	2.5 µg L^{-1}	Mochida et al, 2006
	Pagrus major (fish)	9.3 µg L^{-1}	Mochida et al, 2006
	Aquatic fish	4.3-43.6 µg L^{-1}	Yamada and Kakuno, 2002
	Pinmephales promelas (fish)	4.3 µg ai L^{-1}	NRAAVC, 2001
	Oncorhynchus mykiss (fish)	7.6 µg ai L^{-1}	Okamura et al, 2002
ZPT	*Pinmephales promelas* (fish)	2.6 µg ai L^{-1}	NRAAVC, 2001

Concentrations of diuron that cause mortality in non-target species are 100 to 1000 fold higher than environmental levels previously reported in the U.K. (6.7 μg L^{-1}; Thomas et al, 2001) and in Japan (3.03 μg L^{-1}; Okamura et al, 2003). Thus acute toxicity of diuron does not appear to be an issue in the non target species, *L. variegatus,* at environmentally relevant concentrations.

Although diuron did not appear to elicit acute toxicity in *L. variegatus* at environmental concentrations it did significantly decreased fertilization efficiency. Gametes released from adults exposed for 96 h to concentrations of diuron approximating the acute LC_{50} and gametes from untreated adults that were pre-exposed for 30 minutes to a series of diuron dilutions, both resulted in significantly lower fertilization percentages. The experiments exposing adults to LC_{50} levels are analogous to previously published studies (Sanders and Cope, 1968; Davis and Hidu, 1969; Nebeker and Schuytema, 1998) and show impacts at concentrations 1000 fold higher than reported environmental levels. However, when environmental conditions of broadcast spawners were mimicked, by exposing the egg and sperm to varying levels of diuron before fertilization, we observed significant decreases in percent fertilization at concentrations as low as 0.1 μg L^{-1} (Figure 4.4). These concentrations were 30 to 60 times less than reported environmental levels (Thomas et al, 2001; Okamura et al, 2003).

Fertilization using sperm from unexposed *L. variegatus,* which was then treated *in vitro* with diuron, consistently produced lower percentages of successfully fertilized

embryos than sperm from any other type of cross (Table 4.1). This suggests that diuron may impact male reproduction. This conclusion is bolstered by the findings of Sarkar et al (1997) who reported that some phenylureas (the same class of compounds as diuron) decreased epididymal sperm count and the percentage of motile sperm in rats. They also demonstrated that phenylurea exposure often resulted in increased numbers of morphologically abnormal sperm cells. The mechanism directly responsible for sperm damage is not known.

Sub-lethal effects of exposure in the form of delayed development and abnormalities were observed in embryos at concentrations comparable to those present in the environment. After 90 minutes post-fertilization, concentrations as low as 0.1 μg L^{-1} diuron induced significant developmental delays during one or more exposure periods (Figure 4.4a). The first cell division between 90 and 120 minutes, and the 16-cell stage between 180 and 240 minutes were especially susceptible to delay. Interestingly during these exposure periods, there were relatively lower occurrences of abnormalities compared to other periods (Figure 4.6). The zygote, 8-cell, 64-cell, and hatched blastula stages appeared to be the most sensitive stages for abnormal development (Figure 4.6). Very few abnormalities were seen in the 2- and 4-cell stages between 60 and 150 minutes at the lower concentrations. Although the mode of action of diuron on development in invertebrates is unknown, it does significantly affect fertilization and larval development at concentrations well within the range of current environmental levels.

4.4.2 Copper pyrithione

Copper pyrithione induced mortality in *L. variegatus* at concentrations as low as 5 $\mu g\ L^{-1}$ and resulted in a 96 h LC_{50} of 14.04 $\mu g\ L^{-1}$. No other studies have reported CuPT LC_{50} values for *L. variegatus* however Mochida et al (2006) reported CuPT LC_{50}s of 2.5 and 9.3 $\mu g\ L^{-1}$ for the toy shrimp, *Heptacarpus futilirostris,* and the teleost red sea bream, *Pagrus major*, respectively. These results suggest that *L. variegatus* is equivalent to marine crustaceans and teleosts in their sensitivity to CuPT. Our acute toxicity assays show CuPT is three orders of magnitude more toxic to *L. variegatus* than diuron.

CuPT has not been reported in present environmental samples, however the compound was only recently added to the list of approved biocides and most agencies do not monitor for the presence of this compound or its derivatives. Concentrations of pyrithione molecules (sodium-, zinc-, and copper-) exceed 100 nM in some U.K. waters (Mackie et al, 2004). ZPT is known to transchelate in seawater becoming the more stable compound, CuPT (Dahllöf et al., 2005). Applying current ZPT concentrations and assuming that ZPT and CuPT use in antifoulants will increase in the future, it is reasonable to assume that the concentrations used in this study are applicable regarding sub-lethal effects.

CuPT exposure caused a significant decrease in fertilization success when *L. variegatus* adults were exposed to concentrations approximating the LC_{50} (Table 4.1). All crosses involving CuPT treated adult females resulted in significantly reduced

fertilization. The control-CuPT (female: male) cross did not result in reduced fertilization suggesting that CuPT exposure has less of an effect on males than in females. As seen with diuron, CuPT exposure of the gametes *in vitro* significantly reduced fertilization at concentrations as low as 0.1 $\mu g\ L^{-1}$ (Figure 4.5). This, in conjunction with decreases in percent fertilization observed using adult females exposed to CuPT; suggest that negative effects on fertilization success may be due to compromised eggs. It is possible that the lipophilic egg takes up the organic pyrithione molecule or that it is ovodeposited as are many other organic contaminants. Bellas et al (2005) proposed a similar hypothesis based on their studies showing significant decreases in the percentage of normal larvae in the ascidian, *Ciona intestinalis,* following exposure of unfertilized eggs to 48 $\mu g\ L^{-1}$ ZPT.

As with diuron there are direct relationships between CuPT concentration and both developmental delay (Figure 4.5) and abnormal growth (Figure 4.7). Development of *L. variegatus* embryos exposed to CuPT was delayed at concentrations 5 to 16 fold lower than the reported environmental levels of total pyrithiones (Bragadin et al, 2003). All tested concentrations of CuPT resulted in near total failure of embryos to reach the hatched blastula stage within the 2 h window from 360-480 minutes post-fertilization compared with nearly 100% of the control embryos (Figure 4.5b).

Ramachandran et al (1997) noted that the most sensitive stage of development in the copper-exposed sea urchin, *Diadema setosum* occurred during the first cell cleavage.

This partially agrees with the data presented here. In addition to the first cell cleavage, early morula and late blastula stages of development were also found to be very sensitive to CuPT exposure (Figures 4.5a, 4.7). Treatment with CuPT at concentrations as low as 1 μg L^{-1} resulted in significant abnormal growth in 8-cell and 64-cell stage embryos however exposure to 0.1 μg L^{-1} never significantly elevated levels of abnormalities in any cell stage. Significant abnormal development was seen at 100 μg L^{-1} CuPT during all cell stages except the 2-cell and early 4-cell stages (Figure 4.7) suggesting these stages may be resistant to CuPT-induced abnormalities. These findings for CuPT parallel those we report for diuron as well as those reported by Bellas et al (2005) for *Paracentrotus lividus* urchin embryos exposed to ZPT (sister compound to CuPT). They found significant decreases in the percentage of normal pluteus larvae when fertilized eggs were exposed to various concentration of ZPT (EC_{50} of 2.56 μg L^{-1}). Conversely they report no normal development of larvae at concentrations greater then 3.3μg L^{-1} whereas our study clearly demonstrates the presence of abnormal embryos at 1, 10, and 100 μg L^{-1}.

The specific cause of CuPT toxicity is not well understood. Sub-lethal effects or sensitivities could be a result of DNA protein cross-links (Costa et al, 1993; Garman et al, 1997), altered transcription of regulatory genes required for normal development (Dandapat et al, 2003), cell membrane disruptions (Al-Adham et al, 1998; Dinning et al,

1998), or the interference of ATP synthesis resulting from mitochondrial damage (Bragadin et al, 2003).

Any or all of these interferences could be responsible for mortality, a decrease in fertilization capacity, and developmental effects. Sensitivity in embryonic developmeny appears to occur in all periods from the 1 cell stage to blastula formation. With the unknown mode of action and the numerous biochemical processes occurring throughout larval metamorphosis, it is possible that diuron and CuPT affect multiple pathways in *L. variegatus*. While current environmental concentrations of diuron and CuPT are not yet at levels that can induce mortality in adult marine organisms, we have shown that low concentrations can both reduce fertilization efficiency and impact embryo development. Further examination with other non target organisms should be carried out in order to understand the effects of diuron and CuPT on overall ecological health. In addition, studies examining current levels of these compounds and their behavior in the environment should be conducted in order to evaluate risk.

Chapter 5

Variations in copper pyrithione toxicity among populations and families of the barnacle, *Amphibalanus* (= *Balanus*) *amphitrite*

5.1 Introduction

Fouling, the colonization of man-made surfaces by living and non-living substances, has major strategic, economic, and environmental consequences (Costlow and Tipper,1984). Fouling of boat hulls has historically been controlled by the use of broad spectrum biocides that leach from paint formulations and kill settling organisms. By law, these biocides must be registered and environmental risk evaluated (Voulvoulis et al, 2002; Rittschof et al, 2003). Registering biocides requires a battery of EPA-mandated assays including acute, sub-chronic, and chronic toxicity tests (FIFRA, 1972; Forbes and Forbes, 1994).

Typically regulations governing the registration of biocides are based on laboratory toxicity assays that use organisms with little genetic variability such as *Daphnia sp.*, *Ceriodaphnia sp.*, *Hyalella sp.*, and *Mysis sp*. (Forbes and DePledge, 1992). One concern is that because laboratory populations are far removed from natural populations, regulatory assays may not accurately predict natural impacts. The purpose of this study was to examine the variation in toxic responses following exposure to the biocide, copper pyrithione (CuPT), in the barnacle, *Amphibalanus* (= *Balanus*) *amphitrite* (Pitombo, 2004), which were collected from five different environmentally impacted sites.

A. amphitrite can be found throughout the world in both tropical and subtropical environments and is considered to be a major fouling organism. These organisms thrive

in areas such as marinas, on harbor pilings, and on boat hulls where they are routinely exposed to heavy metals, polycyclic aromatic hydrocarbons (PAHs), and other industrial pollutants. Exposure to such compounds can potentially alter physiological or metabolic activities, or cause changes in detoxification pathways resulting in variation in organisms among and within populations (Barata et al, 2002; Virgilio et al, 2005; Piola and Johnston, 2006).

Antifouling companies have voluntarily withdrawn organotin-based antifoulants from the market in response to restrictions on heavy metal release and have adopted a new approach to coating synthesis. Organic biocides, such as pesticides and broad spectrum biocides are now being incorporated into paint formulation in an attempt to lower overall copper concentrations. Copper pyrithione (2-mercaptopyridine N-oxide copper salt) (CuPT) was introduced into the antifouling market in 1996 (Arch Chemicals; Maraldo and Dahllöf, 2004). It is hypothesized to act as a biocide by interfering with the activity of the primary proton pump, H^+-ATPase (Ermolayeva and Sanders, 1995). This interaction can lead to disruptions of cell membranes, disruptions of pH gradients, and complex binding with metals and proteins (Chandler and Segel, 1978, Dinning et al, 1998).

Here we report on variations in the acute toxicity of CuPT among different populations of the barnacle, *Amphibalanus amphitrite*, collected from four different sites with varying pollutant loads. The four sites were ranked for environmental pollution

load by incorporating two qualitative assessments and two quantitative measurements. Qualitative assessments included: *a*) visual indicators of anthropogenic sources; and *b*) sediment type. Quantitative measurements included: *a*) mud snail imposex rates (a measure directly linked to organotin contaminants); and *b*) the presence of PAHs in the sediments. Furthermore, we characterized the variation in sensitivity to CuPT at the population level using 15 separate barnacle families collected from a fifth site. This was accomplished following the procedures of Holm et al (2000, 2005) to ensure independent maternity and paternity.

5.2 Materials and Methods

5.2.1 Study Sites

Barnacles were collected from four sites (Figure 5.1) in the Newport River estuary in Carteret County, NC. The sites were chosen based on visual evidence of anthropogenic inputs and in order to provide a range of levels of industrial and residential pollutants. The four sites described below are numbered according to their assumed level of pollution with Site 1 being the cleanest and Site 4 the most polluted.

Site 1 (34°42.38N, 76°40.10W) is located within the Rachel Carson Estuarine Research Reserve (RCERR) and is considered to be a AA Quality Shellfish area (North Carolina Department of Environment and Natural Resources). Mud snails collected from this site have historically showed low imposex rates (Oberdörster et al, 1998; Straw

and Rittschof, 2004; Chapter 2, this dissertation) and sediment analysis indicates no discernable organic contamination (P. McClellan-Green, unpublished). The lack of contaminants at this site is due to the absence of both residential and industrial activity and to constant flushing by the coastal ocean. Site 2 (34°42.27N, 76°37.32W) is located on Taylors Creek, a small tidal creek adjacent to public and private boat docks. This creek serves as the main waterway for the town of Beaufort, NC. Site 3 (34°42.56N, 76°39.55W) borders a public boat ramp on Taylor's Creek that is used to launch small recreational boats. In addition a veneer factory and a Menhaden processing plant (closed in 2006) are within close proximity. Site 4 (34°43.18N, 76°42.26W) is located on Calico Creek, a tidal creek in the northeast corner of Morehead City, NC. This creek was visually the most polluted site and is impacted by a private marina, a commercial port, dockage for a NC Army Reserve station, sewage discharge from the town of Morehead City, and previously served as the site for an automobile repair shop.

A fifth low impact site, a seawall at the same tidal height and water quality as Site 1, was used for the collection of 15 independent barnacle families. Site 5 (34°42.54N, 76°40.21W) is located on the southeast facing seawall of the Duke University Marine Laboratory (DUML), Beaufort, NC. This site has been the source of living barnacles used in previous studies of heritable traits (Holm et al, 2000, 2005).

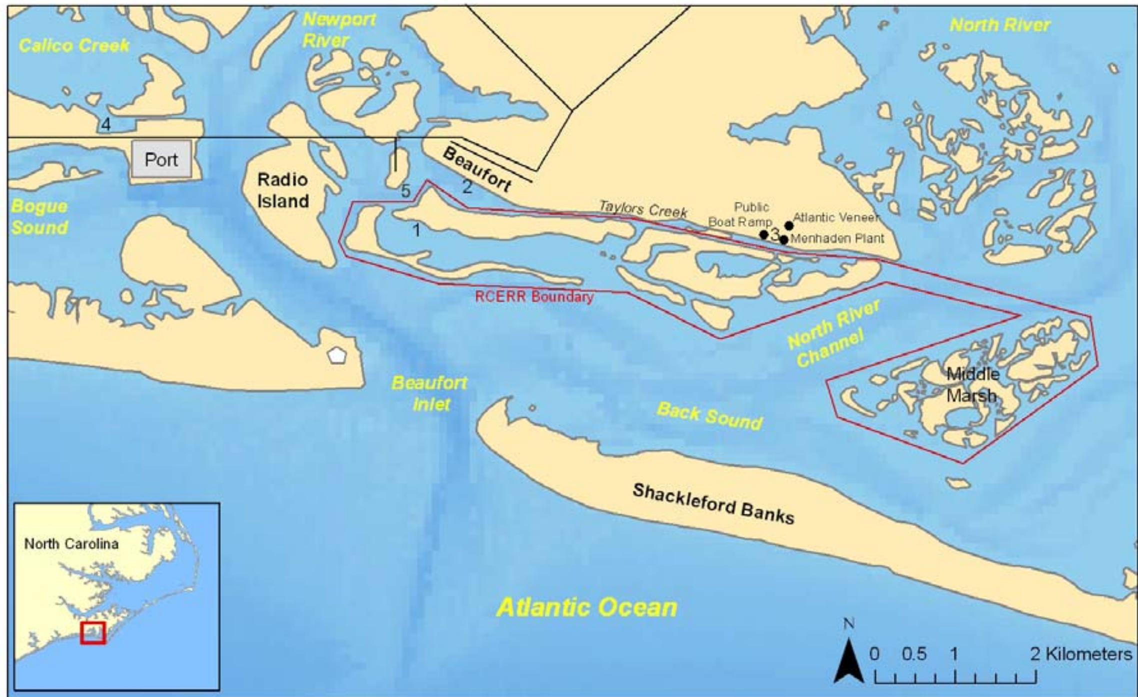


Figure 5.1 Location of the five barnacles sampling sites in the Newport River estuary.

5.2.2 Percent Imposex

The historical presence of antifouling compounds was established for Sites 1-4 by monitoring the imposex rate of resident mud snails. Approximately 50-70 mud snails, *Ilyanassa obsoleta*, were randomly collected during August 2006 from Sites 1-4. Animals were removed from shells and female snails examined for the presence of male accessory sex organs following Oberdörster et al. (1998).

5.2.3 PAH Analysis

Sediment was collected from Sites 1–4 during August 2006 in clean glass containers and transported to the laboratory wrapped in aluminum foil to reduce any degradation of PAHs by ultraviolet light. Three replicate 20 g wet wt. sediment samples from each site were oven dried at 35°C for 24 hours, then ground with a mortar and pestle before PAH extraction (Extraction of PAHs was carried out according to USEPA Protocol 3550B, 1996). A 2 g dry wt. subsample was dissolved in 10 mL of methylene chloride and sonicated (Sonic Dismembrator-60, Fisher Scientific) for 2–3 minutes. Samples were centrifuged at 5000 rpm for 30 minutes and the supernatant dried under nitrogen.

Spectrofluorometry was used to detect PAHs because it is a rapid and inexpensive screening method (Dissanayake and Galloway, 2004). Samples were resuspended in 3 mL of methanol and the fluorescence measured using a Perkin-Elmer (Norwalk, CT) Model LS50B luminescence spectrofluorometer. The following parameters were used to obtain PAH estimates for pyrene, naphthalene, and benzo (a) pyrene (B(a)P), respectively: excitation 341 nm, emission 383 nm; excitation 290 nm, emission 335 nm; and excitation 380 nm, emission 430 nm.

5.2.4 Toxicant

CuPT (Arch Chemicals, Norwood, CT) stock solutions were prepared in dimethylsulfoxide (DMSO) at a concentration of 2,000 mg L^{-1} following Kobayashi and

Okamura (2002). Stock and working solutions were kept for no longer than one week in amber bottles, at room temperature to avoid photodegradation. Working solutions were made immediately before use by diluting stock solutions in filtered seawater (FSW). All working solutions of CuPT contained 0.00005% (v/v) DMSO.

5.2.5 Ranking of Sites

The four sites were ranked for environmental pollution load by incorporating two qualitative (visual impact and sediment type) and two quantitative (imposex levels and total PAH concentration) measurements (Table 5.1). The presence of pollution sources and sediment type associated with the sites were used to establish visual estimates for each site. Mud snail imposex percentages (a biomarker of organotin contamination) and the presence of PAHs in sediments were used as quantitative measurements to rank the four sites with respect to actual anthropogenic contaminants. Local pollution sources, percent imposex in mud snails, and total PAH concentration were ranked from 1–4 (lowest to highest pollution) while sediment type was scored as either 0 (mud) or 1 (sand). Since PAHs sorb better to high organic mud than low organic sand (Schwarzenbach et al, 1992), sand was scored with a 1 to offset likely underestimated PAH measurements based on sandy sediments. The lowest, cleanest total pollution score a site could receive is 3 while the highest, most polluted score is 13.

Table 5.1 Site pollution scores, rankings, and LC_{50} values

Site Number	Rank					Overall Rank	Nauplii LC_{50} $\mu g\ L^{-1}$ (95% CI)
	Sources[a]	Imposex	PAH	Sediment	Sum		
1	1	1	2	0	4	**1**	6.1 (5.0–7.1)
2	2	2	1	1	6	**2**	5.6 (5.3–5.9)
3	3	4	3	0	10	**3**	5.3 (4.6–5.9)
4	4	3	4	0	11	**4**	4.0 (3.6–4.3)

[a]Sources of anthropogenic input in the area

5.2.6 Toxicity Assays

Naupliar assays for the toxicity of CuPT were preformed as described in Rittschof et al (1992). Stage II nauplii of *A. amphitrite* from sites 1–4 were collected within 3 hours of hatching. Four replicate, 250 μL aliquots (20–40 nauplii) were added to glass test tubes containing 4 mL of the test solutions. All test solutions were prepared in 0.22 μm filtered seawater (35‰). Larvae were not fed for the duration of the assay. Exposures were carried out at 22–25°C for 24 h after which time nauplii were examined for swimming activity or movement using a dissecting microscope. The number of live and dead larvae were counted. Nauplii that could no longer move or swim were determined to be dead.

5.2.7 Experiment I – Naupliar Toxicity Assay

Approximately 100 barnacles were collected during June 2006 from hard substrata (dock pilings, PVC pipe) at each site (1–4). Upon return to DUML barnacles were placed in approximately 1 L of 0.22 μm filtered seawater at 35‰ (FSW) and

crushed to facilitate release of nauplius larvae. Nauplii were collected and exposed to FSW, DMSO or 3.76, 5.64, 6.58, and 7.52 μg L^{-1} CuPT in FSW or DMSO.

5.2.8 Experiment II – Variation Between Families

Twenty to thirty barnacle-laden oyster shells were collected at approximately 1 m intervals along the DUML seawall at Site 5 during June 2006. Collection of oyster shell substrata and egg masses ensured that no egg mass from an individual barnacle shared a parent with any egg mass from another barnacle (Holm et al, 2000). Masses of well-developed fertilized eggs were dissected from barnacles (the maternal parents) and placed into separate plastic containers with 100 mL FSW. Nauplii from the most fecund 15 barnacles were used in the toxicity assay. Nauplii larvae that hatched from egg masses were exposed to solutions of DMSO, FSW or to 6.1 μg L^{-1} CuPT. Mortality was scored after 24 h as described above and families were numbered in order of their sensitivity to CuPT with family 1 the least sensitive and family 15 the most.

5.2.9 Statistics

Mortality in the FSW control treatments served as the natural response rate. LC_{50} concentrations for each of the sites were calculated using PROC PROBIT in SAS (SAS/STAT Version 8, SAS Institute Inc., 1999). Differences in larval mortality among families were characterized using the nonparametric Kruskal-Wallis test (PROC NPAR1WAY, SAS/STAT Version 8, SAS Institute Inc., 1999).

5.3 Results

5.3.1 Ranking of sites

Table 5.1 shows the ranking results for each of the two qualitative and quantitative assessments. Site 1 received the lowest score for anthropogenic sources. It had 0% imposex, a low level of total PAH (0.43 μg L^{-1}), mud sediment, and received a total score of 4. Sites 2 and 3 are ranked the second and third cleanest sites with pollution scores of 6 and 10, respectively. Site 2 had the second lowest visual pollution score, 41.2% imposex, the lowest levels of PAH (0.23 μg L^{-1}), and organic/ sand sediment. Site 3 had the second highest visual pollution score, the highest percent imposex (91.2%), total PAH levels of 0.53 μg L^{-1}, and mud sediment. Site 4 had the highest visual pollution score resulting in a total score of 11. It had 81.2% imposex, PAH concentrations (6.22 μg L^{-1}) an order of magnitude greater than other sites, and mud sediment. The pollution scores ranked the sites in the same order as predicted based on visual estimates of anthropogenic activities adjacent to each site.

5.3.1 Variation in LC_{50} among sites

Nauplii toxicity tests from Sites 1, 2 and 3 were statistically similar to one another with CuPT LC_{50} of 6.1 (95% CI, 5.0–7.1), 5.6 (5.3–5.9), and 5.3 (4.6–5.9) μg L^{-1}, respectively (Table 5.1). Site 4 nauplii were significantly ($p<0.001$)) more sensitive to CuPT with an LC_{50} of 4.0 (3.61–4.29) μg L^{-1}. There is an inverse relationship between the pollution rank

of a site and the LC_{50} of its nauplii to CuPT (Table 5.1). Site 1, the cleanest site, had the highest LC_{50}, and the least sensitive larvae while the most polluted Site 4 had the lowest LC_{50} and most sensitive larvae.

5.3.2 Variation in mortality among barnacle families

All 15 families collected from Site 5 showed significant mortality when exposed to 6.1 μg L^{-1} CuPT compared to FSW or DMSO ($p<0.0001$). Mortality ranged from 15.0% (95% CI, 8.78–21.39) for Family 1 up to 98.9% (95% CI, 95.4–100) for Family 15 (Figure 5.2). Families were ordered based on their sensitivity to CuPT exposure.

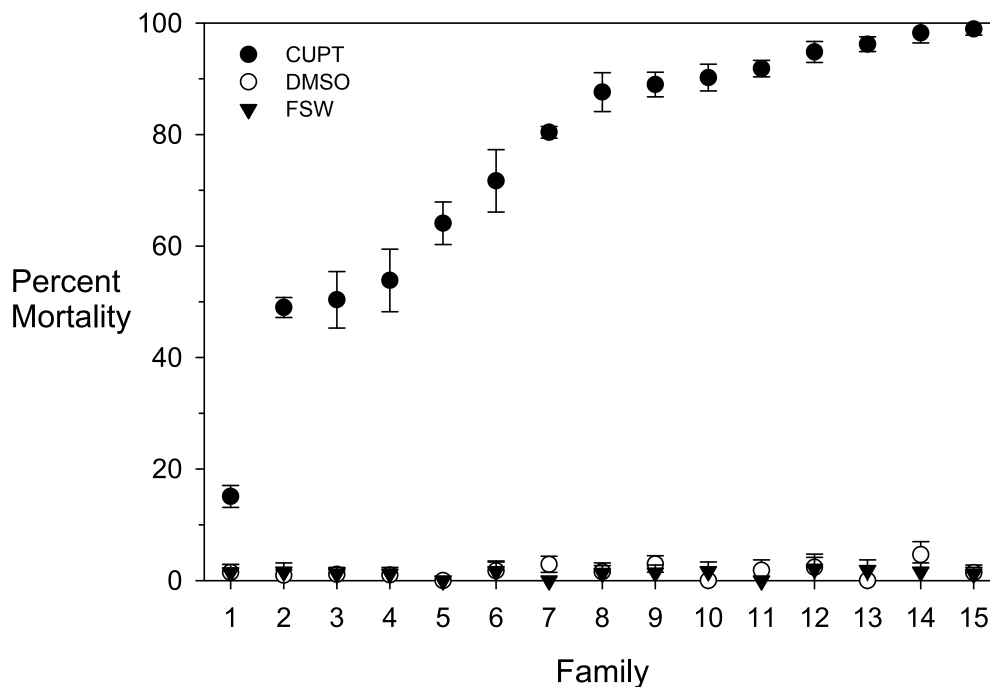


Figure 5.2 Percent mortality of nauplii from 15 *Amphibalanus amphitrite* families after 24 h exposure to controls DMSO (○) and FSW (▼), and 6.1 μg L^{-1} CuPT (●).

5.4 Discussion

Acute toxicity assays using *A. amphitrite* nauplius larvae collected from four sites within Carteret County, NC had LC_{50} values ranging between 4.0 to 6.1 μg L^{-1} CuPT (Table 5.1). These concentrations are similar to the LC_{50} reported for another crustacean, the adult toy shrimp, *Heptacarpus futilirostris* (LC_{50} = 2.5 μg L^{-1}; Mochida et al., 2006). Other marine organisms assayed for CuPT toxicity include the sea urchin *Lytechinus variegatus* (LC_{50} = 14 μg L^{-1}), the mud snail, *Ilyanassa obsoleta* (LC_{50} = 63.2 μg L^{-1}), (Romano et al, unpublished) and the red sea bream, *Pagrus major* (LC_{50} = 9.3 μg L^{-1}; Mochida et al., 2006). Total pyrithiones in harbors have not been reported at concentration higher than 100 nM (Mackie et al, 2004) thus we do not expect CuPT pollution to cause acute toxicity in *A. amphitrite* larvae not associated with boat hulls.

The LC_{50} values (Table 5.1) for the four collection sites were inversely related to the pollution score for each site. Site 1, located within the RCERR with notable AA shellfish quality waters, ranked the cleanest of the four sites and produced nauplii with the most resistance (highest LC_{50}). Site 4, located in a tidal creek surrounded by a commercial port, marina, and other anthropogenic inputs and which has high levels of imposex and PAHs produced nauplii with the lowest resistance to CuPT toxicity (lowest LC_{50}).

One explanation for this susceptibility is that barnacles residing in highly polluted sites are undergoing altered physiological and metabolic processes. It is also

highly probable that their detoxification systems are already at full capacity and that they are unable to handle additional stress (Baird et al, 1990; Lam, 1996). This concept is supported by Wang et al (2006) who reported that fish exposed to organotin and PAHs exhibited increased vulnerability to oxidative stress and had lower glutathione-s-transferase activity, resulting in reduced physiological resilience to new stressors. Brüschweiler et al (1996) found similar results with inhibition of cytochrome P4501A in fish after organotin exposure. In molluscs, similar findings have been reported in oysters exposed to PAH contaminants. Surveys conducted by Auffret et al (2004) report lingering effects of oil spill 'Erika' off the west coast of France are still causing immunological impairment and alterations and decreased defense related functions in the Pacific oyster, *Crassostrea gigas,* three years after the accident. The data suggest that organisms from low impacted sites are better able to withstand toxicant stress than those from more impacted sites.

To examine natural variation between families, nauplii from 15 different barnacle families from a low impacted site were tested for sensitivity to CuPT. After exposing the barnacle families to a single concentration of CuPT, we found that mortality ranged between 15 and 98.9%. We interpret the high variation in mortality as evidence for selection of compound resistant organisms. Similar conclusions have been reached for other model systems such as those exposed to heavy metals. Forbes et al (1995) found that exposure to cadmium increased the variability in growth rate of two species of

gastropods and hypothesized that environmental stress can reduce population response and result in increased variation in biological responses. To further support this supposition Baird et al (1990) reported that clones of *D. magna* exposed to cadmium varied over 3 orders of magnitude in their acute responses with LC_{50} ranging between 0.06–100 ppb.

In this study, two observations were made: 1) that prior exposure to pollutants can increase the sensitivity of *A. amphitrite* to CuPT; and 2) that a large variability in mortality (15-98.9%) was found to exists within a population of *A. amphitrite* exposed to CuPT. These results suggest caution when extrapolating results from laboratory strains that may have little genetic variability. Wild populations are routinely exposed to an assortment of anthropogenic contaminants and other stressors. These conditions, in turn, may select for different genotypes that are more or less sensitive to toxicant insult. When developing protocols for environmental risk assessment, future tests should include assays that examine responses of field populations in addition to those of laboratory strains in order to better represent environmental conditions and variability.

Chapter 6

Conclusions:

Effects of Non-Point Source Pollution

on Invertebrates

6.1 Overview

Non-point source pollution is not generated from any single source of contamination; rather it is a mixture of agricultural, residential, and industrial activities. In a recently released report, the United Nations estimated that several million tons of pesticides and nearly half a million tons of petroleum products are released into aquatic environments annually through intentional or accidental disposal (McGinn, 2004). These man-made contaminants enter into streams, rivers, and ultimately into the worlds oceans, but first they filter through transitory environments such as the Rachel Carson Estuarine Research Reserve (RCERR). The area surrounding the Reserve is one of the fastest growing areas in the state of North Carolina and is within close proximity to numerous agricultural, residential, and industrial developments. According to the latest data available from the USEPA Toxics Release Inventory Program (March 22, 2007), industrial sources in and around Beaufort, NC released a total of 113,859 pounds of styrene, 9,966 pounds of copper, and 6,861 pounds of lead into the environment adjacent to the RCERR in 2005. The exact amounts of pesticides and other anthropogenic compounds released into the area surrounding and entering the RCERR are unknown. Standard water quality assessments within the reserve do not routinely monitor for the presence of anthropogenic contaminants.

This dissertation investigated the hypothesis that non-point source contaminants entering into estuarine systems adversely affects the health, development and

reproductive capacity (productivity) of organisms living in that environment. To accomplish this, a detailed examination of the acute toxicity and sub-lethal effects of four representative compounds that have the potential to enter into the RCERR (based on surrounding development and anthropogenic activities) were first conducted. These compounds included pesticides, antifoulants, petroleum by-products, and industrial solvents. Specifically, the effects of diuron, copper pyrithione (CuPT), benzo(a)pyrene (B(a)P) and styrene exposure on the mud snail, *Ilyanassa obsoleta*, the American oyster, *Crassostrea virginica*, the sea urchin, *Lytechinus variegatus*, and the barnacle, *Amphibalanus amphitrite* were examined. These organisms were selected because they represent ecologically important mollusks, echinoderms, and crustaceans that live within this reserve system. Next, the general effects of non-point source pollution were examined at six sites within the RCERR in order to gain a better understanding of the current health of this unique habitat. Our results show that the pesticide diuron, the antifoulant CuPT, the polycyclic aromatic hydrocarbon B(a)P, and the industrial solvent, styrene all adversely impacted mortality, reproduction, and/or development in each of the model organisms used in these studies.

6.2 Pesticides/Antifoulants

One of the major hypotheses tested in this dissertation was that diuron would exhibit acute and/or sub-lethal affects in non-photosynthesizing organisms at concentration currently observed in the environment. While diuron has been used since

the 1960s as a photosystem II inhibiting herbicide, more recently it has been added to the arsenal of antifouling additives for copper-based paint formulations. Very little information is available on the effects of diuron in the context of its role as an antifoulant additive. Diuron has been reported at concentrations ranging from a few μg L^{-1} to several mg L^{-1} in marinas, harbors, and some U.S. surface waters (Thomas et al, 2001; Moncada, 2004).

The model organisms employed in this study were exposed to increasing concentrations of diuron for a period of 96 hours in a static renewal system. Diuron was not shown to be acutely toxic to adult *C. virginica* or *L. variegatus* at environmentally relevant concentrations (Fig. 6.1). We were also not able to achieve an LC_{50} for *I. obsoleta,* possibly due to a solubility limitation of the pesticide in saltwater at the higher test concentrations. Sub-lethal effects were however observed in *I. obsoleta*, *C. virginica,* and *L. variegatus* at concentrations well within current environmental ranges. A significant reduction in egg capsule production was seen in *I. obsoleta* at concentrations as low as 100 μg L^{-1} and a significant reduction in *C. virginica* condition index was achieved at 1 mg L^{-1}. Larval development and fertilization success in *L. variegatus* was negatively impacted at concentrations as low as 0.1 μg L^{-1}. This concentration has been reported to negatively affect photosynthesis in two species of sea grass (Haynes et al, 2000) and is over one order of magnitude lower that levels which have been recorded in several marinas and harbors (Thomas et al, 2001).

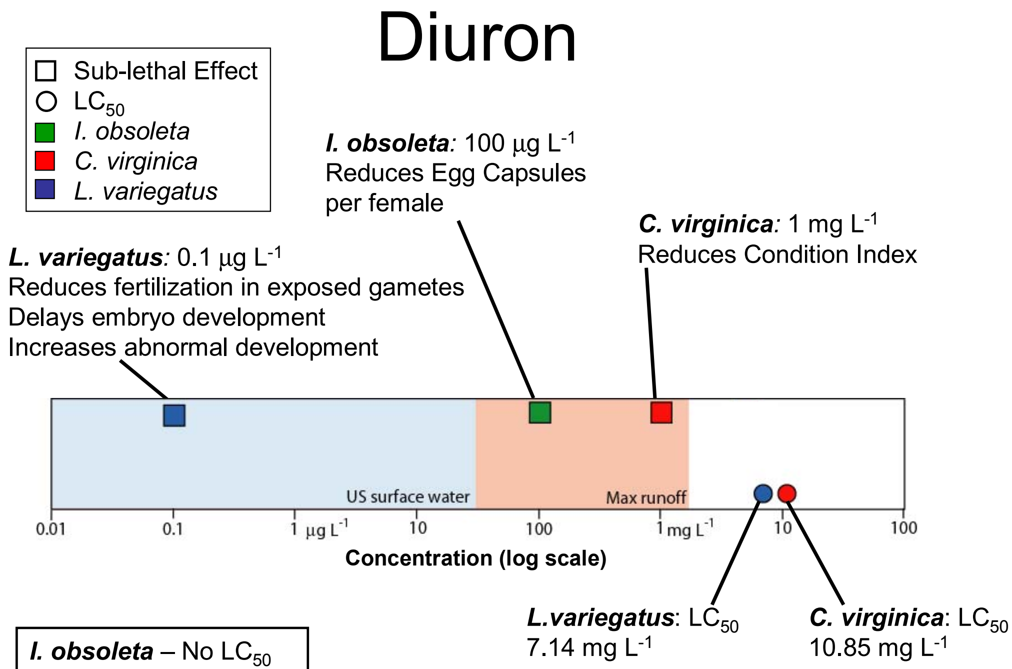


Figure 6.1 Summary of the concentrations at which diuron affects invertebrate models both lethally and sub-lethally in relation to environmental concentrations (shaded blue, common; shaded pink, worst case).

Copper pyrithione is another compound that has recently been added to the antifouling market. Environmental levels of this compound have not been reported however; total pyrithiones (including Cu, Zn, and Na conjugates) have been estimated in excess of 0.3 µg L^{-1} in some U.K. waters (Mackie et al, 2004). The use and environmental concentrations of CuPT and its sister compound zinc pyrithione (ZPT) (which can transchelate to CuPT upon introduction into marine environments) is

predicted to increase as organotin based antifoulants are phased out and replaced with newer paint formulations (Jensen et al, 2000). Presently 70% of all European pleasure crafts are estimated to use antifoulants that include ZPT (Jensen et al, 2000) and approximately half of this level when applied will transchelate into CuPT (Dahllöf et al, 2005).

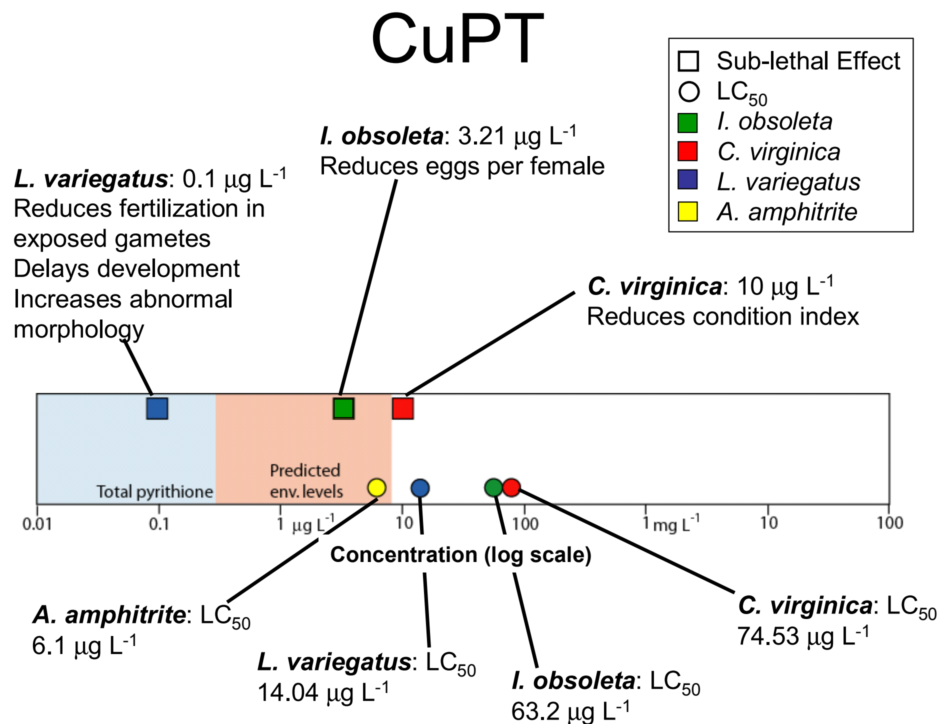


Figure 6.2 Summary of the concentrations at which CuPT affects invertebrate models both lethally and sub-lethally in relation to environmental concentrations (shaded blue, total pyrithiones; shaded pink, predicted future concentration).

At exposure levels equivalent to currently estimated environmental concentrations, CuPT was not acutely toxic to adult *I. obsoleta*, *C. virginica*, *L. variegatus*, or stage II *A. amphitrite* nauplii (Fig. 6.2). CuPT exposure did however, produce sub-lethal affects including decreased fertilization success and increased delays in development and abnormal morphologies. Based on the findings presented in this dissertation, anticipated increases in the use of this compound(s) will acheive environmental concentrations of pyrithione that elicit acute toxicity in stage II *A. amphitrite* nauplii and produces sub-lethal effects in *I. obsoleta*, *C. virginica*, and *L. variegatus*.

6.3 Polycyclic Aromatic Hydrocarbons

B(a)P is a product of both natural and anthropogenic production and is not normally considered to be acutely toxic to most marine organisms at environmental levels. Background levels of B(a)P that are considered typical in drinking water, rainwater, and subterranean water range from 0.2 to 1000 ng L^{-1}, while highly contaminated waters have been reported to exceed 50 µg L^{-1} (Irwin et al, 1997). In our range finding study, we were not able to achieve an LC_{50} in *I. obsoleta* or *C. virginica*; however it should be noted that some death of *I. obsoleta* did occur at concentrations as low as 10 µg L^{-1}. In addition, decreases in egg capsule production and condition index

were observed in *I. obsoleta* and *C. virginica* at the lowest (fecundity and condition index) test concentration of 50 μg L^{-1} (Fig. 6.3).

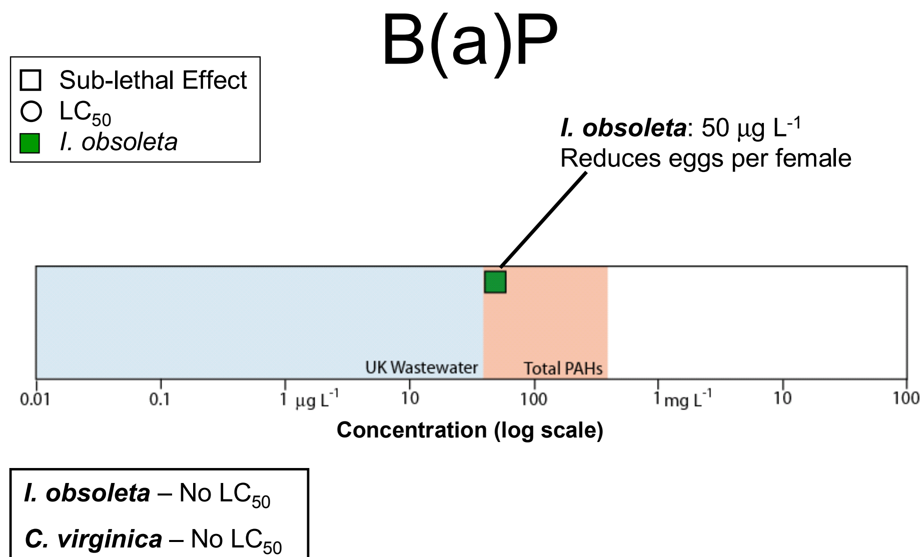


Figure 6.3 Summary of the concentrations at which B(a)P affects invertebrate models both lethally and sub-lethally in relation to environmental concentrations (shaded blue, B(a)P levels reported in UK wastewater; shaded pink, total PAHs).

Based on the findings reported in this dissertation B(a)P was not acutely toxic to adult *I. obsoleta* or *C. virginica*. No LC_{50} could be acheived in *I. obsoleta* or *C. virginica* either, most likely due to solubility issues at higher concentrations in saltwater environments. Acute toxicity due to benzo (a) pyrene exposure is rarely reported in mammals, birds, and aquatic organisms. PAHs in general are more frequently associated with chronic risks that result from reactive metabolites produced within

detoxification pathways (Livingstone et al, 1990; Grundy et al, 1996; Ericson and Balk, 2000; McElroy et al, 2000). These chronic effects often result in biological and physiological changes including inhibition of molting, growth, behavioral alterations, or induction of enzymatic activities such as ethoxycoumarin-O-deethylase (ECOD) (Oberdörster et al, 1999; 2000). In addition, exposure to PAHs has been reported to cause severe immunological alterations such as inhibition of phagocytosis and damage to lysosomes (Grundy et al, 1996; Wootton et al, 2003; Auffret et al, 2004).

6.4 Industrial Solvents

Styrene is globally used as an industrial solvent in the production of polystyrene, copolymers and other forms of resins. According to USEPA's Toxic Chemical Release Inventory, total styrene released into U.S. land and water bodies from 1987-1993 totaled over 2 million lbs. These releases mainly stemmed from the adhesives and sealants industries with the largest releases occurring in Texas and Louisiana (USEPA, accessed 27 April, 2007). While styrene is highly volatile (vapor pressure, 4.5 mmHg at 20°C), it still has the potential to enter into aquatic environments through various exposure scenarios (Cushman et al, 1997). In 1992, Miller et al (1994) estimated a worldwide use of approximately 16 million tons with much of that volume being transported by barge and vessels over large bodies of water. One of the major hypotheses of this dissertation was that exposure of an organism to environmental concentrations of styrene would elicit acute and sub-lethal effects.

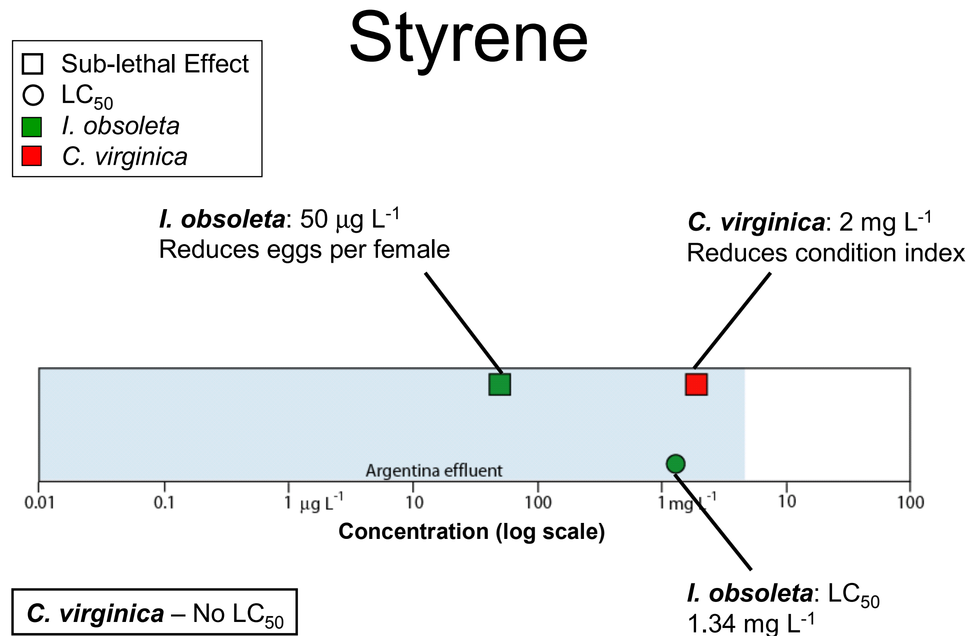


Figure 6.4 Summary of the concentrations at which styrene affects invertebrate models both lethally and sub-lethally in relation to environmental concentrations (shaded blue, from Argentinean wastewater effluent).

Styrene caused mortality in *I. obsoleta* at concentrations as low as 50 µg L^{-1} and resulted in an LC_{50} of 1.34 mg L^{-1} (Fig. 6.4). These concentrations are well within reported environmental levels both within the U.S (exceeding 50 µg L^{-1}; Pelish et al, 2003) and globally (exceeding 5 mg L^{-1}; Gomez et al, 2001). One possible explanation for the inability to achieve an LC_{50} in *C. virginica* exposed organisms may be the exposure methodology for the experiment. Oysters were placed in 10 gallon aquaria with surface areas approximating three square feet. The systems were open and constantly aerated with the use of air stones. It is possible that the styrene was volatilized thereby

decreasing exposure concentrations. In the *I. obsoleta* toxicity assay, while containers were aerated they did not contain air stones and were covered at all times with plastic wrap.

Sub-lethal affects were observed including reductions in fecundity and overall health indices in both species at environmentally relevant concentrations. A reduction in egg capsules laid per female *I. obsoleta* was observed at concentrations as low as 50 µg L^{-1}, the lowest test concentration used in the fecundity study. Lowered condition index values were observed at a concentration of 2 mg L^{-1}, the only concentration tested.

6.5 Health Assessment of RCERR

Developmental, reproductive and health indices were assessed in organisms from six sites surrounding the RCERR. These sites were chosen based on their proximity to potential sources of agricultural, industrial, and residential pollution (Figure 6.5). Site 1, a mud flat consisting of fine sediments and sandy bottom, was chosen as a control site. This site has been previously analyzed for contaminants and has no notable levels of PAHs, PCBs, or heavy metals (Oberdörster and McClellan-Green, 1998, 2000; Straw and Rittschof, 2004; Oberdörster et al, 2005; McClellan-Green et al, 2006) and is considered AA quality shellfish water by the North Carolina Department of Environment and Natural Resources (NCDENR). Site 2 is a sandy bottom environment located along the Carrot Island side of Taylors Creek. This site is directly across from the Beaufort, NC waterfront and has the potential to be impacted by recreational and

residential activities. Site 3 is located on the same tidal creek as Site 2 but receives wastewater discharge from a tertiary sewage treatment pipe located approximately 200 m across the creek. Organisms at this site are potentially exposed to pharmaceuticals, roadway runoff, and residential wastes. Site 4 was the final site chosen along the Carrot Island side of Taylors Creek. This high organic matter site is regularly exposed to persistent and residual contaminant inputs located on the opposite side of the creek including an active boat launch, commercial fishing vessels, and a nearby wood veneer production facility. Site 5 is located along the North River Channel and was chosen in order to evaluate anthropogenic input resulting from agricultural and recreational activities in the North River. Site 6 is situated on the western end of the RCERR across from Radio and Pivers Islands. It receives tidal flows from the North and Newport River estuary and is an active waterway for boats entering the Beaufort Inlet.

Presently, the exact nature and composition of anthropogenic contaminants affecting the RCERR is not known. It does appear however, that some compound or mixture of compounds is impacting the overall health of organisms within the Reserve. A reduction in egg capsules laid per female *I. obsoleta* was observed at Sites 3, 4, and 6 while a reduction in condition index in *C. virginica* was seen at Sites 4, 5, and 6. In addition to the reduction in condition index of oysters from Site 6, an increase in ECOD activity was observed that was approximately 3 times higher than that which was observed in oysters from Site 1.

These site-specific results imply that Sites 4 and 6 are the two most highly impacted sites, possibly a result of petroleum by-products and/or endocrine disrupting compounds contained within the sediments. Sites 3 and 5 appear to be undergoing initial signs of environmental impact as observed by the decrease in fecundity of *I. obsoleta* and the reduction in overall health of *C. virginica*. This may be related to the proximity of Site 3 to the wastewater discharge pipe and the proximity of Site 5 to tidal flushing from the eastern end of Taylors Creek and the North River Channel. No significant impacts or reduction of fecundity in *I. obsoleta* or overall health of *C. virginica* was observed in organisms from Sites 1 and 2. This is most likely a result of the tidal flushing and lack of proximity to anthropogenic sources for Site 1. In addition, Site 2 contains very little organic matter with the majority (>99%) of the sediment consisting of coarse sands and is highly influenced by the strong tidal flow through the Beaufort Inlet.

Environmental assessments such as water quality and sediment analysis need to be performed within the RCERR before any concrete cause and effect relationship can be determined. It is clear based upon the observance of decreased egg capsule production in *I. obsoleta* and decreased condition index in *C. virginica,* that something, most likely xenobiotic compounds of some sort, is adversely affecting invertebrates within the Reserve. With the dramatic increases in coastal development and recreational and commercial activities surrounding the RCERR, the Reserve should be routinely monitored. Current monitoring activities within the Reserve include daily tidal height,

wind direction, water temperatures, salinity, and phosphate and nitrate concentrations. Based on our observations, the presence of contaminants such as pesticides, antifoulants, petroleum by-products and industrial solvents within the Reserve should also be monitored.

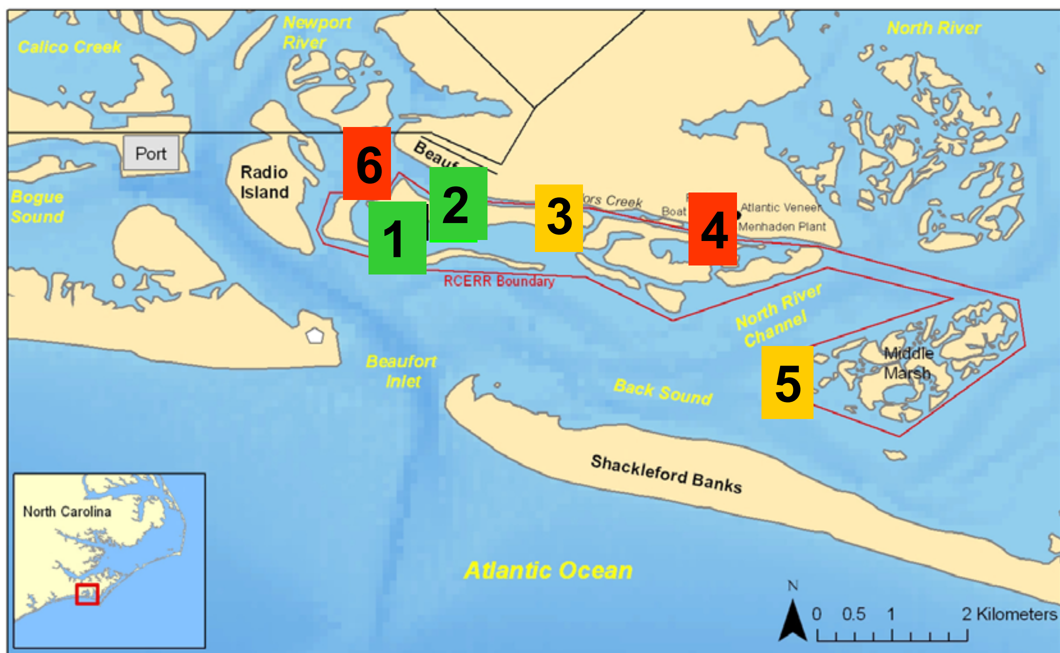


Figure 6.5 Relative health of the six sampled RCERR sites. Healthy, green; slightly impacted, orange; highly impacted, red.

Estuaries provide many wildlife benefits such as nursery habitat, feeding grounds, and sanctuaries. They also contribute millions of dollars to local economies in the form of tourism and commercial and recreational fishing. In general, estuaries serve the public with a suite of resources, benefits, and services and must be maintained

carefully for the shared benefit of all who enjoy and depend upon them. In order to maintain their usefulness and accurately determine whether they continue to function as healthy ecosystems additional recommendations for assessing the health of the Reserve have been formulated. They include 1) expanding field observations to include other invertebrate and vertebrate species commonly found within the RCERR (including different life stages) and 2) conducting contaminant assessments within sediment and water samples and 3) examining seasonal health assessments and yearly trends with regard to reproductive capacity in important Reserve species. Hopefully such recommendations will aid in the assurance of continued estuarine health of this unique and ecologically important environment.

References

Abbe, G., Sanders, J., 1988. Rapid decline in oyster condition in the Patuxent River, Maryland. J. Shellfish Res. 7, 57-59.

Al-Adham, I.S.I., Dinning, A.J., Eastwood, I.M., Austin, P., Collier, P.J., 1998. Cell membrane effects of some common biocides. J. Ind. Microbiol. Biotechnol. 21, 6-10.

Alzieu, C., Sanjuan, J., Deltreil, J.P., Borel, M., 1986. Tin Contamination in Arcachon Bay - Effects on Oyster Shell Anomalies. Mar. Pollut. Bull. 17, 494-498.

Alzieu, C., 1991. Environmental-Problems Caused by TBT in France - Assessment, Regulations, Prospects. Mar. Environ. Res. 32, 7-17.

Alzieu, C., 2000. Impact of tributyltin on marine invertebrates. Ecotoxicology 9, 71-76.Anderson, D.W., Hickey, J.J., 1972. Eggshell changes in certain North American birds. Proc. Int. Ornithol. Congr. 15, 514-540.

Au, D.W.T., Wu, R.S.S., Zhou, B.S., Lam, P.K.S., 1999. Relationship between ultrastructural changes and EROD activities in liver of fish exposed to Benzo[a]pyrene. Environ. Pollut. 104, 235-247.

Auffret, M., Duchemin, M., Rousseau, S., Boutet, I., Tanguy, A., Moraga, D., Marhic, A., 2004. Monitoring of immunotoxic responses in oysters reared in areas contaminated by the "Erika" oil spill. Aquat. Living Resour. 17, 297-302.

Austin, H., Haven, D.S., Moustafa, M.S., 1993. The Relationship between Trends in a Condition Index of the American Oyster, *Crassostrea virginica*, and Environmental Parameters in 3 Virginia Estuaries. Estuaries 16, 362-374.

Baird, D.J., Barber, I., Calow, P., 1990. Clonal Variation in General Responses of *Daphnia magna* Straus to Toxic Stress .1. Chronic Life-History Effects. Funct. Ecol. 4, 399-407.

Barata, C., Baird, D.J., Mitchell, S.E., Soares, A., 2002. Among- and within-population variability in tolerance to cadmium stress in natural populations of *Daphnia magna*: Implications for ecological risk assessment. Environ. Toxicol. Chem. 21, 1058-1064.

Barber, B.J., Mann, R., 1994. Growth and Mortality of Eastern Oysters, *Crassostrea virginica* (Gmelin, 1791), and Pacific Oysters, *Crassostrea gigas* (Thunberg, 1793)

under Challenge from the Parasite, Perkinsus marinus. J. Shellfish Res. 13, 109-114.

Bauer, B., Fioroni, P., Ide, I., Liebe, S., Oehlmann, J., Stroben, E., Watermann, B., 1995. TBT Effects on the Female Genital System of *Littorina littorea* - a Possible Indicator of Tributyltin Pollution. Hydrobiologia 309, 15-27.

Beddingfield, S.D., McClintock, J.B., 1998. Differential survivorship, reproduction, growth and nutrient allocation in the regular echinoid *Lytechinus variegatus* (Lamarck) fed natural diets. J. Exp. Mar. Biol. Ecol. 226, 195-215.

Bellas, J., 2005. Toxicity assessment of the antifouling compound zinc pyrithione using early developmental stages of the ascidian *Ciona intestinalis*. Biofouling 21, 289-296.

Bellas, J., Granmo, A., Beiras, R., 2005. Embryotoxicity of the antifouling biocide zinc pyrithione to sea urchin (*Paracentrotus lividus*) and mussel (*Mytilus edulis*). Mar. Pollut. Bull. 50, 1382-1385.

Bielmyer, G.K., Brix, K.V., Capo, T.R., Grosell, M., 2005. The effects of metals on embryo-larval and adult life stages of the sea urchin, *Diadema antillarum*. Aquat. Toxicol. 74, 254-263.

Bond, J.A., 1989. Review of the Toxicology of Styrene. CRC Crit. Rev. in Toxicol. 19, 227-249.

Bondoc, F.Y., Bao, Z.P., Hu, W.Y., Gonzalez, F.J., Wang, Y.Y., Yang, C.S., Hong, J.Y., 1999. Acetone catabolism by cytochrome P450 2E1: Studies with CYP2E1-null mice. Biochem. Pharmacol. 58, 461-463.

Bouilly, K., Bonnard, M., Gagnaire, B., Renault, T., Lapegue, S., 2007. Impact of diuron on aneuploidy and hemocyte parameters in Pacific oyster, *Crassostrea gigas*. Arch. Environ. Contam. Toxicol. 52, 58-63.

Boxall, A.B.A., Comber, S.D., Conrad, A.U., Howcroft, J., Zaman, N., 2000. Inputs, monitoring and fate modelling of antifouling biocides in UK estuaries. Mar. Pollut. Bull. 40, 898-905.

Bradford, M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72, 248-254.

Brady, J.F., Lee, M.J., Li, M., Ishizaki, H., Yang, C.S., 1988. Diethyl ether as a substrate for acetone/ethanol inducable cytochrome P-450 and as an inducer for cytochrome(s) P-450. Mol. Pharmicol. 33, 148-154.

Bragadin, M., Manente, S., Marton, D., Cima, F., Rigobello, M.P., Bindoli, A., 2003. The interaction of zinc pyrithione with mitochondria from rat liver and a study of the mechanism of inhibition of ATP synthesis. Appl. Organomet. Chem. 17, 869-874.

Britton, J.C., Morton, B., 1994. Marine Carrion and Scavengers, Oceanography and Marine Biology, Vol 32. Oceanography and Marine Biology, pp. 369-434.

Brumbaugh, R.D., Sorabella, L.A., Garcia, C.O., Goldsborough, W.J., Wesson, J.A., 2000. Making a case for community-based oyster restoration: An example from Hampton Roads, Virginia, USA. J. Shellfish Res. 19, 467-472.

Bruschweiler, B.J., Wurgler, F.E., Fent, K., 1996. Inhibition of cytochrome P4501A by organotins in fish hepatoma cells PLHC-1. Environ. Toxicol. Chem. 15, 728-735.

Bryan, G.W., Gibbs, P.E., Hummerstone, L.G., Burt, G.R., 1986. The Decline of the Gastropod *Nucella Lapillus* around Southwest England - Evidence for the Effect of Tributyltin from Antifouling Paints. J. Mar. Biol. Assoc. U.K. 66, 611-640.

Bryan, G.W., Gibbs, P.E., Burt, G.R., Hummerstone, L.G., 1987. The Effects of Tributyltin (TBT) Accumulation on Adult Dog-Whelks, *Nucella Lapillus* - Long-Term Field and Laboratory Experiments. J. Mar. Biol. Assoc. U.K. 67, 525-544.

Burrell, M., 1993. The successful implementation of a solvent recovery program. In: US Department of Energy, US Air Forces (Eds.), Solvent Substitution for Pollution Prevention. Noyes Publications, New Jersey, pp. 194-197.

Callahan, M., Green, B., 1995. Hazardous Solvent Source Reduction. McGraw-Hill, New York, NY.

Calvo, G.W., Luckenbach, M.W., Allen Jr, S.K., Burreson, E.M., 2000. A comparative field study of *Crassostrea ariakensis* and *Crassostrea virginica* in relation to salinity in Virginia. Special Report in Applied Marine Science and Ocean Engineering No. 360. Virginia Institute of Marine Science, 1-25.

Carman, K.R., Fleeger, J.W., Pomarico, S.M., 1997. Response of a benthic food web to hydrocarbon contamination. Limnol. Oceanogr. 42, 561-571.

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., Smith, V.H., 1998. Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecol. Appl. 8, 559-568.

Chandler, C.J., Segel, I.H., 1978. Mechanism of Anti-Microbial Action of Pyrithione - Effects on Membrane-Transport, ATP Levels, and Protein-Synthesis. Antimicrob. Agents Chemother. 14, 60-68.

Chesworth, J.C., Donkin, M.E., Brown, M.T., 2004. The interactive effects of the antifouling herbicides Irgarol 1051 and Diuron on the seagrass *Zostera marina* (L.). Aquat. Toxicol. 66, 293-305.

Cheung, S., Tse, S., 1993. Effects of tidal height on desiccation tolerance, body condition and population structure of the green lipped mussel, *Perna viridis*, Proceedings of the 2nd International Conference on Marine Biology South China Sea, Guangzhou, China, pp. 52-58.

Christian, F.A., Tate, T.M., 1982. Effects of Fluometuron on Embryonic Development and Hatching of *Fasciola hepaticas miracidia*. Bull. Environ. Contam. Toxicol. 28, 353-357.

Clare, A.S., Rittschof, D., 1989. What's in a name? Nature 338, 627.

Clarkson, T.W., 1991. Inorganic and Organometal Pesticides. In: Hayes, W.J., Laws, E.R. (Eds.), Handbook of Pesticide Toxicology. Academic Press, New York, NY, pp. 497-583.

Coeurdassier, M., de Vaufleury, A., Crini, N., Scheifler, R., Badot, P.M., 2005. Assessment of whole effluent toxicity on aquatic snails: Bioaccumulation of Cr, Zn, and Fe, and individual effects in bioassays. Environ. Toxicol. Chem. 24, 198-204.

Costa, M., Zhitkovich, A., Toniolo, P., 1993. DNA-Protein Cross-Links in Welders - Molecular Implications. Cancer Res. 53, 460-463.

Costlow, J.D., Bookhout, C., 1956. Molting and shell growth in *Balanus amphitrite* niveus. Biol. Bull. 110, 107-116.

Costlow, J.D., Tipper, R.C., 1984. Marine Biodeterioration: An Interdisciplinary Study. Naval Institute Press, Annapolis, MD.

Cushman, J.R., Rausina, G.A., Cruzan, G., Gilbert, J., Williams, E., Harrass, M.C., Sousa, J.V., Putt, A.E., Garvey, N.A., St. Laurent, J.P., Hoberg, J.R., Machado, M.W., 1997. Ecotoxicity hazard assessment of styrene. Ecotox. Environ. Safe. 37, 173-180.

Dahllöf, I., Grunnet, K.S., Haller, R., Hjorth, M., Maraldo, K., Petersen, D., 2005. Analysis, fate and toxicity of zinc- and copper pyrithione in the environment, TemaNord, Nordic Council of Ministers, Copenhagen.

Dandapat, J., Chainy, G.B.N., Rao, K.J., 2003. Lipid peroxidation and antioxidant defense status during larval development and metamorphosis of giant prawn, *Macrobrachium rosenbergii*. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 135, 221-233.

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environ. Health Perspect. 107, 907-938.

Davis, H.C., Hidu, H., 1969. Effects of Pesticides on Embryonic Development of Clams and Oysters and on Survival and Growth of Larvae. 67, 393-&.

Depledge, M.H., Billinghurst, Z., 1999. Ecological significance of endocrine disruption in marine invertebrates. Mar. Pollut. Bull. 39, 32-38.

Digiulio, R.T., Behar, J.V., Carlson, D.B., Hasspieler, B.M., Watson, D.E., 1995. Determinants of Species Susceptibility to Oxidative Stress - a Comparison of Channel Catfish and Brown Bullhead. Mar. Environ. Res. 39, 175-179.

Dimitriou, P., Castritsi-Catharios, J., Miliou, H., 2003. Acute toxicity effects of tributyltin chloride and triphenyltin chloride on gilthead seabream, *Sparus aurata* L., embryos. Ecotox. Environ. Safe. 54, 30-35.

Dinning, A.J., Al-Adham, I.S.I., Eastwood, I.M., Austin, P., Collier, P.J., 1998. Pyrithione biocides as inhibitors of bacterial ATP synthesis. J. Appl. Microbiol. 85, 141-146.

Dissanayake, A., Galloway, T.S., 2004. Evaluation of fixed wavelength fluorescence and synchronous fluorescence spectrophotometry as a biomonitoring tool of environmental contamination. Mar. Environ. Res. 58, 281-285.

Downs, C.A., Dillon, R.T., Fauth, J.E., Woodley, C.M., 2001. A molecular biomarker system for assessing the health of gastropods (*Ilyanassa obsoleta*) exposed to natural and anthropogenic stressors. J. Exp. Mar. Biol. Ecol. 259, 189-214.

Edwards, N.T., 1983. Polycyclic Aromatic-Hydrocarbons (PAHs) in the Terrestrial Environment - a Review. J. Environ. Qual. 12, 427-441.

Elder, J.F., Dresler, P.V., 1988. Accumulation and Bioconcentration of Polycyclic Aromatic-Hydrocarbons in a Nearshore Estuarine Environment near a Pensacola (Florida) Creosote Contamination Site. Environ. Pollut. 49, 117-132.

Elkomi, M.M., Kajihara, T., 1991. Breeding and Molting of Barnacles under Rearing Conditions. Mar. Biol. 108, 83-89.

Encomio, V.G., Stickler, S.M., Allen, S.K., Chu, F.L., 2005. Performance of "natural dermo-resistant" oyster stocks - survival, disease, growth, condition and energy reserves. J. Shellfish Res. 24, 143-155.

Ericson, G., Lindesjoo, E., Balk, L., 1998. DNA adducts and histopathological lesions in perch (*Perca fluviatilis*) and northern pike (*Esox lucius*) along a polycyclic aromatic hydrocarbon gradient on the Swedish coastline of the Baltic Sea. Can. J. Fish. Aquat. Sci. 55, 815-824.

Ericson, G., Noaksson, E., Balk, L., 1999. DNA adduct formation and persistence in liver and extrahepatic tissues of northern pike (*Esox lucius*) following oral exposure to benzo[a]pyrene, benzo[k]fluoranthene and 7H-dibenzo[c,g]carbazole. Mutat. Res.-Fundam. Mol. Mech. Mutagen. 427, 135-145.

Ericson, G., Balk, L., 2000. DNA adduct formation in northern pike (*Esox lucius*) exposed to a mixture of benzo[a]pyrene, benzo[k]fluoranthene and 7H-dibenzo[c,g]carbazole: time-course and dose-response studies. Mutat. Res.-Fundam. Mol. Mech. Mutagen. 454, 11-20.

Ermolayeva, E., Sanders, D., 1995. Mechanism of Pyrithione-Induced Membrane Depolarization in *Neurospora crassa*. Appl. Environ. Microbiol. 61, 3385-3390.

Ettensohn, C.A., Wessel, G.M., Wray, G.A. (Eds.), 2004. Development of sea urchins, ascidians, and other invertebrate deuterostomes: Experimental approaches. Methods in Cell Biology, 74. Elsevier Academic Press, San Diego, CA.

Ewart, J., Ford, S., 1993. History and impact of MSX and dermo diseases on oyster stocks in the Northeast region. Fact sheet no. 200, Northeast Region Aquaculture Center.

Fent, K., 1996. Organotin compounds in municipal wastewater and sewage sludge: Contamination, fate in treatment process and ecotoxicological consequences. Sci. Total Environ. 185, 151-159.

FIFRA, 1972. Federal Insecticide, Fungicide, and Rodenticide Act, 7 U.S.C. s/s 136 et seq. (1972). Environmental Protection Agency.

Flory, J., Alber, M., 2005. Marinas: Best Management Practices & Water Quality: Prepared for GA DNR - Coastal Resources Division, Georgia Coastal Research Council.

Forbes, V.E., Depledge, M.H., 1992. Predicting Population Response to Pollutants - the Significance of Sex. Funct. Ecol. 6, 376-381.

Forbes, T.L., Forbes, V.E., Depledge, M.H., 1994. Individual Physiological-Responses to Environmental Hypoxia and Organic Enrichment - Implications for Early Soft-Bottom Community Succession. J. Mar. Res. 52, 1081-1100.

Forbes, V.E., Forbes, T.L., 1994. Ecotoxicology in Theory and Practice. Chapman & Hall, London.

Forbes, V.E., Moller, V., Depledge, M.H., 1995. Intrapopulation Variability in Sublethal Response to Heavy-Metal Stress in Sexual and Asexual Gastropod Populations. Funct. Ecol. 9, 477-484.

Forkert, P.G., Redza, Z.M., Mangos, S., Park, S.S., Tam, S.P., 1994. Induction and Regulation of Cyp2e1 in Murine Liver after Acute and Chronic Acetone Administration. Drug Metab. Dispos. 22, 248-253.

Garman, G.D., Anderson, S.L., Cherr, G.N., 1997. Developmental abnormalities and DNA-protein crosslinks in sea urchin embryos exposed to three metals. Aquat. Toxicol. 39, 247-265.

Geraci, F., Pinsino, A., Turturici, G., Savona, R., Giudice, G., Sconzo, G., 2004. Nickel, lead, and cadmium induce differential cellular responses in sea urchin embryos by activating the synthesis of different HSP70s. Biochem. Biophys. Res. Commun. 322, 873-877.

Gerritsen, J., Holland, A.F., Irvine, D.E., 1994. Suspension-Feeding Bivalves and the Fate of Primary Production - an Estuarine Model Applied to Chesapeake Bay. Estuaries 17, 403-416.

Giacomazzi, S., Cochet, N., 2004. Environmental impact of diuron transformation: a review. Chemosphere 56, 1021-1032.

Giannotti, A.L., McGlathery, K.J., 2001. Consumption of *Ulva lactuca* (Chlorophyta) by the omnivorous mud snail *Ilyanassa obsoleta* (Say). J. Phycol. 37, 209-215.

Gibbs, P.E., Bryan, G.W., Pascoe, P.L., Burt, G.R., 1987. The Use of the Dog Whelk, *Nucella lapillus*, as an Indicator of Tributyltin (TBT) Contamination. J. Mar. Biol. Assoc. U.K. 67, 507-523.

Gibbs, P.E., Spencer, B.E., Pascoe, P.L., 1991. The American Oyster Drill, *Urosalpinx cinerea* (Gastropoda) - Evidence of Decline in an Imposex-Affected Population (R-Blackwater, Essex). J. Mar. Biol. Assoc. U.K. 71, 827-838.

Gibbs, B.F., Mulligan, C.N., 1997. Styrene toxicity: An ecotoxicological assessment. Ecotox. Environ. Safe. 38, 181-194.

Goksoyr, A., Förlin, L., 1992. The Cytochrome-P-450 System in Fish, Aquatic Toxicology and Environmental Monitoring. Aquat. Toxicol. 22, 287-311.

Goldberg, R.N., Averbuj, A., Cledon, M., Luzzatto, D., Nudelman, N.S., 2004. Search for triorganotins along the Mar del Plata (Argentina) marine coast: finding of tributyltin in egg capsules of a snail *Adelomelon brasiliana* (Lamarck, 1822) population showing imposex effects. Appl. Organomet. Chem. 18, 117-123.

Gomez, C.E., Contento, L., Carsen, A.E., 2001. Toxicity tests to assess pollutants removal during wastewater treatment and the quality of receiving waters in Argentina. Environ. Toxicol. 16, 217-224.

Gonzalez, R.J., Tarloff, J.B., 2004. Expression and activities of several drug-metabolizing enzymes in LLC-PK1 cells. Toxicol. Vitro 18, 887-894.

Green, P.G., Young, T.M., 2006. Loading of the herbicide diuron into the California water system. Environ. Eng. Sci. 23, 545-551.

Grinwis, G.C.M., Vethaak, A.D., Wester, P.W., Vos, J.G., 2000. Toxicology of environmental chemicals in the flounder (*Platichthys flesus*) with emphasis on the immune system: field, semi-field (mesocosm) and laboratory studies. Toxicol. Lett. 112, 289-301.

Gross, J.M., McClay, D.R., 2001. The role of Brachyury (T) during gastrulation movements in the sea urchin *Lytechinus variegatus*. Dev. Biol. 239, 132-147.

Gross, J.M., Peterson, R.E., Wu, S.Y., McClay, D.R., 2003. LvTbx2/3: a T-box family transcription factor involved in formation of the oral/aboral axis of the sea urchin embryo. Development 130, 1989-1999.

Grundy, M.M., Ratcliffe, N.A., Moore, M.N., 1996. Immune inhibition in marine mussels by polycyclic aromatic hydrocarbons. Mar. Environ. Res. 42, 187-190.

Grunnet, K.S., Dahllöf, I., 2005. Environmental fate of the antifouling compound zinc pyrithione in seawater. Environ. Toxicol. Chem. 24, 3001-3006.

Hamers, T., Kalis, E.J.J., van den Berg, J.H.J., Maas, L.M., van Schooten, F.J., Murk, A.J., 2004. Applicability of the black slug *Arion ater* for monitoring exposure to polycyclic aromatic hydrocarbons and their subsequent bioactivation into DNA binding metabolites. Mutat. Res.-Fundam. Mol. Mech. Mutagen. 552, 219-233.

Hayes, W.J. (Ed.), 1978. Toxicology of Pesticides. Williams & Wilkins Company, Baltimore, MD.

Haynes, D., Ralph, P., Prange, J., Dennison, B., 2000. The impact of the herbicide diuron on photosynthesis in three species of tropical seagrass. Mar. Pollut. Bull. 41, 288-293.

Heiden, T.K., Hutz, R.J., Carvan, M.J., 2005. Accumulation, tissue distribution, and maternal transfer of dietary 2,3,7,8,-tetrachlorodibenzo-p-dioxin: Impacts on reproductive success of zebrafish. Toxicol. Sci. 87, 497-507.

Hellou, J., Yeats, P., Steller, S., Gagne, F., 2003. Chemical contaminants and biological indicators of mussel health during gametogenesis. Environ. Toxicol. Chem. 22, 2080-2087.

Heslinga, G.A., 1976. Effects of Copper on Coral Reef Echinoid *Echinometra mathaei*. Mar. Biol. 35, 155-160.

Hill, S.K., Lawrence, J.M., 2003. Habitats and characteristics of the sea urchins *Lytechinus variegatus* and *Arbacia punctulata* (Echinodermata) on the Florida gulf-coast shelf. Mar. Ecol.-Pubbl. Stn. Zool. Napoli 24, 15-30.

Holm, E.R., McClary, M., Rittschof, D., 2000. Variation in attachment of the barnacle *Balanus amphitrite*: sensation or something else? Mar. Ecol. Prog. Ser. 202, 153-162.

Holm, E.R., Orihuela, B., Kavanagh, C.J., Rittschof, D., 2005. Variation among families for characteristics of the adhesive plaque in the barnacle *Balanus amphitrite*. Biofouling 21, 121-126.

Hylland, K., 2006. Polycyclic aromatic hydrocarbon (PAH) ecotoxicology in marine ecosystems. J. Toxicol. Env. Health Part A 69, 109-123.

Hynes, D.E., DeNicola, D.B., Carlson, G.P., 1999. Metabolism of styrene by mouse and rat isolated lung cells. Toxicol. Sci. 51, 195-201.

IMO, 2004. Marine Environment: Antifouling Strategies, International Maritime Organization.

Irwin, R.J., Van Mouwerik, M., Stevens, L., Seese, M.D., Basham, W., 1997. Environmental Contaminants Encyclopedia: Benzo [a] Pyrene Entry, National Park Service, Water Resources Division, Fort Collins, Colorado. Accessed electronically at http://www.nature.nps.gov/hazardssafety/toxic//benzoapy.pdf on 26 April 2007.

James, M.O., 1989. Biotransformation disposition of PAH in Aquatic Invertebrates. In: Varanasi, U. (Ed.), Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment. CRC Press, Boca Raton, FL, pp. 69-91.

Jackson, R.B., Carpenter, S.R., Dahm, C.N., McKnight, D.M., Naiman, R.J., Postel, S.L., Running, S.W., 2001. Water in a changing world. Ecol. Appl. 11, 1027-1045.

Jenner, M.G., 1979. Pseudohermaphroditism in *Ilyanassa obsoleta* (Mollusca, Neogastropoda). Science 205, 1407-1409.

Johnson, L.L., Collier, T.K., Stein, J.E., 2002. An analysis in support of sediment quality thresholds for polycyclic aromatic hydrocarbons (PAHs) to protect estuarine fish. Aquat. Conserv.-Mar. Freshw. Ecosyst. 12, 517-538.

Johnson, L.T., Miller, J.A., 2003. Making dollars and sense of nontoxic antifouling strategies for boats. Report no. T-052, California Sea Grant College Program.

Johnson, L.T., Gonzalez, J.A., 2004. Staying afloat with nontoxic antifouling strategies for boats. Report no. T-054, California Sea Grant College Program.

Jones, R.J., Kerswell, A.P., 2003. Phytotoxicity of Photosystem II (PSII) herbicides to coral. Mar. Ecol. Prog. Ser. 261, 149-159.

Kaminsky, L.S., Fasco, M.J., 1992. Small Intestinal Cytochromes P450. Crit. Rev. Toxicol. 21, 407-422.

Kennedy, V.S., 1996. The ecological role of the eastern oyster, *Crassostrea virginica*, with remarks on disease. J. Shellfish Res. 15, 177-183.

Kime, D.E., 1999. A strategy for assessing the effects of xenobiotics on fish reproduction. Sci. Total Environ. 225, 3-11.

Kirso, U., Irha, N., Paalme, L., Reznikov, S., Matveyev, A., 2002. Levels and origin of PAHs in some big lakes. Polycycl. Aromat. Compd. 22, 715-728.

Klaassen, C.D. (Ed.), 2001. Casarett and Doull's Toxicology: The basic science of poisons. McGraw-Hill, New York, NY.

Knapek, R., Lakota, S., 1974. Biological testing to determine toxic effects of pesticides in water. Taqungsber Akad Landwirtschaftswiss DDR 126, 105-109.

Kobayashi, N., 1980. Comparative Sensitivity of Various Developmental Stages of Sea Urchins to Some Chemicals. Mar. Biol. 58, 163-171.

Kobayashi, N., Okamura, H., 2002. Effects of new antifouling compounds on the development of sea urchin. Mar. Pollut. Bull. 44, 748-751.

Kobayashi, N., Okamura, H., 2004. Effects of heavy metals on sea urchin embryo development. 1. Tracing the cause by the effects. Chemosphere 55, 1403-1412.

Koop, D.R., 1992. Oxidative and Reductive Metabolism by Cytochrome-P450-2E1. Faseb J. 6, 724-730.

Kummerer, K., 2001. Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - a review. Chemosphere 45, 957-969.

Lam, P.K.S., 1996. Interpopulation differences in acute response of *Brotia hainanensis* (Gastropoda, Prosobranchia) to cadmium: Genetic or environmental variance? Environ. Pollut. 94, 1-7.

Landis, W.G., Yu, M.-H., 1999. Introduction to Environmental Toxicology: Impacts of Chemicals upon Ecological Systems. Lewis Publishers, New York, NY.

Lannig, G., Flores, J.F., Sokolova, I.M., 2006. Temperature-dependent stress response in oysters, *Crassostrea virginica*: Pollution reduces temperature tolerance in oysters. Aquat. Toxicol. 79, 278-287.

Lauenstein, G.G., O'Connor, R., 1988. Measuring the health of US coastal waters: the first year results of NOAAs mussel watch program highlighted the worth of bivalve molluscs to monitor environmental contaminants along three US coasts Sea Technology, pp. 29-32.

Lauenstein, G.G., Daskalakis, K.D., 1998. US long-term coastal contaminant temporal trends determined from mollusk monitoring programs, 1965-1993. Mar. Pollut. Bull. 37, 6-13.

Lawrence, D.R., Scott, G.I., 1982. The Determination and Use of Condition Index of Oysters. Estuaries 5, 23-27.

Lawrence, A.J., Poulter, C., 1998. Development of a sub-lethal pollution bioassay using the estuarine amphipod *Gammarus duebeni*. Water Res. 32, 569-578.

Liao, C.M., Jou, L.J., Chen, B.C., 2005. Risk-based approach to appraise valve closure in the clam *Corbicula fluminea* in response to waterborne metals. Environ. Pollut. 135, 41-52.

Lieber, C.S., Decarli, L.M., 1972. Role of Hepatic Microsomal Ethanol Oxidizing System (Meos) for Ethanol Metabolism in-Vivo. J. Pharmacol. Exp. Ther. 181, 279-286.

Lieber, C.S., 1999. Microsomal ethanol-oxidizing system (MEOS): The first 30 years (1968-1998) - A review. Alcoholism 23, 991-1007.

Linhart, I., Gut, I., Smejkal, J., Novak, J., 2000. Biotransformation of styrene in mice: Stereochemical aspects. Chem. Res. Toxicol. 13, 36-44.

Little, C., 2000. The biology of soft shores and estuaries. Oxford Press, New York, NY.

Livingstone, D.R., Farrar, S.V., 1985. Responses of the Mixed-Function Oxidase System of Some Bivalve and Gastropod Mollusks to Exposure to Polynuclear Aromatic and Other Hydrocarbons. Mar. Environ. Res. 17, 101-105.

Livingstone, D.R., 1988. Responses of Microsomal Nadph-Cytochrome-C Reductase-Activity and Cytochrome-P-450 in Digestive Glands of *Mytilus edulis* and *Littorina littorea* to Environmental and Experimental Exposure to Pollutants. Mar. Ecol.-Prog. Ser. 46, 37-43.

Livingstone, D.R., Kirchin, M.A., Wiseman, A., 1989. Cytochrome-P-450 and Oxidative-Metabolism in Mollusks. Xenobiotica 19, 1041-1062.

Livingstone, D.R., Martinez, P.G., Michel, X., Narbonne, J.F., Ohara, S., Ribera, D., Winston, G.W., 1990. Oxyradical Production as a Pollution-Mediated Mechanism of Toxicity in the Common Mussel, *Mytilus Edulis*-L, and Other Mollusks. Funct. Ecol. 4, 415-424.

Livingstone, D., 1991. Organic xenobiotic metabolism in marine invertebrates. Adv. Comp. Environ. Physiol. 7, 46-185.

Livingstone, D.R., Nasci, C., Sole, M., DaRos, L., Ohara, S.C.M., Peters, L.D., Fossato, V., Wootton, A.N., Goldfarb, P.S., 1997. Apparent induction of a cytochrome P450 with immunochemical similarities to CYP1A in digestive gland of the common mussel (*Mytilus galloprovincialis* L) with exposure to 2,2',3,4,4',5'-hexachlorobiphenyl and Arochlor 1254. Aquat. Toxicol. 38, 205-224.

Lytle, T.F., Lytle, J.S., 1990. Heavy-Metals in the Eastern Oyster, *Crassostrea virginica*, of the Mississippi Sound. Bull. Environ. Contam. Toxicol. 44, 142-148.

Mackie, D.S., van den Berg, C.M.G., Readman, J.W., 2004. Determination of pyrithione in natural waters by cathodic stripping voltammetry. Anal. Chim. Acta 511, 47-53.

Madany, I.M., Alhaddad, A., Alshirbini, E.S., 1994. Spatial and Temporal Distributions of Aromatic Petroleum-Hydrocarbons in the Coastal Waters of Bahrain. Arch. Environ. Contam. Toxicol. 26, 185-190.

Maraldo, K., Dahllöf, I., 2004. Seasonal variations in the effect of zinc pyrithione and copper pyrithione on pelagic phytoplankton communities. Aquat. Toxicol. 69, 189-198.

McCarthy, D.A., Young, C.M., 2002. Gametogenesis and reproductive behavior in the echinoid *Lytechinus variegatus*. Mar. Ecol.-Prog. Ser. 233, 157-168.

McClellan-Green, P., 2005. Endocrine disruption in gastropods differentially affects sexual morphology, reproduction and behavior, Society of Environmental Toxicology and Chemistry North America 26th Annual Meeting, Baltimore, MD.

McClellan-Green, P., Romano, J., Rittschof, D., 2006. Imposex induction in the mud snail, *Ilyanassa obsoleta* by three tin compounds. Bull. Environ. Contam. Toxicol. 76, 581-588.

McElroy, A.E., Farrington, J.W., Teal, J.M., 1989. Bioavailability of PAH in the aquatic environment. In: Varanasi, U. (Ed.), Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment. CRC Press, Boca Raton, FL, pp. 1-40.

McElroy, A., Leitch, K., Fay, A., 2000. A survey of in vivo benzo[alpha]pyrene metabolism in small benthic marine invertebrates. Mar. Environ. Res. 50, 33-38.

McGinn, A.P., 2002. Reducing our Toxic Burden. In: Starke, L. (Ed.), State of the World 2002: A Worldwatch Institute Report on the Progress Towards a Sustainable Society. W.W. Norton and Company, New York, NY, pp.

McKenney, C.L., Weber, D.E., Celestial, D.M., MacGregor, M.A., 1998. Altered growth and metabolism of an estuarine shrimp (*Palaemoaetes pugio*) during and after metamorphosis onto fenvalerate-laden sediment. Arch. Environ. Contam. Toxicol. 35, 464-471.

Medesani, D.A., Susana, L., Greco, L., Rodriguez, E.M., 2004. Interference of cadmium and copper with the endocrine control of ovarian growth, in the estuarine crab *Chasmagnathus granulata*. Aquat. Toxicol. 69, 165-174.

Meyer, P.A., Quinn, J.G., 1973. Association of Hydrocarbons and Mineral Particles in Saline Solution. Nature 244, 23-24.

Miles, C.J., Delfino, J.J., 1999. Priority pollutant polycyclic aromatic hydrocarbons in Florida sediments. Bull. Environ. Contam. Toxicol. 63, 226-234.

Miller, R.R., Newhook, R., Poole, A., 1994. Styrene Production, Use, and Human Exposure. Crit. Rev. Toxicol. 24, S1-S10.

Mochida, K., Ito, K., Harino, H., Kakuno, A., Fujii, K., 2006. Acute toxicity of pyrithione antifouling biocides and joint toxicity with copper to red sea bream (*Pagrus major*) and toy shrimp (*Heptacarpus futilirostris*). Environ. Toxicol. Chem. 25, 3058-3064.

Moncada, A., 2004. Environmental fate of diuron, California Department of Pesticide Regulation, Sacremento.

Moraga, D., Meistertzheim, A.L., Tanguy-Royer, S., Boutet, I., Tanguy, A., Donval, A., 2005. Stress response in Cu^{2+} and Cd^{2+} exposed oysters (*Crassostrea gigas*): An immunohistochemical approach. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 141, 151-156.

Morcillo, Y., Ronis, M.J.J., Porte, C., 1998. Effects of tributyltin on the Phase I testosterone metabolism and steroid titres of the clam *Ruditapes decussata*. Aquat. Toxicol. 42, 1-13.

Nakata, H., Sakakibara, A., Kanoh, M., Kudo, S., Watanabe, H., Nagai, N., Miyazaki, N., Asano, Y., Tanabe, S., 2002. Evaluation of mitogen-induced responses in marine mammal and human lymphocytes by in vitro exposure of butyltins and non-ortho coplanar PCBs. Environ. Pollut. 120, 245-253.

NC Sea Grant, 2004. Oyster Science on the Edge: Reef Design and Disease, Coastwatch.

NCDMF, 2005. Crabs, Blue, Hard. North Carolina Division of Marine Fisheries, Commercial Statistics. http://www.ncfisheries.net/statistics/comstat/crabhard.htm.

NCDMF, 2005. Oysters. North Carolina Division of Marine Fisheries, Commercial Statistics. http://www.ncfisheries.net/statistics/comstat/oysters.htm.

Nebeker, A.V., Schuytema, G.S., 1998. Chronic effects of the herbicide diuron on freshwater cladocerans, amphipods, midges, minnows, worms, and snails. Arch. Environ. Contam. Toxicol. 35, 441-446.

Nebert, D.W., Nelson, D.R., Feyereisen, R., 1989. Evolution of the Cytochrome-P450 Genes. Xenobiotica 19, 1149-1160.

Notten, M.J.M., Oosthoek, A.J.P., Rozema, J., Aerts, R., 2005. Heavy metal concentrations in a soil-plant-snail food chain along a terrestrial soil pollution gradient. Environ. Pollut. 138, 178-190.

NRAAVC, 2001. Evaluation of the new active zinc pyrithione in the product International Intersmooth 360 Ecoflex Antifouling, National Registration Authority for Agricultural and Veterinary Chemicals, Canberra, Australia.

Nriagu, J.O., Pacyna, J.M., 1988. Quantitative Assessment of Worldwide Contamination of Air, Water and Soils by Trace-Metals. Nature 333, 134-139.

NY Sea Grant, 2001. Nonpoint Source Pollution: New York's Primary Water Quality Program, New York Sea Grant, NEMO Program, Stony Brook, NY.

Oberdörster, E., Rittschof, D., McClellan-Green, P., 1998. Testosterone metabolism in imposex and normal *Ilyanassa obsoleta*: Comparison of field and TBTA Cl-induced imposex. Mar. Pollut. Bull. 36, 144-151.

Oberdörster, E., Martin, M., Ide, C.F., McLachlan, J.A., 1999. Benthic community structure and biomarker induction in grass shrimp in an estuarine system. Arch. Environ. Contam. Toxicol. 37, 512-518.

Oberdörster, E., McClellan-Green, P., 2000. The neuropeptide APGWamide induces imposex in the mud snail, *Ilyanassa obsoleta*. Peptides 21, 1323-1330.

Oberdörster, E., Cheek, A.O., 2001. Gender benders at the beach: Endocrine disruption in marine and estuarine organisms. Environ. Toxicol. Chem. 20, 23-36.

Oberdörster, E., Romano, J., McClellan-Green, P., 2005. The neuropeptide APGWamide as a penis morphogenic factor (PMF) in gastropod mollusks. Integr. Comp. Biol. 45, 28-32.

Oehlmann, J., Fioroni, P., Stroben, E., Markert, B., 1996. Tributyltin (TBT) effects on *Ocinebrina aciculata* (Gastropoda: Muricidae): Imposex development, sterilization, sex change and population decline. Sci. Total Environ. 188, 205-223.

Oehlmann, J., Schulte-Oehlmann, U., Tillmann, M., Markert, B., 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca : Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. Ecotoxicology 9, 383-397.

Oettmeier, W., 1992. Herbicides of photosystem II. In: Barber, J. (Ed.), The Photosystems: Structure, Function, and Molecular Biology, Vol II. Elsevier Press, Amsterdam, pp. 349-408.

Ohtani, H., Ichikawa, Y., Iwamoto, E., Miura, I., 2001. Effects of styrene monomer and trimer on gonadal sex differentiation of genetic males of the frog *Rana rugosa*. Environ. Res. 87, 175-180.

Okamura, H., Watanabe, T., Aoyama, I., Hasobe, M., 2002. Toxicity evaluation of new antifouling compounds using suspension-cultured fish cells. Chemosphere 46, 945-951.

Okamura, H., Aoyama, I., Ono, Y., Nishida, T., 2003. Antifouling herbicides in the coastal waters of western Japan. Mar. Pollut. Bull. 47, 59-67.

Oyama, Y., Arata, T., Chikahisa, L., Umebayashi, C., Tabaru, K., Sakai, K., Hayashi, H., 2003. Effects of A23187 and CaCl2 on tri-n-butyltin-induced cell death in rat thymocytes. Environ. Toxicol. Pharmacol. 13, 29-36.

Padros, J., Pelletier, E., 2000. In vivo formation of (+)-anti-benzo[a]pyrene diol-epoxide-plasma albumin adducts in fish. Mar. Environ. Res. 50, 347-351.

Paez-Osuna, F., Ruiz-Fernandez, A.C., Botello, A.V., Ponce-Velez, G., Osuna-Lopez, J.I., Frias-Espericueta, M.G., Lopez-Lopez, G., Zazueta-Padilla, H.M., 2002. Concentrations of selected trace metals (Cu, Pb, Zn), organochlorines (PCBs, HCB) and total PAHs in mangrove oysters from the Pacific Coast of Mexico: an overview. Mar. Pollut. Bull. 44, 1303-1308.

PAN Pesticide Database, Diuron Factsheet. www.pan.uk.org/pestnews/actives/diuron.htm.

Pelish, J., Slusher, D., Johnson, L., 2003. Spectrum Chemical Factsheet, chemical 100425. Spectrum Laboratories, Inc. www.speclabs.com/compounds/c100425.htm.

Piola, R.F., Johnston, E.L., 2006. Differential tolerance to metals among populations of the introduced bryozoan *Bugula neritina*. Mar. Biol. 148, 997-1010.

Pitombo, F.B., 2004. Phylogenetic analysis of the Balanidae (Cirripedia, Balanomorpha). Zool. Scripta 33, 261-276.

Preuss, M., Hall, E.A., 1995. Mediated herbicide inhibition in a pet biosensor. Anal. Chem. 67, 1940-1949.

Propst, T.L., Fort, D.J., Stover, E.L., Schrock, B., Bantle, J.A., 1997. Evaluation of the developmental toxicity of benzo[alpha]pyrene and 2-acetylaminofluorene using Xenopus: Modes of biotransformation. Drug Chem. Toxicol. 20, 45-61.

Qiu, J.W., Thiyagarajan, V., Cheung, S., Qian, P.Y., 2005. Toxic effects of copper on larval development of the barnacle *Balanus amphitrite*. Mar. Pollut. Bull. 51, 688-693.

Ramachandran, S., Patel, T.R., Colbo, M.H., 1997. Effect of copper and cadmium on three Malaysian tropical estuarine invertebrate larvae. Ecotox. Environ. Safe. 36, 183-188.

Reichert, W.L., Myers, M.S., Peck-Miller, K., French, B., Anulacion, B.F., Collier, T.K., Stein, J.E., Varanasi, U., 1998. Molecular epizootiology of genotoxic events in marine fish: Linking contaminant exposure, DNA damage, and tissue-level alterations. Mutat. Res.-Rev. Mutat. Res. 411, 215-225.

Rheault, R.B., Rice, M.A., 1996. Food-limited growth and condition index in the eastern oyster, *Crassostrea virginica* (Gmelin 1791), and the bay scallop, *Argopecten irradians irradians* (Lamarck 1819). J. Shellfish Res. 15, 271-283.

Rittschof, D., Clare, A.S., Gerhart, D.J., Avelin, M., Bonaventura, J., 1992. Barnacle in vitro assays for biologically active substances: toxicity and settlement inhibition assays using mass cultured *Balanus amphitrite* Darwin. 6, 115-122.

Rittschof, D., Sawardecker, P., Petry, C., 2002. Chemical mediation of egg capsule deposition by mud snails. J. Chem. Ecol. 28, 2257-2269.

Rittschof, D., Lai, C.H., Kok, L.M., Teo, S.L.M., 2003. Pharmaceuticals as antifoulants: Concept and principles. Biofouling 19, 207-212.

Romano, J., McClellan-Green, P., Rittschof, D., 2004. Copper pyrithione and diuron toxicity in the non-target species, *Lytechinus variegatus*. Integr. Comp. Biol. 44, 742-742.

Ronis, M.J.J., Huang, J., Longo, V., Tindberg, N., IngelmanSundberg, M., Badger, T.M., 1998. Expression and distribution of cytochrome P450 enzymes in male rat kidney: Effects of ethanol, acetone and dietary conditions. Biochem. Pharmacol. 55, 123-129.

Rosas, I., Baez, A., Belmont, R., 1983. Oyster (Crassostrea virginica) as Indicator of Heavy-Metal Pollution in Some Lagoons of the Gulf of Mexico. Water Air Soil Pollut. 20, 127-135.

Rosen, G., Rivera-Duarte, I., Kear-Padilla, L., Chadwick, D., 2004. Use of laboratory toxicity tests with bivalve and echinoderm embryos to evaluate the bioavailability of copper in San Diego Bay, California, USA. Environ. Toxicol. Chem. 24, 415-422.

Ruppert, E., Barnes, R., 1996. Invertebrate Zoology. Harcourt College Publications, Philadelphia, PA.

Saltiene, Z., Brukstiene, D., Ruzgyte, A., 2002. Contamination of soil by polycyclic aromatic hydrocarbons in some urban areas. Polycycl. Aromat. Compd. 22, 23-35.

Sanders, H.O., Cope, O.B., 1968. Relative Toxicities of Several Pesticides to Naiads of 3 Species of Stoneflies. Limnol. Oceanogr. 13, 112-&.

Sarkar, S.N., Majumdar, A.C., Chattopadhyay, S.K., 1997. Effects of isoproturon on male reproductive systems: clinical, histological and histoenzyonological studies in rats. Indian J. Exp. Biol. 35, 133-138.

Sarkar, A., Ray, D., Shrivastava, A.N., Sarker, S., 2006. Molecular biomarkers: Their significance and application in marine pollution monitoring. Ecotoxicology 15, 333-340.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., von Gunten, U., Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. Science 313, 1072-1077.

Schwarzenback, R., Geschwend, P.M., Imboden, D., 1992. Environmental Organic Chemistry. Wiley Interscience Publications, New York, NY.

Scott, G.I., Lawrence, D.R., 1982. The American Oyster as a Coastal Zone Pollution Monitor - a Pilot-Study. Estuaries 5, 40-46.

Servia, M.J., Pery, A.R.R., Heydorff, M., Garric, J., Lagadic, L., 2006. Effects of copper on energy metabolism and larval development in the midge *Chironomus riparius*. Ecotoxicology 15, 229-240.

Shcherban, E.P., 1972. The effects of low concentrations of pesticides on the development of some cladocera and the abundance of their progeny. 6, 85-95.

Smith, G.F., Bruce, D.G., Roach, E.B., Hansen, A., Newell, R.T.E., McManus, A.M., 2005. Assessment of recent habitat conditions of eastern oyster *Crassostrea virginica* bars in mesohaline Chesapeake Bay. North Am. J. Fish Manage. 25, 1569-1590.

Sohda, T., Shimizu, M., Kamimura, S., Okumura, M., 1993. Immunohistochemical Demonstration of Ethanol-Inducible P450 2e1 in Rat Brain. Alcohol Alcohol. 28, 69-75.

Soniat, T.M., Finelli, C.M., Ruiz, J.T., 2004. Vertical structure and predator refuge mediate oyster reef development and community dynamics. J. Exp. Mar. Biol. Ecol. 310, 163-182.

Stegeman, J.J., Lech, J.J., 1991. Cytochrome-P-450 Monooxygenase Systems in Aquatic Species - Carcinogen Metabolism and Biomarkers for Carcinogen and Pollutant Exposure. Environ. Health Perspect. 90, 101-109.

Stegeman, J., Brouwer, M., Di Guilio, R.T., Forlin, L., Fowler, B.A., Sanders, B.M., Van Veld, P.A., 1992. Molecular responses to environmental contamination: Enzyme and protein synthesis as indicators of chemical exposure and effect. In: Huggett, R.J., Kimerle, R.A., Mehrle, P.M., Bergman, H.L. (Eds.), Biomarkers, Biochemical, Physiological and Histological Markers of Anthropogenic Stress. Lewis, Boca Raton, FL, pp. 235-336.

Stegeman, J., Hahn, M., 1994. Biochemistry and molecular biology of monooxygenases: Current directions in forms, functions, and regulation of cytochrome P450 in aquatic species. In: Malins, D.C., Ostrander, G.K. (Eds.), Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives. Lewis, Boca Raton, FL, pp. 87-206.

Stewart, C., Demora, S.J., Jones, M.R.L., Miller, M.C., 1992. Imposex in New Zealand Neogastropods. Mar. Pollut. Bull. 24, 204-209.

Stewart, C., Thompson, J.A.J., 1997. Vertical distribution of butyltin residues in sediments of British Columbia harbours. Environ. Technol. 18, 1195-1202.

Straw, J., Rittschof, D., 2004. Responses of mud snails from low and high imposex sites to sex pheromones. Mar. Pollut. Bull. 48, 1048-1054.

Sullivan, C.H., Maugel, T.K., 1984. Formation, Organization, and Composition of the Egg Capsule of the Marine Gastropod, *Ilyanassa obsoleta*. Biol. Bull. 167, 378-389.

Sumpter, J.P., 1998. Xenoendocrine disrupters - environmental impacts. Toxicol. Lett. 103, 337-342.

Tatarazako, N., Takao, Y., Kishi, K., Onikura, N., Arizono, K., Iguchi, T., 2002. Styrene dimers and trimers affect reproduction of daphnid (*Ceriodaphnia dubia*). Chemosphere 48, 597-601.

Ternes, T., 1999. Preface - Drugs and hormones as pollutants of the aquatic environment: determination and ecotoxicological impacts. Sci. Total Environ. 225, 1-2.

Thomas, K.V., Blake, S.J., Waldock, M.J., 2000. Antifouling paint booster biocide contamination in UK marine sediments. Mar. Pollut. Bull. 40, 739-745.

Thomas, K.V., 2001. The environmental fate and behaviour of antifouling paint booster biocides: a review. Biofouling 17, 73-86.

Trucco, R.G., Engelhardt, F.R., Stacey, B., 1983. Toxicity, Accumulation and Clearance of Aromatic-Hydrocarbons in Daphnia pulex. 31, 191-202.

Turley, P.A., Fenn, R.J., Ritter, J.C., 2000. Pyrithiones as antifoulants: Environmental chemistry and preliminary risk assessment. Biofouling 15, 175-182.

Turley, P.A., Fenn, R.J., Ritter, J.C., Callow, M.E., 2005. Pyrithiones as antifoulants: Environmental fate and loss of toxicity. Biofouling 21, 31-40.

US ATSDR, 1995. Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAHs), US Agency for Toxic Substances and Disease Registry, Atlanta, GA.

USEPA, 1980. Investigation of selected potential environmental contaminant: Styrene, ethylbenzene and related compounds. EPA/560/11-80-018, US Environmental Protection Agency, Washington, DC.

USEPA, 1989. Health effects assessment of Styrene. EPA/600/8-88-054, Office of Research and Development, US Environmental Protection Agency, Cincinnati, OH.

USEPA, 1994. EPA's Polluted Brochure, EPA841-F-94-005, US Environmental Protection Agency.

USEPA, 1996. Ultrasonic Extraction. Method 3550B, US Environmental Protection Agency.

USEPA, 2000. Office of Pesticide Programs, US Environmental Protection Agency, Washington, D.C. Accessed through USEPA Ecotox database 9 February 2007.

USEPA, 2002. Polycyclic Organic Matter, US Environmental Protection Agency, Washington, DC.

VanDolah, R., Bobo, M., Levisen, P., Wendt, P., Manzi, J., 1992. Effects of marina proximity on the physiological condition, reproduction, and settlement of oyster populations. 11, 41-48.

Verslycke, T., Vercauteren, J., Devos, C., Moens, L., Sandra, P., Janssen, C.R., 2003. Cellular energy allocation in the estuarine mysid shrimp *Neomysis integer* (Crustacea : Mysidacea) following tributyltin exposure. J. Exp. Mar. Biol. Ecol. 288, 167-179.

Virgilio, M., Maci, S., Abbiati, M., 2005. Comparisons of genotype-tolerance responses in populations of *Hediste diversicolor* (Polychaeta : Nereididae) exposed to copper stress. Mar. Biol. 147, 1305-1312.

Voulvoulis, N., Scrimshaw, M.D., Lester, J.N., 1999. Alternative Antifouling Biocides. Appl. Organomet. Chem. 13, 135-143.

Voulvoulis, N., Scrimshaw, M.D., Lester, J.N., 2002. Comparative environmental assessment of biocides used in antifouling paints. Chemosphere 47, 789-795.

Wallace, R.K., 2001. Cultivating the Eastern Oyster, *Crassostrea virginica*. SRAC Publication No. 432, Southern Region Aquaculture Center.

Wang, C.G., Zhao, Y., Zheng, R.H., Ding, X., Wei, W., Zuo, Z.H., Chen, Y.X., 2006. Effects of tributyltin, benzo[a]pyrene, and their mixture on antioxidant defense systems in *Sebastiscus marmoratus*. Ecotox. Environ. Safe. 65, 381-387.

Ware, G.W., 1978. The Pesticide Book. W.H. Freeman & Co., San Francisco.

Watts, S.A., McClintock, J.B., Lawrence, J.M., 2001. The ecology of *Lytechinus variegatus*. In: Lawrence, J.M. (Ed.), The biology of edible sea urchins. Elsevier Press, Amsterdam, pp. 375-394.

Wcislo, E., 1998. Soil Contamination with Polycyclic Aromatic Hydrocarbons (PAHs) in Poland - a Review. Pol. J. Environ. Stud. 7, 267-272.

Wessel, G.M., Berg, L., Adelson, D.L., Cannon, G., McClay, D.R., 1998. A molecular analysis of hyalin: A substrate for cell adhesion in the hyaline layer of the sea urchin embryo. Dev. Biol. 193, 115-126.

Willingham, G.L., Jacobson, A., 1997. Designing an environmentally safe marine antifoulant. In: DeVito, S.C., Garrett, R.C. (Eds.), Designing Safer Chemicals: Green Chemistry for Pollution Prevention. A.C.S. Symposium Series 640. American Chemical Society, Washington, DC, pp. 224-233.

Wong, J.L., Wessel, G.M., 2005. Reactive oxygen species and Udx1 during early sea urchin development. Dev. Biol. 288, 317-333.

Woodruff, T.J., Axelrad, D.A., Caldwell, J., Morello-Frosch, R., Rosenbaum, A., 1998. Public health implications of 1990 air toxics concentrations across the United States. Environ. Health Perspect. 106, 245-251.

Woods, H., Hargis, W.J., Hershner, C.H., Mason, P., 2005. Disappearance of the natural emergent 3-dimensional oyster reef system of the James River, Virginia, 1871-1948. J. Shellfish Res. 24, 139-142.

Wootton, E.C., Dyrynda, E.A., Pipe, R.K., Ratcliffe, N.A., 2003. Comparisons of PAH-induced immunomodulation in three bivalve molluscs. Aquat. Toxicol. 65, 13-25.

Wronska-Nofer, T., Wisniewska-Knypl, J., Wyszynska, K., Dziubaltowska, E., 1997. Genotoxicity of industrial dyes under the inductive effect of ethanol on monooxygenase system in mice. Mutat. Res.-Fundam. Mol. Mech. Mutagen. 392, 229-235.

Xue, W.L., Warshawsky, D., 2005. Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. Toxicol. Appl. Pharmacol. 206, 73-93.

Yamada, H., Kakuno, A., 2002. Present status on the development of alternative tributyltin-free antifouling paints and toxicity of new biocides to aquatic organisms. 6.

Yamazaki, H., Inoue, K., Mimura, M., Oda, Y., Guengerich, F.P., Shimada, T., 1996. 7-Ethoxycoumarin O-Deethylation catalyzed by cytochrome P450 1A2 and 2E1 in human liver microsomes. Biochem. Pharmicol. 51, 313-319.

Yaroslavtseva, L.M., Sergeeva, E.P., 2002. Sensitivity of sea urchin *Strongylocentrotus intermedius* embryos and larvae to copper ions. 28, 431-435.

Zapata, V., Greco, L.L., Rodriguez, E.M., 2001. Effect of copper on hatching and development of larvae of the estuarine crab *Chasmagnathus granulata* (Decapoda, Brachyura). Environ. Toxicol. Chem. 20, 1579-1583.

Zullo, V., 1979. Marine flora and fauna of northeastern US: Arthropoda, Cirripedia. Technical Report Circ-425, National Oceanic and Atmospheric Administration, National Marine Fisheries Service.

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Oberdörster, E., **Romano, J.A.**, and McClellan-Green, P.D. 2005. The neuropeptide APGWaimde as a Penis Morphogenic Factor (PMF) in gastropod mollusks. *Integrative and Comparative Biology* 45: 28-32.

Aldridge, J.E., Flaherty, M.M., Gibbons, J.A., Kreider, M.L., **Romano, J.A**., and Levin, E.D. 2003. Heterogeneity of Toxicant Response: Sources of Human Variability. *Toxicological Science*. 76: 3-20.

McClellan-Green, P.D., **Romano, J.A**. and Oberdörster, E. (in review). Does gender really matter in contaminant exposure? A case for invertebrate risk assessment. *Environmental Research.*

Presentations:

Romano, J. A., Rittschof, D., Holm, E., and McClellan-Green, P. *Variation in toxicity to copper pyrithione among populations and families of the barnacle,* Balanus amphitrite. Society for Integrative and Comparative Biology, Phoenix, AZ. January, 2007

Osterberg, J.S., **Romano, J.A.** and McClellan-Green, P. *Antioxidant defenses and stress biomarkers in molluscs from Lau and North Fiji Basin hydrothermal vents*. Society for Comparative and Integrative Biology Annual Meeting, Phoenix, AZ. January, 2007

Romano, J.A., Osterberg, J.S. and McClellan-Green, P. *Lipid peroxidation and catalase activity in gill and foot tissue of Lau and North Fiji Basin hydrothermal vent molluscs.* 11th International Deep-Sea Biology Symposium, Southampton, UK. July, 2006

Osterberg, J.S., **Romano, J.A.** and McClellan-Green, P. *Glutathione and superoxide dismutase activity in* Alviniconcha hessleri *and* Bathymodiolus brevior *from Lau and North Fiji Basin hydrothermal vents*. 11th International Deep-Sea Biology Symposium, Southampton, UK. July, 2006

Osterberg, J.S., **Romano, J.A.** and McClellan-Green, P. *Glutathione and Lipid Peroxidation Levels in* Bathymodiolus brevior *and* Alviniconcha hessleri *from the Lau and North Fiji Basins*. Third International Symposium on Hydrothermal Vent and Seep Biology, San Diego, California. October, 2005

Romano, J.A., McClellan-Green, P., and Rittschof, D. *Copper pyrithione and diuron toxicity in the non-target species,* Lytechinus variegatus. Society for Integrative and Comparative Biology, San Diego, California. January, 2005.

Romano, J.A. and McClellan-Green, P. *Tissue specific localization of APGWamide in TBT exposed mud snails,* Ilyanassa obsoleta. 25th Annual Meeting Society of Environmental Toxicology and Chemistry, Portland, Oregon. November, 2004.

Romano, J. A., Rittschof, D., Holm, E., and McClellan-Green, P. *Possible endocrine disruption in* Balanus amphitrite *after exposure to fouling-release coatings*. Society for Integrative and Comparative Biology, New Orleans, Louisiana. January, 2004

Oberdorster, E., **Romano, J.A.**, and McClellan-Green, P. The neuropeptide APGWamide as a Penis Morphogenic Factor (PMF) in gastropod mollusks. Society for Integrative and Comparative Biology, New Orleans, Louisiana. January, 2004

McClellan-Green, P, **Romano, J.A.,** and Rittschof, D. *A comparison of imposed induction in* Ilyanassa obsoleta *by three tin compounds*. 12th International Symposium on Pollution Responses in Marine Organisms, St. Petersburg, FL. May, 2003.

Romano, J.A. and McClellan-Green, P. *Comparison of imposex and intersex in two species of estuarine snails.* 23rd Annual Meeting Society of Environmental Toxicology and Chemistry, Salt Lake City, Utah. November, 2002.

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