1-1-2013

Quantifying the Effects of Antibiotics Upon Benthic Diatoms and Marine Benthic Algal Communities

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QUANTIFYING THE EFFECTS OF ANTIBIOTICS UPON BENTHIC DIATOMS AND MARINE BENTHIC ALGAL COMMUNITIES

by

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Bachelor of Science
Pennsylvania State University, 2004

Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Biological Sciences

College of Arts and Sciences

University of South Carolina

2013

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DEDICATION

Firstly, I dedicate this dissertation to the True Man -- the Logos. A wise man gone before me said, "I believe in Christianity as I believe that the sun has risen. Not only because I see it, but because by it I see everything else." Additional dedications to all those who have shaped and directed the man I have become. Without my parents I would not exist. Without my siblings, I would be a tyrant. Without my wife I would be insensitive and uncaring. Without my children I would be ungenerous and ungentle. Thank you all.

ACKNOWLEDGEMENTS

This research was funded by the University of South Carolina Estuarine Ecology Lab, Slocum-Lunz Foundation, Sigma Xi Society, the F. John Vernberg Bicentennial Fellowship, and the William H. Nolte Graduate Assistant Fellowship. First acknowledgements are directed to my major professor James L. Pinckney, Ph.D. I am deeply grateful to him for his patience and willingness to let me make mistakes in my journey toward the doctoral hood. I would also like to acknowledge the members of my dissertation: Tammi Richardson, Richard Long, Alan Decho, and especially Sally Woodin who was willing to pay so much of her limited and precious time into my development as a scientist and scientific thinker.

I extend a special thanks to the Belle W. Baruch Institute, especially those who staff the USC and Georgetown offices. Without the administrative savvy and first-hand field experience of Margaret and Paul I would have been, literally, up the creek.

I thank my friends and colleagues at USC especially Gabriel J. Swenson for our many and varied conversations. Life is larger than science, isn't it my friend? I include John Alam in these thanks for all of his assistance in locating and obtaining needed technological equipment and welcomed distraction from purely scientific topics.

There are many others with whom I have interacted with during my time at USC and while I can't include them by name here, I thank them all, even those with whom the interactions were heated and strained. I hope I was able to contribute a modicum of the understanding to you that you have to me.

ABSTRACT

Antibiotics are a cornerstone of modern medicine. Their ability to impair prokaryotic metabolism has saved the lives of many millions of humans in the decades since wide antibiotic usage began. Antibiotic compounds are also widely used in the husbandry of animals that are used for food but, unfortunately, much of the antibiotic mass that goes into humans and animals is excreted unchanged. Those excretions go, directly or indirectly, into aquatic ecosystems. The discovery of this fact has led to a growing global concern about the fate of antibiotics in the environment. This concern is driven by no fewer than three primary facts: 1.) Globally, more than 50% the human population lives within 150 km of major coasts. 2.) Wastewater treatment plants do not specifically remove antibiotics. 3.) Antibiotics tend to bind to particles, which leads to elevated antibiotic levels in the benthos of aquatic systems. These facts have come together to create concern about the antibiotic content of streams, rivers, lakes, and the coastal ocean. The question driving this dissertation is, "*Do anthropogenic antibiotics pose a problem for coastal benthic ecosystems?*" The work presented here is specifically concerned with the ubiquitous eukaryotic microbial primary producers, the diatoms. Diatoms are found in virtually every type of aquatic system, contributing no less than 40% of the carbon found in many aquatic and aquatically-associated terrestrial species. This dissertation introduces a novel way to rapidly estimate surface-associated diatom cells using *in vivo* chlorophyll *a* fluorescence. It further shows that three commonly used, and purportedly prokaryote-only antibiotics can have direct negative effects on some

diatom species and that the effects of mixtures of these compounds cannot be predicted *a prioi* by summing the effects of individual compounds. Finally, I show that the intersection of experimental results from laboratory and those that come from using environmental microbial communities is less than clear. This is done using antibiotic-impacted, environmentally-derived microbial communities that were monitored using a combination of traditional physiological measurements and hyperspectral imaging of sediment-surface chlorophyll *a*.

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CHAPTER 1

INTRODUCTION

The last two decades have seen an increasing interest in the ecological effects of pharmaceuticals and personal care products, tons of which are released into the environment every year (Halling-Sørensen *et al*, 1998; Hirsch *et al*, 1999). Antibiotics have received a great deal of scrutiny, especially the aquatic environment, over that time as worries about drug-resistant microorganisms and unintended effects upon non-target species have grown (Daughton and Ternes, 1999; Kim and Aga, 2007). While still relatively new, there has been a global effort to identify compounds of concern and document their toxicological and ecological effects (Halling-Sørensen *et al*, 1998; Hirsch *et al*, 1999; Kolpin *et al*, 2002; Calamari *et al*, 2003; Zuccato *et al*, 2005; Feitosa-Felizzola and Chiron, 2009). This is fitting since all watersheds, by definition, feed into some body of water. The two main sources of antibiotic input are people and food animals, which are often kept in close proximity. As of 2010, 44% of global population lives within 150 km of oceans (UN, 2011). In the United States, including Great Lakes watersheds, the number is 52% (NOAA, 2013) and the numbers are increasing. The vast majority of antibiotic-effect studies have ignored marine systems, focusing instead on the effects of single compounds upon freshwater systems and freshwater species (Halling-Sørensen, 2000; Wollenberger *et al*, 2000; Wilson *et al*, 2003; Halling-Sørensen *et al*, 2003; Eguchi *et al*, 2004; Liu *et al*, 2011; Weber *et al*, 2011). While herbicide effects have been investigated, very few studies have investigated the effects of pharmaceuticals

upon non-target organisms (Pape-Lindstrom and Lydy, 1997; Magnusson *et al*, 2010). Given these facts, a salient question is whether or not the presence of antibiotics is a problem and if so, how so.

This dissertation is concerned with the benthic microalgae (BMA) or microphytobenthos that underpin coastal, specifically estuarine food webs. The coastal oceans, especially estuarine and marsh systems, are among the most productive habitats in the world (Pomeroy, 1959; Bunt *et al*, 1972; Pinckney and Zingmark 1993a,b; Miller *et al*, 1996; Johnston *et al*, 2002). They are also among the most heavily impacted by human presence because they receive wastewater and runoff from inland and coastal human populations (Fulton *et al*, 1996; Paerl *et al*, 2003; Van Dolah *et al*, 2007). This water carries with it a complex mixture of chemicals, including antimicrobial compounds, many of which have an affinity for particles that increases the likelihood of their being deposited in the benthos (Halling-Sørensen *et al*, 2003; Andreozzi *et al*, 2006; Weber *et al*, 2011). This has been confirmed recently by investigators in China who found a suite of anthropogenic antibiotics in the Gulf of Tonkin and Laizhou Bay (Zhang *et al*, 2012; Zheng *et al*, 2012).

Many estuarine species feed directly or indirectly on eukaryotic microalgae. As much as 60% of the carbon found in coastal organisms is fixed by microbial primary production (Sullivan and Moncreiff, 1990; Currin *et al*, 1995; Kwak and Zedler, 1997). In marsh systems, much of this microbial primary productivity is accomplished by the eukaryotic microphytobenthos or benthic microalgae (BMA). In photic waters, BMA are the primary support for benthic consumers with diatoms contributing no less than 40% of the carbon in many species (Evrard *et al*, 2012). BMA communities are composed of

diverse species assemblages of unicellular, filamentous, and colonial photoautotrophs that inhabit surface sediments in aquatic ecosystems. In estuarine sediments, benthic diatoms, cyanobacteria, and chlorophytes are the primary photoautotrophs, but in temperate regions pennate diatoms are numerically and photosynthetically dominant. The bulk of the community biomass is located in the upper few millimeters of sediment and rarely extends below the depth of light penetration (Plough *et al*. 1993; Kuhl *et al*. 1994). These communities are ubiquitous and prolific primary producers in intertidal and shallow coastal habitats (Admiraal, 1984; Cahoon, 1999; Underwood and Kromkamp, 1999) and their importance in trophodynamics, biogeochemical cycling, and sediment-water-atmosphere exchange is well established (Chardy & Dauvin, 1992; Miller *et al*. 1996; Anderson *et al*. 1997). In most habitats, BMA present an abundant, nutritious food source for grazers that is relatively constant over long (monthly to yearly) time scales (Currin *et al*. 1995; Miller *et al*. 1996; Deegan and Garritt, 1997). Because BMA communities are often numerically and photosynthetically dominated by pennate diatoms, diatoms are prominent among those species for which contaminant response(s) must be quantified (Admiraal, 1984; Miller *et al*, 1987; Acs and Kiss, 1993).

Previous work in my study site has demonstrated that BMA are major primary producers (Pinckney & Zingmark, 1993). The average annual benthic microalgal production for North Inlet estuary in 1990-91 was estimated to be 3423 tonnes C/yr. In this estuary, BMA production exceeds phytoplankton and macroalgal production, but is less than Spartina grass species. BMA production is approximately 43% (3.4×10^9 gC/yr) of the maximum estimated total Spartina production (7.9×10^9 gC/yr) in this system. If one considers that all of this production occurs within a 2 mm thick layer of sediment, the

spatial scale of production is greatly compressed relative to other producers. Because much of the carbon fixed by *Spartina* species is not easily assimilated by herbivores, benthic microalgae may be the most abundant and easily accessible source of utilizable carbon for estuarine primary consumers.

Much of the work done thus far regarding antibiotic contamination in aquatic systems has concentrated on the development and proliferation of genetic antibiotic resistance in bacteria (Daughton and Ternes, 1999; Sengeløv *et al*. 2003, Kim and Aga, 2007). While antibiotic resistance is perhaps the most significant human health threat of the 21st century, another large area of concern is that of ecological impact. There have been a number of laboratory-based toxicological studies, most of which suggest that antibiotics in the environment pose a "potential" ecological problem. Wilson *et al*. (2003) found that antibiotics reduce algal genus richness and final biomass in environmental communities. Eguchi *et al*. (2004) found that antibiotics reduced cell growth and that some compounds acted synergistically against eukaryotic algae. Kim and Carlson, (2007) reported high concentrations of antibiotics in freshwater stream sediments. There are numerous other studies indicated potential problems, such as Liu *et al*., (2011_a), (Liu *et al*., 2011_b), Weber *et al*. (2011), Hagenbuch and Pinckney, (2012), and Pinckney *et al*. (2013). A number of studies that have determined that heavily-impacted environments have ecological issues that extend through trophic levels beyond prokaryotes. For example, Wollenberger *et al*. (2000) found that Daphnia magna are subject to reduced reproductive rate at antibiotic concentrations orders of magnitude below "toxic" levels. Chelossi *et al*. (2003) found increased antibiotic resistance in sediments below the site of floating fish cages. Isadori *et al*. (2005) found that in several groups of organisms

including rotifers and crustaceans, toxic concentrations were in the mg/l range but negative effects were observed at µg/l levels. The backdrop to all these studies is a threadbare tapestry of reports indicating a generally low-level presence of antibiotics in various, mostly freshwater, environments (Hirsch *et al*., 1999; Halling-Sørensen *et al*., 1998; Halling-Sørensen *et al*., 2000; Kolpin *et al*., 2002; Calamari *et al*., 2003; Halling-Sørensen *et al*., 2003; Wilson *et al*., 2003; Eguchi *et al*., 2004; Zuccato *et al*., 2005; Kim and Carlson, 2007; Feitosa-Felizzola and Chiron, 2009; Weber *et al*., 2011). With notable exceptions such as Anderson *et al*. (2004) and Weber *et al*. (2011), virtually none of these studies have attempted to directly link laboratory-based cause and effect toxicity studies with in situ communities. The work presented here is a step toward that integration.

In Chapter 2, I have presented a simple, rapid, reliable and inexpensive protocol for quantifying surface-associated microbiota. This is of interest to a wide range of investigators from a variety of microbiological disciplines. I have also demonstrated the applicability of this protocol in antibiotic sensitivity assays.

In Chapter 3, I have quantified the effect of three environmentally common anthropogenic antibiotics and their mixtures upon two species of common eukaryotic marine benthic primary producers.

In Chapter 4 I have addressed several questions: Do environmentally common antibiotics affect the biomass, mobility or physiology (net primary productivity and respiration), of complex and diverse microphytobenthic communities in sediments? At what concentrations do single or combinations of antibiotics affect BMA productivity? What is the approximate time-scale over which potential effects manifest? I have also investigated whether antibiotic contamination impacts diatom migration behavior.

CHAPTER 2

FLUOROMETRIC ESTIMATION OF SURFACE ASSOCIATED DIATOMS USING *IN VIVO* CHLOROPHYLL *A* FLUORESCENCE

INTRODUCTION

A central tenet of microbial investigation of ecology and ecotoxicology involves the quantification of microbial abundance. The capacity to rapidly and accurately enumerate abundance of surface associated microbial cells is imperative for multiple disciplines within the field of microbial ecology. Many observational methods have been employed to assess abundances of these microorganisms. Direct observations include various forms of microscopy (i.e., light, epiflourescence, confocal microscopy, electron microscopy). Though epifluorescence microscopy has been shown in the past to result in density estimation errors (Blackman and Frank, 1996), microscopy remains central in quantifying surface associated microbes (Lad and Costerton, 1990; Lindsay and von Holy, 1997).

Fuches *et al.* (2002) report on a technique using thin light-sheet microscopy (TLSM) for observation of microbes associated with individual particles at the sub-millimeter scale in suspension; however, this is time intensive. Flow cytometry has been shown to be an effective alternative to microscopy (Diaper and Edwards, 1994) but is not applicable for enumerating particle associated cells, bacterial aggregates or biofilm communities while they are surface associated. Wegley *et al.* (2006) recently identified bulk fluorescence as

compared to direct counts via epifluorescence microscopy. However this technique was not employed for quantification of surface associated cells. Müsken *et al.* (2010) report on a protocol to measure bacterial density and antibiotic sensitivity of *Pseudomonas aeruginosa* using fluorescent live-dead stains and automated confocal microscopy equipment to qualitatively and quantitatively evaluate biofilm formation but requires specialized equipment, 16 procedural steps, ~60 hours of preparation time and ~5 hours per plate of data analysis.

Several indirect observational methods such as plate counts coupled with sonication, vortexing and scraping have been examined to enumerate surface associated microbes by first detaching and then quantifying the cells. However, many of these techniques are considered inaccurate, unreliable and inconsistent (Lind, 1985; Poulsen, 1999; Patil and Anil, 2005; Goeres *et al.*, 2009). The use of microtiter plates is an indirect means of microbial observation that is easily modified for biofilm and colonization assays (An and Friedman, 2000). Applications have successfully employed multi-well microtiter plates to assess biofilm formation by measuring absorbance of crystal violet (CV) stained biofilms (Christensen *et al.*, 1985; Djordjevic *et al.*, 2002; Silva *et al.*, 2009). This is a rapid approach to qualifying biofilm formation but is limited in analytical power and sensitivity.

Quantification of benthic microalgal species is especially complex given the heterogeneous nature of sediments and the propensity of cells to form biofilms incorporating multiple species of microalgae and bacteria (Miller *et al.*, 1996). The use of fluorescent staining of environmentally-derived samples has been limited due to the non-specific binding of the dyes and subsequent high background fluorescence (Diaper and

Edwards, 1994). Additionally, this approach requires time intensive microscopy and automated image analysis software for enumeration.

In the current study, we present a simple, rapid, reliable and inexpensive protocol for quantifying surface associated microbiota that will be of interest to a wide range of investigators from a variety of microbiological disciplines. I have eliminated the need to concentrate microbial cells via membrane filtration, mitigated the influence of non-specific dye binding and media derived background noise and improved the reproducibility of abundance estimates using multi-well microtiter plates. Finally, I demonstrate the applicability of this protocol in antibiotic sensitivity assays.

MATERIALS & METHODS

Eukaryotic cultivation

The benthic diatom *Cylindrotheca closterium* (CCMP 1855) was grown in filtered, autoclaved, f/2 + Si enriched seawater at 22° C on a 12 hr light/dark cycle at an irradiance of ~85 µmol $m^{-2}s^{-1}$. During assay setup, subcultures were gently vortexed for 30 sec to disrupt flocs before being distributed into 24-well microtiter plates.

Microalgal culture plate preparation

All preparatory work was done inside a laminar flow hood. Each 6-well row of a 24-well microtiter plate was used as a complete dilution series of an antibiotic, including a control. The following was added to each well and homogenized via resuspension: 4 µl concentrated antibiotic solution, 2 ml f/2 +Si, and 25 µl *C. closterium* culture. Plates

were incubated at 22° C for 2 hrs to allow the diatom cells to settle before initial microscopic enumeration and fluorescence measurements.

Fluorometry

Plates were read on a SpectraMax GeminiEM spectrofluorometer (Molecular Devices; Sunnyvale, CA, USA). The optical parameters were excitation wavelength = 460nm and emission wavelength = 685nm (long band pass filter at 665nm). Fluorescence was detected from below the bottom of clear plates, allowing *in vivo* chlorophyll *a* fluorescence to be measured closer to the cells, improving the signal/noise ratio. Fluorescence data were collected and analyzed using SoftMaxPro™. Each well was read using the following empirically-determined parameters: Well scan = fill, density = 3, spacing = 1.13mm, sensitivity = normal, and total points = 9. Each setting was adjusted and uniformly applied over entire plates, depending on the sample density. All fluorescence results are reported as relative fluorescence units (RFU).

Cell density and growth rate

Direct cell counts were conducted daily using microscopic quadrant (grid) counts. Counts were made prior to fluorometry measurements using an eyepiece micrometer grid attached to an inverted epifluorescent microscope. Five grid counts were in each well and only cells exhibiting chl *a* fluorescence were counted. Plates were allowed to sit in a dark room for 15 min before being scanned in the plate reader to allow the cells to recover from photon saturation. Growth rates were calculated by fitting an exponential function to the population estimates and RFU values.

Antibiotic assays

24-well microtiter plates were prepared and as previously described with the addition of one of three antibiotics: Ciprofloxacin, lincomycin, or tylosin. Because each antibiotic was differently soluble in water, the maximum concentrations of each antibiotic also differed. Final concentrations (g/ml) of antibiotics were ciprofloxacin (1×10^{-2} to 10^{-6}), lincomycin (4×10^{-2} to 10^{-6}) and tylosin (2×10^{-2} to 10^{-6}). Assay concentrations of each compound were normalized to the maximum concentration of that compound to provide a common unit (concentration factor) for all three antibiotics. Assays were run for up to 5 days and measurements were made once per day. Assays were terminated when cultures became too dense to enumerate via epifluorescent microscopy.

Statistical analysis

Linear regression and univariate analysis of variance with post hoc analyses were conducted using SPSS 19.0 (IBM; Armonk, NY, USA). The Ryan-Einot-Gabriel-Welsch F or Q tests were used to determine antibiotic subset identity.

RESULTS & DISCUSSION

Auto-fluorescence of growth medium

The f/2 + Si medium used in microalgal assays exhibited no significant auto-fluorescence and did not affect fluorescent detection even at very low population densities. The mean RFU of 288 wells before algal addition was 0.26 ± 0.04 RFU. This is an order of magnitude below the lowest single RFU value observed after algal cells were

added. This suggests that f/2 + Si enriched seawater is an ideal medium for use in benthic microalgal fluorescent bioassays of this kind because it contributes virtually no auto-fluorescent noise or variability to assay.

Microalgal abundance

The chl *a* fluorescence of the control-wells exhibited a linear relationship to the number of cells present (Figure 2.1, $R^2 = 0.667$, $p < 0.001$). With the exception of three outliers (two from the lincomycin assays and one from the ciprofloxacin assays) the relationship between cell number and fluorescence was consistent across a wide range of cell concentrations and population ages.

Microalgal antibiotic sensitivity

Each antibiotic substantially reduced endpoint chl *a* fluorescence emission of *C. closterium* (Figure 2.2). Endpoint (115-120 hrs) fluorescent emissions were significantly different between compounds and concentrations ($p < 0.001$ in both cases). The compound specific response was unique, with each antibiotic compound having a more pronounced effect on the diatoms. The highest antibiotic concentrations had a significant effect on chl *a* fluorescence, whereas intermediate doses were more difficult to separate and the effect of the two lowest concentrations were indistinguishable from the control.

Cell-specific growth rates of *C. closterium* were consistent with the rates derived using RFU (Figure 2.3). Cell-specific rates span -0.04 to 1.34 r^{-d} and RFU-specific rates ranged -0.56 to 0.82 r^{-d}, with distinct antibiotic and concentration-specific patterns. Tylosin had the most pronounced effect (Figures 2.2 and 2.3_A), followed closely by

lincomycin. These differences were observed in both cell and RFU growth rates (Figure 2.3_B). Two of the ciprofloxacin RFU growth rates exhibited relatively high coefficients of variation of 1.03 and 3.81 (Figure 2.3_C), which may be due to the fluorescence of crystallized ciprofloxacin in treatment wells near the end of the experiment. Nevertheless, the responses induced by ciprofloxacin were consistent between cell counts and chl *a* fluorescence.

Evaluation of fluorometric method

Previous studies have employed benthic diatom microtiter assays to investigate various questions, including heterotrophy (Tuchman *et al.* 2006). However, the use of *in vivo* chl *a* fluorescence to estimate surface-associated diatom populations in microtiter antibiotic assays is novel. The present study shows that the relationship between chl *a* fluorescence and population size is linear and statistically significant. Therefore, microtiter fluorometric assays may be useful for quickly and accurately estimating surface associated microalgal populations.

Because chl *a* concentrations vary depending on environmental and nutrient conditions, previous studies have avoided linking chl *a* concentration to the number of algal cells, focusing instead upon chl *a* concentration (Lorenzen, 1966; Vyhnálek *et al.* 1993). The consistency of microscopic and fluorometric growth rate estimations in this study is therefore a significant finding. Given the time differential between microscopy counts versus fluorometric quantification of chl *a*, it is worth emphasizing the similarity and consistency between growth rates derived using the two methods, especially during chemical stress treatments. This is extremely useful for investigators using dose-response

assays where relative growth rates are desired and quantification (Halling-Sørensen, 2000; Eguchi *et al.*, 2004).

CONCLUSION

In the present study, we have assessed the abundance and growth rate of a surface-associated diatom species across broad concentration gradients and under different chemical treatments using the *in vivo* autofluorescence of chl *a*. We have shown that *in vivo* chl *a* fluorescence reliably facilitates detection and our results shows strong linearity of robust statistical significance. This technique is a valid, inexpensive, and high-throughput means of assessing microbial sensitivity to several antibiotic compounds at various concentrations.

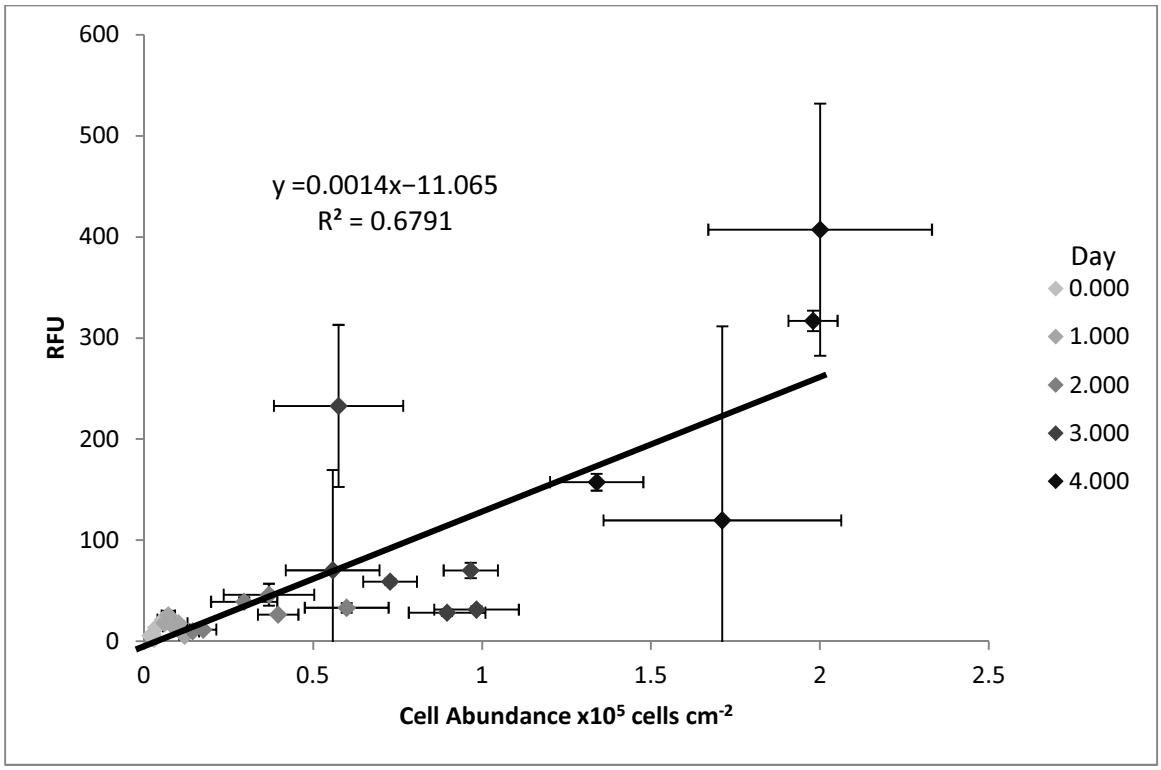

Figure 2.1: Linear regression of algal cells vs. chl *a* fluorescence from day 0 (white diamonds) through day 4 (black diamonds) for the benthic diatom *C. closterium* populations in f/2 enriched seawater in 24-well microtiter plates, y= 0.0014x − 11.065, R^2 = 0.679, $p < 0.001$, (N = 4). Error bars are standard deviation.

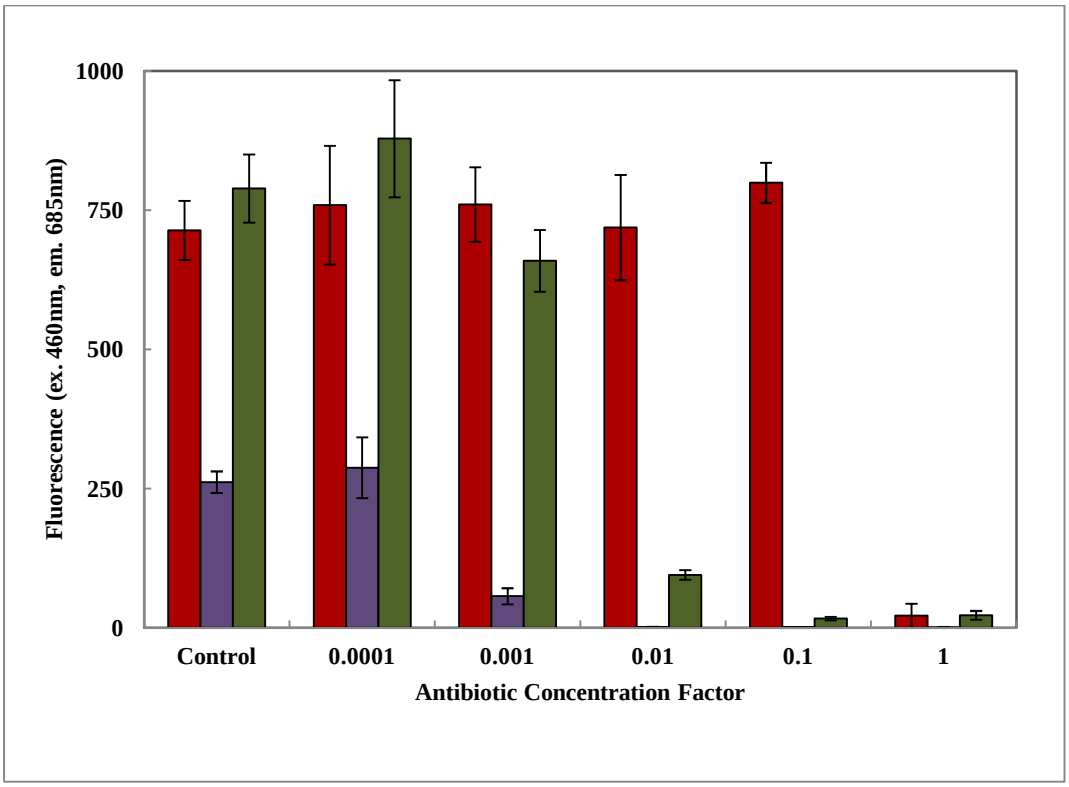

Figure 2.2: Antibiotic sensitivity as measured by fluorescence of *C. closterium* at assay termination (hour 71–120 of exposure). Antibiotic concentrations start at 1 = 1×10^{-2} g ml^{-1} (ciprofloxacin; green bars), 1 = 4×10^{-2} g ml^{-1} (lincomycin; purple bars) and 1=2×10^{-2} g ml^{-1} (tylosin; red bars); (N = 4). Error bars are standard deviation.

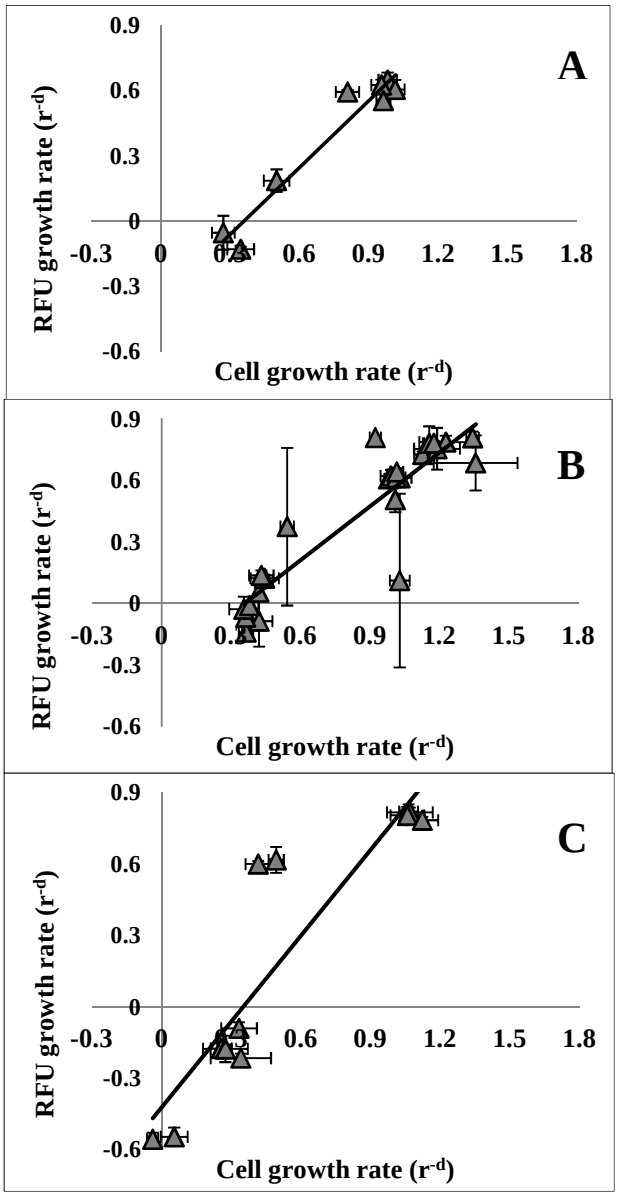

Figure 2.3: Comparison of *C. closterium* growth rates (r^{-d}) in antibiotic exposures and controls as determined by chl *a* fluorescence and microscopy cell counts. With the exception of some few outliers, growth rate estimates under all treatments are consistent and exhibit high coefficients of determination and degrees of significance ($p < 0.001$ in all cases). Y-axis, RFU (r^{-d}) excitation 460 nm and emission 685 nm. Each panel includes the matching controls: A) growth rates under ciprofloxacin, $y = 0.8775x - 0.3164$, $R^2 = 0.8201$; B) lincomycin, $y = 1.026x - 0.3703$, $R^2 = 0.932$; C) tylosin, y =

1.203x − 0.4239, R^2 = 0.810. Antibiotic treatments ranged from 412 to 0.0104 g ml^{-1}.

Error bars are standard deviation.

CHAPTER 3

TOXIC EFFECT OF THE COMBINED ANTIBIOTICS CIPROFLOXACIN, LINCOMYCIN, AND TYLOSIN ON TWO SPECIES OF MARINE DIATOMS[1]

[1] Isaac M. Hagenbuch and James L. Pinckney. *Water Research*, 2012, 46(16): 5028-5036.

ABSTRACT

The role that antibiotics and other "emerging contaminants" play in shaping environmental microbial communities is of growing interest. The use of the prokaryotic metabolic inhibitors tylosin (T), lincomycin (L), and ciprofloxacin (C) in livestock and humans is both global and extensive. Each of these antibiotic compounds exhibits an affinity for sediment particles, increasing the likelihood of their deposition in the benthos of aquatic systems and each are often present in environmental samples. The purpose of this study was to determine if T, L, and C and their mixtures exhibit significant toxicity to two species of marine diatoms, an algal class comprised of ubiquitous eukaryotic primary producers. Subpopulations from laboratory cultures of *Cylindrotheca closterium* and *Navicula ramosissima* were reared in 24-well microtiter plates in the presence of single or combined antibiotics in dilution series. Population growth rates were assessed via epifluorescent microscopic cell counts, from which the half-max inhibitory concentrations (IC_{50}) were calculated and used as part of a toxic unit (TU) method for assessing mixture interactions. The single-compound IC_{50}'s were, for *C. closterium*: T = 0.27 mg/l, L = 14.16 mg/l, C = 55.43 mg/l, and for *N. ramosissima*: T = 0.99 mg/l, L = 11.08 mg/l, C = 72.12 mg/l. These values were generally higher than similar metrics for freshwater species. Mixture IC_{50}'s were generally synergistic against *C. closterium* and additive for *N. ramosissima*. Both single and combined treatments reduced or eliminated diatom motility. Monochemical responses were similar between species and were not useful for predicting mixture interactions. Mixtures had compound-specific and species-specific effects, favoring *N. ramosissima*. These results suggest that anthropogenic antibiotics may play a significant role in the ecology of environmental benthic microbial

communities. They also suggest single-compound/species studies do not yield useful predictions of the ecological impact of anthropogenic pharmaceuticals.

INTRODUCTION

The last two decades have seen an increasing interest in the ecological effects of pharmaceuticals and personal care products, tons of which are released into the environment every year (Halling-Sørensen *et al*, 1998; Hirsch *et al*, 1999). Antibiotics have received a great deal of scrutiny over that time as worries about drug-resistant microorganisms and unintended effects upon non-target species have grown (Daughton and Ternes, 1999; Kim and Aga, 2007). While still relatively new, there has been a global effort to identify compounds of concern and document their toxicological and ecological effects (Halling-Sørensen *et al*, 1998; Hirsch *et al*, 1999; Kolpin *et al*, 2002; Calamari *et al*, 2003; Zuccato *et al*, 2005; Feitosa-Felizzola and Chiron, 2009). The vast majority of these studies have ignored marine systems, focusing instead on the effects of single compounds upon freshwater systems and freshwater species (Halling-Sørensen, 2000; Wollenberger *et al*, 2000; Wilson *et al*, 2003; Halling-Sørensen *et al*, 2003; Eguchi *et al*, 2004; Liu *et al*, 2011; Weber *et al*, 2011). While herbicide mixture effects have been investigated, very few studies have investigated the effects of pharmaceutical combinations upon non-target organisms (Pape-Lindstrom and Lydy, 1997; Magnusson *et al*, 2010).

The coastal ocean, especially estuarine and marsh systems are among the most productive habitats in the world (Pomeroy, 1959; Bunt *et al*, 1972; Pinckney and Zingmark 1993$_{a,b}$; Miller *et al*, 1996; Johnston *et al*, 2002). They are also among the most

heavily impacted by human presence because they receive wastewater and runoff from inland and coastal human populations (Fulton *et al*, 1996; Paerl *et al*, 2003; Van Dolah *et al*, 2007). This water carries with it a complex mixture of chemicals, including antimicrobial compounds, many of which have an affinity for particles that increases the likelihood of their being deposited in the benthos (Halling-Sørensen *et al*, 2003; Andreozzi *et al*, 2006; Weber *et al*, 2011). This has been confirmed recently by investigators in China who found a suite of anthropogenic antibiotics in the Gulf of Tonkin and Laizhou Bay (Zhang *et al*, 2012; Zheng *et al*, 2012).

Many estuarine species feed directly or indirectly on eukaryotic microalgae. As much as 60% of the carbon found in coastal organisms is fixed by microbial primary production (Sullivan and Moncreiff, 1990; Currin *et al*, 1995; Kwak and Zedler, 1997). In marsh systems, much of this microbial primary productivity is accomplished by the eukaryotic microphytobenthos or benthic microalgae (BMA). In photic waters, BMA is the primary support for benthic consumers with diatoms contributing no less than 40% of the carbon in many species (Evrard *et al*, 2012). Because BMA communities are often numerically and photosynthetically dominated by pennate diatoms, they are prominent among those species for which contaminant response(s) must be quantified (Admiraal, 1984; Miller *et al*, 1987; Acs and Kiss, 1993).

Ciprofloxacin (C) is a broad-spectrum antibiotic that has been identified as a contaminant of concern based upon its pattern of use, presence in the environment and ecological impact (Hooper 1998; Halling-Sørensen *et al*, 2000; Kolpin *et al*, 2002; Wilson *et al* 2003; Batt *et al*, 2007; Weber *et al*, 2011). Previous studies have shown that environmentally relevant concentrations of C negatively impact algal biomass and change

algal assemblages in freshwater systems (Wilson *et al*, 2003). C adsorbed to sediment particles shows a 3.5-fold potency increase and has an affinity for adsorption (Tolls 2001; Halling-Sørensen *et al*, 2003).

Tylosin (T) is another antibiotic of interest due to its worldwide use as a veterinary prophylactic and growth factor (De Liguoro *et al*, 2003). T has been found in a wide variety of aquatic habitats and is toxic to some freshwater phytoplankon at near-environmental (μg/l) concentrations (Halling-Sørensen *et al*, 2000; Kolpin *et al*, 2002; Calamari *et al*, 2003; Eguchi *et al*, 2004). Like C, T also has an affinity for sediment particles with an active residence time of 100+ days which suggests that it is present in its active form for long enough to affect benthic communities (Kim and Carlson, 2007; Halling-*Sørensen et al*, 2003).

Lincomycin (L) is commonly used in both veterinary and human medicine and has been found to impact a range of taxa including mycoplasma and some *Plasmodium* species (Powers 1969; Hamdy and Blanchard 1970; Alderman and Hastings, 1998; Serrano, 2005). L inhibits the synthesis of the D1 protein in photosystem II, which reduces the ability of microalgae to recover from light-inhibition (Bergmann *et al*, 2002). Unlike the previous two compounds, L is generally overlooked as a potential environmental toxin despite the fact that is commonly found in the environment alongside T and C (Kolpin *et al*, 2002; Calamari *et al*, 2003).

The purpose of this study was to quantify the effect of three environmentally common anthropogenic antibiotics and their mixtures upon two species of ubiquitous marine eukaryotic benthic primary producers. Effect was assessed using the cell-specific growth rates of diatom populations reared in 24-well microtiter plates as

determined by daily cell counts. The hypotheses to be tested were: each antibiotic will have a measureable IC_{50} , each antibiotic mixture will have a measurable IC_{50} , IC_{50}'s will differ between species, and antibiotic mixtures will not exhibit antagonism.

MATERIALS AND METHODS

Culture selection and maintenance

Cultures of *Cylindrotheca closterium* (CCMP1855) and *Navicula ramosissima* (CCMP2602) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP). These strains were chosen based on their documented presence and ecological role in the coastal ocean, their relatively recent collection/deposit dates (1998 and 2006, respectively), and that they both were collected from temperate benthic sites (Zingmark, 1992; Lewitus *et al*, 1998; Daume *et al*, 1999). Cultures were maintained according to CCMP guidelines in 500 ml acid-washed and autoclaved glass bottles. Growth medium was Guillard's f/2 + Si prepared by amending then autoclaving salinity-adjusted natural seawater collected from North Inlet Estuary, Georgetown, South Carolina. Cultures and experimental well plates were kept in an incubator at 23 ^{o}C and a photon flux of ~75 µmol/m^2/s for a 12:12 photoperiod.

Antibiotic selection and preparation

Crystalline antibiotics or their salts were acquired from MP Biomedicals, LLC. (tylosin tartrate, ≥ 91.3% pure), TCI America (lincomycin hydrochloride, monohydrate, ≥ 98% pure) and Enzo Life Sciences (ciprofloxacin, ≥ 98% pure). The T and L salts were both very soluble in water whereas crystalline C was insoluble in water but very soluble

in dilute HCl. The maximum solubility of each antibiotic in autoclaved ultrapure water and 0.1N HCl was empirically determined and formed the upper-limit for the single-compound assays.

Assay construction

Experimental assays were conducted in sterile 24-well tissue culture plates (Advangene®). All plates were opened and filled aseptically in a laminar flow hood. Each row formed a complete dilution series (10^{th}s) including a control. Each well was loaded with concentrated 4.0 µl aliquots of antibiotic, followed by 2.00 ml of growth medium, then 25.0 µl of algal culture which was added and dispersed evenly by gentle pipetting. Aliquots were taken from exponentially growing cultures and contained a consistent number of cells as verified by microscopy (coefficient of variation < 0.25). Aliquots were vortexed before being added to the wells to break up flocs and keep the diatom cells from settling during pipetting. Control wells were treated identically to treatment wells excepting the 4.0 µl aliquots which contained either autoclaved ultrapure water or 0.1 N HCl.

Algal population quantification

Algal populations and growth rates were quantified using microscopic population estimates. Growth rates were determined by least-squares nonlinear regression of the following form:

$$N(t) = N_0 e^{rt}$$

N(t) is the number of algal cells at time *t* (day). N_0 is the initial number of cells, *r* is the growth rate d^{-1}. The toxic effect of each compound was objectively quantified by determining the half-maximum inhibitory concentration (IC_{50}). IC_{50} is based on the % inhibition (%I) of *r* in treatments:

$$\% \, Inhibition = 100\left(1 - \frac{r_{treatment}}{r_{control}}\right)$$

Assays continued until cell densities precluded counting, when stationary-phase was achieved, or there were no fluorescent cells to count; generally 4-5 days. Values of *r* were estimated using daily cell counts made using an inverted compound microscope and eyepiece micrometer grid at magnifications ranging from 100x-400x. Magnification was determined by cell density. Five random grids were counted in each well, the mean of which was used to estimate the total population. As benthic species, both *C. closterium* and *N. ramosissima* exhibited a 99% sinking rate within 1 hour of being placed in an assay which allowed populations to be quantified by area rather than well volume. At no time were individuals observed attached the walls of the wells.

Quantification of single and mixture effect

A toxic unit (TU) approach was used to examine mixture activity and toxicity and to compare antibiotic sensitivity across compounds and between species (Faust *et al*, 1993; Pape-Lindstrom and Lydy, 1997; Magnusson *et al*, 2010). The IC_{50} value for each replicate dilution series was determined by fitting a four parameter sigmoidal curve to the time-series population estimates (GraphPad Software, Inc.®), where the constraint (IC_{50} > 0.00001 mg/l) was applied to ensure model convergence:

$$\%Inhibition = \min + \frac{(r_{min} - r_{max})}{1 + \left(\frac{Log(concentration)}{IC_{50}}\right)^{Hillslope}}$$

In the Hill-Slope model, r_{min} and r_{max} refer to the minimum and maximum growth rates. These values were not constrained because treatment populations declined in some assays, yielding negative values of *r* and leading to %I values greater than 100%. This was not a problem for the model, however, which converged in virtually every case. IC_{50} values with $R^2 \leq 0.65$ or 95% confidence intervals spanning 1.5 orders of magnitude were excluded from further analyses. Effect of treatments was determined using full-factorial, two-way ANOVA.

Toxicity of binary and tertiary mixtures were determined by replacing single compounds with antibiotic mixtures ranging from 3.0 - 0.13 TU where 1.0 TU = 1.0 IC_{50}. The interaction of the components of a mixture's was determined by the value of that mixture's IC_{50} (Pape-Lindstrom and Lydy, 1997; Magnusson *et al,* 2010). For mixtures, $IC_{50} > 1.0$ is denoted as antagonistic, $IC_{50} = 1.0$ is additive, and $IC_{50} < 1.0$ is synergistic. Deviation from 1.0 was determined using one-sample t-tests (IBM SPSS Statistics© 19).

RESULTS

Single compound responses

Two-factor ANOVA of the IC_{50}'s indicated that antibiotic treatment and species were significant influences ($p < 0.0001$, power = 1.000). There was also a significant treatment*species interaction, suggesting that species did not respond identically to all treatments ($p < 0.0001$, power = 1.000). Variance was not homogenous, precluding the use of post-hoc analysis (Levine's, $p < 0.01$).

C. closterium and *N. ramosissima* differed only slightly in their response to the single antibiotic treatments (Figure 3.1). Tylosin (T; protein synthesis inhibitor) had the most negative effect, with an IC_{50} less than 1.0 mg/l in both species. An increase of two orders of magnitude was required to see the same impact when using lincomycin (L; protein synthesis inhibitor). Ciprofloxacin (C; DNA synthesis inhibitor) was the least toxic of the three, having coefficients of variation of 0.38 (*C. closterium*) and 0.85 (*N. ramosissima*). This contrasted against T and L which ranged 0.51 - 1.28 with *N. ramosissima* always being at least 30% higher. As concentrations were changed by factors of 10, both T and L elicited a typical sigmoidal dose-response. In contrast, C exhibited a very narrow effect gradient, exhibiting a break-point spanning only one order of magnitude (140 - 14 mg/l; Figure 3.2).

Mixture responses

Mixtures were included with the single-compound treatments in the ANOVA, where treatment, species, and treatment*species had significant effects ($p < 0.0001$, OP = 1.000). Mixture concentrations were reported in toxic units (TU), where 1.0 TU is equal to 1.0 IC_{50}. Thus, in binary mixtures, a 2.0 TU treatment includes 1.0 IC_{50} (mg/l) of each compound. Similarly, a 1.0 TU treatment includes 0.5 IC_{50} of each compound and so on. A mixture IC_{50} equal to 1.0 is additive, less than 1.0 is synergistic, and greater than 1.0 is antagonistic. Deviations from 1.0 were determined using one-sample t-tests.

There were marked differences of response between the two diatom species. Three of the four antibiotic mixtures *C. closterium* (T+L, L+C, T+L+C) exhibited a synergistic toxic effect ($p < 0.001$). The effect of the fourth mixture, T+C, was found to

be additive (p = 0.380). This contrasts against the response of *N. ramosissima* where all mixtures behaved additively (p = 0.094 - 0.424). There was also a clear difference in variability between the species where *N. ramosissima* had near-extreme outliers and large coefficients of variation (0.61 - 1.24). The elevated variability of *N. ramosissima* in both single-compound and mixed assays is likely an artifact of spatial distribution. While both diatom species were motile, *N. ramosissima* did not spread as thinly as *C. closterium*. *C. closterium* cells were very motile and tended to separate, forming a uniform monocellular layer on the bottom of the wells. In contrast, *N. ramosissima* spread more slowly and tended to form clumps of 5-50 cells.

DISCUSSION

Tylosin (T) exhibited the most negative effect, with an IC_{50} of 0.27 mg/l in *C. closterium* and 0.96 mg/l for *N. ramosissima*. Lincomycin (L) following distantly behind with 14.2 and 11.1 mg/l, respectively. The lesser effect exhibited by *N. ramosissima* was consistent across all treatments, except L. Because post-hoc analyses were not done, it is not possible to be certain of whether the difference between the species is statistically significant but it is possible, given the wide standard deviation of the *N. ramosissima* IC_{50} (CV = 1.00) that the responses did not significantly differ. Ciprofloxacin (C), long recognized as a compound of concern, when applied alone, it is less toxic to benthic diatoms than other common compounds (Figure 1.1). This is likely due to its mechanism of action (DNA synthesis inhibition), which would presumably have the most pronounced effect when the diatom or its chloroplasts are dividing. In contrast, protein synthesis inhibitors could be significantly active any time proteins are being assembled

by diatom or, more likely, chloroplast or mitochondrial ribosomes. It is generally thought that antibiotics act against eukaryotes by interfering with the function of mitochondria and chloroplasts (Mitsuhashi, 1971; Halling-Sørensen, 2000; Brain *et al*, 2004; Liu *et al*, 2011). It is also possible that the toxicity of C is lessened by the nature of our assays, which exclude sediment. Further experiments are needed to determine if the 4-fold potency increase in sediment-bound-C reported by Tolls (2001) and Halling-Sørensen *et al*, (2003) holds true for eukaryotic microbial species as well as prokaryotes.

The IC_{50}'s of L and C reported in this present study differ from previously reported EC_{50} values from freshwater species (Table 3.1). In the case of L, the values that we report (11.08 and 12.16 mg/l) are at least an order of magnitude higher for both benthic diatom species versus the literature values. In the case of C, our IC_{50} values (55.43 and 72.12 mg/l) are again several times higher than those previously reported. These differences are probably not simply due to the differences in algal species, given the ionic profile of the amended seawater used in these assays. Some quinolones are reversibly antagonized by magnesium and calcium ions, reducing their efficacy against bacteria (Marshall and Piddock,1994). Such antagonism can increase the minimum inhibitory concentration of some quinolones, including C, by a factor of 2-8 (compared to freshwater) for some pathogenic bacteria (Smith, 1989 and 1990; Li *et al*, 2000). The mechanism of this antagonism hasn't been fully described, though it is known that chelation with metal ions occurs at a ratio of 1:1, reducing the accumulation of antibiotic in the cytoplasm but not via porin channel size-exclusion (Marshall and Piddock, 1994; Barnes *et al*, 1995). Martinsen and Horseberg (1995) also noted that not all antibiotics within a given class behave uniformly, which is an important point given the lack of data

on the chemistry of T and L in seawater. Like many antibiotics, both T (a macrolide) and L (a lincosimide) are susceptible to photodegradation, however there hasn't been any notable work on their chemistry in seawater and this degradation process is unlikely to be active below the top millimeter of many sediments (Halling-Sørensen *et al,* 2003; Andreozzi *et al,* 2006; Hu and Coats, 2007; Werner *et al,* 2007).

The most interesting results were those of the mixture assays, which showed up differences in compound interactions that were dependent upon algal species (Figure 3.3). In *C. closterium* all antibiotic mixtures, with the exception of T+C, worked together to elicit a synergistically (i.e. multiplicative) negative response. This sharply contrasts against the response of *N. ramosissima* in which all mixtures behaved additively. *N. ramosissima*'s response to T+L and T+L+C were variable (CV = 1.20 and 1.24) which is probably why the very high IC_{50}'s for those treatments (6.98 and 10.22 TU) were not determined to be significantly different from 1.00 (p = 1.18 and 1.25). Regardless, this suggests that benthic primary producers exposed to multiple antimicrobial compounds in the environment may be subject to selection pressures from that pharmaceutical profile. This is consistent with the few other studies that have investigated antimicrobial interaction via microalgal exposure assays (Eguchi *et al,* 2004).

The species-specific components of these results suggest that different species may respond differently to different antibiotics. Although expected, this result has implications for benthic ecology. Evrard *et al,* (2012) found that benthic consumers subsist almost entirely on diatom-dominated BMA communities, which opens an avenue for upward-propagating trophic effects either through the reduction of available carbon or through alteration of preferred food abundance (Vanden Berghe and Bergmens, 1981;

Carman and Thistle, 1985). As a corollary, microalgal species are unequally productive either through differences in cell physiology or migratory behavior (Underwood *et al,* 2005). The anthropogenic antimicrobial compounds used in this study may be at play at either of these avenues, given that diatoms exposed to moderate antibiotic concentrations ceased motility. Even a moderate reduction in benthic diatom movement rate can be sufficient to significantly impact benthic primary productivity given the tight coupling between benthic diatom migration and tide stage (Hay *et al,* 1993; Underwood and Kromkamp, 1999). Finally, biodiversity of algal species serves to improve water quality and may confer resilience to benthic microbial communities (Elmqvist *et al,* 2003; Cardinale *et al,* 2006, Cardinale, 2011).

The study of the presence and ecological impact of pharmaceuticals in the environment is in an intermediate period. It has been noted by Kolpin *et al,* (2002) and others that there is a conspicuous dearth of data regarding the presence of antibiotics in the environment (Halling-Sørensen *et al,* 1998; Daughton and Ternes, 1999; Calamari *et al,* 2003; Zuccato *et al,* 2005; Feitosa-Felizzola and Chiron, 2009). The extant data is somewhat sparse and the sampling overwhelmingly consists of the analysis of water rather than sediments, and freshwater rather than marine environments (Table 3.2), however two recent studies have confirmed the transport of antibiotics into the coastal ocean (Zhang *et al,* 2012; Zheng *et al,* 2012). The disparity in coverage between the two major aquatic environments and classes seems to be due to the cost and methodological difficulty of extraction and quantification of antibiotics in the benthos and a focus on drinking water.

Anthropogenic antibiotics, including those used in this study, are present in the environment as mixtures (Kolpin *et al,* 2002; Calamari *et al,* 2003). Previous studies investigating eukaryotic antibiotic toxicity have focused on single species' responses to single compounds. Given the chemical and biological heterogeneity of natural aquatic systems, monochemical approaches are unlikely to yield useful ecological predictions or information directly applicable outside the laboratory. Heterogeneity aside, previous model algal organisms have been free-floating pelagic rather than benthic species. Environmental concentrations vary by location but they are universally higher in the sediment than in the overlaying water due to the affinity of many antibiotics for sediment particles (Kolpin *et al,* 2002; Kim and Carlson. 2007). Contrary to conventional wisdom, it is not universally true that the adsorption of antibiotic compounds diminishes their potency. In at least one case, sediment binding increases the effectiveness of antibiotics (Weber *et al,* 2011).

We have shown that algal species of the same class (diatoms, *Bacillariophyceae*) are similarly inhibited by single antibiotic compounds (Figure 3.1). However, single-compound responses cannot be combined to form *a priori* predictions of combined effects (Figure 3.3). Species-specific inhibition of algal growth rates may constitute a selection pressure in environmental communities, resulting in altered community composition or the proliferation of antibiotic resistance genes (Harris *et al,* 1989; Lopez-Rodas *et al,* 2001). This potential cannot be minimized by appealing to low environmental concentrations given the complex nature of antibiotic mixture effects and the potential for adsorbative potency enhancement.

Many avenues of future work have yet to be explored. Questions regarding the stability and bioavailability of T and L in seawater and in marine sediment should be addressed**.** Similarly, future studies should determine whether different strains of the same algal species respond differently. There may be a difference antibiotic sensitivity between benthic and pelagic algal species, which could be interesting in light of sediment stockpiling of many antibiotics. Investigators may also wish to determine whether the formation of multi-species eukaryotic biofilms confers resistance as is commonly reported in prokaryotic biofilms. Similarly, there needs to be greater understanding of how sediment influences the effect of antibiotics on benthic algal species, including the presence of lipopolysaccharide (LPS). Some of these questions might be answered using a design similar to what we have outlined here.

CONCLUSIONS

Benthic diatoms are a key component of coastal marine food webs. Globally, anthropogenic antibiotics are present in continental surface waters which drain into the costal ocean. Some of these so-called prokaryotic-specific antimicrobial compounds are toxic to eukaryotic algae. These facts strongly suggest that anthropogenic antibiotics are playing a significant role in the ecology of the coastal marine benthos. In the present study, we have shown that common antibiotics toxic to benthic diatoms and antibiotic mixtures favor some species over others in ways that cannot be predicted by single-compound approach.

ACKNOWLEDGEMENTS

Bulk of support was provided by the University of South Carolina Estuarine Ecology Lab, with initial work partially funded by a Sigma Xi dissertation improvement grant. Innumerable thanks go to Drs Tammi Richardson, Sarah Woodin, Richard Long, and Alan Decho for their editorial services and advice on experimental questions, design, and measurement. Thanks also to two anonymous reviewers who significantly improved the text, clarity, and scope of this manuscript. This is publication no. 1647 from the Belle W. Baruch Institute for Marine and Coastal Research.

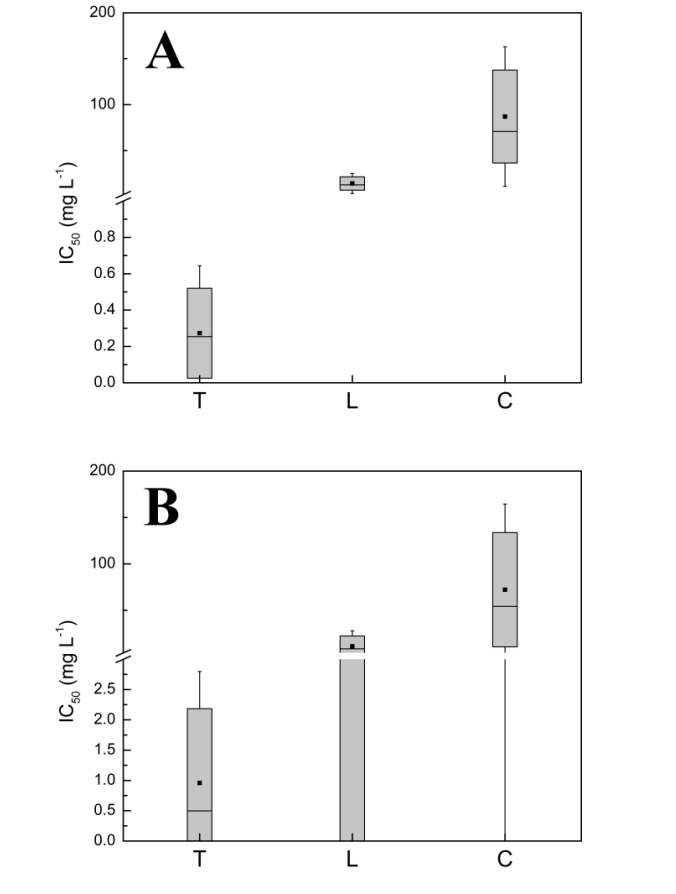

Figure 3.1: Summary of single antibiotic IC_{50}'s for *Cylindrotheca closterium* and *Navicula ramisissima*. A) Response of *C. closterium* to tylosin (T), lincomycin (L), and ciprofloxacin (C). Boxes are standard deviation (SD), center line is median, ■ is mean, and bars are 0.5 SD. B) Response of *N. ramosissima* to T, L, and C. In both panels, note the break in the Y-axis necessitated by the large difference between T and C.

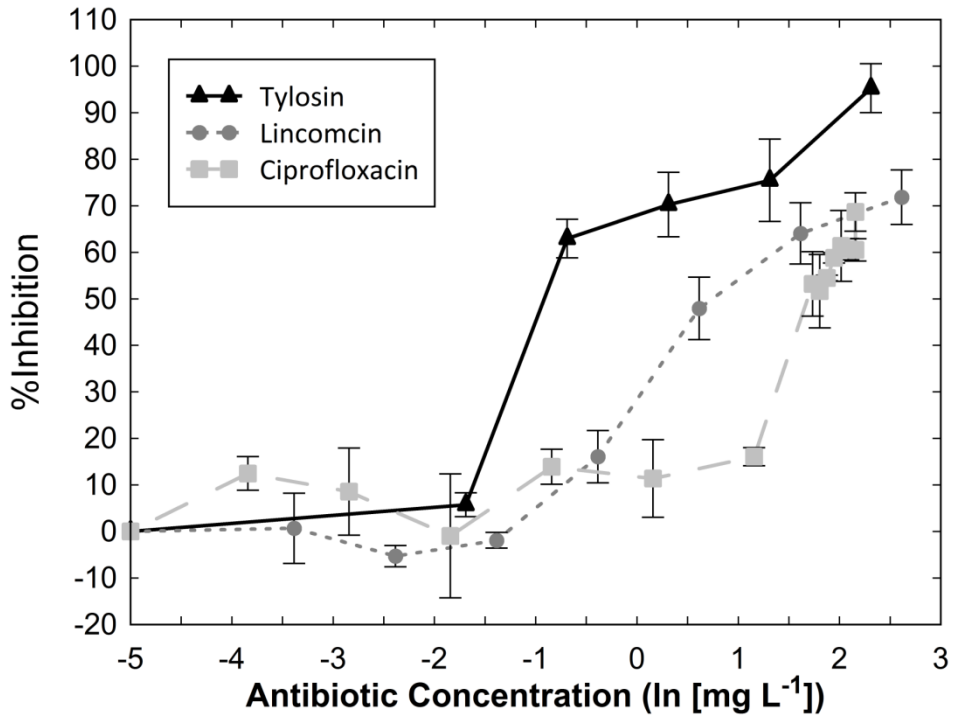

Figure 3.2: Representative dose-response assays of *Cylindrotheca closterium* exposed to three antibiotics. Both tylosin and lincomycin curves represent single N = 4 assays. The ciprofloxacin curve represents two N = 3 assays; one high concentration and one low. Error bars on all curves are SD. Tylosin and lincomycin elicited typical sigmoidal responses as concentrations were changed by 10^1. The response to ciprofloxacin was different, with the response being compressed into one order of magnitude from 1-2 ln(mg/l).

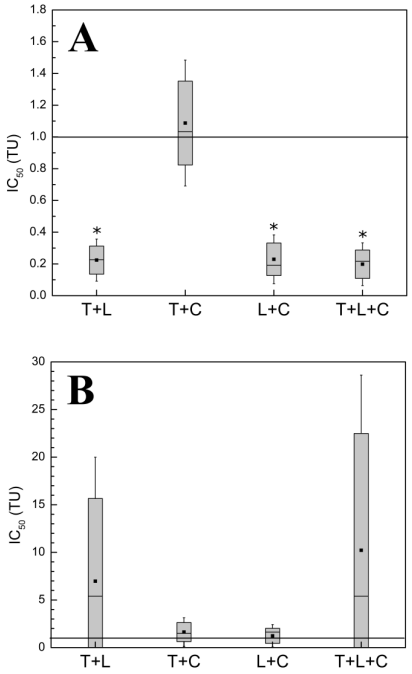

Figure 3.3: Summary of antibiotic mixtures IC_{50}'s for *Cylindrotheca closterium* and *Navicula ramisissima*. A) Response of *C. closterium* and B) response of *N. ramosissima* to mixtures of tylosin (T), lincomycin (L), and ciprofloxacin (C). Y-axis is concentration in Toxic Units (TU), boxes are standard deviation (SD), center line is median, ■ is mean, bars are 0.5 SD and ***** denotes mean-values significantly different from 1.00. In both panels, the reference line is IC_{50} = 1.00. Means values above the line denote antagonistic mixture interaction, not different from the line denotes additive interaction, and below the line denotes synergistic interaction.

Table 3.1 — Toxicity of tylosin, lincomycin, and ciprofloxacin to various algal species

Compund	EC50 (mg L^{-1})	Species	Source
Ciprofloxacin	0.313	*Chlorella vulgaris*	Nie *et al.* (2008)
	0.005	*Microcystis aeruginosa*	Halling-Sørensen *et al.* (2000)b
	2.970	*Selenastrum capricornutum*	Halling-Sørensen *et al.* (2000)b
Lincomycin	0.163	*Cyclotella meneghiniana*	Andreozzi *et al.* (2006)
	1.510	*Pseudokirchneriella subcapitata*	Andreozzi *et al.* (2006)
	0.195	*Synechococcus leopoliensis*	Andreozzi *et al.* (2006)
Tylosin	0.034	*Microcystis aeruginosa*	Halling-Sørensen (2000)a
	1.380	*Selenastrum capricornutum*	Halling-Sørensen (2000)a
	0.411	*Selenastrum capricornutum*	Eguchi *et al.* (2004)

Table 3.2 — Reported environmental concentrations of antibiotics in water

	($ug\ L^{-1}$)	Source
Ciprofloxacin	0.03	Kolpin *et al.* (2002)
	0.03	Calamari *et al.* (2003)
	0.03	Zucco *et al.* (2005)
	0.10	Feitosa-Felizzola and Chiron (2009)
	0.90	Daughton and Ternes (1999)
	0.10	Batt *et al.* (2006)
Lincomycin	0.73	Kolpin *et al.* (2002)
	0.25	Calamari *et al.* (2003)
	0.25	Zucco *et al.* (2005)
Tylosin	0.28	Kolpin *et al.* (2002)
	0.01	Calamari *et al.* (2003)

CHAPTER 4

EFFECTS OF CIPROFLOXACIN AND TYLOSIN UPON THE FORMATION AND PHYSIOLOGY OF MARINE BENTHIC MICROALGAL COMMUNITIES[1]

[1]Isaac M. Hagenbuch, Nils Volenborn, Lubos Polerecky, and James L. Pinckney, (2013) To be submitted to *Environmental Science and Technology*.

ABSTRACT

Currently, there is great interest in the effects and fate of antibiotics in the environment, especially the aquatic environment. The two main sources of antibiotic input are people and food animals, which are often kept in close proximity. Given that 52% of global population lives within 150 km of oceans or the Great Lakes questions about effects of antibiotics upon coastal ocean primary producers is an important one. Even so, there are essentially no studies investigating the links between laboratory cause-and-effect toxicity studies with *in situ* communities. This study is concerned with the benthic microalgae (BMA) or microphytobenthos communities that underpin coastal/estuarine food webs. In temperate regions benthic diatoms are numerically and photosynthetically dominant. In the laboratory, single antibiotics are capable of reducing cell division, photophysiology, and motility in common diatom species at concentrations that approach those found in the environment. This study focuses on the effects of ciprofloxacin which inhibits prokaryotic DNA synthesis and tylosin which inhibits prokaryotic protein synthesis. This study presents a method for using hyperspectral imaging of surface chlorophyll to capture temporal variations in addition to traditional pigment extraction and quantification methods. Only relatively high antibiotic concentrations had any measurable effect upon BMA communities. Tylosin (T) and ciprofloxacin (C) had opposite effects on BMA biomass as detected by hyperspectral imaging. Environmentally-relevant concentrations of T and C elicited no measureable effects in any of the physiological, pigment, or hyperspectral parameters. This suggests that over the short term, the primary productivity of marine BMA communities exposed to these antibiotics is indifferentiable from un-impacted communities. T and C, regardless

of concentration, failed to cause any measureable effect on net primary productivity and respiration. It is possible that the outcome of antibiotic exposure assays may strongly depend on the species composition of BMA communities. The only parameters affected by the antibiotic treatments were those associated with surface chl *a* biomass. Half-max inhibitory (IC_{50}) concentrations of T and C Significantly affected surface chlorohyll but in opposite ways: T_{IC50} pushed it down and C_{IC50} pushed it up and in the combined treatment, the effects cancelled each other. Time-to-effect was different as well. The inhibitory effect of T took twice as long to manifest as positive effect of C. Future studies should employ some metric of community composition/diversity to see if these communities really are changing.

INTRODUCTION

Currently, there is great interest in the fate and effect of antibiotics in the environment, especially the aquatic environment. This interest isn't new, but it has grown in breadth and visibility over the last two decades as studies reporting the presence of human and veterinary antibiotics in the environment have multiplied. The concern regarding antibiotic presence is global, which is fitting since all watersheds, by definition, feed into some body of water. The two main sources of antibiotic input are people and food animals, which are often kept in close proximity. As of 2010, 44% of global population lives within 150 km of oceans (UN, 2011). In the United States, including Great Lakes watersheds, the number is 52% (NOAA, 2013) and the numbers are increasing. Given these facts, a salient question is whether or not the presence of antibiotics is a problem and if so, how so.

Much of the work done thus far regarding antibiotic contamination has concentrated on the development and proliferation of genetic antibiotic resistance in bacteria (Daughton and Ternes, 1999; Sengeløv *et al*. 2003, Kim and Aga, 2007). While antibiotic resistance is perhaps the most significant human health threat of the 21st century, another large area of concern is that of ecological impact. There have been a number of laboratory-based toxicological studies, most of which suggest that antibiotics in the environment pose a "potential" ecological problem. Wilson *et al*. (2003) found that antibiotics reduces algal genus richness and final biomass in environmental communities. Eguchi *et al*. (2004) found that antibiotics reduced cell growth and that some compounds acted synergistically against eukaryotic algae. Kim and Carlson, (2007) reported high concentrations of antibiotics in freshwater stream sediments. There are numerous other studies suggesting potential problems, such as Liu *et al*. (2011a), (Liu *et al*. 2011b), Weber *et al*. (2011), Hagenbuch and Pinckney, (2012), and Pinckney *et al*. (2013). A number of studies that have determined that heavily-impacted environments have ecological issues that extend through trophic levels beyond prokaryotes. For example, Wollenberger *et al*. (2000) found that *Daphnia magna* are subject to reduced reproductive rate at antibiotic concentrations orders of magnitude below "toxic" levels. Chelossi *et al*. (2003) found increased antibiotic resistance in sediments below the site of floating fish cages. Isadori *et al*. (2005) found that in several groups of organisms including rotifers and crustaceans, toxic concentrations were in the mg/l range but negative effects were observed at µg/l levels. The backdrop to all these studies is a threadbare tapestry of reports indicating a generally low-level presence of antibiotics in various, mostly freshwater, environments (Hirsch *et al*. 1999; Halling-Sørensen *et al*.

1998; Halling-Sørensen *et al*. 2000; Kolpin *et al*. 2002; Calamari *et al*. 2003; Halling-Sørensen *et al*. 2003; Wilson *et al*. 2003; Eguchi *et al*. 2004; Zuccato *et al*. 2005; Kim and Carlson, 2007; Feitosa-Felizzola and Chiron, 2009; Weber *et al*. 2011). With notable exceptions such as Anderson *et al*. (2004) and Weber *et al*. (2011), virtually none of these studies have attempted to directly link laboratory cause-and-effect toxicity studies with *in situ* communities. The present study is a step toward that integration.

This study is concerned with the benthic microalgae (BMA) or microphytobenthos that underpin coastal, specifically estuarine food webs. BMA communities are composed of diverse species assemblages of unicellular, filamentous, and colonial photoautotrophs that inhabit surface sediments in aquatic ecosystems. In estuarine sediments, benthic diatoms, cyanobacteria, and chlorophytes are the primary photoautotrophs, but in temperate regions benthic diatoms are numerically and photosynthetically dominant. The bulk of the community biomass is located in the upper few millimeters of sediment and rarely extends below the depth of light penetration (Plough *et al*. 1993; Kuhl *et al*. 1994). These communities are ubiquitous and prolific primary producers in intertidal and shallow coastal habitats (Admiraal, 1984; Cahoon, 1999; Underwood and Kromkamp, 1999) and their importance in trophodynamics, biogeochemical cycling, and sediment-water-atmosphere exchange is well established (Chardy & Dauvin, 1992; Miller *et al*. 1996; Anderson *et al*. 1997). In most habitats, BMA present an abundant, nutritious food source for grazers that is relatively constant over long (monthly to yearly) time scales (Currin *et al*. 1995; Miller *et al*. 1996; Deegan and Garritt, 1997). Food web studies in a variety of estuarine settings have demonstrated that BMA contribute between 30 and 60% (or more) of the primary production

supporting resident estuarine species (Peterson & Howarth, 1987; Sullivan and Moncreiff, 1990; Currin *et al*. 1995; Page, 1997; Creach *et al*. 1997). Furthermore, BMA production can be traced into the food webs supporting birds and commercially important fish and shellfish (Sullivan and Moncreiff, 1990; Kwak and Zedler, 1997; Deegan and Garritt, 1997). Previous work in our study site has demonstrated that BMA are major primary producers (Pinckney & Zingmark, 1993). The average annual benthic microalgal production for North Inlet estuary in 1990-91 was estimated to be 3423 tonnes C/yr. In this estuary, BMA production exceeds phytoplankton and macroalgal production, but is less than Spartina grass species. BMA production is approximately 43% (3.4×10^9 gC/yr) of the maximum estimated total Spartina production (7.9×10^9 gC/yr) in this system. If one considers that all of this production occurs within a 2 mm thick layer of sediment, the spatial scale of production is greatly compressed relative to other producers. Because much of the carbon fixed by Spartina is not easily assimilated by herbivores, benthic microalgae may be the most abundant and easily accessible source of utilizable carbon for estuarine herbivores.

In the laboratory, single antibiotics are capable of reducing cell division, photophysiology, and motility in common diatom species at concentrations that approach those found in the environment (Hagenbuch and Pinckney 2012; Pinckney *et al*. 2013). Antibiotics work by interfering with purportedly prokaryote-specific cellular chemistry. Ciprofloxacin, a fluoroquinolone, inhibits DNA synthesis by interfering with the activity of DNA gyrase. Others, such as tylosin, a macrolide, inhibits protein synthesis by binding to the 50S ribosomal subunit. While antibiotics are generally regarded as prokayrotic-

specific, diatoms are not the only non-target organisms that respond negatively to antibiotics. Negative effects in have been catalogued in many other species (Table 4.1).

This study is intended to address several questions: Do environmentally common antibiotics affect the biomass, mobility or physiology (net primary productivity and respiration), of complex and diverse microphytobenthic communities in sediments and how do these findings compare to laboratory studies with cell cultures? At what concentrations do single or combinations of antibiotics affect BMA productivity? What is the approximate time-scale over which potential effects manifest?

Some of our questions involve temporal changes. In order to document the temporal response of the BMA biomass to antibiotic treatments we used hyperspectral imaging of surface chlorophyll. In contrast to traditional sediment sampling followed by chlorophyll extraction, the optical detection of chlorophyll has the benefit that the same location can be repeatedly measured. Additionally, consecutive scans at daytime (chl *a* max) and nighttime (chl *a* min) allow us to analyze vertical migration behavior.

MATERIALS AND METHODS

Study Site & Sample Collection

North Inlet estuary in Georgetown, SC is a bar-built, mixo-euhaline/metahaline estuary on the mid-Atlantic coast of the United States. North Inlet has been used as a reference site in many studies. It is a suitable reference site because of its limited human-impacted freshwater inputs and its net material export which minimize any background contaminant presence. Sediments were collected from North Inlet in January and February 2013. The top 1 cm of sediment from an ~1 m^2 area of sandy mud was collected

and transported back to the University of South Carolina, Columbia before being homogenized, sieved (500 µm), and distributed into 2.2 cm diameter core tubes. Additional sediment that was not immediately used was spread evenly over the bottom of a ~80 cm x ~30 cm plastic tide-simulating microcosm. The microcosm had a drain and was fed by a timed reservoir pump with head-tank that simulated tides. This holding tank was illuminated by approximately the same light intensity of the experimental cores.

Assays

We used the antibiotics ciprofloxacin and tylosin in this study. Ciprofloxacin (C) is a broad-spectrum antibiotic that has been identified as a contaminant of concern based upon its pattern of use, presence in the environment and potential ecological impact (Hooper, 1998; Halling-Sørensen *et al*. 2000; Kolpin *et al*. 2002; Wilson *et al*. 2003; Batt *et al*. 2007; Weber *et al*. 2011). Tylosin (T) is another antibiotic of interest due to its worldwide use as a veterinary prophylactic and growth factor (De Liguoro *et al*. 2003). T has been found in a wide variety of aquatic habitats and is toxic to some freshwater phytoplankton at near-environmental (mg/l) concentrations (Halling-Sørensen *et al*. 2000; Kolpin *et al*. 2002; Calamari *et al*. 2003; Eguchi *et al*. 2004). Like C, T also has an affinity for sediment particles with an active residence time of 100 days which suggests that it is present in its active form for long enough to affect benthic communities (Kim and Carlson, 2007; Halling-Sørensen *et al*. 2003). Moreover, they can be found together in environmental sediments (Kolpin *et al*. 2002; Calamari *et al*. 2003). We elected to use the environmental and half-max inhibitory (IC_{50}) concentrations reported by Hagenbuch and Pinckney (2012).

Initial experiments uncovered a number of important factors. The first is that 1500 µE was photoinhibitory to the BMA community. We also uncovered a 3-fold variability in surface chl *a* despite having collected the sediment cores from an apparently homogenous area (Figure 4.1). This intrinsic variability could have masked effects due to antibiotic addition. We also found that small infauna were capable of significantly reducing the standing stock of chl *a* in the cores. Given these facts, we decided to homogenize the collected sediments, sieve them through a 500 µm mesh, and reduce the illumination of the cores to a value less than 500 µE. These manipulations are comparable to what intertidal sediments undergo during a strong resuspension event such as during heavy precipitation or a spring tide.

Antibiotic exposure incubations were accomplished using an 18-chamber microcosm located in an environmental chamber. Each chamber was physically partitioned and separate from the others with its own 1.0 L reservoir and re-circulating diaphragm water pump. The microcosm was kept at a constant 20^{o} C ambient temperature and was lit by two 60 W incandescent growth lamps. Each chamber received 240 ± 6 µE at the sediment surface. The content of the reservoirs, including the given antibiotic treatments, was exchanged and renewed every two days. Temperature and microcosm light field homogeneity were ensured using temperature loggers (iButton®) and a 1.3 cm diameter PAR sensor (Biospherical® QSL2101).

The experiment was set up in a 2 x 3 fully crossed factorial design with cyproflaxacin and tylosin as main factors, both applied at 3 different concentrations: environmentally-realistic and half-maximum inhibitory (IC_{50}) concentrations (T_{Enviro} = 0.28 µg/l, T_{IC50} = 0.62 mg/l, C_{Enviro} = 155 µg/l, C_{IC50} = 63.8 mg/l (Hagenbuch &

Pinckney, 2012). A block factor was introduced to account for the two successive experimental runs with 2 replicates of each antibiotic combination in each run.

Parameters & Calculations

We measured several parameters: aerial surface chl *a* density and heterogeneity, proportional migration, net primary productivity (NPP), respiration, and aerial photopigments. Surface chl *a* and heterogeneity were tracked using a hyperspectral (HS) camera (Resonon® Pika II) mounted on a linear-motor. Initial work indicated that daily HS scans weren't required, thus the entire microcosm was scanned immediately after loading to ensure homogeneity, then every 2-3 days until the end of the experiment. Scans were done at the same time (± 30 min) on each day of the experiment. Spectral hypercubes were processed to isolate and display aerial chl *a* (HSchla) absorbance data as bitmap images using Look@MOSI (Polerecky *et al*. 2009). Mean surface HSchla and heterogeneity (HSCV) were quantified using ImageJ ver. 1.46r (Rasband, 2013). Diatom migration was calculated as:

$$Proportional\ Migration = \frac{HSchla_{min}}{HSchla_{max}}$$

$HSchla_{max}$ was at ~12:00 and $HSchla_{min}$ at ~00:00 of the final experiment day (day 13). HSchla homogeneity (HSCV) for each core is estimated by that core's HSchla coefficient of variation (N = 3852 pixels/core).

Net primary productivity and community respiration were measured using Clark-style microelectrodes in a light/dark bottle method. On the final day of the experiment,

experimental cores were removed from the microcosm and sub-cored. Sub-cores (0.50 cm^2 x 1.0 cm) were taken from the center of each experimental core. The bottom of each sub-core was sealed with paraffin to restrict gas exchange to the sediment surface. The prepared cores were then placed within a 5 ml glass vial. Each vial contained a magnetic stir bar and was filled with water from the appropriate experimental reservoir and sub-aerially capped to exclude gas bubbles. Each cap had a conical 1 mm sampling port for insertion of the probe. Initial experiments with deoxygenated seawater indicated that oxygen exchange was negligible using this apparatus over the duration of incubation and measurement (data not shown). After capping, the initial dissolved O_2 concentration (mg/l) was measured within each vial, then each vial was sealed with Parafilm® and placed in complete dark for 1 hr. After the dark incubation, each vial was unwrapped and final O_2 concentration was immediately measured. Respiration was calculated as:

$$\frac{DO_{Initial} - DO_{Final}}{Core\ Surface\ Area} \qquad \text{(Lind, 1985)}$$

Following the final O_2 measurements, each core in turn was exposed to a fiber optic halogen light source for 5 minutes of continuous O_2 measurement. The rate of oxygen production for each sub-core was determined using the slope of a linear regression fit to the complete 5-minute dataset (N = 900). The oxygen rates were converted to NPP using a conservative photosynthetic quotient of 1.4 (Grant, 1986).

Aerial chemosystematic photopigments were quantified using high performance liquid chromatography (HPLC). Immediately after the NPP measurements, sub-cores were sectioned and the top 2 mm of sediment was retained and stored at -80° C pending

analysis. Prior to pigment quantification, samples were lyophilized for 24 hrs at -50^{o} C, placed in 90% acetone, and extracted at -20^{o} C for 24 hrs. Extracts were centrifuged and filtered to remove sediment particles, then injected into a Shimadzu HPLC (Pinckeny *et al*. 1996; Pinckney *et al*. 2013). BMA biomass (mg chl *a*/m^{2}) was converted to carbon units assuming a C:chl *a* ratio of 47.6 (de Jonge, 1980). To estimate change in community composition, we estimated the ratio of diatom biomass to that of cyanobacterial biomass by using the ratio of fucoxanthin, a diatom biomarker pigment, to zeaxanthin, a cyanobacterial marker pigment (Pinckney *et al*. 2013).

Statistical Analysis

In all statistical analyses, the data either fit parametric test assumptions such as normality and equality of variance or were transformed to do so. The data transformations included natural log, reciprocal, and square root. Dependant variables were included in each statistical model in a manner designed to preserve independence of factors. For example, because HSchla is used to calculate Migration, these data were analyzed separately.

Analysis of the antibiotic addition assay results was accomplished using a combination of multivariate analysis of variance (MANOVA), univariate analysis of variance (ANOVA), and linear regression. Analyses of variance included the 2 antibiotics: tylosin (T), and ciprofloxacin (C). with three levels (none, environmental, and IC_{50}), their interactions, and a block factor to account for variance between the two consecutive experimental runs. HSchla and HSCV were investigated using a repeated-measures ANOVA to account for within-sample variance and time.

RESULTS

Chlorophyll biomass

HSchla increased exponentially throughout the duration of the experiment (Figure 4.2) indicating that the experimental conditions supported BMA growth. Repeated-measurement ANOVA indicated significant day* antibiotic treatment interactions (Table 4.2-a). The significant Day*T interaction was caused by lower rate of HSchla increase in the IC50 treatments, leading to significant lower Hschla values by Day 13 (Figure 4.3-A). The opposite was true for the Day*C interaction, where HSchla was significantly higher in IC_{50} treatments from Day 6 onward (Figure 4.3-B). Simultaneous exposure to both antibiotics did not cause a change in Hschla. In no cases did the environmentally-relevant concentrations of T and C have a significant effect on HSchla (Table 4.2). HSCV was unaffected by all antibiotic treatments at all levels but there was a significant Day effect (Table 4.2-b). HSCV was most homogeneous on Day 6 and most heterogeneous on Day 13 (Figure 4.3-C). Migration was not significantly affected by any of the treatments (Table 4.2).

Physiology

Two Resp points from Assay 2 (enviro-T and enviro-T + $IC_{50\text{-}C}$) were found to be significant outliers. To maintain an equal number of points for all treatments, these values were replaced with the mean of the three matching replicates prior to statistical analysis. None of the antibiotic concentrations significantly affected community Resp or NPP (Table 4.3).

Pigments

None of the antibiotic treatments significantly affected BMA community biomass or diatom:cyanobacteria ratio (Table 4.2).

DISCUSSION

The issue of antibiotic contamination and its effects on non-target organisms is an important one. It is generally assumed by scientists and regulators alike that detectable environmental presence of any human-origin chemical compound is a danger. As a general assumption, it is probably a good one since the effect of such an assumption will be to minimize the number of detectable anthropogenic chemicals in the environment. However, the scientific questions of first importance are not, "What negative impacts could chemical X have in the environment," but rather, "Does chemical X have detectible environmental effects and at what exposure?" The latter question is the one we have addressed in this study.

Only the IC_{50} concentrations had any measurable effect upon BMA communities and tylosin (T) and ciprofloxacin (C) had opposite effects on BMA biomass as detected by hyperspectral imaging. The environmentally-relevant concentrations of T and C elicited no measureable effects in any of the physiological, pigment, or hyperspectral parameters (Table 4.2). This suggests that over the short term, the primary productivity of marine BMA communities exposed to these antibiotics is indifferentiable from un-impacted communities.

There are a number of possible reasons that the environmental concentrations didn't have a measureable effect. The low concentrations used in this study are orders of

magnitude lower than those reported to cause measurable effects in diatom cultures, so it may be that concentrations such as these have no measurable negative effects (Hagenbuch and Pinckney, 2012). Additionally, this study included diverse BMA communities associated with their native sediment. Sediment binds chemically active compounds like T and C, possibly making them less available to the eukaryotes inhabiting the spaces between sediment grains (Xu *et al*. 2010). It may be that the species that comprise these communities share a high degree of functional equivalence so that when one constituent species is negatively affected or eliminated, other species might make up the difference. This might be expected given that BMA communities are significant estuarine producers regardless of weather and season (Pinckney and Zingmark, 1993). Finally, it is possible that the exopolysaccharide (EPS) produced by these biofilms acts as a barrier and a binder of large molecular weight compounds such as these (Decho, 1990). Given the relatively short length and breadth of this study, it is impossible to definitively say that there are no physiological or ecological effects of ambient antibiotic concentrations. In fact, we suspect that there may be a significant reorganization in the BMA or sediment bacterial community in response to low-level exposures such as these.

T and C, regardless of concentration, failed to cause any measureable effect in the physiological measurements. Using a MANOVA model including Assay, NPP, and Resp the multivariate p-values were above 0.280 in all cases (Tables 4.3 and 4.4). This is a surprising finding given that Pinckney *et al*. (2013) found a modest effect upon gross community primary productivity (GPP) in BMA communities exposed to tylosin at concentrations similar to what we have used here. Regarding the difference seen between

our experimental runs and with Pinckney *et al*. (2013), this conflict could be attributed to differences in BMA community at different times. BMA communities are neither monolithic nor unchanging. Their character changes markedly over the yearly seasons (Mitbavkar and Anil, 2002; Patil and Anil, 2005; Mitbavkar and Anil, 2006). Hagenbuch and Pinckney (2012) found that different North Inlet species may have widely variable productivity, motility rates, and sensitivity to antibiotics, thus it is possible that differing reports are caused by different BMA communities. It is possible that the outcome of antibiotic exposure assays may strongly depend on the species composition of BMA communities and therefore assays should preferentially be performed during different times of the year. Additionally, the difference between our findings and those of Pinckney *et al*. (2013) could be due to the innate difference between measuring gross (GPP) and net primary productivity (NPP). We measured NPP through the medium of the overlaying water whereas GPP measurements are made at single points within the sediment itself. The most likely reason for the difference is the sub-sampling of the cores for the physiological measurements.

Because of the destructive nature of the sampling, the sub-samples were taken at the end of the experiment on Day 13. HSchla is the mean hyperspectral chlorophyll absorbance of a given surface area. A surface area is composed of N pixels at a resolution of 0.099 mm^2 per pixel. Thus, the area of an experimental core is N = 3852 and for the sub-cores is N = 316. The different surfaces are denoted as: surface of the entire experimental core (Entire), the subsampled area before subcoring (Pre), and the subsampled area after coring (Post). Linear regressions of Pre vs. Post and Pre/Post subcores vs. HPLC chl *a* were non-significant (p = 0.6047, 0.4705, 0.8377) which is

surprising since Pre = Post and Pre/Post was the sediment from which HPLC chl *a* was extracted (Figure 4.4). While the sub-cores didn't match well with HPLC chl *a*, Entire vs. HPLC was significant, if not very predictive (Figure 4.5, $p < 0.001$). Entire vs. Pre also revealed a significant positive relationship (Figure 4.5, $p = 0.006$). Finally, Entire vs. Post indicated a significant but negative relationship (Figure 4.5, $p = 0.006$). These results are perhaps best explained by what we observed when the physical subcoring was done. We observed that the cores with the highest HSchla had a thin (~1 mm) biofilm crust on their surface. When the subcore tube was inserted into these high-HSchla cores the crust was broken, revealing grey, low-HSchla sediment below. This phenomenon was not apparent in cores of low or intermediate HSchla. Therefore the insertion of the sub-core tube redistributed the photosynthetic biomass and therefore negatively affected HPLC pigment extraction and the physiological measurements.

Regarding community composition differences, initial exploratory experiments during from May-August, 2012 we noted that BMA community responses and sediment grain size distribution changed depending on the season. Changes in sediment grain size are indicative of changes in sediment composition. Sediment composition may affect both the BMA community present and the activity of antibiotics by changing the rate of adsorption and release from sediment particles (Pinckney and Zingmark, 1993; Xu *et al*. 2009). While we endeavored to avoid these complications by collecting sediment for both experimental runs at only one point in time , we were apparently unable to completely mitigate the effects. Finally, Weber *et al*. (2011) showed that the activity of C can be increased in aquatic sediments but this study shows that this does not hold true in all instances.

The only parameters affected by the antibiotic treatments were those associated with surface chl *a* biomass. The IC_{50} concentrations of T and C Significantly affected HSChla but in opposite ways: T_{IC50} pushed it down and C_{IC50} pushed it up (Figure 4.3-A and 4.3-B) while in the combined treatment the effects cancelled each other. Not only did the antibiotics cause opposite effects, the time required to cause detectable effects was different. The inhibitory effect of T_{IC50} didn't become significant until day 10-13 whereas C_{IC50} was manifested after 6 days which suggests that future chronic-effect studies should be at least 14 days in duration. The negative effect of T was congruent with previous laboratory culture studies in a very general way. In unialgal cultures of diatom species typical of those found in North Inlet sediments, T is toxic even at ~0.10 mg/l concentrations (Hagenbuch & Pinckney 2012; Pinckney *et al*. 2013). The positive effect of C is surprising, however.

The opposite effects of the two antibiotics are interesting for a number of reasons. The first of which is the differing molar masses of T and C. C has a molar mass equivalent to 33% of T and it is still orders of magnitude less toxic to diatoms than T is (Hagenbuch & Pinckney, 2012). The major reason for this vast difference in toxicity is probably the different mechanisms of action. In target prokaryote organisms, C acts as a DNA synthesis inhibitor and T acts as a protein synthesis inhibitor.

Ciprofloxacin inhibits DNA synthesis in prokaryotes by inhibiting DNA gyrase (Drlica and Zhao, 1997). The chloroplasts of some higher plants contain a form of DNA gyrase and it plays a role in nucleoid partitioning (Lam and Nam-Hai 1987; Cho *et al*. 2004). Since microbial chloroplasts are significantly different from those of higher-plants (such as in the presence of chl *b*), one cannot assume that chloroplast DNA biochemistry

is precisely the same between the two kingdoms. This is supported by the fact that DNA gyrase has not been reported in microbial chloroplasts, with the notable exception of *Cyanidioschyzon merolae*, whose chloroplast contains many genes not found in other algae (Itoh *et al*. 1997; Ohta *et al*. 2003).

In contrast, Tylosin is a protein synthesis inhibitor that binds the 50S subunit of bacterial ribosomes (Omura *et al*. 1983). The 50S ribosomal subunit is widely found in the chloroplasts and mitochondria of both higher plants and microbes (Smith and Bourque, 1985; Kowallik *et al*. 1995). Thus, it is likely that the effect of T is manifested in those organelles. This conclusion is further supported by the loss of motility and *in vivo* chl *a* fluorescence that diatoms exhibit under moderate T exposure (Hagenbuch and Pinckney, 2012). We didn't detect any affect upon motility in this study and that may be due to the max/min method that we used to estimate motility. In our initial trials, we tracked surface chl *a* over a 12-hour daylight cycle, which revealed that 0:00 and 12:00 were appropriate minimum and maximum time points. Thus, in this study, we calculated motility using only those two points, but given the time-sensitive nature of the BMA community motility it is possible we weren't actually measuring the maximum and minimum surface chl *a* values because of a different community motility rate.

An additional factor may play a role in the relative toxicities of these compounds is the chelation of antibiotics by metal ions in seawater. Hagenbuch and Pinckney, (2012) discuss the potential for seawater antagonism of T and C in some detail. However, given that the effect in these assays was positive and negative rather than non-significant, saltwater chelation is not a good explanation for the results presented here. The same can be said for the possibility that the effects here reported are indirect, through the

prokaryotic community rather than direct effects upon the diatoms themselves. That these are indirect effects is certainly possible, however, the IC_{50} concentrations at least have direct negative effects and mechanisms for those effects have been proposed.

While C is much less toxic to diatoms than T, we know of no studies to date have shown that any concentration of C to produce ecological effects that could be interpreted in a positive manner. It may be that C can function in these benthic communities much like it functions when used in humans and animals by exerting pressure against competitive or parasitic prokaryotic species, at least in the short term. A longer study of the chronic effects, especially one that includes metrics for the eukaryotic and prokaryotic communities, is likely to show up negative effects including an increase in antibiotic resistance.

It is equally interesting that with the increase in observed surface biomass we saw no increase in NPP. The reverse was true in T treatments, where low-biomass cores had essentially the same NPP as those with higher biomass. This could be expressed in terms of carbon turnover time where C increases turnover time and T decreases it. When viewed in this way, our results are again surprisingly positive where T can be said to increase aggregate productivity. We think this view is spurious, however and that a better way to explain the results is that there is simply that chl *a* biomass is not what drives productivity. There is some other factor or set of factors that determines how productive a given BMA community is. Hagenbuch & Pinckney, (2012) suggested that antibiotic exposure might cause changes in community productivity as high-productivity species were supplanted by lower-productivity species with better antibiotic resistance. This study lends some limited support for that hypothesis. Future studies should employ some

metric of community composition/diversity to see if these communities really are changing.

Recommendations

Methodologically, we have confirmed that homogenization of sediments is advisable in contaminant exposure assays and we have also shown that mixing is not without measurable effects (Burgess and McKinney, 1997; Anderson *et al*. 2004). Given the possibility of fine-level changes in benthic community structure, future studies should include indexes of eukaryotic and prokaryotic community composition. Studies assessing the impact of season/community-shift should also be done, including assays performed during different times of the year. However, replicates should be run concurrently to minimize founder-effect community shifts and "natural" temporal community variability. Given that the effects of T were not significantly manifest until day 10-13, future chronic-effect studies should be at least 14 days in duration. It is possible to miss the surface chl *a* peak/trough with a two-point calculation, therefore community motility rates should be calculated using iterative scans covering individual diurnal periods. Follow-up studies might investigate whether the negative effects that we have reported can be mitigated via the addition of nutrients (N, P, etc.), inorganic carbon (CO_2), or vitamin cofactors which might help narrow down the efficient cause(s). Finally, those interested in the general activity of the prokaryotic community under antibiotic stress might investigate whether there is a build-up of soluble EPS in antibiotic-treated benthic biofilms.

CONCLUSION

We have shown in this study that the low levels of antibiotics typically reported in environmental water bodies are unlikely to produce measurable effects in ecologically-relevant parameters such as net primary productivity in benthic microalgal communities over time scales of 0-14 days. We have also shown that toxicity values such as half-max inhibitory concentrations that are developed using unialgal laboratory cultures are capable of producing measurable effects in these same communities over the same time scale.

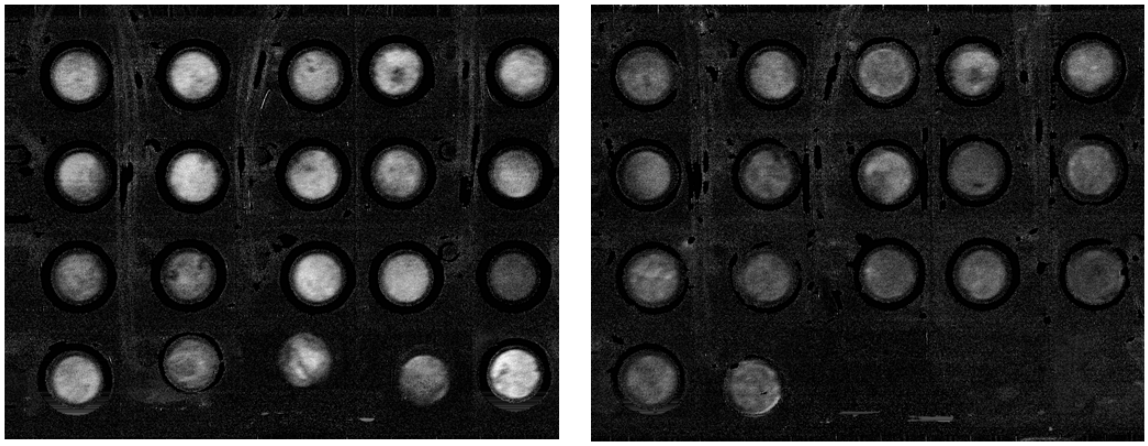

Figure 4.1: Grayscale images of hyperspectral chl *a* absorbance of assay cores. Brighter areas indicate higher chl a concentrations. The cores in the left image have not been homogenized. The cores in the right image the same sediment cores after half of them had been homogenized. The natural variation (left image) was three-fold between the highest and lowest cores and was reduced to 20% after homogenization (right image).

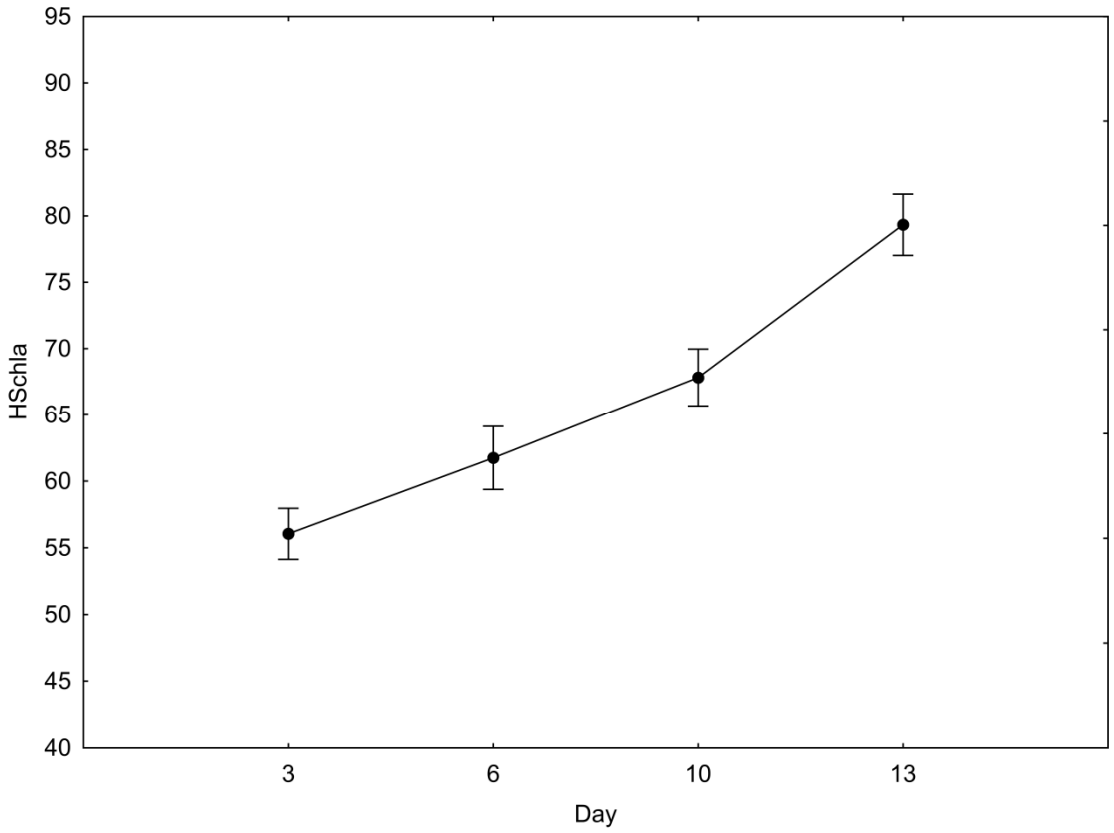

Figure 4.2: Hyperspectral chl *a* (HSchla) throughout the antibiotic addition assay. Error bars are 95% confidence intervals.

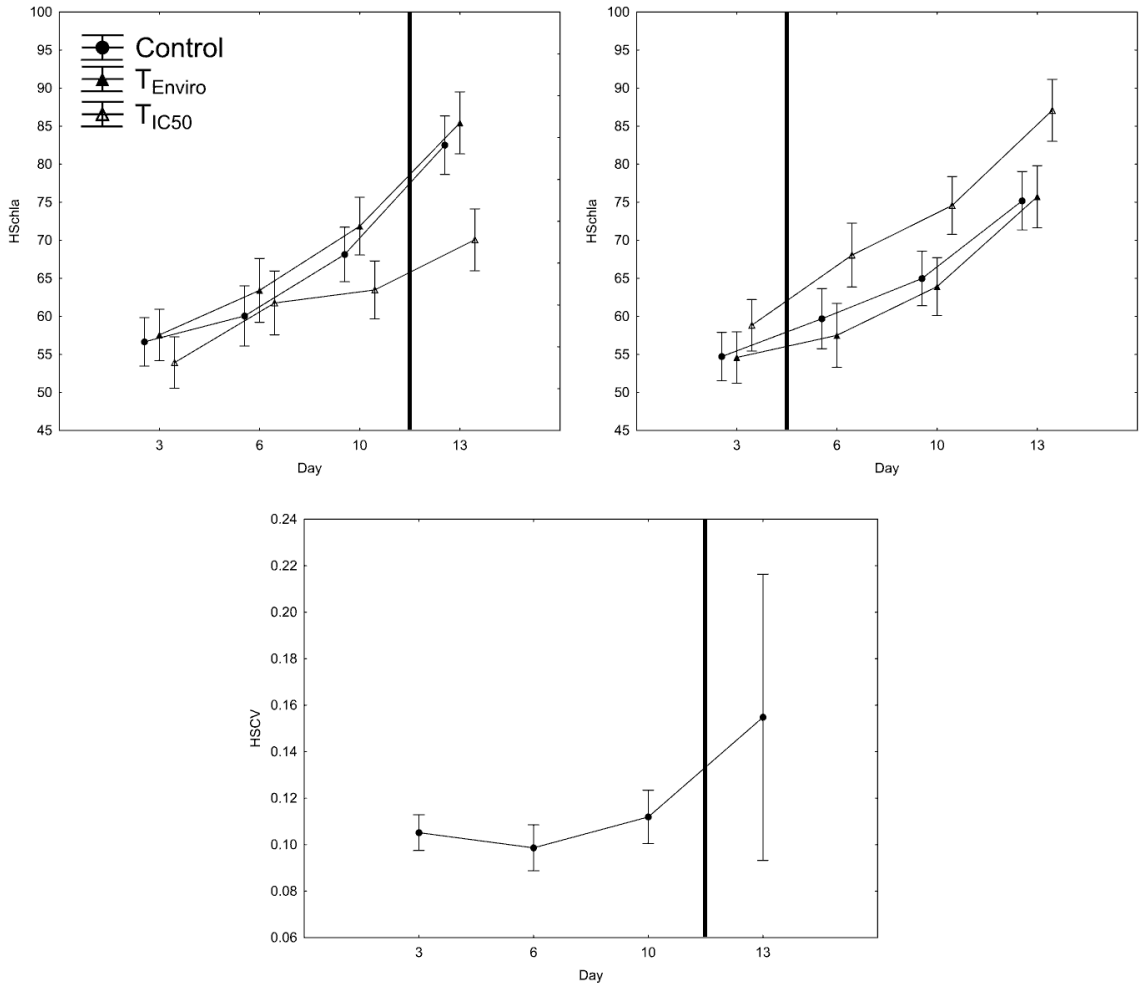

Figure 4.3: HSchla during the antibiotic assays. Vertical lines indicate days that significantly differ. differences. **A)** HSchla in cores exposed to environmentally an relevant concentration of tylosin (T_{Enviro}) and the half-max inhibitory concentration (T_{IC50}). T_{IC50} significantly depressed HSchla from day 10 through the end. **B)** HSchla in cores exposed to ciprofloxacin. By day 6 the C_{IC50} cores had significantly more surface chl a than the other two treatments. **C)** HSCV of all the antibiotic treatment cores. The effect of Day was caused by the increase in heterogeneity on day 13. Error bars are 95% confidence intervals.

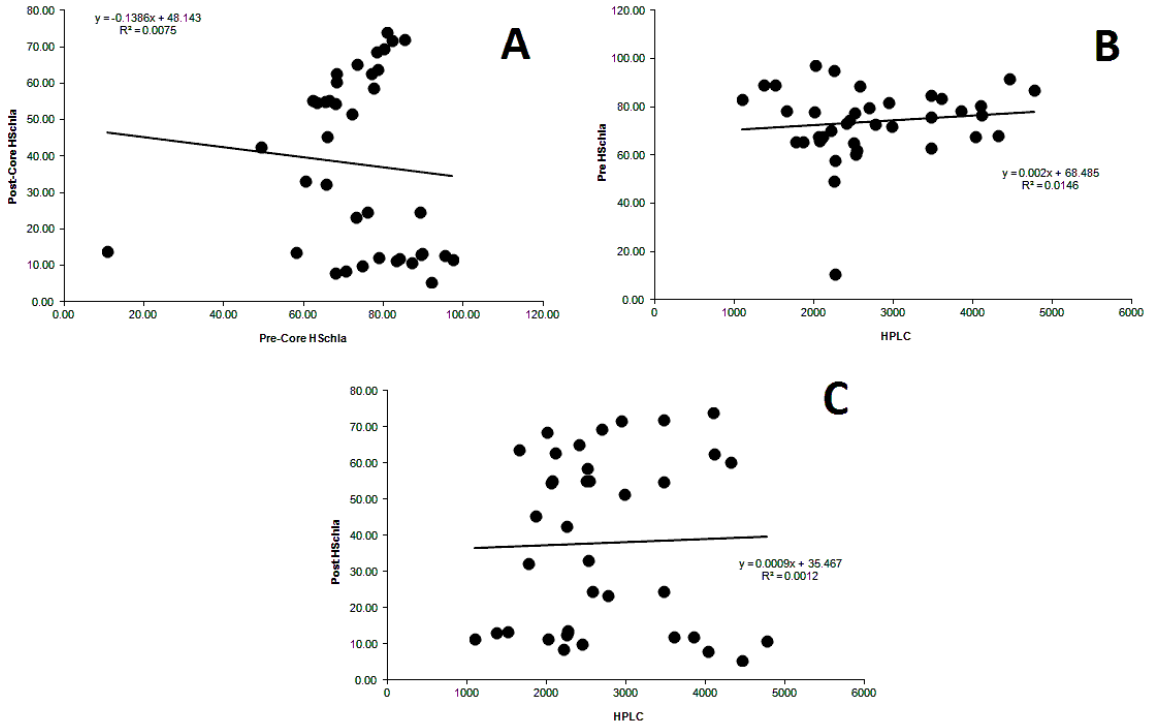

Figure 4.4: Regressions of surface chlorophyll (HSChla) and extracted chlorophyll (HPLC). Data come from the experimental core before (Pre) and after (Post) the sub-core had been inserted. **A)** Pre vs. Post HSChla. **B)** Pre HSChla vs. HPLC. **C)** Post vs. HPLC.

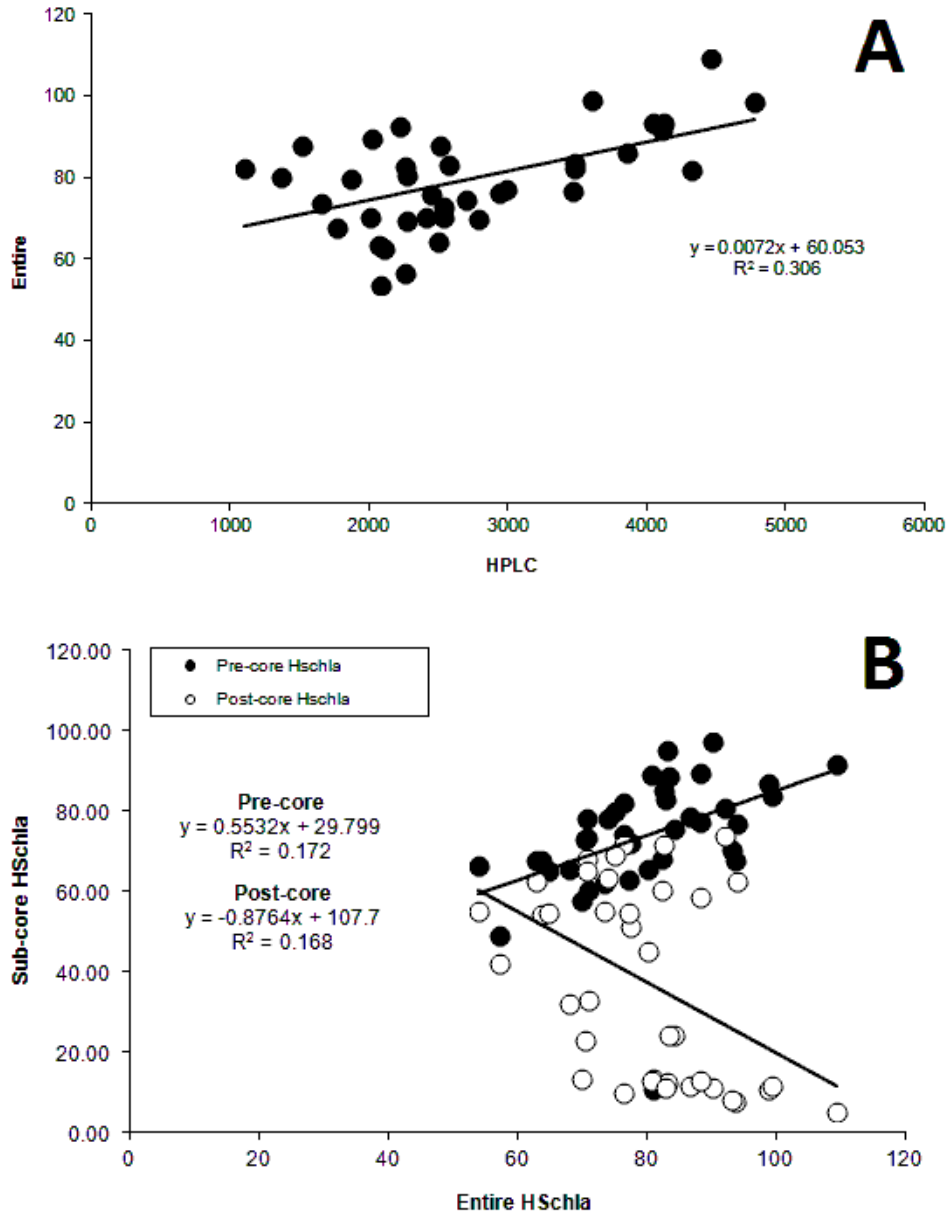

Figure 4.5: Regressions surface (HSChla) and extracted (HPLC) chlorophyll in the experimental and sub-cores. **A)** HSChla of the entire experimental core surface vs. extracted chlorophyll of sub-cores. **B)** Pre and Post sub-core HSChla vs. HSChla of entire experimental cores.

TABLE 4.1: Antibiotics and non-target species

Organism	Classification	Antibiotic Compound	Reference
Daphnia magna	Crustacean	metronidazole olaquindox oxolinic acid oxytetracycline streptomycin sulfadiazine tetracycline tiamulin tylosin	Wollenberger *et al.* (2000)
Chlamydomonas reinhardtii		erythromycin neamine/kanamycin streptomycin spectinomycin	Harris *et al.* (1989)
Vibrio fischeri *Brachionus calyciflorus* *Thamnocephalus platyurus* *Daphnia magna* *Ceriodaphnia dubia* *Danio rerio*	Bacterium Rotifer Crustacean Crustacean Crustacean Fish	clarithromycin erythromycin lincomycin sulfamethoxazole ofloxacin oxytetracyclin	Isadori *et al.* (2005)
Selenastrum capricornutum *Microcystis aeruginosa* *Daphnia magna* *Danio rerio*	Green algae Cyanobacterium Crustacean Fish	mecillinam trimethoprim ciprofloxacin	Halling-Sørensen *et al.* (2000)
Selenastrum capricornutum	Green algae	ciprofloxacin erythromycin sulfamethoxazole	Liu *et al.* (2011)
Euglena gracilis	Protist	144 compounds	Ebringer (1972)
Brachionus calyciflorus *Brachionus plicatilis*	Rotifer Rotifer	streptomycin tetracycline tylosin	Araujo and McNair (2007)
Lemna gibba	Higher plant	22 compounds	Brain *et al.* (2004)
Cylindrotheca closterium *Navicula ramosissima*	Diatom Diatom	ciprofloxacin lincomycin tylosin	Hagenbuch and Pinckney (2012)
Pseudokirchneriella subcapitata *Anabaena sp.*	Green algae Cyanobacterium	amoxicillin erythromycin levofloxacin norfloxacin tetracycline	Gonza´ lez-Pleiter *et al.* (2013)

Table 4.2: Antibiotic Assay Statistics

a. Hschla - Univariate Repeated Measures

	SS	df	MS	F	p
T	1293	2	647	5.7	**0.009**
C	2554	2	1277	11.2	< **0.001**
T*C	865	4	216	1.9	0.139
Assay	33	1	33	0.3	0.592
DAY	1.11E+04	3	3715	193.1	< **0.001**
DAY*T	902	6	150	7.8	< **0.001**
DAY*C	283	6	47	2.5	0.031
DAY*T*C	214	12	18	0.9	0.526

b. HSCV - Univariate Repeated Measures

	SS	df	MS	F	p
T	0.029	2	0.015	1.275	0.295
C	0.031	2	0.016	1.366	0.272
T*C	0.065	4	0.016	1.406	0.258
Assay	0.018	1	0.018	1.544	0.224
DAY	0.072	3	0.024	2.907	**0.039**
DAY*T	0.059	6	0.01	1.194	0.317
DAY*C	0.062	6	0.01	1.25	0.289
DAY*T*C	0.119	12	0.01	1.195	0.300

Table 4.3: Antibiotic Assay Physiology

a. NPP & Resp - Multivariate

	SS	df	MS	F	p
T	0.833	1.29	4.00	54.00	0.285
C	0.884	0.86	4.00	54.00	0.492
T*C	0.742	1.09	8.00	54.00	0.387
Assay	0.475	14.94	2.00	27.00	< 0.001

b. NPP - Univariate

	df	SS	MS	F	p
T	2	31.22	15.61	1.08	0.354
C	2	2.52	1.26	0.09	0.917
T*C	4	22.97	5.74	0.40	0.810
Assay	1	0.92	0.92	0.06	0.803

c. Resp - Univariate

	df	SS	MS	F	p
T	2	5.21	2.61	1.07	0.358
C	2	7.68	3.84	1.57	0.226
T*C	4	18.14	4.53	1.85	0.146
Assay	1	72.56	72.56	29.67	**0.000**

Table 4.4: Antibiotic Assay Pigments

a. Biomass & Community Ratio - Multivariate

	Wilks	F	dfe	Edf	p
T	0.855	1.10	4	54	0.366
C	0.893	0.79	4	54	0.538
T*C	0.927	0.26	8	54	0.976
Assay	0.646	7.40	2	27	0.003

b. Biomass - Univariate

	SS	df	MS	F	p
T	1.57E+09	2	7.85E+08	2.1	0.145
C	1.04E+09	2	5.21E+08	1.4	0.270
T*C	1.97E+08	4	4.93E+07	0.1	0.970
Assay	2.39E+09	1	2.39E+09	6.3	**0.018**

c. Community Ratio - Univariate

	SS	df	MS	F	p
T	28.36	2	14	0.1	0.925
C	196.67	2	98	0.5	0.586
T*C	250.49	4	63	0.3	0.844
Assay	871.10	1	871	4.8	**0.036**

FINAL CONCLUSION

The following list of questions was used as an outline for the focus group questions. Benthic diatoms are a ubiquitous and indispensible component of coastal marine ecosystems. As the coastal ocean is more and more impacted by human activities, it is important that we determine how benthic diatom communities may respond. The goal of this dissertation research was to determine if anthropogenic antibiotics negatively affect marine diatoms and the benthic communities of which they are a part.

Chapter 2 was an investigation of a new method to quantifying surface-associated diatoms. I demonstrated that Guillard f/2 + Si enriched seawater to be an ideal medium for use in benthic microalgal fluorescent bioassays. It contributes virtually no auto-fluorescent noise or variability to the assays. I also showed that the relationship between cell number and chl a fluorescence to be consistent across a wide range of cell concentrations and population ages.

I was also able to show that tylosin, ciprofloxacin, and lincomycin substantially reduce chl *a* fluorescence emission of the benthic diatom *C. closterium*. The highest antibiotic concentrations had a significant effect on chl *a* fluorescence, but intermediate doses were more difficult to separate and the effect of the two lowest concentrations were indistinguishable from the control. Tylosin had the most pronounced effect, followed closely by lincomycin.

Microtiter fluorometric assays are a possible method for quickly estimating surface associated microalgal populations. This might have been extremely useful for

low-resolution dose-response assays where relative growth rates are desired and quantification such as those done by Halling-Sørensen, (2000) and Eguchi et al. (2004). As nice as this method might look, I decided to do standard microscopy counts in future work.

In Chapter 3, I assessed the toxicity of the same three antibiotics used in Chapter 2, but against two benthic diatom species. Tylosin (T) exhibited the most negative effect, with an IC_{50} of 0.27 mg/l in *C. closterium* and 0.96 mg/l for *N. ramosissima*. Lincomycin (L) following distantly behind with 14.2 and 11.1 mg/l, respectively. The lesser effect exhibited by *N. ramosissima* was consistent across all treatments, except L. Because post-hoc analyses were not done, it is not possible to be certain of whether the difference between the species is statistically significant but it is possible, given the wide standard deviation of the *N. ramosissima* IC50 (CV = 1.00) that the responses did not significantly differ. Ciprofloxacin (C), long recognized as a compound of concern, when applied alone, it is less toxic to benthic diatoms than other common compounds.

The most interesting results in Chapter 3 are the species-specific differences in the antibiotic interaction assays (Figure 3.3). In C. closterium all antibiotic mixtures, with the exception of T+C, worked together to elicit a synergistically (i.e. multiplicative) negative response. This sharply contrasts against the response of *N. ramosissima* in which all mixtures behaved additively. *N. ramosissima*'s response to T+L and T+L+C were variable (CV = 1.20 and 1.24) which is probably why the very high IC_{50}'s for those treatments (6.98 and 10.22 TU) were not determined to be significantly different from 1.00 (p = 1.18 and 1.25). Regardless, this suggests that benthic primary producers exposed to multiple antimicrobial compounds in the environment may be subject to

selection pressures from that pharmaceutical profile. This suggest that different species may respond differently to different antibiotics and this result has implications for benthic ecology. Evrard *et al*. (2012) found that benthic consumers subsist almost entirely on diatom-dominated BMA communities, which opens an avenue for upward-propagating trophic effects either through the reduction of available carbon or through alteration of preferred food abundance (Vanden Berghe and Bergmens, 1981; Carman and Thistle, 1985). As a corollary, microalgal species are unequally productive either through differences in cell physiology or migratory behavior (Underwood *et al*. 2005). The anthropogenic antimicrobial compounds used in this study may be at play at either of these avenues, given that diatoms exposed to moderate antibiotic concentrations ceased motility. Even a moderate reduction in benthic diatom movement rate can be sufficient to significantly impact benthic primary productivity given the tight coupling between benthic diatom migration and tide stage (Hay *et al*. 1993; Underwood and Kromkamp, 1999). Finally, biodiversity of algal species serves to improve water quality and may confer resilience to benthic microbial communities (Elmqvist *et al*. 2003; Cardinale *et al*. 2006, Cardinale, 2011).

In Chapter 4, I investigated the effects of antibiotic contamination at laboratory-derived concentrations (IC_{50}) and environmentally-relevant concentrations upon benthic algal communities. I showed that the low levels of antibiotics typically reported in environmental water bodies are unlikely to produce measurable effects in ecologically-relevant parameters such as net primary productivity in benthic microalgal communities over time scales of 0-14 days. I have also shown that toxicity values such as half-max inhibitory concentrations that are developed using unialgal laboratory cultures are

capable of producing measurable effects in these same communities over the same time scale.

Only the IC_{50} concentrations had any measurable effect upon BMA communities and tylosin (T) and ciprofloxacin (C) had opposite effects on BMA biomass as detected by hyperspectral imaging. The environmentally-relevant concentrations of T and C elicited no measureable effects in any of the physiological, pigment, or hyperspectral parameters. This suggests that over the short term, the primary productivity of marine BMA communities exposed to these antibiotics is indifferentiable from un-impacted communities.

Regarding community composition differences, initial exploratory experiments during from May-August, 2012 we noted that BMA community responses and sediment grain size distribution changed depending on the season. Changes in sediment grain size are indicative of changes in sediment composition. Sediment composition may affect both the BMA community present and the activity of antibiotics by changing the rate of adsorption and release from sediment particles (Pinckney and Zingmark, 1993; Xu *et al*. 2009). While we endeavored to avoid these complications by collecting sediment for both experimental runs at only one point in time, we were apparently unable to completely mitigate the effects. Finally, Weber *et al*. (2011) showed that the activity of C can be increased in aquatic sediments but this study shows that this does not hold true in all instances.

The only parameters affected by the antibiotic treatments were those associated with surface chl a biomass. The IC_{50} concentrations of T and C Significantly affected HSChla but in opposite ways: T_{IC50} pushed it down and C_{IC50} pushed it up while in the

combined treatment the effects cancelled each other. Not only did the antibiotics cause opposite effects, the time required to cause detectable effects was different. The inhibitory effect of T_{IC50} didn't become significant until day 10-13 whereas C_{IC50} was manifested after 6 days which suggests that future chronic-effect studies should be at least 14 days in duration. The negative effect of T was congruent with previous laboratory culture studies in a very general way. In unialgal cultures of diatom species typical of those found in North Inlet sediments, T is toxic even at ~0.10 mg/l concentrations (Hagenbuch & Pinckney 2012; Pinckney *et al*. 2013).

While C is much less toxic to diatoms than T, I know of no studies to date have shown that any concentration of C to produce ecological effects that could be interpreted in a positive manner. It may be that C can function in these benthic communities much like it functions when used in humans and animals by exerting pressure against competitive or parasitic prokaryotic species, at least in the short term. A longer study of the chronic effects, especially one that includes metrics for the eukaryotic and prokaryotic communities, is likely to show up negative effects including an increase in antibiotic resistance.

Thus I have shown that high concentrations of anthropogenic antibiotics have negative physiological and ecological effects on marine benthic diatoms and their communities. I have also shown that chronic exposure to low-levels of antibiotics may have no detectible effects.

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APPENDIX A: COPYRIGHT RELEASES

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