TOXICITY AND AVAILABILITY OF COPPER AND ZINC TO QUEEN CONCH: IMPLICATIONS FOR LARVAL RECRUITMENT IN THE FLORIDA KEYS

by

Amber L. Garr

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This dissertation was prepared under the direction of the candidate's dissertation advisor, Dr. Megan Davis, Harbor Branch Oceanographic Institute, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

SUPERVISORY COMMITTEE:

Megan Davis, Ph.D.
Dissertation Advisor

C. Edward Proffitt, Ph.D.

Paul Wills, Ph.D.

Brian Lapointe, Ph.D.

Nancy Denslow, Ph.D.

Rodney K. Murphey, Ph.D.
Chair, Department of Integrative Biology

Gary W. Perry, Ph.D.
Dean, The Charles E. Schmidt College of Science

Barry T. Rosson, Ph.D.
Dean, Graduate College

March 29, 2012
Date

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ABSTRACT

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The presence of heavy metals and other pollutants is detrimental to marine ecosystems. The queen conch, once an important fisheries species in the Florida Keys, has not sufficiently recovered after a 25-year fishery closure. Research has shown high levels of copper and zinc in the gonads and digestive glands of adult conch found in the nearshore waters. Four sites relevant to queen conch larval recruitment were tested in 2010 for the presence of copper and zinc in the water, phytoplankton, sediment, and seagrass epiphytes over seven months. Both metals were detected in all sample types and no seasonal or geographical differences were detected. Surface water concentrations from the field were used to conduct acute and chronic toxicity tests on various ages of queen conch larvae and their phytoplankton food source. When zinc concentrations (0-40 µg/L) similar to those measured in situ were used, there was no significant impact on conch larval survival although some velar lobe development was impaired. However,

field concentrations of copper (0-15 μg/L), which often surpassed water quality standards, negatively impacted growth, survival, and development of the larvae. Chronic exposure to copper, through the water and food, disrupted the metamorphic success of competent larvae and decreased post-metamorphosis survival. Exposure to copper at later life stages increased mortality, suggesting that heavy metals have a negative effect on larval recruitment in localized areas of the Florida Keys. Structural equation modeling revealed that copper and zinc are moving through the systems differently and are best represented by two different models. The Florida Keys National Marine Sanctuary, NOAA's Office of National Marine Sanctuaries, and the Environmental Protection Agency will be able to use this data to better understand the extent of copper and zinc contamination in the Florida Keys and its effect on an important invertebrate species. It is suggested that long-term sampling of metals be incorporated into the already established water quality monitoring programs. Further research on the synergistic behavior of heavy metals with other marine pollutants should be examined to determine the long term effect on juvenile conch growth and development.

DEDICATION

This work is dedicated to my family, friends, and colleagues who have encouraged me along the way, but especially to my husband Bryan, who was instrumental to this entire process. It was wonderful to have him by my side as my boat captain, field assistant, and partner while I tackled this important step in my life.

TOXICITY AND AVAILABILITY OF COPPER AND ZINC TO QUEEN CONCH: IMPLICATIONS FOR LARVAL RECRUITMENT IN THE FLORIDA KEYS

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CHAPTER ONE: GENERAL INTRODUCTION

Environmental Concerns in the Florida Keys

The Florida Keys in Monroe County is a popular wintering and tourist destination. With nearly three million visitors each year and 80,000 permanent residents, the chain of small islands has been drastically impacted by residential and commercial development. Mangrove forests and seagrass beds have been filled and dredged to create land space and to build canals. These processes have increased nearshore turbidity and altered the natural tidal flows. Many of the canals were dredged to provide fill material for development and most of them are now dead-end channels with little to no tidal flushing (Kruczynski, 1999). Additionally, stormwater runoff, wastewater (onsite and cesspit), agricultural inputs, and pesticides are all sources of nutrient pollution and overload in the Keys (Lapointe et al., 1990; Lapointe and Clark, 1992; Lapointe and Matzie, 1996; Kruczynski, 1999). The Florida Keys National Marine Sanctuary (FKNMS), Mote Marine Laboratory (MML), Florida International University's Southeast Environmental Research Center (FIU SERC), and the Environmental Protection Agency (EPA) have been involved in establishing short-term and long-term water quality and ecosystem health monitoring programs, which are primarily focused on identifying locations and sources of nutrient-rich and turbid waters.

Despite the Florida Keys being designated as an "Outstanding Florida Waters" area, relatively few sites have been tested for heavy metal contamination in surface water, sediments, or primary producers, and only six of those sites have included multiple samples (Glazer et al. 2008; Lapointe and Clark, 1990; Lewis et al., 2007). Little information exists on concentrations of metals in these sources and on their bioavailability to marine consumers in the Keys. Although copper and zinc are essential metals found naturally in the environment, there are several anthropogenic sources that may be impacting the Florida Keys. Runoff from agriculture, marinas, or local weather proofing materials may all contribute to the presence of copper and zinc in the Florida Keys ecosystems.

With approximately 2.5% of Florida's state land designated for citrus culture (U.S. Department of Agriculture, Agricultural Chemical Usage Summary, 2002), the use of copper containing fungicides and herbicides can significantly contribute to the amount of copper released into the environment. Copper hydroxide and copper sulfate are two of the most common fungicides and nearly one million pounds of these were dispersed in Florida in 2005 (U.S. Department of Agriculture, Agricultural Chemical Usage Summary, 2006). Discharge into the Florida Bay from excess runoff can reach the lower Keys reef tract due to tidal passes and the dredged canals (Kruczynski, 1999).

Research with antifouling paint commonly used on boat hulls and other submerged structures has shown that metals leech from the particles in the sediment in quantities high enough to accumulate in marine macroalgae (Turner et al., 2009).

Likewise, in the case of ship groundings, the stripping of antifouling materials caused by the collision can have localized impacts of copper and zinc in the surrounding sediments (Jones, 2007). These fine particles released into the environment can pose a threat to grazing invertebrates, as up to 1% by weight of the sediment can be contaminated at severely polluted sites such as marinas and harbors (Turner et al., 2009). With over 60 commercial marinas, hundreds of private docks, and a three ship cruise port, water and sediment along the entire Florida Keys' chain are potentially exposed to many types of antifouling agents.

Leaching from coated materials and other outdoor structures is another direct pathway for zinc and copper to enter aquatic environments (Heijerick et al., 2002). Most often the metal is present as a free zinc ion (Zn^{2+}), which is the most bioavailable speciation form. However, zinc can also bind to dissolved organic content, turning it into an unavailable form (De Schamphelaere et al., 2005). Similar to zinc, ionic copper (Cu^{2+}) can bind with dissolved organic matter and become unavailable for animal uptake (Meador, 1991). In addition, water quality parameters, such as hardness, alkalinity, and pH will have an impact on metal toxicity (DiToro et al., 2001). Runoff into the numerous marinas in the Keys potentially creates point and non-point sources for localized heavy metal contamination (Kruczynski, 1999). Therefore, it is important to determine the sources and availability of these metals to better understand how they may be reacting individually and synergistically in the environment.

Extensive sampling of heavy metals in Florida and specifically in the Everglades, Biscayne Bay, and Spring Creek, has shown that metal contaminants are present in areas of point-source pollutants such as marinas and fresh water canal outflows (Carnahan et al., 2008; Georgiadis et al., 2001; Lapointe and Clark, 1990; Mitchell-Tapping & Mitchell-Tapping, 1997). Heavy metal surveys in the surface water and sediment of the Florida Bay have regularly found copper and zinc at detectible levels (Caccia and Millero, 2003; Cantillo et al., 1999). Sampling in Biscayne Bay found that copper and zinc were in highest concentrations in areas of the bay with the muddiest sediments as well as in areas closest to the mouth of canals (Carnahan et al., 2008). Lewis et al. (2007) found elevated copper levels (16.8-17.8 ppb) in the Keys that exceeded the water quality criteria (< 2.9 μg/L, 1996 standard) in the surface water of two nearshore sites. Copper and zinc were found in the sediment at nearshore and offshore areas in the Middle and Lower Keys (Lapointe & Clark, 1990; Glazer et al, 2008) as well as in the seagrasses *Thalassia testudinum* and *Halodule wrightii* in several locations throughout the Keys (Lewis et al., 2007).

The implication of heavy metal toxicity on the Florida Keys ecosystem is not fully understood. It is known that clear waters low in nutrients and pollutants are necessary to maintain the dynamic equilibrium that exists amongst the natural communities in the Keys (Kruczynski, 1999), and this equilibrium has been disrupted in the nearshore and offshore sites. Anthropogenic impacts from costal development and overfishing, along with natural fluctuations such as tidal flow and geography, cause changes in the ecosystem health of coral reef and seagrass habitats. There is a strong need to examine these impacts in the nearshore and offshore habitats in the Florida Keys. This research took an approach to focus on the impacts of heavy metal toxicity and availability of metal contamination in the Florida Keys to understand the effects of anthropogenic inputs on ecosystem health. The queen conch, *Strombus gigas*, an important molluscan herbivore in seagrass beds and coral reef environments, was chosen as a model species to study in this approach. Since tourism and fisheries in Monroe County account for the majority of the Florida Keys economy (Leeworthy and MacMinn, 2003), studies that help aid in the recovery of queen conch populations could have a major impact on the economy of the region where a recreational or commercial conch fishery could be reopened. The queen conch is also an important fisheries species in the Caribbean and was once a prime fishery in the Florida Keys.

The Model Species: Queen Conch

The queen conch is a large marine gastropod and important fisheries species throughout the Florida and Caribbean waters (Appeldoorn, 1994). For hundreds of year, queen conch have been used as tribal tools, decorations, building materials, and as one of the main protein food sources (Berg, 1976). In the 1970's, the commercial queen conch industry increased rapidly due to more efficient fishing techniques and the availability of freezer storage (Brownell and Stevely, 1981). Conch are considered the second most valuable resource in the region, after spiny lobsters (Appeldoorn, 1994), and the fishery is valued at nearly $40 million USD (Chakallal et al., 2007).

However, this increased demand has severely depleted many of the wild queen conch populations (Berg and Olsen, 1989; Appeldoorn, 1994). With annual commercial landings once as high as 25,500 kg per year in the late 60s, the conch fishery in the

Florida Keys drastically declined to just 14 kg per year by 1970 (FDNR,1976). This prompted the state to completely close its conch fishery in 1986. Many Caribbean countries followed suit and have established regulations such as quotas, SCUBA bans, bag limits, and closed seasons (Chakalall and Cochrane, 1997). In 1992, queen conch was added to Appendix II of the Convention of International Trade of Endangered Species (CITES) to ensure that the species is harvested at sustainable levels (Theile, 2001).

Marine protected areas in Florida and the Caribbean, especially in the Bahamas, have provided a refuge for spawning populations of queen conch (Glazer and Delgado, 2003; Glazer et. al., 2003; Chiappone and Sullivan Sealey, 2000), and larval densities are often higher within the protected areas (Stoner and Ray-Culp, 1996). However, if spawning adult populations are at a density less than 48 conch per hectare, mating and egg laying will not occur (Stoner and Ray-Culp, 2000). Although adult conch spawning aggregations densities are estimated to be around 800 conch per hectare (Glazer and Delgado, 2003), the allee effect in nearshore areas may be a possible explanation for the lack of significant population recovery in the Florida Keys, despite the long-term fishery closure. Likewise, larval densities and juvenile aggregations are strongly tied to the size of the local spawning stock (Stoner and Davis, 1997), and if that stock has been depleted, as is the case in many areas of the Florida Keys, then local population recruitment will be limited.

In the Florida Keys, queen conch have experienced very slow population recovery since the fishery closed. There are two distinct populations of conch, nearshore and offshore, that are separated by the silt-laden Hawk's Channel. The benthic conch do not cross this barrier, yet larvae are capable of drifting between the sites during their larval cycle (Delgado et al., 2008). Queen conch that settle and mature in the nearshore regions throughout the Keys are not capable of spawning, but when they are moved to offshore areas they regain this capability within six months (Delgado et al., 2004). Some conch in nearshore waters also have reduced external sex organs, a reduced lifespan, and instances of imposex, which may add the slow population recovery.

Heavy metal accumulation is one possible explanation for the lack of reproduction nearshore. Studies have shown that adult queen conch in nearshore waters had a significant increase in zinc concentrations in their digestive glands and copper concentrations in their gonads compared to offshore conch (Spade et al., 2010). These data suggest that nearshore conch accumulate copper in the gonad and zinc in the digestive gland at a greater rate than offshore conch. Sediment analyses indicated elevated levels of copper nearshore, relative to offshore, but the reverse was true for zinc (Spade et al, 2010). The presence of heavy metals may also be disrupting larval survival and metamorphic success of competent conch, as large juvenile aggregations have not been seen in nearshore waters of the Keys (Delgado et al., 2008). Since metals are accumulating in adults, it is reasonable to assume they may also be accumulating in juveniles.

Previous research has shown that water quality will impact conch larvae in the Florida Keys. Studies where larvae exposed to increased ammonia (NH_3) levels found in the nearshore Keys environments showed poor survival and impaired development

(McIntyre, 2005), and larval conch exposed to nearshore sediment from the Florida Keys had lower metamorphic success rates than those exposed to offshore sediments (Kowalik et al., 2006).

The preliminary analyses conducted by Spade et al. (2010) were based on a small sample size of adult conch, water, and sediment. My study aimed to expand the sampling area and increase replication to better understand the extent of heavy metal contamination.

Metals and Invertebrates

Molluscs are often used as bioindicators because their metal concentrations generally parallel those in their environment (O'Conner and Lauenstein, 2005). Copper is widely known to be toxic to invertebrates, and copper and zinc have been linked to reproductive impairment. Toxic effects of copper and zinc have been described in a number of gastropod species, although the mechanisms creating the toxic effects are not known. Stark (1998) determined that spiking sediment with Cu EDTA in a 56-day experiment led to reduced numbers of gastropods in an experimental population. Copper and zinc have been shown to reduce fecundity of *Helix aspersa* (Laskowski and Hopkin, 1996). Zinc can reduce fecundity in *Lymnaea pallustris* (Coerdassier et al., 2005). Copper oxychloride ($Cu_2Cl(OH)_3$) reduces *Helix aspersa* oocyte production (Snyman et al., 2004), and zinc reduces reproduction and population growth rate in *Valvata piscinalis* (Ducrot et al., 2007). Also, copper exposure results in reduced fecundity in *Pomacea paludosa*, the Florida apple snail (Rogevich et al., 2008).

Heavy metal accumulation may be a logical explanation for the lack of conch reproduction in nearshore waters, and it may also be negatively impacting survival, development, and metamorphosis for conch larvae recruiting to the nearshore habitats. Queen conch have a 21-day larval cycle during which they feed on phytoplankton, and a benthic stage when they feed on macroalgae, epiphytes, and detritus (Davis, 1998; Davis and Shawl, 2005). Being primary consumers, conch larvae and early juveniles serve as a food source for a variety of fish and other invertebrates. Therefore, to begin to understand the consequences of copper and zinc on early life stages of conch and other species, it is important to identify the sources and availability of heavy metals that conch are exposed to in these nearshore habitats. Several studies have examined the bioaccumulation and effects of copper and zinc in land snails (Laskowski and Hopkin, 1996); freshwater snails (Rogevich et al., 2008); and marine bivalves (Conner, 1972; Martin et al., 1981; Marmolejo-Rivas and Paéz-Osuna, 1990; Ruelas-Inzunza and Pàez-Osuna, 2000; Jara-Marini et al., 2008; Frías-Espericueta et al., 2008). Only Sanders (1984) and Spade et al. (2010) have examined copper and zinc accumulation in the benthic stages of queen conch. No studies have addressed effects on the early life stages of conch.

Research with larval marine oysters and clams show that the veligers are affected by copper and zinc (EC_{50} of 9.1 ug/L Cu and 129 ug/L Zn) (Beiras and Albentosa, 2004). Several studies have found toxic effects from copper and zinc on mussel and oyster embryos (Martin et al., 1981; Fernández and Beiras, 2001), and it has been shown that mollusc larvae are much more susceptible to copper and zinc toxicity than adults

(Connor, 1972). Given that queen conch have a similar larval stage as these bivalves, they may be sensitive as well.

Since elevated levels of copper and zinc have been found in the Florida Keys nearshore habitats and in adult queen conch, it is important to determine how these metals may impact the critical early life stages of queen conch. Research has shown that nearly 80% of the larvae from offshore sites recruit to a corresponding nearshore site, yet few juvenile conch are found in the nearshore habitats (Delgado et al., 2008). It is possible that water quality issues may help to explain the limited recruitment seen in nearshore habitats in the Florida Keys.

Research Goals and Project Impacts

The FKNMS Management Plan (2007) specifically addresses the need for research on wastewater pollutant loadings and their impacts (Strategy W.23), with particular reference to heavy metals. Although there have been extensive nutrient monitoring programs in the Florida Keys, there are very little data currently available on the bioavailability and toxicity of copper and zinc to marine organisms in this ecosystem. The goal of this research was to determine the availability and the toxic effects that copper and zinc have on the early life stages of the queen conch. **The overall hypothesis of this study is that elevated copper and zinc in the Florida Keys nearshore environment contributes to impaired growth, development, and survival of conch larvae and early juveniles.** The specific goals of this project were:

1. to quantify the amount of copper and zinc present in the water, sediment,

phytoplankton, and epiphytes in selected nearshore and offshore sites in the Florida Keys that have, or historically had, queen conch aggregations;

2. to determine the tolerance of early life stages of queen conch to copper and zinc using LC_{50} levels for conch larvae and IC_{50} levels to assess metal uptake and tolerance for cultured microalgae;
3. to determine the effects of intermittent and chronic copper exposure on growth, survival, development, and metamorphic success for the early life stages of queen conch;
4. to test models describing the potential pathways of copper and zinc bioaccumulation during the larval recruitment and settlement period for queen conch and provide recommendations for marine park managers and water quality monitoring programs.

The data from this project were shared with FKNMS park managers. This research complements ongoing work with conch reproduction and histology in relationship with heavy metal accumulation being conducted by the Florida Fish and Wildlife Conservation Commission (FWC) and the University of Florida (UF). Results from the larval studies will be of importance to FWC and their ongoing monitoring of queen conch recovery. Heavy metal concentrations found in the nearshore and offshore habitats in the Keys will contribute to the Water Quality Monitoring Network of the Southeast Environmental Research Center, provide a toxicity reference point for FWC queen conch biologists, and can be used as baseline sampling for future monitoring programs in the Keys.

CHAPTER TWO: COPPER AND ZINC PROFILES AT FOUR SITES RELEVANT TO QUEEN CONCH RECRUITMENT IN THE FLORIDA KEYS

ABSTRACT

Spatial and temporal sampling of copper and zinc at four sites in the middle and lower Florida Keys was conducted from April to October 2010. Two offshore-nearshore site pairs were selected based on predictive larval flow and retention patterns for the queen conch. Sombrero Reef (SR) and Bahia Honda (BH) were the first site pair where it is predicted that 91% of the SR larvae will reach BH. Looe Key (LK) and Boca Chica (BC) were chosen as the second site pair as 93% of the LK larvae should drift to the BC seagrass beds. Samples of surface water, phytoplankton, seagrass epiphytes, and sediment were collected at each site and analyzed for copper and zinc content. Surface water levels of copper (0-15 µg/L) often surpassed water quality standards, but both copper and zinc remained below total effects levels for the sediment. The heavy metals accumulated in the phytoplankton and the epiphytes, both an important food source for juvenile queen conch. There were no distinct differences in copper and zinc concentrations between the nearshore and offshore sites, and seasonal patterns were not detected. Queen conch larvae and newly-settled juveniles are likely to be exposed to potentially toxic levels of copper and zinc both nearshore and offshore, which may have an impact on population recruitment.

INTRODUCTION

The Florida Keys is a popular tourist destination, and with nearly three million visitors each year and 80,000 permanent residents, the chain of small islands has been drastically impacted by residential and commercial development. Mangrove forests and seagrass beds have been filled and dredged to create land space and to build canals. These processes have increased nearshore turbidity and altered the natural tidal flows. Additionally, stormwater runoff, wastewater (onsite and cesspit), and pesticides are all sources of nutrient pollution and overload in the Keys (Kruczynski, 1999). The Florida Keys National Marine Sanctuary (FKNMS), Mote Marine Laboratory (MML), Florida International University's Southeast Environmental Research Center (FIU SERC), and the Environmental Protection Agency (EPA) have been involved in establishing short-term and long-term water quality and ecosystem health monitoring programs, which are primarily focused on identifying locations and sources of nutrient-rich and turbid waters.

The Keys have been greatly impacted from nutrients from Florida Bay discharge, oceanic and Gulf of Mexico upwelling and currents, rainwater, and anthropogenic sources (Kruczynski, 1999). Most of the point-source contamination is concentrated in populated areas. High nutrient levels have been recorded at Key Largo and Big Pine Key and were linked to the 24,000 septic tanks and treatment facilities in those areas (Kruczynski, 1999). Human waste has been found in nearshore waters and canals and has effected coral reefs in the lower and middle Keys (Lipp et al., 2002). There is a general agreement that canal and nearshore waters are affected by these nutrients

(Kruczynski, 1999), but there has been very little data collected in the Keys that compares nearshore conditions with offshore water quality (Lapointe et al., 2004a).

Heavy metal contamination has been a concern for numerous Florida water systems including the Florida Keys, Florida Bay, the Everglades, and Lake Okeechobee (Caccia et al, 2003; Cantillo et al., 1999; Georgiadis et al., 2001). Sediment studies have discovered high concentrations of metals associated with petroleum around Tavernier marina (Caccia et al., 2003). Extensive sampling of heavy metals in the Everglades, Biscayne Bay, and Spring Creek have also shown increased metal contaminants present in areas of point-source pollutants such as marinas and fresh water canal outflows (Carnahan et al., 2008; Georgiadis et al., 2001; Mitchell-Tapping & Mitchell-Tapping, 1997). Heavy metal pollution has been linked to ship groundings and areas with heavy industrial activities in other countries as well (Bi, et al., 2007; Caruso et al., 2011; Jones. 2007). Since dissolved metals are often transported to the open ocean and most particulate metals are retained on the continental shelf (Caccia & Millero, 2003), there is concern that the alterations to the Florida Keys natural water flow has impacted the accumulation of metals in once pristine habitats.

Water movement in the Florida Keys is dependent upon the Florida current, onshore Ekman transports, coastal countercurrents, and cyclonic circulation of the Tortugas gyre (Lee and Williams, 1999). Tidal channels in the middle Keys allow for exchange between Florida Bay and Hawks Channel, potentially carrying nutrients and contaminants from the Bay and into the nearshore and offshore areas (Haus et al., 2000; Smith and Lee, 2003). Drifters released in the Shark River region move southeastward through the Florida Bay, to the middle Keys channels, and into the Keys coastal zones (Willemsen, 2005). Although there may be seasonal variations in intensity, there is evidence that water is moving from the Florida Bay to the Keys habitats via the transport of major and minor currents.

Heavy metal surveys in the surface water and sediment of the Florida Bay have been conducted, and copper and zinc are regularly found at detectable levels (Caccia et al., 2003; Cantillo et al., 1999). These metal concentrations are affected by numerous variables such as turbidity, currents, interactions with sediments, temperature, and oxygen; all of which are associated with seasonal fluctuations (Caccia & Millero, 2003). Surface water will often have metal concentrations orders of magnitude lower than sediments since metals are quickly absorbed by suspended matter and precipitate to the bottom (Caccia & Millero, 2003). Likewise, sediment high in $CaCO_3$, such as that found in the Florida Bay and under the dense seagrass beds in the Keys, tends to be high in organic content. Seagrass beds will often trap suspended particulate matter which may be high in organics and trace metals. Research has shown that the concentration of several heavy metals is strongly correlated with the organic content in the sediment as well as the presence of seagrass (Caccia et al, 2003).

Seagrass beds are also the primary habitat of the commercially threatened queen conch, *Strombus gigas*. Despite a 25-year fishery closure in the Florida Keys, conch populations have shown little recovery and adults in the nearshore waters have reduced external sex organs, a shorter lifespan, and instances of imposex (Delgado et al., 2004). A recent study discovered that adult queen conch in nearshore waters had a significant increase in zinc in their digestive glands and copper in their gonads compared to offshore conch (Spade et al., 2010), suggesting that conch in nearshore habitats are accumulating copper and zinc at a greater rate than offshore conch.

Heavy metals have the capacity to disperse throughout the trophic levels (Arunakumara & Xuecheng, 2008). Numerous studies have demonstrated the accumulation of metals by plants and invertebrates exposed to metal contaminated field sites (Bi et al., 2007; Cantillo et al., 1999; Lewis et al., 2007; MacFarlane et al., 2007; Romero Núñez et al., 2011; Ruelas-Inzunza & Paéz-Osuna, 2006). Sanders (1984) showed that juvenile queen conch are also capable of accumulating heavy metals directly from their diet in the laboratory. Queen conch have a 21-day larval cycle where they feed on microalgae, followed by metamorphosis to a benthic stage where they consume diatoms and epiphytes in seagrass beds. Therefore, conch may be exposed to copper and zinc through the water, phytoplankton, epiphytes, or sediment during their early life stages. Since the reproductive capability of adult conch is impaired nearshore and there has been little juvenile recruitment in the nearshore seagrass habitats of the Keys (Delgado et al., 2004; Spade et al., 2010), it has been suggested that copper and zinc may be part of the reason why these populations are not quickly recovering.

Despite the Florida Keys being designated as an "Outstanding Florida Waters" area, only six sites have been tested in replication for heavy metal contamination in surface water, sediments, or primary producers. Lewis et al. (2007) found elevated copper levels (16.8-17.8 ppb) in the Keys that exceeded the water quality criteria (< 2.9 μg/L, 1996 standard) in the surface water of two nearshore sites. Copper and zinc have been detected in the sediment at Looe Key, Sombrero Reef, and Boca Chica (Lapointe & Clark, 1990) and more recently been found in the sediment of nearshore and offshore areas important to queen conch aggregations (Glazer et al, 2008). Copper and zinc have been discovered in *Thalassia testudinum* and *Halodule wrightii* in several locations throughout the Keys (Lewis et al., 2007). Since elevated levels of copper and zinc have been found in the Florida Keys nearshore habitats and in adult queen conch, it is important to determine how these metals may impact the critical early life stages of queen conch. Research has shown that nearly 80% of the larvae from offshore sites recruit to a corresponding nearshore site, yet few juvenile conch are found in the nearshore habitats (Delgado et al. 2008). Likewise, local seasonal gyres and eddies present along the Keys coastline have been credited with larval transportation and retention (Lee et al., 1992; Lee and Williams, 1999) and therefore, should be assisting with conch population recruitment. It is possible that water quality issues may help to explain the lack of recruitment seen in nearshore habitats in the Florida Keys.

Due to the limited sampling of metals in the Keys, as well as the established differences in conch recruitment, reproductive abilities, and physical appearances in the nearshore areas, there is an increased need to study and compare these sites to offshore areas where queen conch appear to be unaffected. This study quantified the amount of copper and zinc present in the water, sediment, epiphytes, and primary producers (microalgae) in nearshore and offshore sites in the Florida Keys that have, or historically had, queen conch aggregations. It was hypothesized that heavy metals will accumulate at a higher frequency nearshore rather than offshore. Samples were collected over a seven month period to capture seasonal differences and correlations between and within sites and type of sample are presented. The goal of this research was to determine the possible sources of copper and zinc that would affect the early life stages of queen conch and how this might relate to juvenile survival and recruitment. Results from this study will contribute to the Water Quality Monitoring Network of the Southeast Environmental Research Center, provide a toxicity reference point for FWC queen conch biologists, and can be used as baseline sampling for future monitoring programs in the Keys.

MATERIALS AND METHODS

Site Selection

Two offshore and two nearshore sites were selected with input from the Fish and Wildlife Florida Marine Research Institute (FWC-FMRI) scientists. Previous research with drift vials and plankton tows (Delgado et al., 2008) had delineated source and sink populations

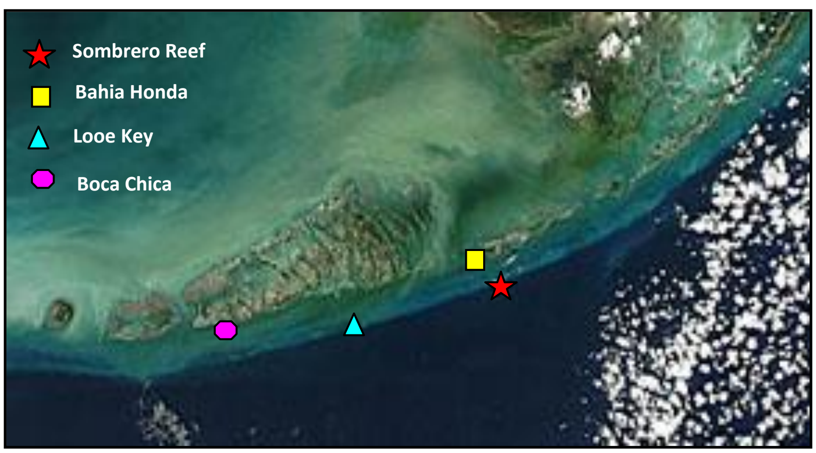

Figure 2.1. Locations of the two nearshore-offshore site-pairs sampled. The first site-pair is Sombrero Reef (SR)–Bahia Honda (BH), and the second site-pair is Looe Key (LK)-Boca Chica (BC).

of queen conch larvae. Based on these results, two site-pairs (middle and lower Keys) were selected where the offshore site potentially provide the nearshore site with the majority of the larvae. Sombrero Reef (SR, offshore) and Bahia Honda (BH, nearshore) were the first site-pair where 91% of the larvae from SR should settle at BH. Looe Key (LK, offshore) and Boca Chica (BC, nearshore) were the second pair selected, where 93% of the LK larvae should be retained at BC (Delgado et al. 2008). The SR and LK sites are within the Keys National Marine Sanctuary SPA's, and the BH site is located within the state park boundaries. The BC site is located approximately two miles southwest of the US Naval Reserve base (**Fig 2.1**).

Sample Collection

Field samples were collected once each month for seven months, April through October 2010 (**Table 2.1**) to coincide with the queen conch reproductive season (Delgado et al., 2004). All collections were done on an outgoing tide, as close to slack tide as possible. Weather conditions prevented sampling offshore in April and September with the exception of water and phytoplankton from SR in April. A center sampling point was delineated at each 100 m^2 site based upon the seagrass bed habitat and the presence of conch or history of conch at the site. Once the location was obtained, GPS coordinates were recorded and used to mark the location for return each month. Basic water quality parameters were measured at the center starting point (temperature, pH, salinity, and dissolved oxygen). From this location, a 0.5 m^2 PCV grid was tossed at least 10 m in distance in a random direction to identify the first sampling point. Six grid locations

Table 2.1. Field locations and water quality parameters collected at the study sites over the seven month experiment.

Location	GPS	April	May	June	July	Aug	Sept	Oct
Sombrero Reef								
Temp (°C)	N 24°37.440'	-	23.9	27.5	31.7	30.5	-	27.5
pH	W081°06.435'	-	8.2	8.4	8.0	8.2	-	8.4
Salinity (ppt)		-	34	35	31	35	-	30
DO (mg/L)		-	-	8.8	11.4	8.4	-	8.8
Looe Key								
Temp (°C)	N 24 °2.909'	-	23.8	27.5	31.6	31.1	-	27.5
pH	W081°24.398'	-	8.2	8.5	8.1	8.2	-	8.5
Salinity (ppt)		-	34	35	32	35	-	35
DO (mg/L)		-	-	8.6	12.4	8.2	-	8.6
Bahia Honda								
Temp (°C)	N 24°39.342'	24.9	26.2	27.8	32.7	36.0	33.0	27.8
pH	W081°16.606'	8.6	8.1	8.4	8.2	8.4	8.4	8.4
Salinity (ppt)		35	35	30	30	35	34	30
DO (mg/L)		-	-	10.3	12.7	7.9	8.5	10.3
Boca Chica								
Temp (°C)	N 24°33.400'	24.1	26.3	28.5	35.0	33.1	33.4	28.5
pH	W081°43.000'	8.3	8.1	8.6	8.3	8.4	8.5	8.6
Salinity (ppt)		35	35	30	35	35	34	30
DO (mg/L)		-	-	9.6	11.8	7.8	8.4	9.6

were sampled each month, allowing variable sampling within the same 100 m^2 area each time. All samples were obtained using snorkeling gear. Mean precipitation recorded at the Marathon Airport during the sampling period was gathered from the National Weather Service and used to observe trends in metal concentrations.

Sediment samples were collected with a polyethylene 50 ml Corning centrifugal tube. Within each grid, one sample of surface sediment (top 1 cm) was scraped into the sample vial, until at least 20 mm of sediment was collected. This often meant that three scrapes were necessary. Care was taken to try and collect sediment only, so any large shells or plant material was removed and excess water was poured out. The samples were immediately sealed in plastic bags, placed on ice for the return trip, and then stored in a freezer in the laboratory. Defrosted wet sediment was weighed to the nearest 0.001 g and dried for 48 hrs at 80ºC in a Fisher Scientific Isotemp oven. The dry samples were weighed, filtered through a 625 µm sieve, and placed in a 15 ml Corning centrifugal tube for storage at room temperature.

Roughly ten *Thalassia testududinum* seagrass blades were collected within each sample grid and placed into polyethelene bags. Water was removed and the samples were immediately placed on ice. At the laboratory, epiphytes were scraped from both sides of the blade using a razor blade (Davis and Stoner, 1994, Smith et al., 2008) so that at least 5 g of wet epiphytes was collected. Samples were dried for 48 hr at 80ºC in a Fisher Scientific Isotemp Oven. Dried epiphytes were weighed (0.001 g), ground into fine particles using a mortar and pestle, and stored in a 15 ml Corning centrifugal tube for storage at room temperature.

Water samples were collected in pre-washed 1L high density polyethelene bottles approximately 1 m below the surface and above the center of the grid. This depth was chosen for consistency between sites since bottom depths ranged from 1.5 to 6 m. Care was taken to collect against the current to prevent possible contamination from the boat (offshore sites). Immediately after all six samples were collected, water was measured to exactly 1L (with a graduated cylinder) and placed into another 1 L sample bottle with 10 ml (1%) of nitric acid for preservation (EPA Method 3005, 1989). Samples were stored in the dark on ice until being returned to the laboratory. Using a 10 µm filter, all water samples were filtered to remove any large suspended particles or phytoplankton that would be too large for larval conch consumption. Water was placed into 50 ml Corning centrifugal tubes for storage.

A subsample of the water was used to collect phytoplankton. Once the water was filtered with the 10 µm mesh, 500 ml (of the 1 L sample) was processed through a vacuum filter. A 0.45 µm nitrocellulose membrane filter pad (Millipore, 47 mm dia) was used to collect algae cells between 0.45 – 10 µm that would be available as a queen conch larval food source (Davis, 1998; Jara-Marini et al., 2008). The filters were weighed and dried for 48 hrs at 80ºC in a Fisher Scientific Isotemp Oven. Several blank nitrocellulose pads were processed in the same manner with de-ionized water in order to establish a base filter pad wet and dry weight. Dried samples of phytoplankton plus filter pads were placed in 15 ml Corning centrifugal tubes for dry storage at room temperature.

Metal Analyses

All glassware and sample bottles were washed with a protocol established for metal processing (EPA Method 3005, 1989). Sediment, epiphyte, and phytoplankton samples were processed using an acid digestion technique similar to Ward et al. (2005). Briefly, 500 mg of sample was weighed to the nearest 0.0001 g and placed into a 100 ml glass digestion tube. For the phytoplankton samples, an average weight of the blank filter pads was used to calculate the actual weight of phytoplankton. These samples averaged less than 0.01 mg.

A 5 ml aliquot of 70% nitric acid (Certified ACS Plus) was added to each digestion tube and placed on a heating plate. Samples were heated to 110ºC for at least 30 minutes until the material was completely digested. At that time, 5 ml of 30% hydrogen peroxide (Acros Organics) was added to the samples and allowed to heat for an additional 30-45 minutes, or until the solution was clear. Digested material was allowed to cool to room temperature, and then poured through a 1 µm filter pad into a 50 ml graduated flask. The samples were diluted up to a volume of 50 ml with de-ionized water and placed into a 50 ml Corning centrifugal tube for storage. For each group of digestions (replicates 1-2, replicates 3-4, replicates 5-6) a blank (no sample) was processed in the same manner and used during the analysis.

All samples (water, sediment, phytoplankton, and epiphytes) were transferred into 15 ml Corning centrifugal sample tube for metal analysis using A ten point standard curve (0, 0.001, 0.005, 0.01, 0.05, 0.10, 0.25, 0.50, 0.75, 1.0 µl/L) was used to quantify samples. Copper was measured at 324.8 wavelength and Zn at 213.9 wavelength. The minimum detection limit was 0.001 ppb. Blank samples containing only the decomposition acid was used with each digested group and was subtracted from both elements in the sediment, epiphyte, and phytoplankton samples (Ward et al., 2005).

Statistical Analysis

All ICP-MS data were analyzed for differences ($p < 0.05$) in means using a Repeated Measures ANOVA to examine overall differences and the changes in metal presence over time with SAS (v 9.2) (SAS, Cary, NC, USA). Differences between site pairs were examined with ANOVA testing within each sample type. Data were tested for homogeneity of variances (Levene's test) and normality of distribution (Shapiro-Wilk test) and were log(x+1) transformed to meet assumptions.

Following these analyses, structural equation modeling (SEM) was used to test for direct and indirect effect of variables and assess model fit with MPlus (v 6, 2010). This is a multivariate technique that allows for examination of these effects on manifest and latent variables (Grace, 2006; Mitchell, 1992). SEM is commonly used to examine causal relationships between two or more variables, and is typically based on *a priori* hypotheses (Shipley, 1999). It is used to examine causal hypotheses in ecological traits and behaviors such as variation in offspring size (Abell, 1999), pairing success (Bart and Earnst, 1999), or sex ratios in a population (Shine, 1996). Often these traits are linked to habitat type, food quantity and quality, or other environmental parameters. In biological studies, the theory is not always well developed, and therefore links between every variable may not be possible or will require prior biological knowledge to determine orderings of dependent and independent variables (Shipley, 1997). Path analysis is based on a linear equation system and is a subset of the multivariate Structural Equation Modeling (SEM) (Ullman, 1996). A path analysis is used to examine a single indicator for each variable in the causal model, whereas SEM can then test the descriptive ability of different models, allowing them to be compared (Mitchell, 1992; Ullman, 1996). Even though the "true model" is usually unattainable, researchers can use SEM to identify models which do not contradict their best data, are consistent with the results of experimental manipulations, and have predictive values independent studies (Shipley, 1999). For this study, SEM was used to examine whether or not metal concentrations in one (independent) variable may have a relationship or an effect on concentrations in

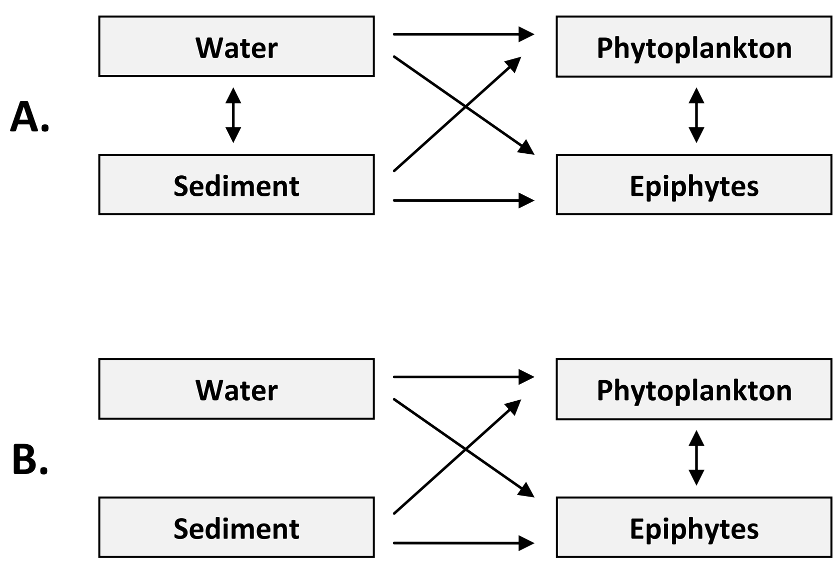

Figure 2.2. Proposed models tested with structural equation modeling (SEM) to examine the causal relationships between the sampled variables.

another (dependent) variable and assess the model fit for each scenario.

Two models were proposed and tested using manifest variables only (water, sediment, phytoplankton, and epiphytes (**Fig. 2.2**). Each model was tested for separately for copper and zinc at different combination of sites: all sites combined, nearshore only, offshore only, and at each of the four individual sites. It was hypothesized that metals in the water and sediment will be correlated and will act as a driver for metals taken up by the phytoplankton and epiphytes, which are also correlated to each other (Model A). A second model was tested to examine water and sediment independently as individual drivers for metal concentrations found in phytoplankton and epiphytes (Model B).

RESULTS

Concentrations of copper and zinc were found in all samples at the four sites with a few exceptions where the metals were below detectible limits (**Table 2.2a, b**). Although there were some differences throughout the sampling period, overall, there were no significant differences in copper ($F_{3,578}$ = 0.66, p=0.7791) and zinc ($F_{3,578}$ = 2.01, p=0.1108) levels between sites. There were monthly differences in concentrations of copper and zinc that varied at each site depending on the sample type. The analysis showed that there were no significant differences in copper concentrations in all phytoplankton samples collected from each site over time (**Fig. 2.3**). Even within the same site, there were no differences from month to month for all four locations. However, there was a differences in zinc levels at SR from April-May ($F_{1,3}$ = 24.52, p=0.0158) and May-June ($F_{1,3}$ = 12.27, p=0.0394) where zinc was highest in April. No differences were detected at the other sites.

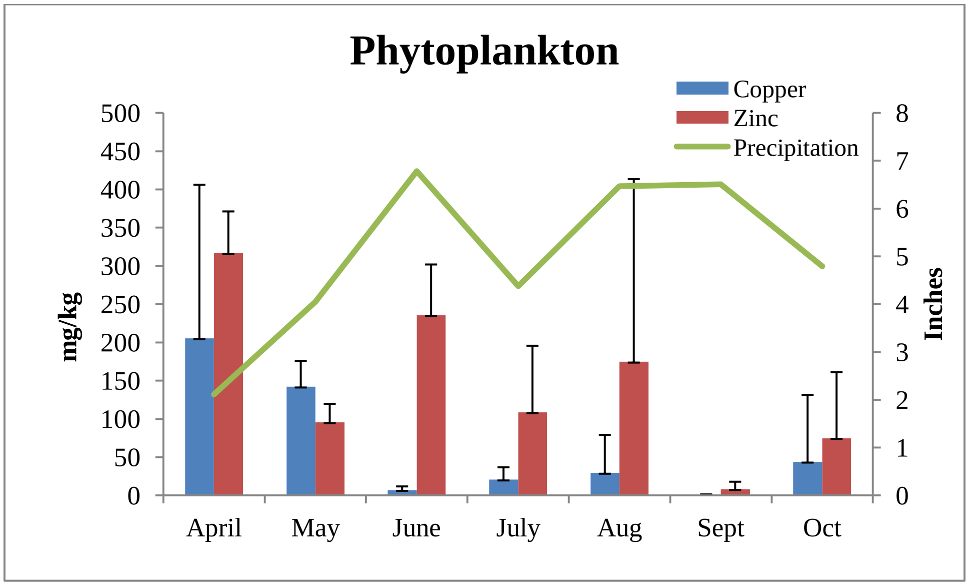

Figure 2.3. Concentrations of copper and zinc in the phytoplankton sampled in 2010 for all sites combined. Mean (±SD) and n=12-24. Monthly precipitation values at Marathon Airport obtained from The National Weather Service.

Copper concentrations in the sediment samples were relatively consistent but did vary significantly at SR (July–August, $F_{1,5}$ = 27.70, p=0.0033), BH (June–July, $F_{1,5}$ = 13.63, p=0.0141), and BC (July–August, $F_{1,5}$ = 10.76, p=0.0219) when levels decreased (**Fig 2.4**). Zinc also varied between months, but only at one site (BC) and only between September–October ($F_{1,5}$ = 7.77, p=0.0385) where the concentration decreased in October.

Metal concentration in the epiphytes differed significantly throughout time at each individual site (**Fig. 2.5**). At SR, copper levels varied from May-June ($F_{1,5}$ = 7.47,

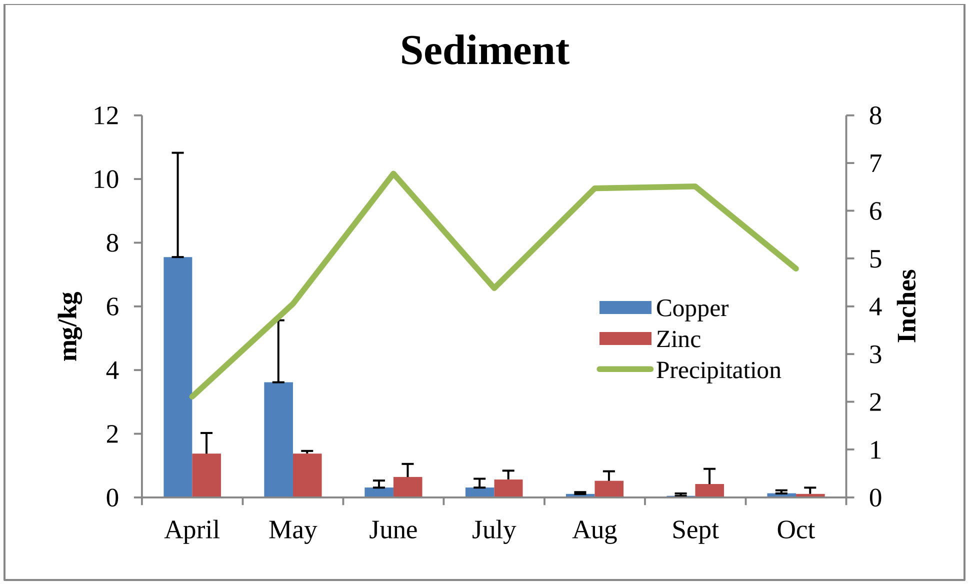

Figure 2.4. Concentrations of copper and zinc in the sediment sampled in 2010 for all sites combined. Mean (±SD) and n=12-24. Monthly precipitation values at Marathon Airport obtained from The National Weather Service. Threshold effect levels in marine sediment for copper is 18.7 mg/kg and is 124 mg/kg for zinc (MacDonald et al., 1996).

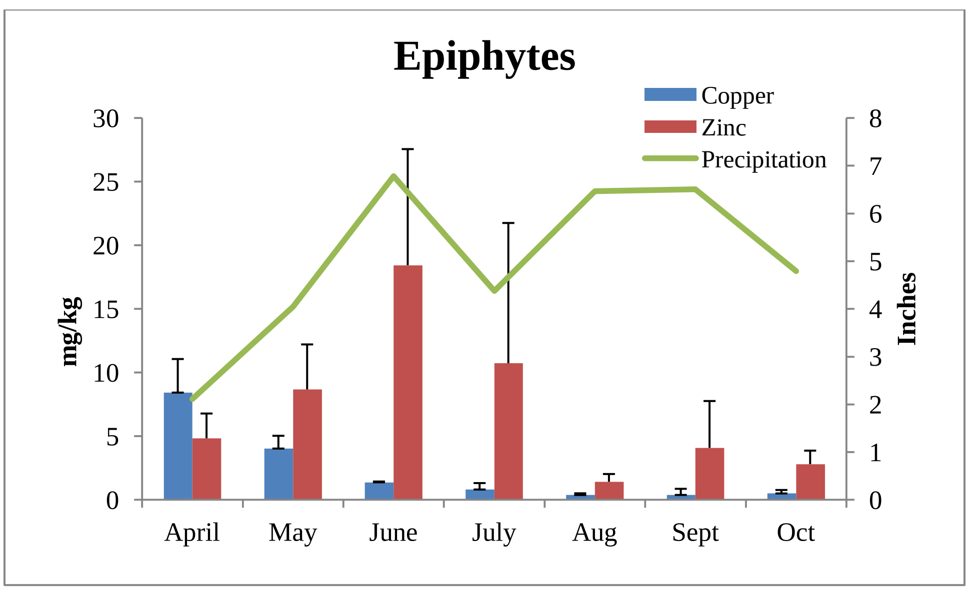

Figure 2.5. Concentrations of copper and zinc in the epiphytes sampled in 2010 for all sites combined. Mean (±SD) and n=12-24. Monthly precipitation values at Marathon Airport obtained from The National Weather Service.

p=0.0411) and July–August ($F_{1,5}$ = 8.68, p=0.0321) as concentrations continued to decrease. Zinc levels were significantly different in June–July ($F_{1,5}$ = 14.34, p=0.0128) and in July-August ($F_{1,5}$ = 50.22, p=0.0009). The corresponding site pair, BH, also reflected differences in copper levels between June-July ($F_{1,5}$ = 47.05, p=0.0010) and July–August ($F_{1,5}$ = 8.18, p=0.0354) due to a decrease in copper in July. Zinc levels in the epiphytes fluctuated at BH significantly during May-June ($F_{1,5}$ = 52.58, p=0.0008), June-July ($F_{1,5}$ = 58.31, p=0.0006), August-September ($F_{1,5}$= 17.21, p=0.0089), and September-October ($F_{1,5}$ = 15.54, p=0.0109). Metal levels in the epiphytes from the second site pair also differed significantly during certain months. At LK, copper varied from July–August ($F_{1,5}$ = 61.06, p=0.0006) as levels decreased. Zinc levels were different from July–August ($F_{1,5}$ = 9.64, p=0.0267) and between August–October ($F_{1,5}$ = 20.53, p=0.0062). At the corresponding nearshore site (BC), copper levels varied from July–August ($F_{1,5}$ = 29.62, p=0.0028) and between August–September ($F_{1,5}$ = 72.30, p=0.0004). Zinc fluctuated during the same time periods of July–August ($F_{1,5}$ = 9.14, p=0.0293), August–September ($F_{1,5}$ = 33.74, p=0.0021), and between September-October ($F_{1,5}$ = 40.92, p=0.0014).

The concentration of copper in the water at SR varied significantly between May-June ($F_{1,5}$ = 722.50, p<0.0001), June-July ($F_{1,5}$ = 35.59, p=0.0019), July-August ($F_{1,5}$ = 16.00, p=0.0103), and August-October ($F_{1,5}$ = 15.00, p=0.0117). Zinc levels were only significantly different between April-May ($F_{1,5}$ = 76.82, p=0.0003). At BH, water copper concentrations varied between May-June ($F_{1,5}$ = 250.00, p<0.0001), June-July ($F_{1,5}$ = 27.00, p=0.0035), and August- September ($F_{1,5}$ = 22.86, p=0.0050). Zinc was

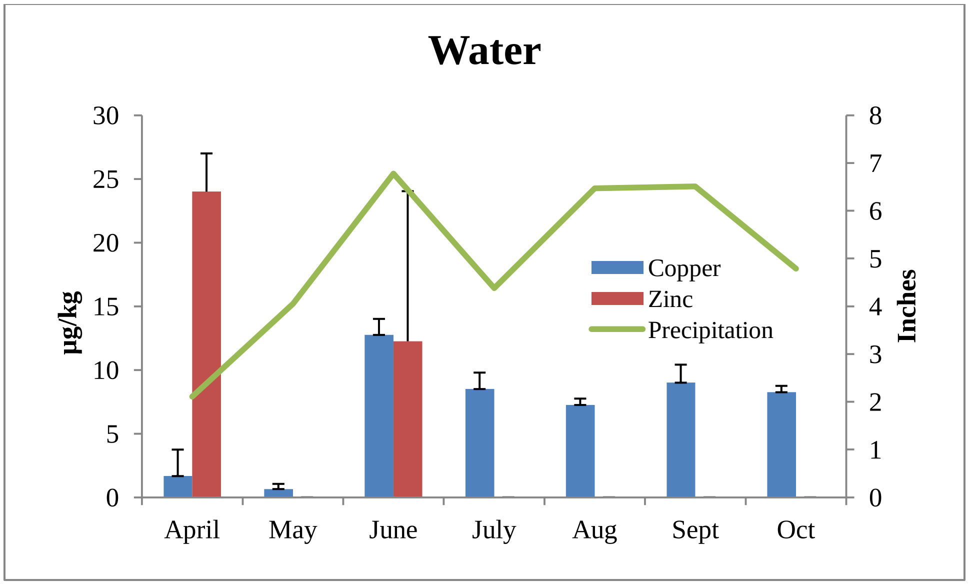

Figure 2.6. Concentrations of copper and zinc in the surface water sampled in 2010 for all sites combined. Mean (±SD) and n=12-24. Monthly precipitation values at Marathon Airport obtained from The National Weather Service. Water quality criteria for copper is <3.7-3.8 µg/L and is <86 µg/L for zinc (FDEP, 2010 and Schuler et al., 2008).

significantly different each month from April to June, until it was no longer detected for the remaining sampling periods (**Fig. 2.6**). Copper only varied from May-June ($F_{1,5}$ = 20.39, p=0.0063) at LK, and there were no differences in zinc concentrations. In the corresponding site pair (BC), copper varied significantly from May-June ($F_{1,5}$ = 855.62, p<0.0001) and June-July ($F_{1,5}$ = 23.82, p=0.0045) and zinc was significantly different from April to June, until levels were no longer detected in the remaining months.

An ANOVA was used to test for differences between site pairs (offshore-nearshore) within sample types and months (**Table 2.3**). Of all the samples collected, only fourteen showed significant differences between sites. No differences were found in water samples anywhere, and those that were detected in the epiphytes, phytoplankton,

Table 2.2a. Copper and zinc concentrations found at site pair Looe Key-Boca Chica in the Florida Keys from April - October 2010 (± SD, n=6). Levels in the sediment, ephiphytes, and phytoplankton are presented in mg/kg (ppm) and water concentrations are in ug/L (ppb).

	April 2010	May 2010	June 2010	July 2010	August 2010	September 2010	October 2010
Site Pair 1							
Boca Chica							
Sediment (Cu mg/kg)	9.86 ± 10.68	3.23 ± 3.89	0.63 ± 0.15	0.66 ± 0.37	0.10 ± 0.09	0.16 ± 0.10	0.23 ± 0.16
Epiphytes (Cu mg/kg)	6.56 ± 5.03	3.70 ± 3.55	1.29 ± 0.32	0.97 ± 0.13	0.38 ± 0.23	1.00 ± 0.14	0.09 ± 0.19
Phytoplankton (Cu mg/kg)	118.56 ± 156.71	122.17 ± 156.61	7.06 ± 8.91	16.04 ± 16.94	103.75 ± 196.43	1.77 ± 4.34	0.0 ± 0.0
Water (Cu µg/L)	4.0 ± 3.0	1.0 ± 1.0	13.0 ± 1.0	8.0 ± 2.0	8.0 ± 2.0	8.0 ± 2.0	8.0 ± 2.0
Sediment (Zn mg/kg)	1.83 ± 0.092	1.43 ± 1.07	1.23 ± 1.57	0.26 ± 0.43	0.94 ± 1.04	0.83 ± 0.38	0.04 ± 0.15
Epiphytes (Zn mg/kg)	6.21 ± 2.56	10.76 ± 5.67	17.51 ± 13.78	10.13 ± 5.22	2.02 ± 1.58	7.16 ± 1.18	4.07 ± 0.59
Phytoplankton (Zn mg/kg)	256.24 ± 377.56	65.8 ± 14.27	273.60 ± 203.72	85.08 ± 121.76	527.10 ± 866.77	20.01 ± 18.7	0.0 ± 0.0
Water (Zn µg/L)	24.0 ± 22.0	0.0 ± 0.0	4.0 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Looe Key							
Sediment (Cu mg/kg)	n/a	3.28 ± 3.41	0.23 ± 0.17	0.36 ± 0.14	0.18 ± 0.19	n/a	0.18 ± 0.45
Epiphytes (Cu mg/kg)	n/a	3.10 ± 3.84	1.43 ± 0.31	1.07 ± 0.12	0.20 ± 0.23	n/a	0.28 ± 0.10
Phytoplankton (Cu mg/kg)	n/a	144.41 ± 195.67	13.61 ± 21.09	37.54 ± 42.18	8.33 ± 18.64	n/a	0.0 ± 0.0
Water (Cu µg/L)	n/a	0.3 ± 1.0	11.0 ± 6.0	9.0 ± 2.0	7.0 ± 2.0	n/a	8.0 ± 2.0
Sediment (Zn mg/kg)	n/a	1.46 ± 2.08	0.49 ± 0.69	0.81 ± 1.36	0.23 ± 0.48	n/a	0.0 ± 0.0
Epiphytes (Zn mg/kg)	n/a	4.85 ± 1.4	18.07 ± 23.6	5.71 ± 3.47	0.58 ± 0.65	n/a	1.87 ± 0.53
Phytoplankton (Zn mg/kg)	n/a	120.78 ± 29.66	250.63 ± 288.72	217.13 ± 250.24	13.89 ± 31.06	n/a	0.67 ± 1.51
Water (Zn µg/L)	n/a	0.0 ± 0.0	29.0 ± 63.0	0.0 ± 0.0	0.0 ± 0.0	n/a	0.0 ± 0.0

Table 2.2b. Copper and zinc concentrations found at site pair Sombrero Reef- Bahia Honda in the Florida Keys from April - October 2010 (± SD, n=6). Levels in the sediment, epiphytes, and phytoplankton are presented in mg/kg (ppm) and water concentrations are in ug/L (ppb).

	April 2010	May 2010	June 2010	July 2010	August 2010	September 2010	October 2010
Site Pair 2							
Bahia Honda							
Sediment (Cu mg/kg)	5.23 ± 5.33	6.30 ± 10.79	0.23 ± 0.15	0.002 ± 0.01	0.10 ± 0.19	0.02 ± 0.04	0.05 ± 0.08
Epiphytes (Cu mg/kg)	10.29 ± 9.71	3.86 ± 3.64	1.36 ± 0.45	0.06 ± 0.00	0.38 ± 0.28	0.53 ± 0.26	0.41 ± 0.12
Phytoplankton (Cu mg/kg)	434.91 ± 410.51	112.74 ± 175.86	2.84 ± 4.41	0.02 ± 0.03	4.9 ± 12.01	0.0 ± 0.0	0.0 ± 0.0
Water (Cu µg/L)	1.0 ± 0.0	1.0 ± 1.0	14.0 ± 2.0	7.0 ± 2.0	7.0 ± 2.0	10.0 ± 1.0	9.0 ± 2.0
Sediment (Zn mg/kg)	0.91 ± 0.79	1.30 ± 1.27	0.55 ± 0.82	0.78 ± 0.90	0.43 ± 0.42	0.11 ± 0.13	0.34 ± 0.48
Epiphytes (Zn mg/kg)	3.45 ± 2.21	6.62 ± 2.23	30.21 ± 9.45	0.84 ± 0.05	1.43 ± 1.18	5.08 ± 1.10	3.29 ± 0.55
Phytoplankton (Zn mg/kg)	362.91 ± 375.71	87.15 ± 46.42	138.04 ± 101.77	7.99 ± 6.65	117.56 ± 195.65	11.99 ± 13.57	0.0 ± 0.0
Water (Zn µg/L)	27.0 ± 24.0	0.0 ± 0.0	4.0 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sombrero Reef							
Sediment (Cu mg/kg)	n/a	1.64 ± 2.92	0.13 ± 0.19	0.20 ± 0.09	0.03 ± 0.08	n/a	0.03 ± 0.08
Epiphytes (Cu mg/kg)	n/a	5.45 ± 3.75	1.42 ± 0.48	1.14 ± 0.24	0.54 ± 0.13	n/a	0.41 ± 0.06
Phytoplankton (Cu mg/kg)	61.94 ± 107.89	118.78 ± 263.31	4.00 ± 7.03	28.49 ± 28.08	0.0 ± 0.0	n/a	175.25 ± 350.00
Water (Cu µg/L)	0.0 ± 0.0	0.3 ± 1.0	13.0 ± 1.0	10.0 ± 1.0	7.0 ± 2.0	n/a	8.0 ± 2.0
Sediment (Zn mg/kg)	n/a	1.31 ± 1.57	0.28 ± 0.61	0.40 ± 0.62	0.47 ± 0.60	n/a	0.0 ± 0.0
Epiphytes (Zn mg/kg)	n/a	12.46 ± 6.79	7.94 ± 3.51	26.25 ± 10.56	1.64 ± 1.46	n/a	1.99 ± 1.00
Phytoplankton (Zn mg/kg)	330.49 ± 175.68	108.31 ± 45.52	280.12 ± 323.54	124.35 ± 158.97	26.79 ± 31.07	n/a	149.53 ± 123.92
Water (Zn µg/L)	21.0 ± 6.0	0.0 ± 0.0	12.0 ± 14.0	0.0 ± 0.0	0.0 ± 0.0	n/a	0.0 ± 0.0

Table 2.3. Significant differences detected between offshore and nearshore site pairs using ANOVA (DF=1, Error =10, n=6).

Site Pair	Month	Sample Type	Metal	p value	F value	Site with higher concentration	Offshore or Nearshore
SR - BH	May	Epi	Zinc	0.0466	5.15	SR	offshore
	June	Epi	Zinc	0.0001	34.94	BH	nearshore
	July	Epi	Copper	<0.0001	195.24	SR	offshore
	July	Epi	Zinc	<0.0001	278.41	SR	offshore
	July	Phyto	Copper	0.0021	16.90	SR	offshore
	July	Sed	Copper	0.0002	34.57	SR	offshore
	Oct	Epi	Zinc	0.0143	8.75	BH	nearshore
	Oct	Phyto	Zinc	0.0008	22.75	SR	offshore
LK-BC	May	Epi	Zinc	0.0076	11.08	BC	nearshore
	May	Phyto	Zinc	0.002	17.28	LK	offshore
	June	Sed	Copper	0.0014	18.91	BC	nearshore
	Oct	Epi	Copper	<0.0001	60.96	LK	offshore
	Oct	Epi	Zinc	<0.0001	41.63	LK	offshore
	Oct	Sed	Zinc	<0.0001	57.18	BC	nearshore

and sediment in the site pair SR-BH, were often higher offshore than nearshore (**Table 2.3**). Differences in the LK-BC site pair were evenly split amongst nearshore and offshore samples, where little differences were observed between the sites during the summer months (June, July, August, September).

Results from the SEM analysis indicated that Model A was a better fit for copper and Model B was a better fit for zinc (**Table 2.4**). The difference between the two models was the correlation between water and sediment. For copper, the model best fit at three (BH, SR, LK) of the four sites. At BH, the inverse relationship between water and sediment was significant along with the pathways driven by copper in the sediment. At SR, only the pathways showing a relationship with epiphytes were significant. The model fit best at LK, showing a significant causal relationship for all pathways except for water to phytoplankton (**Fig 2.7**). In this example, the highest R^2 values for phytoplankton (0.460) and epiphytes (0.656) were observed.

Model B, where water and sediment were not hypothesized to be correlated, showed the best fit for predicting the flow of zinc through the system. However, the model only fit at SR (**Fig 2.8**) and not at the remaining three sites. For zinc, it appeared that sediment was the driver for metal being present in the phytoplankton and epiphytes, because the effect of water on those variables did not show any significant relationship. At all of the sites where the model fit (all sites combined, offshore sites, and SR), the R^2 values were under 0.075, indicating that other factors may also be contributing to the levels of metal recorded in the phytoplankton and epiphytes.

DISCUSSION

The results from this study provide a spatial and temporal view of copper and zinc concentrations at four sites in the Florida Keys. The anticipated differences between the nearshore sites and offshore sites were not observed, and in some cases, the offshore site (SR) had higher levels of copper and zinc (**Table 2.3**). However, this lack of difference,

Table 2.4. SEM results showing best fit models for copper (Model A) and zinc (Model B) as tested with several sites and combinations of sites. Bold p values indicate a significant fit of the model.

Model	Site(s)	χ^2	p value	DF
A	All sites	32.389	0.0000	1
(copper)	Nearshore	25.987	0.0000	1
	Offshore	9.87	0.0017	1
	Boca Chica (NS)	31.647	0.0000	1
	Bahia Honda (NS)	1.163	**0.2802**	1
	Looe Key (OS)	3.692	**0.0547**	1
	Sombrero Reef (OS)	0.853	**0.3558**	1
B	All sites	2.195	**0.3338**	2
(zinc)	Nearshore	16.825	0.0002	2
	Offshore	0.083	**0.9593**	2
	Boca Chica (NS)	11.062	0.0040	2
	Bahia Honda (NS)	6.249	0.0440	2
	Looe Key (OS)	12.460	0.0020	2
	Sombrero Reef (OS)	0.419	**0.8108**	2

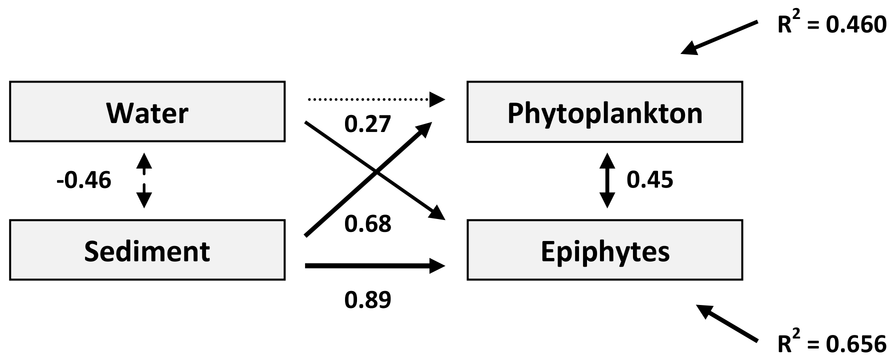

Figure 2.7. Path diagram for Model A (copper) at Looe Key (LK). Width of each arrow is proportional to the standardized path coefficient. Large dashed lines indicate a negative relationship and small dashed line (water to phytoplankton) indicates that the path was not significant (p=0.0547).

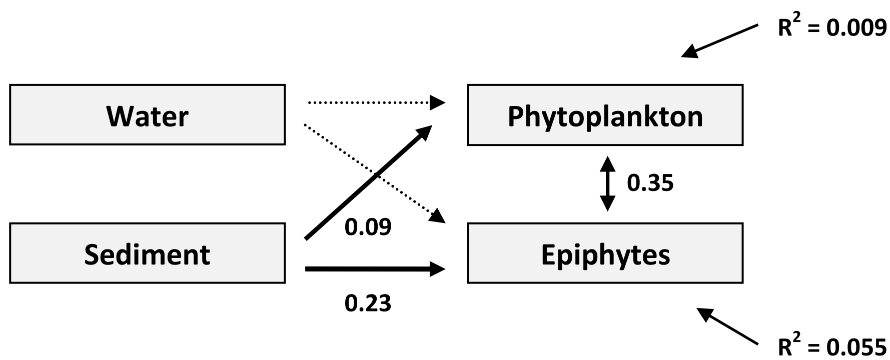

Figure 2.8. Path diagram for Model B (zinc) at Sombrero Reef (SR). Width of each arrow is proportional to the standardized path coefficient. Small dashed line (water to phytoplankton and epiphytes) indicates that the path was not significant (p=0.8108).

most likely due to a mixing of water, has been shown before at LK. Lapointe et al. (2004a) found an increase in nutrients at LK following a rain event showing that land-based nutrient enrichment is impacting offshore sites. Both offshore SR and nearshore BC are exposed to high boating activity (SR) and industrial activity (BC – U.S. Naval Reserve base) perhaps contributing to some of the differences compared to their corresponding nearshore site pairs. However, LK is also a popular snorkeling destination like SR, but the tidal flushing events may be different at each location and warrants further investigation.

Although the deepest site of the four sampled areas, the presence of the Sombrero Key lighthouse may impact heavy metal concentrations and should be examined as a potential source. SR is also located near the Seven Mile Bridge at Moser Channel where Florida Bay water is entering the coastal system (Smith and Lee, 2003). This might explain why copper and zinc were found at higher levels in the offshore (SR) site compared to its nearshore counterpart (BH), since tidal flushing and transportation of nearshore waters to offshore are more likely. Regardless of these oceanographic differences, copper and zinc in the water and phytoplankton was not discretely different from a queen conch egg mass hatch site (offshore) to a potential larval settlement site (nearshore).

The strength and shape of the Florida Current varies seasonally along the Keys coastline (Lee and Williams, 1999; Leichter and Miller, 1999). Part of that fluctuation is due to the movement of the Tortugas gyre that is pushed along the Florida Keys and inshore of the Florida Current (Willemsen, 2005). When this occurs, coastal eddies develop along portions of the Keys and will be present for two to four weeks at a time. These gyres are often well defined in the Lower Keys but begin to shrink as it passes through the narrowing Hawks Channel in the Middle Keys. They have usually disappeared by the time they reach the Upper Keys where the Florida Current dominates the water flow again (Willemsen, 2005). In the summer season, onshore currents will also transport offshore water to the inner shelf in some areas of the Upper Keys (Haus et al., 2004). The presence of these gyres at different strengths throughout the season would provide a means for seasonal and site differences in surface water metal concentrations. However, differences were not seen in this study.

Although the concentration of copper in the surface waters did not differ significantly between sites and throughout the season, the levels found in this study often exceeded the water quality criteria concentrations set by the Florida Department of Environmental Protection (≤ 3.7 µg/L, 2010) and the U.S. Environmental Protection Agency (3.8 µg/L, Schuler et al. 2008). At SR, the levels were higher than allowable in 66% of the samples and in 71% of the samples from BH. Offshore, at LK, copper in the water was higher than allowable levels in 80% of the samples, whereas all (100%) of the mean copper values at BC were higher than the recommended 3.7 µg/L. The range of copper (0.0-14.0 µg/L) found in the water in this study were comparable to the high concentrations found by Lewis et al. (2007) in the Florida Keys (16.8-17.8 µg/L). These levels have the potential to severely impact numerous saltwater species. A sensitivity index conducted for saltwater fish, crustaceans, mollusks, plants, and other invertebrates showed that the average acute copper toxicity level for 90% of these species is 9.8 µg/L, and as low as 2.0 µg/L for mollusks (Schuler et al., 2008). The toxic concentration of copper for chronic exposure was 3.9 µg/L, also lower than values seen in the Florida Keys surface water in this study and others (Schuler et al., 2008).

Increased metal concentrations in surface waters have been linked to warm water events (summer), increased dissolved oxygen, and lower pH values, as these factors can increase metal ion availability (Donlagic et al., 2007). Although levels of copper and zinc were slightly higher in the warmer summer months in this study, there were still no distinct seasonal differences detected. This is in contrast to copper concentrations in the Florida Bay, which showed higher levels in the summer months which was thought to be due to increased precipitation (Caccia & Millero, 2003). Seasonal uptake of zinc in bivalves and marine algae has been demonstrated before, yet copper did not have the same seasonal variations as zinc (Ruelas-Inzunza and Páez-Osuna, 2000; Kalesh and Nair, 2006). The variability in copper seen in this study is similar to what has been observed before in several locations in Florida and the Bahamas and can not necessarily be linked to increased boating activities or nutrient input during periods of high precipitation (Caccia & Millero, 2003).

Levels of copper and zinc in the sediment decreased in all sites throughout the sampling period, with the highest levels observed in April and May (**Table 2.2a,b**). The range of metals found in this study (0.002-9.86 mg/kg for copper and 0-1.83 mg/kg for zinc) are similar to values obtained at the same locations (LK, SR, BC) by Lapointe and Clark (1990) and Glazer et al. (2008). However, when compared to research conducted in Florida Bay (Caccia et al., 2003), Biscayne Bay (Carnahan et al, 2008), the Everglades

(Georgiadis et al., 2001), and selected contaminated Florida sites (Yoon et al., 2006), copper and zinc found in this study were sometimes tenfold lower. Copper in the Florida Bay (15-30 ppm), Biscayne Bay (1-100 ppm), Everglades (0-30 ppm), and throughout contaminated Florida sites (21-990 ppm) often exceeds the TEL (Threshold Effects Level) of 18.7 ppm for Florida coastal waters (MacDonald et al., 1996). The TEL for zinc is 124 ppm (MacDonald et al., 1996), and sediment levels were under this criteria in the Florida Bay (30-48 ppm) and the Everglades (0-80 ppm), but not at Biscayne Bay (0-129 ppm), and throughout contaminated Florida sites (195-2,200 ppm). Copper and zinc remained below TEL at all four sites tested in this study.

Heavy metals can be in several forms in sediment which can directly impact their bioavailability and toxic effect (Donlagic et al., 2007). Copper, in particular, can be devastating not only to benthic grazers, but also to the macrobenthic assemblages (Stark 1998). Research in the Florida Bay has suggested that low copper levels (in water) and the increased levels in the sediments in the middle of the Bay in the summer could have been due to seagrass uptake or precipitation that occurs at high salinities (Caccia et al., 2003). Plant biota will accumulate heavy metals and are often considered for their capacity to eliminate metals from the environment (Hansen et al., 2011; Perales-Vela et al., 2006; Yoon et al., 2006). Frequently, high metal concentrations in the sediment will result in high concentration in roots of associated macrophyte and plant species (MacFarlane et al., 2007; Romero Núñez et al., 2011). Likewise copper and zinc has been found to be higher in sediment from seagrass vegetated sites than non vegetated sites (Lewis et al., 2007).

Results from the SEM in this study indicate a strong causal relationship between sediment and phytoplankton and epiphytes at several sites, further supporting this research. In this study, phytoplankton and epiphytes were collected to test for the presence of heavy metals in conch food sources. Research with other macrophytes and their associated epiphytic biomass has shown that they will accumulate heavy metals at differing capacities (Kljakovic-Gaspic et al., 2006; Lakatos et al., 1999, Schlacher-Hoenlinger and Schlacher, 1998) and water flow rates will be a factor in metal uptake (Hansen et al., 2011). Although not explicitly identified in this experiment, some of the common epiphytes that may be associated with the *Thalassia testudinum* blades in Florida include diatoms from the genera *Pleurosigma*, *Licmophora, Nitzschia, Thalassionema, Synedra, Rhopalodia, Mastogloia, Navicula* and *Amphora* (Smith et al., 2008). As microalgae species, both the seagrass epiphytes and the phytoplankton collected in this study predictably accumulated metals with only a few phytoplankton sample exceptions (**Table 2.2a,b**). Epiphytes accounted for five of the twelve site pair differences observed (**Table 2.3**) and three of the five times, the metal concentration was higher offshore. This occurred most notably at SR, perhaps due to the frequency of boat visitors to that site.

The concentrations of copper and zinc in the epiphytes was slightly higher than the levels in the sediment although they were similar to concentrations found by Lewis et. al. (2007) in the roots and blades of *Thalassia testudinum* and *Haldule wrightii* in a Florida Key seagrass bed near Little Duck Key. Metals in the phytoplankton were highly variable throughout the sampling period as may be expected for a sample type that is easily transported and has a quick turnover time. This may have also been due to the relatively small sample size used in the analytical process. Using known cell counts and cell weights extrapolated from laboratory microalgae processed in the same manner, it is estimated that phytoplankton counts in the field (those algae between 0.45-9.9 µm) averaged around one million cells per ml. Knowing that algae can accumulate heavy metals at a rapid pace (Kaewsarn, 2002; Levy et al., 2007; Sandau et al., 1996), the high values seen in some of the samples may be representative of the bioavailability for queen conch larvae.

In April and September, there were windy and turbulent weather conditions. At the two nearshore sites (BH and BC), copper and zinc were slightly higher in April for most samples, but the same effect was not seen in September when conditions once again prevented sampling offshore. June was a record warm month in the Keys and July, August, September, and October were record breaking rain months (NOAA, 2011). Most of the variations from month to month detected by the RM-ANOVA were seen in the summer and early fall months (wet season). An increase in epiphytes and chlorophyll levels during the rainy season has been recorded in the Keys before both nearshore and offshore (Lapointe et al., 2004a). In this study, zinc levels in the phytoplankton and epiphytes fluctuated with rainfall, but copper concentrations in both sample types appeared to be unrelated to precipitation. Adding additional variables to the model, such as precipitation, distance from canal, mean water depth, and current speed may assist in explaining varying metal accumulation at each site.

Copper occurs naturally in the environment and is an essential metal for plants and animals. It will not break down in nature and often attaches to particles made out of organic matter, clay, soil, or sand (ATSDR, 2004). The SEM showed that copper in the sediment was more of a significant driver of metal in the phytoplankton and epiphytes than water was. This was most likely due to the inverse relationship of copper concentration in the water in comparison to the other variables, as the levels in the water increased during the summer months and decreased in the other sample types. However, the model that included a significant negative correlation between copper in the water and sediment did fit at three of the four sites.

The limited number of significant model fit both offshore and nearshore suggests that there are other sources where zinc is accumulating that were not tested here. The difference in the flow of copper in the environment versus zinc may be due to the availability of the metal to break free of bonds. Zinc binds to sediment and will most likely not dissolve in water (ATSDR, 2005). Research has shown that zinc will accumulate in phytoplankton (Muyssen and Janssen, 2001) and it will also build up in animals (ATSDR, 2005). Zinc is an essential element for most animals, but the uptake rates differ even within gastropod species, partially because they have the ability to regulate accumulation (Moolman et al., 2007).

Although the proposed pathways did not explicitly explain how zinc was moving through the system, it was still present at all sites. High levels of zinc can damage metabolic processes (Muyssen and Janssen, 2001), disrupt fertility (Ducrot et al., 2007; ATSDR, 2005), and induce hypocalcaemia and bone resorption (WHO, 1996). Since adult conch nearshore have been found with reduced gonads and instances of imposex (Glazer et al., 2008), future research should still consider zinc toxicity as one of the explanations as to why nearshore conch are not reproducing. The conch larvae were tolerant of water zinc concentrations well above what was recorded in the field, and is therefore, not thought to be linked to poor larval recruitment at this time.

Copper levels often exceeded the surface water quality criteria which also surpassed toxicity levels seen for mollusks. Although zinc was present, it did not appear in concentrations that surpassed water and sediment criteria. Since molluscs in general are known to bioacculuate heavy metals from both their food and surrounding environment (Beiras and Abbentosa, 2004; Coerdassier et al., 2005; Connor, 1972; Ducrot et al., 2007; Fernández & Beiras, 2001; Laskowski & Hopkin, 1996; Martin et al., 1981; O'Conner and Lauenstein, 2005; Rogevich et al., 2008; Snyman et al., 2004), queen conch of all life stages will be exposed to metals at each of the sites tested, which may have a negative effect on population growth of this species in the Florida Keys.

CHAPTER 3: ACUTE TOXICITY OF COPPER AND ZINC ON QUEEN CONCH LARVAE AND A PHYTOPLANKTON FOOD SOURCE

ABSTRACT

Acute toxicity of copper and zinc was determined for queen conch *Strombus gigas*, larvae and the marine phytoplankton *Isochrysis sp*. as a representative food source. Three separate age groups of larvae were exposed to four concentrations of copper (1, 5, 10, 15 µg/L) and zinc (5, 10, 20, 40 µg/L) that encompassed the metal range found in surface water sampled in nearshore and offshore conch habitats in the Florida Keys. The LC_{50} values were determined at 24, 48, 72, and 96 hrs. The initial tests showed copper acute toxicity (LC_{50}) levels as low as 1µg/L at 96 hrs for all larval stages, however when the experiment was repeated with fed larvae, the mortality was not high enough to determine LC_{50} levels despite differences in survival rates. Zinc was not as toxic to the larvae at all age stages, and LC_{50} values (8.9 – 24.3 µg/L) could not be calculated until 96 hrs. *Isochrysis sp.* was also exposed to the field concentrations of copper (1, 5, 10, 15 µg/L), and growth rates as well as percent motility were recorded at 24, 48, 72, and 96 hrs. The IC_{50} values were calculated using linear extrapolation and copper concentrations higher than 5 µg/L had a significant negative impact on cell division and motility. The results of this study show that queen conch larvae and one of their phytoplankton food sources may

be impacted by the copper levels found in the Florida Keys surface water, but that zinc toxicity may not be a major factor in larval survival and recruitment.

INTRODUCTION

Waste water discharges, habitat destruction, and other human activities have been linked to environmental changes in estuarine and saltwater ecosystems of the southeastern United States (Reish et al., 1998). Many of these types of practices increase pollutants, including the heavy metals that are the focus of this study (Fernández & Beiras, 2001; Scordino et al., 2008). Strom water runoff and the numerous marinas in the Keys potentially creates point and non-point sources for localized heavy metal contamination (Kruczynski 1999). Copper, in particular, is often present in fungicides and herbicides commonly used in the Florida citrus industry (Alva et al., 1995; Leslie, 1990). Dredging and filling of canals and other waterways have changed the natural tidal flow which has resulted in an increased amount of pollutants flowing from the Florida Bay (Kruczynski 1999).

Heavy metals such as copper and zinc are essential at low levels as they are critical components of enzymes and respiratory proteins crucial to normal cellular functions (Gorski & Nugegoda, 2006a; Nagajyoti et al., 2010). However, at increased levels these metals will also have detrimental impacts on the survival and development of invertebrates, particularly in the early life stages (His et al., 1999). Because of this sensitivity, molluscs are often used as bioindicators since their accumulated metal concentrations generally parallel those in their environment (O'Conner and Lauenstein, 2005).

Toxicity of both metals has been described in a number of gastropod species, although the mechanisms creating the toxic effects (physiological) are not well understood. Copper is widely known to be toxic to invertebrates, and both copper and zinc have been linked to reproductive impairment. Research with *Lymnaea pallustris*, *Helix aspersa*, *Valvata piscinalis*, and *Pomacea paludosa* adults have all shown reduced fecundity when exposed to copper and zinc (Coerdassier et al., 2005; Ducrot et al., 2007; Laskowski & Hopkin, 1996; Rogevich et al., 2008; Snyman et al., 2004). Studies with larval marine oysters and marine clams showed that veligers are affected by copper and zinc (EC_{50} of 9.1 ug/L Cu and 129 ug/L Zn) (Beiras and Abbentosa, 2004). Several studies have found toxic effects from copper and zinc on mussel and oyster embryos (Fernández & Beiras, 2001; Martin et al., 1981), and it has been shown that mollusc larvae are much more susceptible to copper and zinc toxicity than adults (Connor, 1972).

Although heavy metal accumulation has been documented in queen conch juveniles and adults (Sanders, 1984; Spade et al., 2010), no studies have addressed effects on the early life stages of conch. Since queen conch have a similar larval stage as many of the bivalves observed in other studies, it is thought that they may be equally sensitive. Fluctuations in other water quality and environmental parameters will impact conch larval development and metamorphic success. Larvae exposed to increased ammonia (NH_3) levels show poor survival and impaired development (McIntyre 2005), and those exposed to nearshore sediment from the Florida Keys had lower metamorphic success rates than those exposed to offshore sediments (Kowalik et al., 2006).

Copper and zinc are also known to accumulate in marine and freshwater algae at levels that can reduce growth, delay luminescence, and inhibit photosynthesis (Baumann et al., 2009; Moreira et al., 2006; Sandau et al., 1996; Schmitt et al., 2001; Scordino et al., 2008), and often these assimilation rates are related to water quality. Research with the freshwater microalgae, *Chlorella sp.*, showed a decrease in algae growth (higher IC_{50} value) as pH decreased (Wilde et al., 2006). The marine algae *Padina* sp. adsorption rates were also closely linked to pH and 90% of the uptake of copper (II) occurred within 15 minutes (Kaewsarn 2002). Due to these bioabsortion capabilities, both microalgae and macroalgae can be used to assist in the removal and recovery of heavy metal ions in aqueous solutions like waste water (Hammouda et al., 1995; Kaewsarn 2002; Matheickal & Yu, 1999; Wong et al., 1995).

Copper and zinc were detected at all four sites tested in the Florida Keys and in areas important to conch reproduction and larval recruitment (CHAPTER 2). The current project studied the acute (24, 48, 72, and 96 hr) toxicity of copper and zinc on developing queen conch larvae on various life stages using the concentrations of metals in the surface waters observed in the field. Since motile and healthy (cell division occurring) microalgae cells are needed to sustain conch through their critical larval cycle (Davis, 1998), it is important to understand the potential impacts of metal contaminated algae on larval development and metamorphic success. The acute toxicity (24, 48, 72, and 96 hr) of copper on the growth (IC_{50}) and motility of the marine microalgae *Isochrysis galbana* (clone T-iso) was determined. *Isochrysis sp*. is a common food source for conch larvae cultured in the laboratory (Davis and Shawl, 2005), and strains of the microalgae are found throughout Florida and Caribbean waters (R.A. Glazer, FWC, *personal communication*). It was hypothesized that copper and zinc will accumulate in water and microalgae at sufficient concentrations to cause toxicity in queen conch larvae and in cultured microalgae. The results were expected to identify the role heavy metal contamination may be playing in the slow population growth of queen conch in the Florida Keys.

MATERIALS AND METHODS

Larval LC_{50} Experiments

Three pieces of queen conch egg masses were collected in May, June, July, and August 2010 and June 2011 from Sombrero Reef and Looe Key (see CHAPTER 2 for approximate coordinates). Female conch actively laying eggs were identified by a diver at the water's surface while being towed behind the boat. Once a spawning female was located, a portion (4-8 cm, 25-50%) of the egg mass was collected from directly underneath her leading lip edge. Care was taken to minimally disturb the female to allow for her to finish laying the complete mass. Each piece of egg mass was placed in a 2L thermos filled with saltwater from the collection site and returned to the HBOI laboratory within 24hrs. The eggs were combined together and allowed to hatch in filtered and UV treated laboratory saltwater inside a temperature controlled incubator, and hatching always occurred 96 to 120 hrs after collection.

Larvae that hatched in May, June, and July were utilized for preliminary range tests. The larval cycle was divided into three developmental phases indicative of the main lobes stages of queen conch larvae (Davis, 1998): Days 1-4, Days 8-12, and Days 15-19. Queen conch typically metamorphose between 21 and 26 days depending upon temperature (Davis, 1998). The metamorphic success of competent larvae exposed to varying copper concentrations was determined in the 2011 experiments (CHAPTER 4).

All larvae not being exposed to the metals were cultured in 12L buckets following methods previously established (Davis & Shawl, 2005). In short, the larvae were raised in static culture containers filled with filtered and UV sterilized saltwater, and a total water change was conducted every other day. The larvae were poured through a sieve (120 µm for first 10 days and 250 µm for the remaining cycle), the buckets were washed with a mild solution of muriatic acid then freshwater rinsed, and the larvae were returned to the buckets using a gentle stream of saltwater from a squirt bottle. Larvae were fed *Isochrysis sp.* cultured at HBOI at levels determined daily (Davis & Shawl, 2005) and adjusted according to density and larval age.

For all studies, copper chloride (ACROS Organics, 97% pure) and zinc chloride (ACROS Organics, 98% pure) granules were weighed on a digital scale (306.92 mg copper choride and 412.81 mg zinc chloride) and a 100 ppm (mg/L) solution was prepared. This was further diluted to a 1 ppm stock solution for both copper and zinc. Serial dilutions were then used to create the experimental concentrations using the formula $C_1V_1=C_2V_2$; where C_1 and V_1 are the stock concentration and volume and C_2 is the desired concentration at a particular volume (V_1). Although not explicitly tested in this experiment, other research has shown there to be a minimal loss in metal concentrations in prepared solutions over 96 hrs (Gorski and Nugegoda, 2006a). All glassware used during the studies was washed using the standard operating procedures for metal contaminants highlighted in CHAPTER 2 (EPA Method 3005).

Range Tests

Initial range tests were developed based on acute toxicity experiments with other marine invertebrate larvae and embryos (**Table 3.1**). In the first series of tests conducted in May and June, 2010, five levels of copper (0, 1, 5, 10, 50 µg/L) and zinc (0,1, 50, 150, 400 µg/L) were examined with all three age groups. Fifteen larvae were stocked in 1L glass beakers filled to 600 ml of the solution at three replicates each. Only swimming (e.g., healthy) larvae were selected and the number of dead, swimming, or lethargic larvae were recorded every 24 hrs for 96 hrs. The larvae were not fed during the study.

A second range test was conducted in June and July, 2010 on all three larval stages to narrow down the concentrations. Copper levels of 0, 5, 10, 15, 20 µg/L and zinc levels of 0, 10, 25, 75, 350 µg/L were tested with six replicates and fifteen larvae in each beaker. The number of dead, swimming, and lethargic (on the bottom) larvae were counted every 24 hrs for 96 hrs. The larvae were not fed during the study. Dead larvae were removed and no larvae were used for more than one test. For both range tests, all beakers were held in a temperature (28°C) and light controlled (12hr light: 12 hr dark) incubator.

Acute Toxicity Experiments - Unfed Larvae

Once the copper and zinc analyses were completed for the surface water collected during the first few months of sampling, the acute toxicity ranges were adjusted to reflect a range of concentrations that were found in the field. Eggs collected in August 2010 were used to conduct the first acute toxicity test in August and September, 2010. Five levels of copper (0, 1, 5, 10, 15 µg/L) and zinc (0, 5, 10, 20, 40 µg/L) were examined with all three age groups at six replicate each. In addition to the zero metal control, a separate control (no metal) where the larvae were fed daily was also included. For this control, *Isochrysis sp.* algae was counted each day and fed at a concentration of 3, 5 or 7 million cells/ml depending on the life stage. Fifteen swimming larvae were placed in the treatment containers and held in an incubator controlling both temperature (28ºC) and light (12 hr photoperiod). The initial (Time 0) and final (Time 96) shell length (SL) of the larvae were recorded to the nearest 25 µm for each test using an eyepiece micrometer and a dissecting scope (40X). Daily growth rates (µm/d) were calculated at 96 hrs for surviving larvae. Temperature, pH, salinity, and dissolved oxygen were checked each day during the experiment, and the water was not changed. A PROBIT analysis was used to determine the LC_{50} values and survival and growth rates (µm/d) were compared among treatments using ANOVA followed by a Tukey's Studentized range test for pairwise comparisons (SAS v. 9.2, SAS 2005).

Acute Toxicity Experiments - Fed Larvae

Based on the results of the first acute toxicity test, and following the analysis of the remaining field samples (September and October 2010, CHAPTER 2), a second acute toxicity test using the same copper concentrations (0, 1, 5, 10, 15 µg/L) was conducted with larvae that were fed each day during the toxicity test. *Isochrysis sp.* algae was counted each day and fed to the larvae at a concentration of 3, 5 or 7 million cells/ml depending on the life stage. This was done to determine if the differences in larval survival and daily growth rates between the control and the copper treatments were due to the lack of food or to the acute toxicity. Since the zinc levels consistently recorded in the field were significantly lower than the previously determined Lc_{50} levels, additional studies with zinc were not conducted.

Using similar methods, a 1ppm stock solution of cooper ($CuCl_2$) was prepared and serial dilutions were used to create the test concentrations. Three pieces of egg masses were collected in June 2011 from Looe Key and returned to the laboratory. The eggs were combined and allowed to hatch in filtered and UV treated laboratory saltwater inside a temperature controlled incubator. Fifteen swimming larvae were added to the five treatments at six replicates each. Each age group was observed every 24 hrs for 96 hrs and the number of dead larvae were recorded. A PROBIT analysis was used to determine the LC_{50} values and survival rates were compared among treatments using ANOVA followed by a Tukey's Studentized range test for comparative differences between treatments (SAS v. 9.2, SAS 2005). Percent survival data were arcsine (

transformed to meet assumptions of normality.

Table 3.1. Known effective and lethal copper and zinc concentrations with molluscs, other marine invertebrates, and algae species.

Exposure Type	Organism	Habitat	Value	[Cu]	[Zn]	Reference
Water	*Strombus gigas*	Marine	LC_{50}	1.0-8.4 ug/L	8.9 - >40 ug/L	This study
Water	*Isochrysis sp.*	Marine	IC_{50}	2.0 - 4.9 ug/L	-	This study
Water	*Haliotis rubra*	Marine	LC_{50}	87 ug/L	1730 ug/L	Gorski and Nugegoda 2006
Water	*Haliotis diversicolor supertexta*	Marine	LC_{50}	-	1,200 ug/L	Liao and Lin 2001
Water	*Haliotis cracherodii*	Marine	LC_{50}	50 ug/L	-	Martin et al. 1977
Water	*Haliotis rufescens*	Marine	LC_{50}	65 ug/L	-	Martin et al. 1977
Water	*Crassostrea gigas* embryos	Marine	EC_{50}	5.3 ug/L	119 ug/L	Martin et al. 1981
Water	*Mytilus edulis* embryos	Marine	EC_{50}	5.8 ug/L	175 ug/L	Martin et al. 1981
Water	*Cancer magister* larvae	Marine	EC_{50}	49 ug/L	232 ug/L	Martin et al. 1981
Water	Sea Urchin (*P. lividus*) embryos	Marine	EC_{50}	66.7 ug/L	-	Fernandez and Beiras 2001
Water	*Crangon crangon* larvae	Marine	ET_{50}	330 ug/L	-	Conner 1972
Water	*Carcinus maenas* larvae	Marine	ET_{50}	600 ug/L	1000 ug/L	Conner 1972
Water	*Homarus gammarus* larvae	Marine	ET_{51}	330-1000 ug/L	-	Conner 1972
Water	Bivalve embryos and larvae (*a review*)	Marine	EC_{50}	24 ug/L	320 ug/L	His et al. 1999
Water	*Hydrozoa* sp.	Marine	LC_{50}	25-84 ug/L	11,000 - 14,000 ug/L	Karntanut and Pascoe 2002
Water	*Ruditapes decussatus* embryos	Marine	EC_{50}	8.6-9.6 ug/L	107.3-156.7 ug/L	Bieras and Albentosa 2004
Water	*Mytilus galloprovincialis* embryos	Marine	EC_{50}	9.1-10.9 ug/L	160-320 ug/L	Bieras and Albentosa 2004
Water	*Pomacea paludosa*	Freshwater	LC_{50}	19.47-182 ug/L	-	Rogevich et al. 2008
Water	*Raphidocelis subcaptitata* (microalgae)	Freshwater	E_bC_{50}	-	39-117 ug/L	Muyssen and Janssen 2001
Water	*Chlorella vulgaris* (microalgae)	Freshwater	E_bC_{50}	-	34-105 ug/L	Muyssen and Janssen 2001

Microalgae IC_{50} Experiment

This experiment was conducted in July 2011. Monocultures of *Isochrysis sp.* were obtained from the HBOI Aquaculture algae facility at when cell counts reached 9 to 12 million cells/ml. This microalgae is commonly used to feed *Strombus* larvae in culture and is one of their natural food sources in the wild (Davis, 1998; Davis, 2000; Davis and Shawl, 2005). Using the same range of copper concentrations found in field surface waters (CHAPTER 2), a 250 ml Erlenmeyer flask was filled to 200 ml with sterilized seawater, an aliquot of copper solution, and approximately 20 ml of algae (based on the actual count) to equal a starting cell count of 0.5 x 10^6 cells/ml. The seawater used to make the 1 ppm copper stock solution and the clean water was UV sterilized, autoclaved, and inoculated with commercially available microalgae nutrients. A 5 ml plastic pipet was inserted into each flask to bubble in carbon dioxide from the existing lines in the laboratory. The experimental flasks were placed in a temperature controlled room and exposed to light 24 hrs a day. The flasks were distributed in three rows to ensure even lighting amongst treatments.

There were six replicates of each copper concentration (0, 1, 5, 10, 15 µg/L) and using a hemocytometer, the algae was examined every 24 hrs for 96 hrs for motility (percent) and cell division (actual cell count). The IC_{50} values, concentration at which growth is 50% inhibited, were calculated using a linear extrapolation method (Levy et al., 2007; 2008). Significant differences in percent motility amongst treatments was determined by ANOVA followed by a Tukey's Studentized range test for comparative differences between treatments (SAS v. 9.2). Growth data were log(x+1) transformed to meet assumptions of normality.

RESULTS

Larval LC_{50} Experiments

Range Tests

Larvae exposed to the zinc solutions had better survival during the two initial range tests than those subjected to higher levels of copper. Newly hatched larvae exposed to the higher range of copper solutions (>10 µg/L) rarely survived until 96 hrs. Results were mixed with the two and three week old veligers, although most larvae did not survive past 24 or 48 hrs in concentrations higher than 15 µg/L. At these levels, the soft body parts often appeared to "melt" into the shell so that no distinguishable lobes or anatomical features could be identified. This often occurred within the first 24 hrs after exposure.

Survival was over 80% for larvae of all age groups when exposed to zinc concentrations as high as 400 µg/L. However, at levels ≥75 µg/L, larvae were observed swimming erratically in circles at 24 hrs, and their lobes were shriveled by 48 hrs. At concentrations ≥ 225 µg/L, some of the dead larvae appeared to be "melted" as was observed in the high copper concentrations and the survivors had shriveled lobes. Levels in the field were never more than 40 µg/L, therefore, that was the highest solution used in the acute toxicity tests.

Acute Toxicity Tests

The LC_{50} levels for copper decreased over time with the highest tolerance observed in the beginning (**Table 3.2**) and the lowest LC_{50} level at 96 hr. Newly hatched larvae (Days 1-4) did not appear to be any more susceptible to copper toxicity than the older larvae (**Table 3.2**) for all four time periods (24, 48, 72, 96 hr). However, there were differences in behavior and development and most larvae were completely deformed ("melted") in concentrations ≥5 µg/L (**Table 3.3**). The oldest larvae seemed to be slightly more sensitive to copper after 72 and 96 hrs. Survival rates differed significantly within each age group ($F_{5,30}$ = 53.35, p<0.0001 for Days 1-4 larvae; $F_{5,30}$ = 38.35, p<0.0001 for Days 8-12 larvae; and $F_{5,30}$ = 25.89, p<0.0001 for Days 15-19 larvae). Survival rates were lower for larvae exposed to copper concentrations above 5 µg/L (**Fig. 3.1**).

All larvae had a high tolerance to zinc and a LC_{50} could not be calculated until 96 hrs (**Table 3.2**). Newly hatched larvae had the lowest LC_{50} (8.9 µg/L) compared to two week (24.3 µg/L) and three week old larvae (17.7 µg/L). Survival rates were significantly lower in the 40 µg/L than all other treatments, but only for veligers two ($F_{5,30}$ = 12.64, p<0.0001) and three ($F_{5,30}$ = 12.07, p<0.0001) weeks old (**Fig. 3.2**). No significant difference in survival was observed for Days 1-4 larvae ($F_{5,30}$ = 1.83, p=0.1377). Concentrations ≥40 µg/L had the most detrimental effect on the larvae, causing body deformities at 96 hrs (**Table 3.**3).

However, even though there were not always differences in survival between fed and unfed larvae, there were significant differences in growth rates (Time 0 – Time 96 hr) for Days 1-4 ($F_{6,20}$ = 12.89, p<0.001) and Days 8-12 ($F_{6,10}$ = 31.81, p<0.001) but not during Days 15-19 ($F_{6,18}$ = 1.01, p=0.4467) (**Fig. 3.3**). Water quality for all tests remained within acceptable levels for queen conch culture (Davis and Shawl, 2005). Temperature average 27.1 ± 0.31 (SD)°C, pH was 7.6 ± 0.12 (SD), dissolved oxygen was 5.1 ± 0.62 (SD), and salinity averaged 36.0 ± 1.79 (SD).

Table 3.2. Acute toxicity values of copper and zinc for queen conch larvae at varying age groups(LC_{50}) and the marine microalgae *Isochrysis sp.* (IC_{50}). The copper concentrations tested were 1, 5, 10, 15 µg/L and the zinc concentrations tested were 5, 10, 20, 40 µg/L. n=6.

	24 hr	**48 hr**	**72 hr**	**96 hr**
Copper (µg/L)				
Unfed larvae				
Days 1-4	8.4	3.3	2.3	1.2
Days 8-12	8.6	4.3	2.3	1.2
Days 15-19	8.3	2.4	1.4	1.0
Fed larvae				
Days 1-4	> 15	> 15	> 15	> 15
Days 8-12	> 15	> 15	> 15	> 15
Days 15-19	> 15	> 15	> 15	> 15
Isochrysis sp.	4.9	2.6	2.0	2.0
Zinc (µg/L)				
Unfed larvae				
Days 1-4	> 40	> 40	> 40	8.9
Days 8-12	> 40	> 40	> 40	24.3
Days 15-19	> 40	> 40	> 40	17.7

Table 3.3. Developmental observations of conch larvae subjected to 96 hr acute toxicity tests where larvae were not fed. The copper concentrations tested were 1, 5, 10, 15 µg/L (Cu-1, 5, 10, 15) and the zinc concentrations tested were 5, 10, 20, 40 µg/L (Zn-5, 10, 20, 40).

Larval Group	**Controls**	**Copper Exposure**	**Zinc Exposure**
Days 1-4	**Control No Feed:** most larvae swimming through 96 hrs, developed four lobes, guts empty by 96 hrs **Control Feed**: most larvae swimming though 96 hrs, full guts, and four developed lobes by 96 hrs	**Cu-1**: most larvae continued swimming until 96 hrs, never developed four lobes and by 72 hrs the lobes began to shrivel **Cu-5**: no larvae swimming and lobes reduced by 24 hrs **Cu-10, 15**: no larvae swimming by 24 hrs and most larvae "melted" by 72 hrs	**Zn-5**: most larvae swimming until 96 hrs, developed four lobes **Zn-10**: most larvae swimming until 72 hrs, developed four lobes but were they were small **Zn-20**: most larvae swimming until 72 hrs, some had four lobes but they were stunted **Zn-40**: most larvae only swimming first 24 hrs and they did not develop four lobes
Days 8-12	**Control No Feed:** most larvae swimming through 96 hrs, continued to develop six lobes, guts empty by 96 hrs **Control Feed**: most larvae swimming though 96 hrs, full guts, and continued to develop six lobes	**Cu-1**: most larvae swimming at 24 hrs but had lobes decreased from six to two, swimming ceased by 72 hrs **Cu-5**: no larvae swimming and lobes reduced and shriveled by 24 hrs, by 48 hrs bodies were "melted" **Cu-10, 15**: no larvae swimming by 24 hrs and most larvae "melted" by 24 hrs	**Zn-5**: most larvae swimming until 96 hrs, six lobes continued developing like controls **Zn-10**: most larvae swimming until 96 hrs, maintained six lobes but they were small **Zn-20**: most larvae swimming until 72 hrs, stunted six lobes **Zn-40**: no larvae only swimming by 24 hrs and most had "melted" bodies
Days 15-19	**Control No Feed:** most larvae swimming through 96 hrs, normal development although smaller than feed control **Control Feed**: most larvae swimming though 96 hrs, full guts, normal development	**Cu-1**: few larvae swimming by 24 hrs, six reduced lobes **Cu-5**: no larvae swimming and lobes reduced and shriveled by 24 hrs, by 48 hrs bodies were "melted" **Cu-10, 15**: no larvae swimming by 24 hrs and most larvae "melted" by 24 hrs	**Zn-5**: most larvae swimming until 96 hrs, six lobes continued developing like controls **Zn-10**: most larvae swimming until 96 hrs, reduced six lobes **Zn-20**: most larvae swimming until 72 hrs, stunted six lobes **Zn-40**: most larvae swimming until 48 hrs, lobes shriveled by 48 hrs, and most had "melted" bodies by 72 hrs

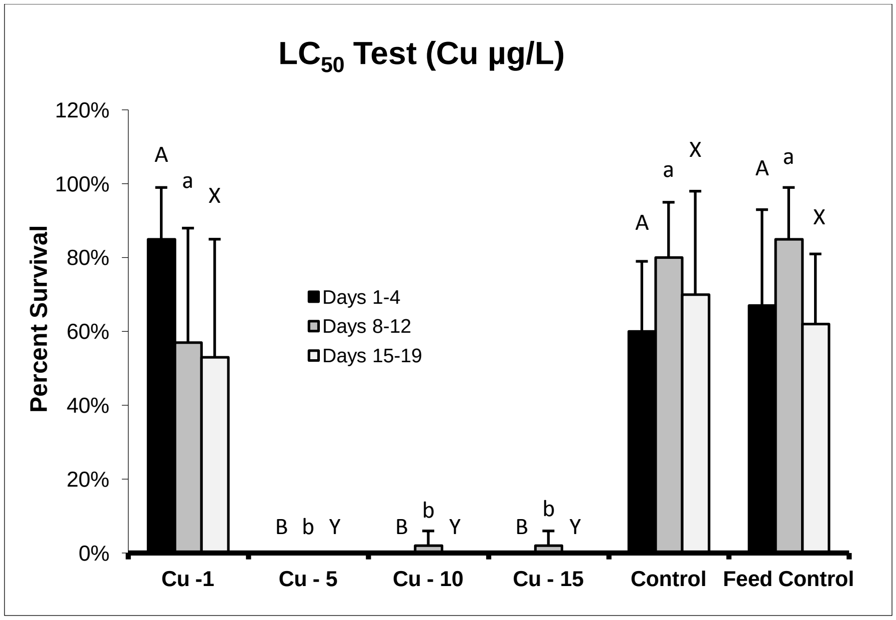

Fig. 3.1. Percent survival for queen conch larvae exposed to copper solutions (1, 5, 10, 15 µg/L). Letters above columns denote statistical differences within each age group as determined by an ANOVA (n=6).

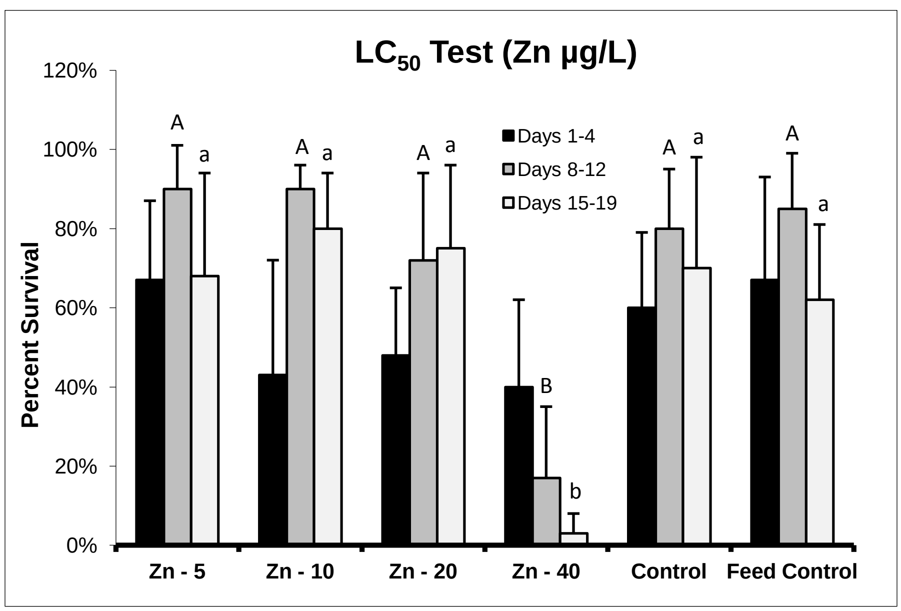

Fig. 3.2. Percent survival for queen conch larvae exposed to zinc solutions (15, 10, 20, 40 µg/L). Letters above columns denote statistical differences within each age group as determined by an ANOVA (n=6).

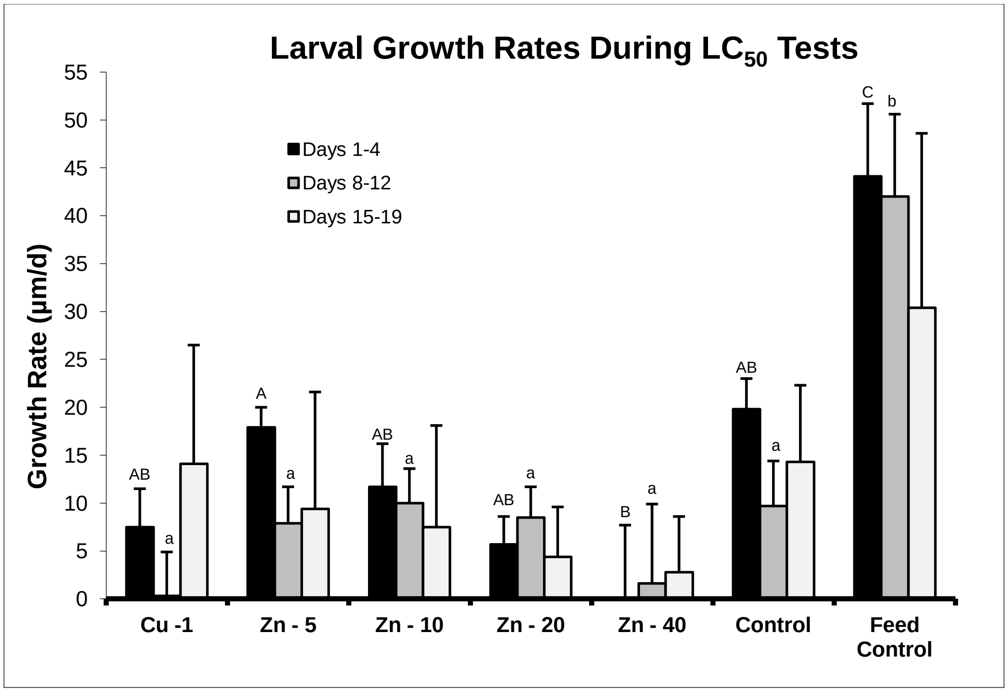

Fig. 3.3. Growth rates (µm/d) of surviving larvae after 96 hrs in copper and zinc solutions. Letters above columns denote statistical differences within each age group as determined by an ANOVA (n=6).

When the experiment was repeated in 2011 and the larvae were fed each day, LC_{50} levels for copper could not be calculated as the mortalities never reached 50%, even after 96 hr (**Table 3.2**). Total percent survival did not significantly differ ($F_{4,25}$ = 1.79, p=0.1631) for the newly hatched larvae or the oldest group of larvae ($F_{4,25}$ = 0.92, p=0.4700) (**Fig. 3.4**). However, two week old larvae had significantly lower ($F_{4,25}$ = 4.30, p=0.0087) survival in the 1 µg/L compared to the control, although this was not seen for the higher concentrations (**Fig. 3.4**). Even though there were no significant differences in survival detected, there did seem to be more pronounced developmental differences within the oldest group of larvae (**Table 3.4**). Reduced lobes and slower swimming was seen within 24 hrs for conch exposed to 10 and 15 µg/L in the two older age groups. The newly hatched larvae appeared to more tolerant to copper when being fed, as their swimming behaviors weren't as disrupted until 72 hrs, despite seeing reduced lobes in copper ≥5 μg/L. Whereas, Day 15-19 aged larvae began the experiment with six long lobes, but those had been completely reduced to less than two well defined lobes after 96 hrs of exposure.

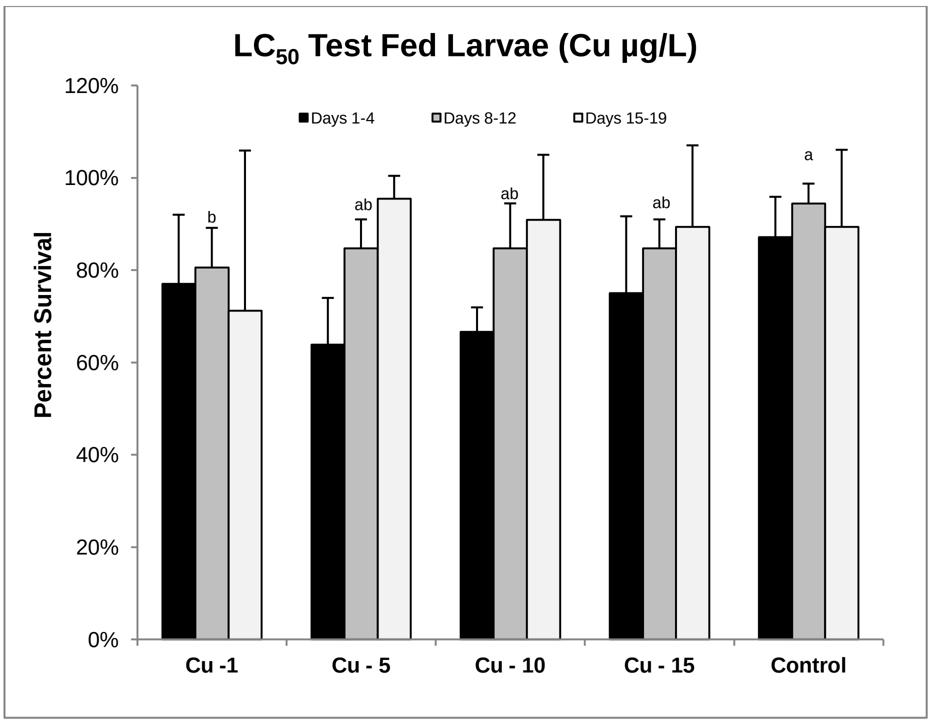

Fig. 3.4. Percent survival for queen conch larvae exposed to copper solutions while being fed. Letters above columns denote statistical differences within each age group.

Table 3.4. Development and behavioral observations of fed conch larvae subjected to 96 hr acute toxicity tests. The copper concentrations tested were 1, 5, 10, 15 µg/L (Cu-1, 5, 10, 15).

Larval Group	**Control**	**Copper Exposure**
Days 1-4	**Control**: most larvae swimming though 96 hrs, full guts, and four developed lobes by 96 hrs	**Cu-1**: most larvae continued swimming until 72 hrs, developed four lobes but they were small **Cu-5**: few larvae swimming after 48 hrs and lobes reduced by 72 hrs **Cu-10:** most larvae not swimming at 48 hrs, although they seemed to recover by 96 hrs, reduced lobes **Cu-15**: most larvae swimming by 96 hrs, reduced lobes
Days 8-12	**Control**: most larvae swimming though 96 hrs although some were lethargic at 96 hrs, full guts, continued six lobe development	**Cu-1**: most larvae swimming until 72 hrs, lobes were reduced **Cu-5**: few larvae not swimming and reduced lobes by 24 hrs **Cu-10**: few larvae swimming by 96 hrs, reduced lones by 72 hrs **Cu-15**: few larvae swimming by 72 hrs, reduced lobes, swimming on bottom
Days 15-19	**Control**: most larvae swimming though 96 hrs, full guts, and six long developed lobes through 96 hrs	**Cu-1**: few larve swimming by 96 hrs although lobe development resembled controls, full guts **Cu-5**: few larvae swimming by 96 hrs, lobes continued developing, guts full at 48 hrs **Cu-10**: shriveled lobes at 24 hrs and swimming slowly, lobes reduced at 48, 72, and 96 hrs **Cu-15**: small, shriveled lobes and suspended in middle, not really swimming at 24 hrs, reduced to four lobes by 48 hrs, smaller body and lobes at 72 hrs, lobes less than two stage by 96 hrs although still alive.

Microalgae IC_{50} Experiment

Although none of the algae cultures completely died, cell division was greatly inhibited at copper concentrations less than 5 µg/L (**Table 3.2**). Algae exposed to 5, 10, and 15 µg/L had very little cell division over the 96 hr study (**Fig. 3.5**). The control algae continued to reproduce at rates that are consistently seen with laboratory cultures, and algae exposed to 1 µg/L of copper grew at rates similar to the control for the entire experiment. Algae in the 5 µg/L solutions had similar growth rates as the control and 1 µg/L at the 24 hr sampling, however, the growth diminished after that point. Motility decreased significantly by 48 hr (**Fig. 3.6**), where the algae in the control and 1 µg/L solutions had motility above 80%, and motility significantly dropped in the 5 µg/L ($p<0.0001$), 10 µg/L ($p<0.0001$.), and 15 µg/L ($p< 0.0001$) concentrations when compared to the control. By 96 hr, the motility decreased in all copper solutions although there were still no differences detected between the control and 1 µg/L treatments ($p=0.1189$), the 1 µg/L and 5 µg/L ($p=0.2516$) and between all three high concentrations. No instances of cell lysing or cell bloating was observed.

DISCUSSION

Copper and zinc were present in the surface water of all four sites sampled in the Florida Keys (CHAPTER 2), and the copper levels regularly exceeded the state and federal water quality criteria (<3.7 µg/L). Queen conch larvae that were not fed throughout the four day experiment demonstrated acute toxicity of copper at levels from 1.0 – 8.6 µg/L each day and zinc toxicity by 96 hrs (8.9 – 24.3 µg/L) (**Table 3.2**).

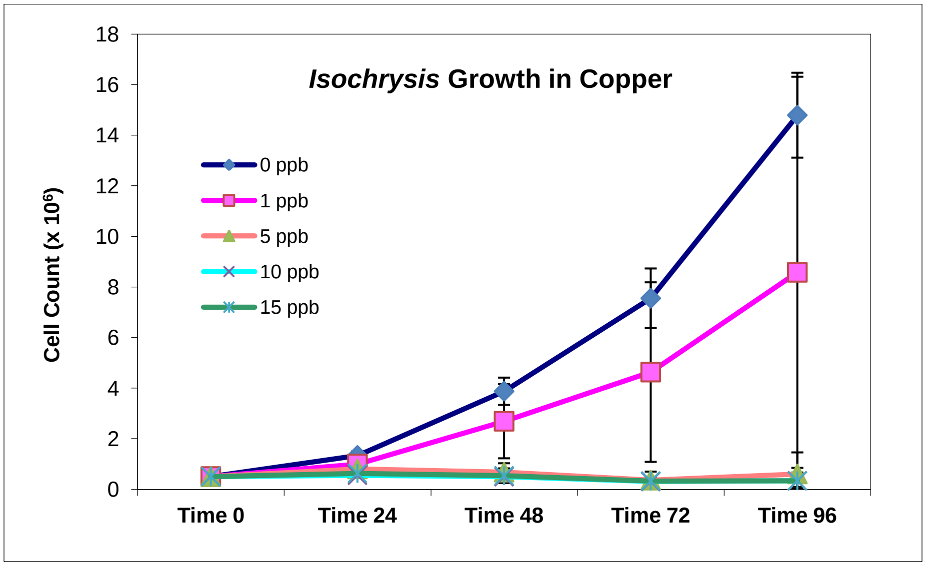

Fig. 3.5. Mean growth of *Isochrysis sp.* over 96 hrs for algae exposed to copper solutions as determined by an ANOVA (n=6, ±SD).

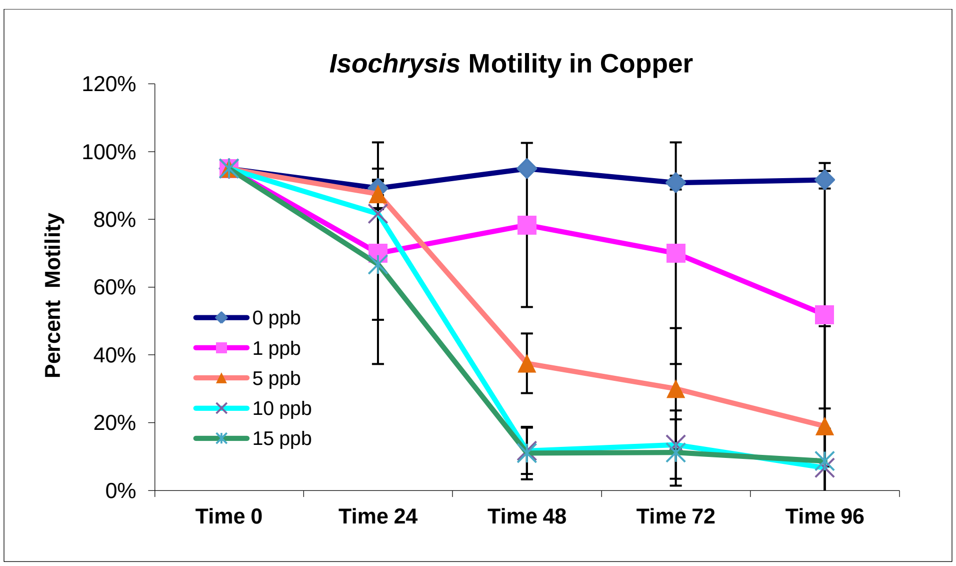

Fig. 3.6. Mean percent motility counts of *Isochrysis sp.* over 96 hrs for algae exposed to copper solutions as determined by an ANOVA (n=6, ±SD).

These values are similar to those observed for oyster, mussel, and clam embryos (Bieras & Albentosa, 2004; Martin et al., 1977; **Table 3.1**). In this study, unfed queen conch larvae seemed to be more susceptible to copper and zinc toxicity than unfed abalone, urchin, hydroza, and crustacean and bivalve larvae in general (Conner, 1972; Fernández & Beiras, 2001; Gorski & Nugegoda, 2006a; Karntanut & Pascoe, 2002; Liao & Lin, 2001; Martin et al., 1977).

When food was added in minimal increments each day, the acute toxicity of copper was above the tested 15 µg/L and therefore, an LC_{50} value could not be calculated. This suggests that conch larval development and survival is closely related to feed uptake and may play an important role in their ability to recruit when exposed to metal contaminated waters. Acute toxicity tests (48-96 hrs) generally do not include a feeding regime (Gorski & Nugegoda, 2006a; Ong & Din, 2001; Rogevich et al., 2008), therefore, based on the results of the unfed study, queen conch larvae are more susceptible to heavy metal toxicity than larvae of other molluscan taxa. Unlike bivalve larvae, queen conch are incapable of completely closing their shell and preventing the penetration of toxic solutions. This closing reaction and the ability to stay closed will often account for greater resistance of bivalves to metal contamination (Wisely & Blick, 1967). However, bivalves with closed shells are also unable to feed. In cases of higher copper concentrations (often ≥5 µg/L) queen conch larvae had shriveled velar lobes and were not as motile as conch exposed to little or no copper. Like the bivalves, a reaction like this may also hinder their ability to feed sufficiently (Davis, 1998). In the experiment where larvae were fed, it did appear that there was food in their guts despite differences in lobe length development and motility.

In this study, *Isochrysis sp.* was impacted by levels of copper as low as 2.0 µg/L, which is lower than the water quality criteria (<3.7 µg/L). Cell division was severely reduced at concentrations over 1 µg/L and motility decreased after 48 hr of exposure. Reduced cell division has been seen in other phytoplankton species exposed to copper (Brand et al., 1986; Moreira et al., 2006). Levy (2008) found growth inhibition at 8 µg/L copper for *Phaeodactylum tricornutum* diatoms. Algae motility and density is important for queen conch larvae who utilize their lobes to capture microalgae cells. Non-motile algae will sink and is an unsuitable food source for this planktotropic larvae (Davis, 1998).

Marine algae can effectively remove heavy metals from their environment (Kaewsarn, 2002; Matcheickal & Yu, 1999). The addition of small quantities of algae to the copper solutions during the second acute toxicity experiment may have reduced the copper available to the conch through the water, thus increasing the LC_{50} values. Studies conducted with marine cyanobacteria have demonstrated their strong chelating properties, and ability to modify the copper chemistry in seawater (Moffett et al., 1997; Moffett & Brand, 1996). Freshwater microalgae can develop an increased tolerance to copper when exposed to high levels, and the same species developed a parallel tolerance to zinc (Gustavson and Wangberg, 1995). The mechanism by which algae cells uptake heavy metals is a two step process, and is well defined but can be impacted by numerous environmental variables (Arunakumara and Xuecheng, 2008). Therefore, under field conditions, copper uptake by the conch from water and algae may be affected by other phytoplankton and environmental parameters in the system that were not explicitly tested in these experiments.

In studies comparing the sensitivity between larval and adult marine molluscs, the early life stages are typically more sensitive than adults (Conner, 1972). Larvae in this experiment not only had higher mortality with increased copper and zinc concentrations, but they also demonstrated slower swimming behavior and their lobes were stunted or deformed. Juvenile queen conch exposed to copper (400 and 1,100 µg/L) in flow through bioassays had reduced grazing rates and had a longer righting response time (Sanders, 1984). Although behavior has not been reported, adult queen conch will accumulate copper and zinc when exposed to contaminated sediment (Díaz Rizo et al., 2010; Spade et al., 2010). Since there has been less recruitment of juvenile queen conch in the Keys than would be expected after a prolonged fisheries closure, it is quite possible that heavy metal contamination may be playing a role. Conch that are incapable of feeding (due to lobe impairment or grazing reduction) or that are unable to right themselves for protection, will have shorter life spans and may not survive until reproductive age.

This study has demonstrated the toxic impacts of copper and zinc on the early life stages of queen conch and a representative phytoplankton food source. Although conch capable of feeding may have a higher resistance to copper, the levels examined in this study are sometimes exceeded in the surface water criteria in the Keys (Lewis et al., 2007). Future experiments that examine the effect of intermittent exposure and recovery capabilities should be examined to understand the impacts of heavy metals on queen conch larval survival and recruitment as they drift in and out of contaminated surface water in the Florida Keys.

CHAPTER 4: EFFECTS OF CHRONIC AND INTERMITTENT EXPOSURE OF COPPER ON QUEEN CONCH LARAVAL DEVELOPMENT

ABSTRACT

Copper is present in the surface water and phytoplankton food source for queen conch larvae in the Florida Keys. Due to its patchiness, an experiment was designed to test the effects of copper on growth, development, and survival of queen conch veligers during their complete larval cycle (chronic, 21-20 d)) and through intermittent (7-10 d) exposure during different developmental stages. Sublethal (3.9 µg/L) levels of copper were added to the water and food and larvae were exposed during the entire larval cycle (27 d), week 1 only, week 2 only, week 3 only, or no exposure at all. They were monitored daily from hatch to one week post-metamorphosis. Additionally, unexposed larvae were induced for metamorphosis in one of four copper concentrations (0, 3.9, 10, or 15 µg/L) and assessed for settlement success and survival. The presence of copper had a greater impact on larvae that were exposed later in their cycle (Days 15 and higher) or chronically exposed. These larvae had slower growth, disrupted lobe development, and poor survival one week post-metamorphosis when compared to larvae exposed earlier in their life. Although there were no differences in survival during settlement for larval exposed during the cycle, larvae exposed to copper during metamorphosis had significantly decreased success rates than the controls. The results of

these experiments demonstrate that juvenile conch may not be surviving long enough to recruit to the adult populations of conch in the Florida Keys and their reaction to copper toxicity is dependent upon age and exposure duration.

INTRODUCTION

The presence of copper in the Florida Keys is a concern for queen conch development and survival. Metals in the surface water and phytoplankton can directly impact the larvae, whereas copper in the sediment and seagrass epiphytes can be consumed by grazing juveniles and adults (CHAPTER 2, 3). Studies with adult and juvenile queen conch have shown that chronic intake of copper through the food (Sanders, 1984) or from exposure to contaminated sediments (Díaz Rizo et al., 2010; Spade et al., 2010) impacts grazing behavior and will accumulate in the muscle tissue and digestive glands. Research with other gastropod species such as land snails (*Helix* sp.) and freshwater apple snails (*Pomacea* sp.) has shown that copper absorbed from the sediment or ingested with food will accumulate in the tissues, inhibit growth, change cellular structures in the digestive gland, and affect oocyte maturity (Gomot and Pihan, 1997; Swaileh et al., 2002; Scheifler et al., 2003; Snyman et al., 2004; Snyman et al., 2005; Hoang et al., 2008a,b). Aside from looking a depuration periods, none of these studies evaluated the impact of intermittent exposure in terms of survival and recovery.

Research to understand the impacts of chronic metal toxicity on invertebrates has been mostly limited to a few species of mollusks (Gorski and Nugegoda, 2006a,b), and no research has been conducted with queen conch (or any *Strombus* sp.) larvae. The effect of copper on the larvae may provide answers to the biological success or failure of queen conch populations in a polluted environment. Since the conch populations in the Florida Keys have not recovered despite the quarter century fishery closure, and conch nearshore are incapable of reproduction (Delgado et al., 2004), it is reasonable to assume that population recruitment is in jeopardy. Results from CHAPTER 2 indicate that copper is available to the larvae in the water and phytoplankton, and concentrations of copper vary on a spatial and temporal scale at the four sites sampled. Therefore, it is important to consider the exposure times during the larval cycle and duration of that exposure to better understand the implications on larval recruitment.

Within the Florida Keys, there are numerous gyres that contribute to the local retention of queen conch larvae (**Fig. 4.1**; Delgado et al., 2008). This implies that conch populations in the Keys are somewhat reliant upon self recruitment and that the lack of recovery could be attributed to local environmental factors. Conch can disperse for a period of 2 to 8 weeks in Florida and the Caribbean (Davis, 1998). As conch larvae potentially drift between nearshore and offshore habitats in the Florida Keys, they may be exposed to varying degrees of copper toxicity (CHAPTER 2), which is known to effect development and survival (CHAPTER 3).

Research on larval stages of abalone (Hunt and Anderson, 1989; Conroy et al., 1995; Gorski and Nugegoda, 2006b), bivalves (Phelps and Mihursky, 1986; Beaumont et al., 1987; Beiras and Albentosa, 2004), polychaetes (Xie at al., 2005; Gopalakrishnan et al., 2007), sea urchins (Fernández and Beiras, 2001), horseshoe crabs (Itow et al., 1998), and midges (Jannssens de Bisthoven et al., 1998) have all shown detrimental effects on

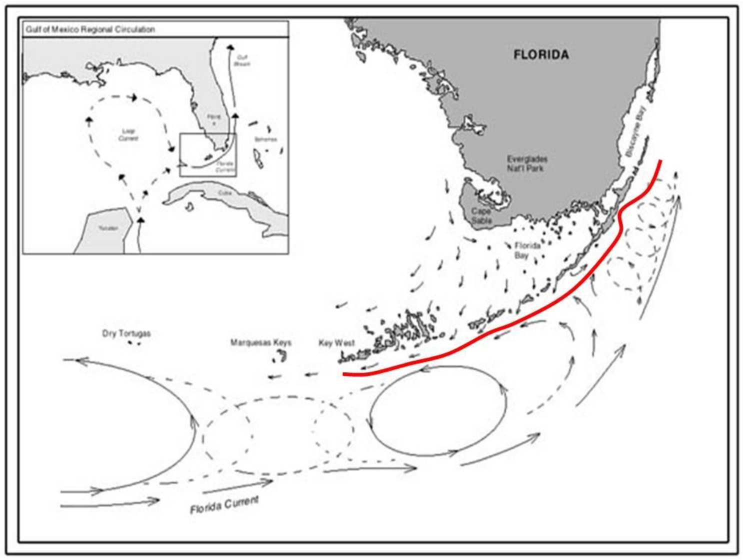

Figure 4.1. Schematic map of the Florida Keys indicating major current and gyre circulation. The red line shows Hawk's Channel that physically separates nearshore and offshore conch populations. From Klein and Orlando, 1994.

development, growth, survival, and metamorphic success. Lethal concentrations of copper were found to exist in the Florida Keys in areas where conch larval recruitment should be occurring (CHAPTER 2), and it is hypothesized that sublethal levels will have an impact on all larval stages.

This experiment tested whether or not chronic and intermittent exposure of sub-lethal copper levels to the larvae through water and food will have different effects at varying developmental stages. Since zinc was not found at concentrations deemed to be toxic to the larvae (CHAPTERS 2, 3), it was decided that only copper would be tested since it was present in toxic levels higher than state water quality standards ($\geq$ 3.7 µg/L) at all sites throughout the year. Results from this study will help determine how copper in the local environment may be impacting successful larval recruitment both nearshore and offshore in the Florida Keys.

MATERIALS AND METHODS

Three pieces of queen conch egg masses were collected from Looe Key, lower Florida Keys, in August 2011. Each piece was placed in a 2L thermos filled with field saltwater and returned to the laboratory within 24hrs. The eggs were combined and allowed to hatch in a container with filtered and UV sterilized laboratory saltwater inside a temperature (28ºC) controlled incubator, and hatching occurred 96 to 120 hrs after collection.

Immediately after hatch, a subsample of 30 larvae were measured for initial shell length (µm) using an eyepiece micrometer on a dissecting microscope (40X). Measurements of all larvae were conducted weekly and growth rate was calculated at µm/d. Copper chloride (ACROS Organics, 97% pure) granules were weighed on a digital scale (306.92 mg) and a 100 ppm (mg/L) solution was prepared. This was further diluted to a 1 ppm stock solution, followed by a serial dilution using the formula $C_1V_1=C_2V_2$ to obtain the desired experimental concentration. Analytical analyses (ICP) conducted by the Harbor Branch Environmental Laboratories were used to verify the stock concentrations (actual values of 1.2–1.3 ppm) and to test the laboratory seawater (under detectable limits).

Larvae were exposed to the copper solutions at different stages of their development. This was to emulate the larvae being exposed to varying levels of copper concentrations that were observed in the field (CHAPTER 2). It is likely that the larvae drift in and out of these areas during their three to four week larval cycle. The larval exposure treatments chosen were:

Treatment 1: Early Exposure (during Days 1-7)

Treatment 2: Middle Exposure (during Days 8-14)

Treatment 3: Late Exposure (during Days 15 – metamorphosis)

Treatment 4: Chronic Exposure (exposed Day 0 – metamorphosis)

Treatment 5: Control (no exposure)

Conch were exposed to a copper concentration of 3.9 µg/L (ppb) through both the water and their microalgae diet. This level was chosen since it is the surface water quality criteria for the Florida Keys and is similar to other EPA guidelines for surface saltwater nationwide (3.1 – 3.8 µg/L chronic), and because 3.9 µg/L is the chronic concentration at which 90% of molluscs are affected (Schuler et al., 2008). Since the conch larvae are planktotrophic, they were fed a microalgae cultured in a 3.9 µg/L copper solution during the timeframe when they were exposed to contaminated water. Copper was added to the microagle *Isochrysis sp.* cultures, following methodology outlined in CHAPTER 3. The ICb_{50} for copper and *Isochrysis sp.* was above 5 µg/L, so the tested concentration did not have a significant impact on cell division and motility. Cultures were no more than four days old, and cell motility and cell count was always assessed before feedings. If the larvae were in clean seawater, they were fed clean microalgae with no copper.

Fifteen larvae were added to 1L glass beakers filled to 600 ml with the test solution at six replicates each. The beakers were held in a temperature and light controlled incubator at 28ºC and 12 hr light:12 hr dark cycle. Larvae were fed microalgae (*Isochrysis sp.*) each day as previously established for laboratory static culture (McIntyre, 2005). Every other day, the water was changed and new solutions were prepared. This was done by carefully pouring the water and larvae through a 100 µm sieve and rinsed back into the treatment with laboratory seawater. Beakers were washed with alconox, hydrochloric acid, and then rinsed with deionized water before being refilled. Larvae were observed under the microscope for mortalities and gut fullness every other day, shell length weekly, and photographed on Days 8 and 15 to observe lobe development.

Larvae in the Treatment 1 and Treatment 2 were exposed to copper contaminants for seven days. Larvae in Treatment 3 (late exposure) were exposed from Day 15 through metamorphosis, which was Day 27. Typically, larvae are competent for metamorphosis by Day 21-25 days (Davis, 1994; Davis and Shawl, 2005). The larvae in all replicates of each treatment were induced to metamorphose when the majority of the larvae showed signs of competence (Davis and Stoner, 1994). To induce metamorphosis, the larvae were set in 100 ml glass watch dishes in 50 ml of clean seawater. Two pieces of seagrass blades (5 cm) collected from a local *Thalassia testudinum* bed in the Indian River Lagoon were placed in each dish. The diatoms and epiphytes on the seagrass blades were used to trigger metamorphosis (Davis and Stoner, 1994). Several test batches to verify the efficiency of this method were completed earlier in the summer.

After 24 hr, the larvae were removed from the glass dishes and observed for survival and metamorphosis. Mortalities were removed and the remaining larvae were placed into clean seawater and observed again 24 hrs later for a final metamorphosis count (48 hr post-set). One week post metamorphosis, survival was again assessed for all treatments.

An additional experiment was conducted to determine the effects of copper on metamorphic success of conch not previously exposed to the metal during their larval cycle (Treatment 6). Extra larvae from the initial experiment were cultured in 13L polyethylene containers using methods described in CHAPTER 3 (Davis and Shawl, 2005). Once competent, ten larvae were placed in one of three treatments (3.9, 10, or 15 µg/L copper), plus a control at three replicates each. Two pieces of seagrass blades (5 cm) were added to 50 ml of seawater. Larvae were removed from the solution at 24 hrs and added to clean seawater. Final observations of metamorphosis and survival were recorded after 48 hrs.

Data for both experiments was tested for homogeneity of variances with Levene's test and assumptions normality of distribution using the Shapiro-Wilk test. No values needed to be transformed. Differences in survival, daily growth rates, size at metamorphosis, percent metamorphosis, and one-week post metamorphic survival were tested with an ANOVA followed by a Tukey's Studentized range test for comparative differences between treatments (SAS v. 9.2).

RESULTS

Water temperature remained constant in the incubator (27-28°C), and laboratory seawater did not contain any detectable levels of copper. The age at which the larvae

Table 4.1. Results from the chronic and intermittent exposure experiments. Results are expressed in mean ± SD (n=6) and letters denote significant differences ($p<0.05$) in each row.

Exposure level = 3.9 ppb Cu	**Control (No Exposure)**	**Treatment 1 (Early Exposure)**	**Treatment 2 (Middle Exposure)**	**Treatment 3 (Late Exposure)**	**Treatment 4 (Chronic Exposure)**
Final survival (larvae)	56.7 ± 22.6 ^{a}	88.9 ± 5.4 ^{b}	83.3 ± 11.0 ^{b}	82.2 ± 10.0 ^{b}	77.8 ± 13.8 ^{b}
Days to metamorphosis	30	21	25	27	27
Growth rate (µm/d)-Day 1-7	50.8 ± 4.6 ^{ab}	58.7 ± 3.1 ^{a}	48.9 ± 5.2 ^{b}	49.3 ± 7.7 ^{b}	58.0 ± 2.9 ^{a}
Growth rate (µm/d)-Day 8-14	23.2 ± 4.2 ^{a}	48.6 ± 1.8 ^{b}	49.8 ± 7.0 ^{b}	33.4 ± 5.8 ^{c}	51.4 ± 3.2 ^{b}
Growth rate (µm/d)-Day 15-Meta	20.0 ± 2.7 ^{a}	19.7 ± 5.3 ^{a}	20.8 ± 6.9 ^{a}	23.3 ± 5.5 ^{a}	4.9 ± 3.3 ^{b}
Size at metamorphosis (µm)	1022.3 ± 11.0 ^{ac}	1136.3 ± 13.3 ^{b}	1072.2 ± 17.1 ^{a}	991.0 ± 16.9 ^{c}	1058.2 ± 9.2 ^{a}
Percent metamorphosis	45.0 ± 13.5 ^{ab}	58.4 ± 26.8 ^{a}	57.4 ± 13.6 ^{a}	15.0 ± 15.2 ^{b}	32.1 ± 13.8 ^{ab}
One week survival post-metamorphosis	58.1 ± 31.3 ^{ab}	68.5 ± 16.2 ^{a}	88.6 ± 12.7 ^{a}	51.7 ± 42.3 ^{c}	18.9 ± 32.8 ^{cb}

exposed to copper did have an impact on their development, metamorphic success, and one-week post metamorphic survival. The final number of larvae that survived to metamorphic competence differed amongst treatments ($F_{4,23} = 4.72$, $p=0.0063$) and was slightly lower in the controls. This was due to a bacterial infection in one replicate resulting in the loss of the majority of the larvae. It also took longer for the control animals to reach competency (30 d) when compared to all copper treatments (**Table 4.1**). Growth rates during the first ($F_{4,23} = 6.07$, $p=0.0017$), second ($F_{4,23} = 40.52$, $p<0.0001$), and third ($F_{4,23} = 12.59$, $p<0.0001$) week varied within treatments. Larvae exposed to copper during the first week of their cycle reached competency faster (21 d) and were larger than all other treatments at metamorphosis competency ($F_{4,23} = 11.50$, $p<0.0001$). In the first week, larvae exposed to copper (Treatment 1 and Treatment 4) grew at a faster rate than the larvae in Treatment 2 and Treatment 3 that had not been exposed to the metal yet. However, none of the treatments differed from the control. Although there were no differences during the first week of growth, all larvae in the copper treatments grew faster than those in the control during the second week. The growth rate of larvae that had been subjected to copper for the entire cycle (Treatment 4) slowed greatly during the third week and differed from all other treatments.

The velar lobe development of larvae exposed to copper appeared to be hindered in some instances. After one week of exposure, larvae in Treatment 1 and Treatment 4 were behind in their lobe development in comparison to the controls (**Fig. 4.2**). By Day 15, larvae in Treatment 4 had recovered in their lobe development and continued to visually resemble the control larvae for the remainder of the experiment.

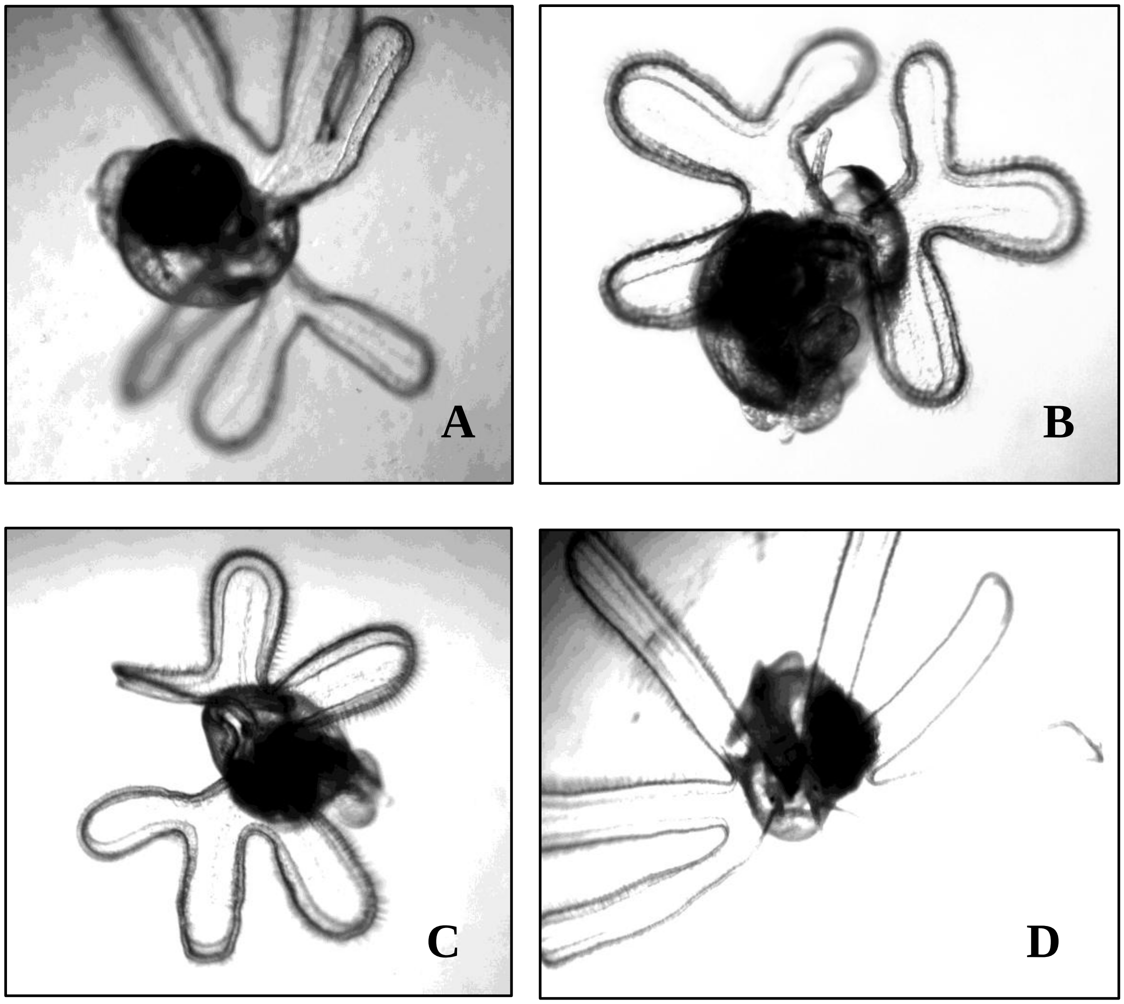

Figure 4.2. Lobe development differences observed during the chronic and intermittent exposure experiment. On Day 8, the larvae not exposed to copper (A, control) had six complete lobes, whereas the larvae exposed during the first week (Days 1-7) had not fully developed six separate lobes (B, Treatment 1). Photo C shows an 8 day old larvae chronically exposed to copper (Treatment 4), where lobe development is behind the control (A); by Day 15 their morphological characteristics (D) are identical to the controls (not shown).

Table 4.2. Metamorphic success and percent survival of conch larvae exposed to copper during metamorphosis (48 hrs post-set). Results are expressed as mean ± SD (n=3) and different letters denote significant differences (p<0.05).

	Control	3.9 ppb Cu	10 ppb Cu	15 ppb Cu
Percent metamorphosis	43.3 ± 5.8 [a]	6.7 ± 11.5 [b]	3.3 ± 5.8 [b]	3.3 ± 5.8 [b]
Percent survival	93.3 ± 5.8	86.7 ± 15.3	83.3 ± 15.3	86.7 ± 5.8

Mean percent metamorphosis ranged from 15.0 to 58.4 percent amongst the treatments and significant differences were detected ($F_{4,23} = 5.69$, p=0.0025). Larvae exposed to copper during the last week of the cycle had the poorest metamorphic success (15%), but the rate was not significantly different than the control and larvae in Treatment 4. However, larvae exposed to copper during week 1 and week 2 had a higher metamorphic success rate than those exposed in week 3. Survival one-week post metamorphosis also differed among treatments ($F_{4,23} = 9.80$, p<0.0001), where larvae either chronically exposed or subjected to copper during the last week, had lower survival than larvae exposed earlier in life (**Table 4.1**).

When competent larvae were exposed to copper during induction, their percent metamorphosis success was significantly reduced ($F_{3,8} = 19.57$, p=0.0005, **Table 4.2**). Rates for the control larvae (43%) were similar to that observed in the first experiment (45%), and were much higher than the success of the animals in the copper treatments. Metamorphic success of the competent larvae exposed to 3.9 µg/L copper for 24 hrs had a mean rate much lower (6.7 ± 11.5) than those larvae that were exposed to that level for the duration of the cycle (Treatment 4; 32.1 ± 13.8; **Table 4.1**). Although percent metamorphosis was affected by the presence of copper, there was no difference in larval survival during the 48 hr period after induction among treatments ($F_{3,8} = 0.40$, $p=0.7597$). Larvae were able to withstand the exposure concentrations, but were unable to complete metamorphosis.

DISCUSSION

Exposure to copper at sublethal levels did impact metamorphic success and post settlement survival of queen conch. Although larval survival did not appear to be hindered by copper exposure, lobe development was delayed. In the case where larvae were exposed to copper from Day 1-7 (Treatment 1), the time to metamorphic competency was faster than the other treatments (**Table 4.1**). Typically, copper inhibits normal development of invertebrate larvae. Embryogenesis success rate and the size of sea urchin larvae decreased at higher concentrations after 48 hrs of exposure (≥ 64 µg/L) (Fernández and Bieras, 2001). In mussel larvae, continuous exposure to copper (20 µg/L) produced 100% mortality within a month, and exposure at 10 µg/L caused changes in growth and behavior of mussel larvae (Beaumont et al., 1987). Beiras and Albentosa (2004) found that at levels as low as 5µg/L will reduce the embryonic success of blue mussels and carpet shell clams. Copper concentrations over 4 µg/L inhibits normal morphological development in blacklip abalone (Gorski and Nugegoda, 2006b), and was found to be the most toxic of the heavy metals. Obvious deformities, such as foot development and muscle attachment to shell, in the abalone larvae were observed after 48 hrs exposed to 3.7 µg/L of copper.

Copper can also negatively affect egg and larval development in non-molluscan invertebrate species. In polychaetes, the presence of copper at concentrations ≥10 µg/L blocked egg fertilization, reduced the survival of released oocytes, and hindered development of the newly hatched larvae (Xie et al., 2005; Gopalakrishnan et al., 2007). However in this study, chronic (27 d) exposure to a sublethal level of copper did not have as much of an impact as sporadic weekly exposure did. Although there were some observed differences in lobe development and growth rates on occasion, the biggest impact appeared to be in post metamorphic survival for queen conch.

Since copper concentrations in the water and phytoplankton vary throughout the nearshore and offshore habitats in the Keys (CHAPTER 2), it is important to determine if there is a critical period when copper is most detrimental during the larval cycle. Queen conch larvae exposed to copper during the first week of their cycle grew faster, were larger at metamorphosis, and had a shorter time to competency (**Table 4.1**). Whereas larvae exposed during the last part of their cycle seemed to be more inhibited by copper, particularly in their metamorphic success and one-week post metamorphosis survival. Studies with other heavy metals (zinc) have shown that the timing to red abalone metamorphosis was similar regardless of exposure concentration (Conroy et al., 1995), but larvae chronically exposed can have delayed competency (Hunt and Anderson, 1989). Horseshoe crab larvae exposed to zinc through pulse treatments one week at a time, had better limb regeneration than those chronically exposed (Itow et al., 1998).

Queen conch larvae that were cultured in clean seawater before metamorphosis were larger at settlement, had better metamorphic success and higher one-week post survival. However, when larvae were only exposed to copper during metamorphosis induction, success rates were severely hindered despite surviving the exposure (**Table 4.2**). Oysters also have slightly lower settlement success when exposed to copper solutions during metamorphosis (Phelps and Mihursky, 1986). Likewise, exposure to other heavy metals can also have a similar impact on gastropod metamorphic success. It has been shown that in red abalone, zinc inhibited metamorphosis and larvae also did not recover and complete metamorphosis when moved to clean seawater (Conroy et al., 1995). Those with deformed shells did not metamorphose and it is thought that metal contamination may play an important role in inhibiting or delaying metamorphosis (Hunt and Anderson, 1989; Conroy et al. 1995). For queen conch in this study, there were differences in shell length at competency, but they were similar in size to what is seen in the wild (Davis, 1998) and in culture (Davis and Shawl, 2005) and no shell deformities were observed.

In general, mollusc larval stages are more susceptible to chronic copper toxicity than adults, although there have been exceptions. Adult abalone susceptible to a nuclear power plant discharge facility had a lower tolerance to copper than their larvae exposed in the laboratory (Martin et al., 1977). Beaumont et al. (1987) found that early stage mussel larvae had higher tolerances to copper than adults, although that trend disappeared with later-stage larvae. As an explanation, it was suggested that mussel larvae metabolism may be based on lipid rather than glycogen storage early on, which will produce more metal-binding metallothioneins, and thus decrease copper toxicity (Beaumont et al., 1987). Likewise, studies with polycheates showed that, when fed microalgae, the copper concentration in the water decreased within 2 hrs from algal absorption (Xie et al., 2005). This could potentially explain why conch larvae in the early life stages (Treatment 1) performed better than those exposed during later stages. Although all treatments were fed identical amounts of microalgae, the newly hatched larvae will not always eat for the first couple of days, thus allowing the algae cells to absorb the copper from the water column.

For the most part, larvae chronically exposed to copper or those exposed in the later stages, had poorer post-metamorphic survival. This is a critical factor when considering successful larval recruitment with queen conch. Previous studies have shown that larval conch exposed to nearshore sediment from the Florida Keys had lower metamorphic success rates than those exposed to offshore sediments (Kowalik et al., 2006). Results from the current study suggest that perhaps the exposure to copper just prior to settlement may be one factor in why large juvenile aggregations have not been found nearshore.

Since queen conch in the Florida Keys are heavily reliant upon self-recruitment (Delgado et al., 2008), the results of this study indicate that the presence and availability of copper may be hindering successful juvenile recruitment into the population. Further research and long term monitoring for the presence of heavy metals (i.e., copper) in conjunction with the annual population assessments should be conducted to monitor conch recovery.

CHAPTER 5: COPPER AND ZINC IN THE FLORIDA KEYS AND THEIR CONTRIBUTION TO MARINE POLLUTION

The Impacts of Marine Pollution in the Florida Keys

As the list of marine pollutants continues to grow, their bioavailability and toxicity is brought into question. Although the focus of this research has been on two heavy metals, many types of pollutants have damaging effects on aquatic life, and often those responses are increased when multiple pollutants are present. For example, rock crabs exposed to waterborne xenobiotics in areas with heavy metal contamination showed energy compensation changes in an attempt to counter the competitive effect of metals and electrolytes (Mayrand and Dutil, 2008). Additionally, there was an initial delay in gonadal growth and the digestive glands were larger since that is where metal detoxification occurs. This means that although the crabs were adjusting to a polluted environment (i.e., low mortalities), there was a high energy cost associated with the physiological acclimatization.

Thyroid function and reproduction abnormalities in American alligators have been linked to pesticide and other chemical pollutants in some of the freshwater Florida lakes that empty into the Everglades and filter through to the Florida Bay (Hewitt et al., 2002). High nutrient levels have been recorded at Key Largo and Big Pine Key and were tied to the 24,000 septic tanks and treatment facilities in those areas (Lapointe et al., 1990). Human waste has been found in nearshore waters and canals and has affected coral reefs in the lower and middle Keys (Lipp et al., 2002). Sewage outflows in Key Largo and Big Pine Key also contained estrogen in concentrations that were fivefold higher than oceanic waters (Atkinson et al., 2003). With the hormone present at these levels, it is predicted that invertebrates, like corals, will have a net uptake of estrogen that may accumulate into the reef benthos. Estradiol injected into squirrelfish disrupted the accumulated zinc distribution in the ovaries (Hogstrand et al., 1996). Estradiol and ethynylestradiol (EE2) were found in measurable quantities both offshore and nearshore around conch aggregations (Glazer et al. 2008). Endocrine disruption has long been thought a possible cause for the lack of nearshore reproduction with queen conch, and the presence of hormones in the waters should be of concern.

Perhaps one of the most studied and a worrisome marine pollutant is mercury. Known for its ability to bioaccumulate, cycling patterns and mass budget estimates have been conducted for the Everglades and in Florida Bay. Mercury deposition, transport, accumulation, and biomagnification are higher during the wet season in the Everglades (Liu et al., 2008). Florida Bay has been under a health advisory since 1995 that recommends limited consumption of fish from the bay due to high levels of mercury (Rumbold et al., 2003). There is also evidence that mercury may be acting as an endocrine-disrupting chemical in Florida waters. Thyroid hormone levels (T4 and T3) were reduced in Atlantic Bottlenose dolphins exposed to mercury in the Indian River Lagoon (Schaefer et al., 2011) and in Sarasota Bay (Woshner et al., 2008). Effects on thyroid activity usually occur after continuous exposure (Sin and The, 1992). It is thought that runoff from the mainland contributes to the mercury found in water and sediment where it is accumulated by the lower tropic levels that ultimately reach higher trophic species.

Although copper and zinc are essential metals found naturally in the environment, there are several anthropogenic sources that may be impacting the Florida Keys. Increased concentrations of copper and zinc in the sediment have been found in developed nearshore canal sites in the Florida Keys (Lapointe and Clark, 1990). Research with antifouling paint commonly used on boat hulls and other submerged structures has shown that copper leeches from the particles in the sediment in quantities high enough to accumulate in marine macroalgae (Turner et al., 2009). These fine particles may pose a threat to grazing invertebrates, as up to 1% by weight of the sediment can be contaminated at severely polluted sites such as marinas and harbors (Turner et al., 2009). Runoff from zinc coated materials and outdoor structures is known to enter aquatic environments (Heijerick et al., 2002; Lapointe and Clark, 1990). Most often, the metal is present as a free zinc ion which is the most bioavailable speciation form. However, zinc can also bind to dissolved organic content, turning it into an unavailable form (De Schamphelaere et al., 2005). With over 60 commercial marinas, hundreds of private docks, and a three ship cruise port, water and sediment along the entire Florida Keys' chain are potentially exposed to many types of antifouling agents. Therefore, it is important to determine the sources and availability of these metals to best understand how they may be reacting individually and synergistically in the environment.

Molluscs require copper and zinc as essential nutrients, but they also have naturally occurring metallothioneins (MTs) that help bind excess amounts of metals. Although also present in mammals, these non-enzymatic proteins appear to be particularly important to invertebrates and are often saturated by several metal atoms at a time (Amiard et al., 2006). They are believed to play a role in the homeostatic control of essential metals, like copper and zinc, but will also function in the detoxification of essential and non-essential metals (Amiard et al., 2006). Due to this characteristic, the presences of MTs can be used as a biomarker for metal exposure. Based on previous research in the Florida Keys (Spade et al., 2010), plans to study MT production in queen conch both nearshore and offshore are underway (Glazer, pers. comm.).

Confounding Effects of Metal Toxicity

The availability of copper and zinc at the four sites tested is better understood along with their inherent effects on larval growth, survival, development, and settlement success; however, this research only examined two metals and their independent toxicities. It has been suggested that in some cases the combination of metals can be more detrimental to biological functions than a single metal alone. A mixture of zinc, copper, lead, and cadmium in the diet affected consumption rates and fecundity of land snails, where only the growth rate was impacted by a mix of the metals (Laskowski and Hopkin, 1996). Mixtures of cadmium and zinc caused a reduction in the carbohydrate and protein reserves (i.e., energy reserves) of two freshwater gastropods over a two week exposure period (Moolman et al., 2007). The addition of copper to cadmium spiked water at varying levels increased the cadmium uptake significantly in periwinkles (Daka et al., 2006).

The presence of polycyclic aromatic hydrocarbons (PAHs) has been documented in the Florida Keys (Glazer et al., 2008). Although they don't explicitly seem to be linked to conch reproductive impairment, their presence can have interaction effects with copper and other metals. Polychaetes and clams in Thailand were impacted more by copper when PAHs were present (Hungspreugs et al., 1984). Likewise, a synergistic relationship between PAHs and other heavy metals was found to exist with two copepod species, suggesting that measuring one pollutant alone will not provide a greater picture of the exposures happening at the site (Fleeger et al., 2007).

Ammonia in the surface water has been shown to impact queen conch larval growth and development (McIntyre, 2005), and its presence in sediment porewater disrupts the embryological development of sea urchins (Carr et al., 2006). When heavy metals are present in bioactivated sludge, the rate of ammonia uptake decreases (You et al., 2009). This phenomenon has also been seen in soybeans, clover, and lichens (Porter and Sheridan, 1981). The amount of nitrate in nearshore waters of the Florida Keys has increased since 1995 along with the amount of total organic carbon (Boyer and Briceño, 2007). Additionally, dissolved inorganic nitrogen has been found at both nearshore and offshore sites in the Florida Keys (Lapointe et al., 2004a). If the presence of metals inhibits nitrification processes and metal ions bind to organic content, the toxicity of excess ammonia and the accumulation of metals in the sediments nearshore may be of concern.

Heavy metals also have synergistic effects on the production of anti-proliferative aldehydes in microalgal species. This has a direct impact on grazer response and is an area of concern when considering confounding effects and organism interactions in the field (Taylor et al., 2005). Nutrient enrichment will increase the availability of heavy metals in plant material (Devliegher and Verstraete, 1996), and heavy metals can inhibit photosynthesis in phytoplankton (Hongve et al., 1980). In the nitrogen rich nearshore waters of the Florida Keys (Boyer and Briceño, 2009), the presence of heavy metals may be having direct and indirect impacts on the grazing behavior of juvenile and adult conch. Marine herbivores will select plant material with elevated nitrogen content (Barile et al., 2004), and this has been seen for queen conch (Lapointe et al., 2004b). If nitrogen uptake processes and responses are being affected by heavy metals, the food source for conch and other herbivores could be negatively modified.

Project Conclusions and Summary

The results of this study indicate that heavy metals are present in the Florida Keys and may be impacting the recruitment success of juvenile queen conch. Although there were not distinct nearshore and offshore differences as anticipated, the flow of copper and zinc through the system differs at each site tested. Copper was more toxic to the larvae than zinc, and the timing and duration of exposure will effect metamorphic success.

The Florida Keys National Marine Sanctuary, NOAA's Office of National Marine Sanctuaries, and the Environmental Protection Agency will be able to use this data to better understand the extent of copper and zinc contamination in the Florida Keys and its effects on a local marine invertebrate. It is suggested that heavy metal monitoring, at least for copper, be incorporated into the water quality programs already developed. This may require a monitoring strategy developed specifically for each site or for nearshore and offshore locations since the presence and flow of the metals throughout the system differed at each site. Further research on the synergistic behavior of heavy metals with other marine pollutants (like ammonia, PAHs, or estrogen), may help to explain the lack of adult conch reproduction nearshore and should also be examined to determine the long term effect on juvenile conch growth and development.

Although capable of tolerating low levels of heavy metal toxicity, queen conch are being exposed to copper and zinc at each stage of their life cycle in the Florida Keys. It would be worthwhile to compare accumulation in the digestive gland and muscle tissue to that occurring in conch in a more pristine or undeveloped environment, such as the Bahamas. If accumulation in the muscle is too high, reopening the recreational fishery in the Keys may not ever be possible. Despite the research trying to understand the lack of recovery, metal levels in the muscle tissue may be higher than human consumption standards would allow.

In conclusion, copper and zinc are impacting queen conch, one of the greatest icons of the Florida Keys. Increased monitoring, mitigation, and coastal conservation should be incorporated into long-term management plans in order to protect the queen conch and other valuable invertebrates. A decrease in marine pollution overall and specifically copper and zinc as indicated by these studies will assist queen conch population growth and will ultimately benefit the economical and ecological value of the Florida Keys.

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