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## Drivers of Extracellular Polysaccharide Production by a Mat-Forming Diatom

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

DRIVERS OF EXTRACELLULAR POLYSACCHARIDE PRODUCTION BY A MAT-  
FORMING DIATOM

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGY

by

Kaitlin Auguste Stansbury

2021

To: Dean Michael Heithaus  
College of Arts, Sciences and Education

This thesis, written by Kaitlin A. Stansbury, and entitled Drivers of Extracellular Polysaccharide Production by a Mat-Forming Diatom, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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Evelyn Gaiser, Major Professor

Date of Defense: November 9, 2021

The thesis of Kaitlin A. Stansbury is approved.

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Dean Michael Heithaus  
College of Arts, Sciences and Education

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Andrés G. Gil  
Vice President for Research and Economic Development  
and Dean of the University Graduate School

Florida International University, 2021

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## ABSTRACT OF THESIS

### DRIVERS OF EXTRACELLULAR POLYSACCHARIDE PRODUCTION BY A MAT- FORMING DIATOM

by

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Florida International University, 2021

Miami, Florida

Professor Evelyn Gaiser, Major Professor

Microbial biofilms are held together by extracellular polymeric substances (EPS), which can facilitate gamete exchange, nutrient sequestration, and desiccation resistance. Microbial mats (periphyton) of the Everglades contain EPS generated by mat-dwelling algae, cyanobacteria, and bacteria, including the most abundant Everglades diatom, *Mastagloia calcarea*. This diatom is characterized by production of an EPS ‘halo’ around the frustule, but the cellular and environmental triggers of EPS production are unknown. In this study, I examined how environmental conditions, reproduction, and bacteria affect the presence and EPS halo area around *M. calcarea* by analyzing *M. calcarea* cells in field and cultured samples. I found that small, paired (likely copulating) cells were more likely to have EPS halos in low ionic conductivity, higher pH, and low phosphorus concentrations with bacteria present. These results indicate that EPS production by diatoms is maintained in ultraoligotrophic, karstic wetlands where beneficial associations with bacteria support population turnover and copulation.

## TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION.....	1
II. METHODOLOGY.....	5
III. RESULTS.....	12
IV. DISCUSSION.....	14
LIST OF REFERENCES.....	21

## LIST OF FIGURES

FIGURE	PAGE
1. Site Map of Field Survey Sample Sites.....	28
2. Measurements of <i>Mastogloia calcarea</i> cells.....	30
3. Paired and Single Cells with and without EPS Halo.....	31
4. Size Declination of Cells with and without EPS.....	33
5. Distribution of Measurements in Cells from Field Samples.....	34
6. Interaction Plot and Predicted Values for EPS Area from GAM.....	36
7. Distribution of Mean EPS Halo Area at Each Field Site.....	38
8. Interaction Plot and Predicted Values for Percentage of EPS Cells from GAM...39	
9. Distribution of Measurements in Cells from Culture Samples.....	40
10. Distribution of Measurements in Paired and Single Cells.....	41
11. Paired Cell Count and Percentage of Cells with EPS over 6 Weeks.....	42
12. Percentage of Cells with EPS in Untreated and Antibiotic Treated Cultures.....	43
13. Paired Cell Count Comparison Between Culture Treatments.....	44

## ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
CERP	Comprehensive Everglades Restoration Plan
DIC	Differential Interface Contrast
EDEN	Everglades Depth Estimation Network
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
FIU	Florida International University
GAM	Generalized Additive Model
MAP	Monitoring and Assessment Program
MeCoCo	Metabolically Cohesive Consortia
PSU	Primary Sampling Unit
SEM	Scanning Electron Microscope
TP	Total Phosphorus



## I. INTRODUCTION

Production of extracellular polymeric substances (EPS) is a distinctive feature of ‘metabolically cohesive consortia’ (MeCoCo) – interdependent microbial communities that engineer their environment by creating and consuming their own resources (Pascual-Garcia et al. 2020). In benthic aquatic ecosystems, MeCoCos may form biofilms and mats containing up to 90% EPS secreted by cyanobacteria, bacteria, and algae (O’Toole et al. 2000; Jain et al. 2007). While EPS secretion is associated with MeCoCo maturation and is understood to increase mutualistic interactions among microbes (O’Toole et al. 2000; Flemming et al. 2000), less is known about what regulates its formation, particularly in ultraoligotrophic systems where EPS-rich MeCoCos are common.

Ultraoligotrophic karstic wetlands like the Florida Everglades harbor well-developed MeCoCos, forming expansive ‘periphyton’ mats of algae, bacteria, fungi, and detritus that float on the water surface or coat sediment and macrophyte surfaces (Browder et al. 1994; Gaiser et al. 2015). These mats supply food and refuge for small organisms at the base of aquatic food webs, contribute to the production of detritus and marl soils, and control gas and nutrient concentrations in the water column (Williams & Trexler 2006; Neto et al. 2006; Gaiser et al. 2006). EPS can comprise up to 90% of the volume of benthic periphyton mats (Donar et al. 2004), which is produced by filamentous and coccoid cyanobacteria and the most abundant diatom, *Mastogloia calcarea* (previously called *M. smithii* and *M. smithii* var. *lacustris*; Gaiser et al. 2006; Gaiser et al. 2010; Lee et al. 2014). Hagerthey et al. (2011) suggests that EPS can mediate calcium carbonate deposition (Merz 1992), increase attachment of enzymes to cell surfaces (i.e., alkaline phosphatase producing inorganic phosphate for cells) (Wright & Reddy 2001),

and aid in desiccation resistance by the binding of water in the EPS molecular structure (Gaiser et al. 2011). Determining the drivers of EPS production by microbes comprising periphyton mats will help unravel the ‘paradox of production’ (Gaiser et al. 2012) wherein the abundance of EPS-rich periphyton is much higher than expected in the ultraoligotrophic conditions that characterize karstic wetlands.

This study focuses on the drivers of EPS production by *M. calcarea*, which comprises 31% of the diatom species richness in the Everglades and 0.02-0.08% of the biovolume of Everglades periphyton mats (Gaiser et al. 2010; Lee et al. 2014), and dominates the flora of other karstic wetlands (La Hée & Gaiser 2012). *M. calcarea* is abundant in mats in the most oligotrophic settings in the Everglades, reaching greatest abundance in the shallow, limestone-rich areas of that desiccate for several months each year during the subtropical dry season (Gaiser et al. 2010). *M. calcarea* may be excreting EPS as a photosynthetic exudate under phosphorus scarcity much like the diatom *Didymosphenia geminata* (Kilroy & Bothwell 2012). The release of EPS by *D. geminata* occurs when phosphorus limitation prevents metabolism of photosynthetically fixed carbon (Shniukova & Zolotareva 2015) so it is catabolized and exuded extracellularly (Kilroy & Bothwell 2012; Shniukova & Zolotareva 2015). EPS is released outside of the cell membrane through openings in the frustule, including the raphe or apical pore field (Wang et al. 2000). In *M. calcarea*, scanning electron microscopy revealed strands of EPS extending from partectal pores (Lee et al. 2014). The presence of EPS may also be influenced by ion concentration due to the ability of EPS to form cation bridges within the mat (Swift & Nicholas 1987; Bellinger et al. 2010). Bellinger et al. (2010) found that reduced concentrations of divalent cations decreased formation of the rigid matrices of

polymers that allow EPS to coagulate (Bellinger et al. 2010). Lower water pH in areas or times of high rainfall-driven hydrology, by high rates of ecosystem respiration, or in areas not underlain by limestone is also associated with low abundances of EPS-rich periphyton in karstic wetlands (Bellinger et al. 2010). Acidophilous diatom and desmid-rich wetland periphyton communities contain low abundances of *M. calcarea* and other diatoms known to secrete copious EPS and those that do are unable to form rigid structures of polymers (Bellinger et al. 2010). Finally, desiccation stress in short hydroperiod (flooded <6 mo. per year) wetlands where *M. calcarea* and periphyton mats are most abundant may trigger EPS production by *M. calcarea* (Iwaniec et al. 2006; Ewe et al. 2006; Gaiser et al. 2010). Increased EPS production in desiccation-prone habitats has been found in other species of algae, including *Nostoc* spp. (Tamaru et al. 2005). Determining how phosphorus availability, ionic conductivity, pH, and desiccation influence EPS production or indirectly trigger other processes that require EPS by this common diatom will help determine why productive MeCoCos rich in EPS can form and be sustained in ultraoligotrophic conditions.

While the environmental settings of karstic wetlands may encourage physicochemical conditions that promote EPS production, they may also create optimal conditions for diatom reproduction that may also benefit from EPS. Other *Mastogloia* species, such as related *Mastogloia smithii* (Stickle 1986) and other EPS-generating species such as *Cymbella* sp. (Stevenson 1990), have been found producing EPS during copulation. *Mastogloia smithii*, which is a species closely related to *M. calcarea*, was found undergoing copulation with pairs of cells encased in an EPS 'halo' (Stickle 1986). These species and others produce EPS in the Golgi apparatus which is then transported in

vesicles to the cell membrane where it is excreted as an envelope to facilitate the movement of motile gametes between the two parent cells (Wetherbee et al. 1998; Amato 2010). Containing the two parent cells within the EPS halo guarantees the zygote/frustule bond, while providing a stable environment for this process to occur.

Finally, EPS production may foster mutually beneficial relationships between algae and bacteria through passive signaling that induces carbohydrate secretion to form the matrix of the biofilm (Bruckner et al. 2011). The process of passive signaling involves bacteria producing different proteins that can bind to a receptor on the surface of diatoms that trigger complex signal cascades to produce more extracellular carbon (Clark et al. 1997; Bruckner et al. 2011). EPS production by some diatom taxa has been shown to decline in the absence of bacteria in culture (Amin et al. 2012; Windler et al. 2015). In addition to exchanging metabolites, bacteria that excrete alkaline phosphatase (Wright & Reddy 2001) may provide necessary inorganic phosphate for *M. calcarea* or signal the diatom to produce EPS when conditions are favorable to continue the formation and growth of the biofilm.

This study was conducted to determine potential drivers of EPS halo formation in the mat-forming, EPS-secreting diatom *M. calcarea*. A survey approach was used to determine whether the appearance of EPS halos in *M. calcarea* increases with decreasing phosphorus availability and ionic conductivity, and to measure interactions with pH and hydroperiod (as a measure of desiccation exposure). Cultured cells were also measured and cell pairs were noted to determine whether EPS halos were associated with the critical minimum cell size triggering sexual reproduction. Finally, to determine if EPS halos are reduced in the absence of bacteria, *M. calcarea* was grown in culture with and

without antibiotic treatment. Understanding the conditions under which this diatom produces EPS will improve concepts of why EPS-production by diatoms tends to be high in benthic, oligotrophic MeCoCos.

## II. METHODOLOGY

### **Sample Site**

The Everglades is a karstic wetland located in southern Florida. It was formed over 5,000 years ago due to rising seas that flooded a limestone depression (Davis & Ogden 1994). Due to its karstic bedrock, this wetland is very oligotrophic and rich in calcium carbonate, which is also precipitated by the periphyton mats that cover most of the landscape (McCormick & O'Dell 1996; McCormick et al. 1996). This concept is applicable to mat-forming communities distributed globally, especially in the Caribbean, where karstic and non-karstic mats are present (La Hée & Gaiser 2012). The Everglades ecosystem has a broad range of environmental conditions, with freshwater wetlands inland to mangrove ecosystems closer to the coast. Freshwater inlands saturated with calcium carbonate contain mats abundant in EPS producing coccoid cyanobacteria and diatoms, including *M. calcarea* (Gottlieb et al. 2006).

### **Field- Based Assessment of EPS Halos in *M. calcarea***

#### *Field Collection*

Representative periphyton samples were collected from 84 locations (Figure 1) during the 2020 wet sampling season (September to November) as part of the Comprehensive Everglades Restoration Plan (CERP) Monitoring and Assessment

Program (see Lee et al. 2013 for details). Two randomly chosen samples were collected at each site, totaling 168 samples. Briefly, the Everglades landscape was divided into a 800 m x 800 m grid stratified by landscape units and sampling locations drawn as primary sampling units (PSU) (Philippi 2005). The normal wet season occurs from May to November, but sampling occurs towards the end of the wet season due to cooler temperatures and shallower water depths. At each sampling site, water depth was measured, and a water sample was taken to determine ionic conductivity ( $\mu\text{S cm}^{-1}$ ) and pH (Table 1). Days since last dry-down for each site were calculated from calibrated water level measurements that were estimated from nearby water level gauges using digital elevation models provided by the Everglades Depth Estimation Network (EDEN) (<http://sofia.usgs.gov/eden/stationlist.php>). Periphyton was removed from a 1 m<sup>2</sup> area with a seine, homogenized by hand, and two 120 ml samples were removed and placed on ice. One sample was frozen at -4°C freezer before further processing for diatom relative abundances, and the second was placed in a 4°C refrigerator until microscopic examination.

### *Sample Processing*

Frozen periphyton samples were thawed in a 4°C refrigerator and then placed in a dish where all non-periphyton material and debris was removed using forceps. After homogenizing with a hand blender, a 10 ml subsample was frozen for later diatom analysis, a 40-ml subsample was dried at 50°C for 3 d and then combusted at 500°C for 1 h for dry and ash-free dry mass, and a 40-ml subsample was dried in a 50°C oven and ground in a Wiley mill for analysis of total phosphorus by the FIU CACHE Nutrient Analysis Core Facility using colorimetric analysis on the Shimadzu 2451 UV-VIS

Spectrophotometer following procedures from Solórzano & Sharp (1980). In oligotrophic wetlands, periphyton total phosphorus is used as a measure of phosphorus availability because water column concentrations are typically below instrumental detection limits (Gaiser et al. 2004).

#### *EPS Measurements*

Refrigerated samples were hand-homogenized in the sample bag and water was removed by pipette. A drop of the remaining water from the homogenized samples was used for microscopic examination. Each *M. calcarea* cell encountered was photographed under brightfield and DIC filters using the Zeiss AX10 with Imager.A2 light microscope under 400x magnification. A total of 100 *M. calcarea* cells were photographed unless densities were too low to achieve. Images were uploaded into ImageJ 4 and calibrated with a 10  $\mu\text{m}$  scale bar. The following attributes were recorded: orientation (valve or girdle view), EPS halo (present/absent), and if present, number of cells per halo, valve length and width (using “straight” tool), and cell and cell plus halo area (using “freehand” tool) (Figure 2 and 3). The EPS halo area was then calculated subtracting the cell area from total area.

#### *EPS Extractions*

A second fresh periphyton subsample was homogenized using a hand blender and dried for one week in a 50°C oven. A ~1 g subsample was weighed and placed into a beaker with 20 mL of deionized water and a stir bar. The homogenized solution was placed in a 50 mL tube and left for 1 h to extract water soluble EPS (Bertocchi et al. 1990). The tube was then centrifuged at 1200 x g for 15 m to separate the supernatant containing water-soluble EPS from the non-water soluble material (Bertocchi et al.

1990). The supernatant was removed by pipette and placed in a beaker for later EPS precipitation. Next, 20 mL of 0.1M EDTA was added to the non-water soluble material for 1 h to extract EPS from algal and bacterial cell walls and mucilage sheaths (Decho 1990, Underwood et al. 1995, Decho et al. 2005). This solution was centrifuged at 1200 x g for 15 m, and the EDTA-soluble supernatant was pooled with the water-soluble supernatant. EPS was extracted from the pooled supernatant by ethanal precipitation using 80 mL of 90% ethanol and placed in a 4°C refrigerator for 24 h. The solution was then transferred to a centrifuge tube and centrifuged at 3000 x g for 15 m. The supernatant was discarded. The EPS pellet was washed three times using 70% ethanol to remove any remaining EDTA (Underwood et al. 2004). The pellet was transferred into a poly-con container, lyophilized, and weighed. The lyophilized EPS sample was then ground by hand and sent to the Blue Carbon Analysis Laboratory at Florida International University for total phosphorus, nitrogen, and carbon analysis. Total carbon and nitrogen in the EPS were analyzed on the Thermo Flash 112 Elemental Analyzer using standard procedures. Total phosphorus was analyzed using colorimetric analysis on the Shimadzu 2451 UV-VIS Spectrophotometer following procedures from Solórzano & Sharp (1980).

#### *Data Analysis*

Cell length, width, and area were compared between cells with and without visible EPS using a two-sample t-test ( $n = 6961$ ) in R (R Core Team 2020 version 1.3.1093). Assumptions of normality, independent and random sampling, continuous data values, and equal variances were met. Pearson correlation coefficients were calculated comparing ionic conductivity, periphyton total phosphorus concentration, pH, days since last dry, and proportion of organic content. Generalized additive models (GAMs) were



used to evaluate the impact of environmental, spatial and periphyton mat characteristics on EPS production and the percentage of cells producing EPS at each PSU using R. This method was used because it allows us to account for nonlinear relationships in my data, which occurred with the included environmental variables in my models. The assumptions of additive functions, smooth components, and independence of observations were met. Deviance explained (analogous to variance in linear regression) was calculated for each model. Variables were selected based on its significance within the model and the change in deviance explained. The interaction effect between ionic conductivity and pH was significant and used in both GAM models. Random effects (time and location) were analyzed using a GAM, but the model was not significant, so the model was not used. An alpha of 0.05 was used to denote significance.

### **EPS Halo Production by *M. calcarea* in Culture with and without Bacteria**

#### *Culturing*

Periphyton collected once on May 12, 2021 from a pond located outside of the Florida City entrance to Everglades National Park (25.858244°, -81.385071°) was sieved through a 45 µm sieve to concentrate *M. calcarea* cells for culturing. The sieved material was either pipetted and spread onto solid media (BG-11 or filter-sterilized Everglades source water, using either agar or agarose) in sterile petri dishes or inoculated into liquid media (BG-11, filter-sterilized Everglades source water, and a 1:1 mixture of the two) in sterile glass culture tubes and 100 mL flasks. Cultures were incubated at 25°C under direct light (40 µmol photons m<sup>-2</sup> s<sup>-1</sup>) or indirect (10 µmol photons m<sup>-2</sup> s<sup>-1</sup>) fluorescent light continuously for 3 weeks to determine the best illumination conditions for growth.

Each culture was then microscopically examined for viable and reproducing *M. calcarea* cells to determine the best media for culturing this taxon. Agarose provided the best environment for sustained *M. calcarea* colony growth, therefore six additional petri dishes containing Everglades source water with agarose were inoculated with subsamples from the Everglades agarose culture and were incubated at 25°C under 10  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  indirect fluorescent light continuously for 1 week. The six cultures were then treated in triplicates with either a ½ dilution of an antibiotic mix with deionized water (5 mg mL<sup>-1</sup> penicillin and streptomycin, and 10 mg mL<sup>-1</sup> neomycin; Gibco, Life Technologies) or with deionized water only. The control and treatment cultures were then subsampled weekly for microscopic examination and continued to be incubated under the same conditions for 6 weeks.

#### *EPS Measurements*

A subsample of each culture was examined using a Zeiss AX10 with Imager.A2 light microscope using 400x magnification. Each *M. calcarea* cell was photographed under brightfield and DIC filters to provide enough contrast to view the EPS halo if present. During the first week, 200 cells per culture were photographed due to small colony size. For the following 5 weeks, 300 cells per viable culture or at least 100 cells per senescent colony were photographed for measurements. Observation of dead cultures ceased after all *M. calcarea* cells were dead, which was determined by lack of chloroplast and other visible organelles.

Images taken from the microscope examination of the samples were uploaded into ImageJ (V4) and calibrated with a 10  $\mu\text{m}$  scale bar. Each *M. calcarea* cell was examined, and the following was recorded: cell viability (live or dead), motility (moving/still), EPS

halo (present/absent) and number of cells per halo, cell length and width (using “straight” tool), cell and cell plus EPS halo area (using “freehand” tool) (Figure 1). EPS halo area was then calculated subtracting the cell area from total area.

### *Data Analysis*

Welch’s two sample t-test was used to compare the length, width, and area of paired cells and single cells to determine if there was a significant difference between cells producing EPS and cells without EPS (n = 6405). This approach was used because assumptions of normality and independent, random samples were met, but there were unequal variances between samples. Difference in EPS production between single and paired cells were also evaluated using the same approach due to unequal variances. The number of paired cells per week were counted for each culture and analyzed over time using a repeated measures ANOVA. Paired cell count was also compared to the percentage of cells with EPS in the culture by finding the correlation coefficient. This was conducted to determine if a correlation between these two variables supported that EPS production increased during reproduction to the percentage of cells with EPS in the culture.

Motility, percentage of living cells per sample, percentage of cells with an EPS halo per sample, and the number of paired cells per sample were tested using a repeated measures ANOVA in SPSS Statistics for Mac, Version 27 (SPSS Inc., Chicago, Ill., USA) to determine if antibiotics significantly affected cell colony growth and EPS production. The assumptions of independent observations, normality, and sphericity were met for almost all variables. Two variables (cell area and EPS area) were not normally distributed even after transformations, so a Wilcoxon Rank Sum test was used to compare

culture cells grown with an antibiotic and with no treatment. The assumptions of independence and equal variance were met. An alpha of 0.05 was used to denote significance.

### III. RESULTS

#### **Field-Based Assessment of EPS Halos in *M. calcarea***

Across the surveyed sites, periphyton total phosphorus concentration was positively correlated with organic content of the mat (p-value = <0.001) and negatively correlated with pH (p-value = 0.02318) (Table 2). Water pH was also negatively correlated with days since last dry down (p-value = 0.04648) and the proportion of organic matter (p-value = <0.001). Ionic conductivity was not correlated with any of the environmental variables.

Cells with EPS halos were longer (p-value = 0.001), wider (p-value = <0.001), and had a greater area (p-value = <0.001) than cells without EPS halos (Figure 4 and 5). Cells with no EPS were on average 0.997  $\mu\text{m}$  longer, 0.1  $\mu\text{m}$  wider, and 9.6  $\mu\text{m}^2$  larger in area than cells with EPS. Ionic conductivity (p-value = 0.1468), periphyton total phosphorus (p-value = 0.0725), and water pH explained 45.2% of the deviance in EPS area (Table 3). EPS halos were larger in higher pH, lower ionic conductivity (Figure 6a), and lower periphyton TP (<600  $\mu\text{g g}^{-1}$ ) (Figure 6b) settings. There was an interaction effect between pH and ionic conductivity (p-value = 0.0093) (Figure 6c). Periphyton total phosphorus (p-value = 0.00827) (Figure 7b), ionic conductivity (p-value = 0.295) (Figure 6c), and pH also explained variability in frequency of cells with EPS halos (42.8 % deviance explained, Table 4). The frequency of *M. calcarea* cells with EPS halos increased with a

higher pH, lower ionic conductivity (Figure 7a), and lower periphyton TP ( $<600 \mu\text{g g}^{-1}$ ) (Figure 7b). There was also an interaction effect between pH and ionic conductivity (p-value = 0.0419) (Figure 7c). Variability in halo area and frequency was not associated with days since last dry down, location (Figure 8), ash free dry mass, dried g of EPS per dried g of sample, or the periphyton organic content. These variables were removed from the final model explaining EPS halo size and frequency.

### **EPS Halo Production by *M. calcarea* in Culture with and without Bacteria**

Cell length (p-value =  $<0.001$ ) and area (p-value = 0.001) were significantly greater in cells producing EPS than cells without EPS in the untreated culture samples (Figure 9). Cells without EPS was on average  $0.1 \mu\text{m}$  longer and  $5.938 \mu\text{m}^2$  larger in area than cells with EPS. Cell length (p-value =  $<0.001$ ), width (p-value =  $<0.001$ ), and area (p-value =  $<0.001$ ) were significantly greater in single cells than in paired cells in the untreated culture samples (Figure 10). Single cells were on average  $1.3 \mu\text{m}$  longer,  $1.9 \mu\text{m}$  wider, and  $33.8 \mu\text{m}^2$  larger in area than paired cells. There was a decrease in the number of paired cells after Week 1, but by Week 3-4, there was an increase in the total number of pairs although this increase was not significant (p-value = 0.1383) (Figure 11a). With this increase in total number of pairs per plate, percentage of cells with an EPS halo decreased from Week 1 until Week 3/Week 4, but again this was not significantly different (p-value = 0.597) (Figure 11b). Over the 6 week observation period, paired cells were more likely to have EPS halos than single cells in untreated cultures (41 vs 16% respectively).

The percentage of living cells and cells producing EPS were significantly greater in cultures with no treatment than cultures treated with an antibiotic (p-value = 0.01)

(Figure 12). All *M. calcarea* cells in antibiotic treated cultures appeared dead by Week 4, while most cells in the untreated cultures were viable for the entire observation period. The percentage of motile cells did not differ between treatments (p-value = 0.309). The number of paired cells in cultures with no treatment was greater than cultures treated with antibiotics (p-value = 0.00556). The treatment of the cultures explains 87.5% of the variance in the number of paired cells (Figure 13).

EPS area in treated cultures was less than in untreated cultures (Week 1 p-value = <0.001, Week 2 p-value = <0.001, Week 3 p-value = <0.001, Week 4 p-value = <0.001). Cell area was greater in antibiotic treated cultures in Weeks 1 (p-value = 0.02876) and 2 (p-value = <0.001), but cell area was not different between antibiotic treated and untreated cultures in Weeks 3 (p-value = 0.1974) and 4 (p-value = 0.9201). Overall, antibiotic treatment affected both cell area and EPS area over the observation period.

#### IV. DISCUSSION

Cells of the most abundant mat-forming diatom, *M. calcarea*, were closely studied from field and culture materials to determine relationships between EPS area and environmental gradients, reproductive stage, and bacterial presence. In the field samples, EPS halo area was greatest in regions of low periphyton phosphorus content where *M. calcarea* was also most abundant. Previous studies examining the EPS-producing, oligotrophic diatom *D. geminata* found that EPS production similarly increased with decreasing phosphorus availability (Kilroy & Bothwell 2011). Under phosphorus limitation, cells release excess carbon as EPS that would normally be used towards cellular division (Kilroy & Bothwell 2011). Although phosphorus limitation increases

EPS production, it limits cellular division (Bothwell & Kilroy 2011). When concentrations increase, *D. geminata* colonies dissipate, much like the mats of the Everglades (Bothwell & Kilroy 2011). Much of the phosphorus that enters in the Everglades is either immediately taken up by cells within periphyton mats or coprecipitated with calcium carbonate in the mats or in the marl soils that result from mat formation (Price et al. 2006). Increases in phosphorus inputs above the ultraoligotrophic background ( $\sim 6\text{-}10 \mu\text{g L}^{-1}$ ) result in changes in algal community composition, including decreased abundance of *M. calcarea*, and eventual mat disaggregation (Gaiser et al. 2006; Bellinger et al. 2010; Marazzi et al. 2017). Our data suggest that *M. calcarea* EPS halos may also represent an external carbon reservoir under phosphorus limitation, and if other mat-dwelling taxa are secreting EPS for similar reasons, this phenomenon may well explain why EPS is so prevalent in mats and collapse upon exposure to increased phosphorus.

EPS halos in *M. calcarea* also increased with declining ionic conductivity in contrast to observations from Bellinger et al. (2010) who found that decreasing ionic conductivity negatively affected the ability for the rigid matrices of polymers that structure EPS to form. In the Everglades, ionic conductivity is largely driven by calcium ion abundance (Scheidt & Kalla 2007), which explains why calcareous periphyton mats with high abundances of *M. calcarea* are often found in areas of high ionic conductivity. High ionic conductivity regions are also typically phosphorus limited (Scheidt & Kalla 2007), which was also a contributing factor to the presence of EPS halos in our study. Other areas of the Everglades located near manmade canals vary in water chemistry (Harvey & McCormick 2009), including ionic conductivity, but a spatial GAM found that

there was no correlation between the proximity of the sample site to the canals and mean EPS area. Ionic conductivity was not correlated with any environmental variable tested in the model, including periphyton total phosphorus. This may contribute to why ionic conductivity was included as an isolated variable in the GAM along with an interaction with pH. While our study did not support the findings of Bellinger et al. (2010) that showed increasing ionic conductivity enhanced EPS production, another study found that areas with the lowest ionic conductivity had shorter hydroperiods and lower concentrations of phosphorus in the water column, which creates a favorable environment for *M. calcarea* (Browder et al. 1994).

In the GAMs, the interaction between ionic conductivity and pH was significant in explaining patterns in EPS halo area and percentage of cells producing EPS. Water pH alone did not increase the deviance explained in our models, but in areas with low ionic conductivity and higher pH, more cells produced EPS and had a larger EPS halo. While ionic conductivity and pH were not correlated in our study, ionic conductivity and pH in the Everglades ecosystem are often related; the majority of the carbonate-based ecosystem ranges from pH 5.7-8.3 (Scheidt & Kalla 2007) with *M. calcarea* most abundant around pH 7.6 (Gaiser et al. 2010; Lee et al. 2014). Areas with a lower pH have little to no mat formation with no calcium carbonate precipitation, resulting in conditions unfavorable to *M. calcarea* communities and thus little EPS production (Gaiser et al. 2006, 2010). Where pH is higher, calcium carbonate is precipitated, primarily by the mat-forming filamentous cyanobacteria *Schizothrix calcicola* and *S. hofmanni* (Browder et al. 1994). This process occurs by active cell membranes releasing  $\text{Ca}^{+3}$  ions in exchange for protons outside of the cell, which increases the pH within the mat and the likelihood of



CaCO<sub>3</sub> precipitation as well as increasing ionic conductivity (Merz 1992; Dittrich & Obst 2004). Imaging of EPS in Everglades periphyton has shown that the linked polymers within EPS of various species serve as a template for calcium carbonate deposition (Bellinger et al. 2010). EPS production by *M. calcarea* under higher pH might contribute to mat cohesion through this interaction with carbonates.

Contrary to the expectation that EPS halos would increase with decreasing hydroperiod (Gaiser et al. 2010), suggesting adaptation to desiccation-prone conditions, no relationship between halo area and hydroperiod was observed. Similarly, the organic content of the mats, which decreases as hydroperiod increases, was also unrelated to halo area. Research has shown that total organic carbon found within the mat is partially made up of EPS (Decho 1990; Sutherland 2001). Increases or decreases in EPS production by other species may have contributed to organic content not being included in our analysis.

By examining *M. calcarea* in culture, cell size declination and replenishment during the cycle of cell division and reproduction as well as copulating cells were observed. Paired cells in EPS halos, indicating copulation, were more abundant than single cells as the culture aged. Paired cells usually occurred during asexual or sexual reproduction when cells were dividing and the colony was growing. Different species of diatoms that produce EPS have been observed producing extracellular materials during copulation. Other taxa, including *Placoneis* sp., *Cymbella* sp., and *Gomphonema angustatum*, have been observed producing an EPS envelope or other EPS structures such as tubes while undergoing copulation (Mann & Stickle 1995; Stevenson 1990). These EPS structures may aid in protecting the gametes as they are transferred from one cell to another or to keep the copulating cells together during this process (Mann 1986; Mann

1988; Danielidis & Mann 2002; Mann et al. 2003). While increases in paired cell counts and the percentage of cells producing EPS were not significantly different, there was a significant correlation between the number of cell pairs and the percentage of cells producing EPS. This correlation could potentially indicate that as cells begin to pair up, potentially during sexual reproduction, the cells begin to produce increased EPS halos.

The antibiotic treatment used to compare EPS halo area around *M. calcarea* cells to untreated cultures suggests bacteria enhance the production of *M. calcarea* EPS and overall cell survival. However, it is not clear whether the antibiotic stopped the cellular process of EPS production which then led to the death of the culture or if it slowly killed the cells through indirect routes and led to a halt in EPS production. Windler et al. (2015) found that the EPS producing diatom *Achnantheidium minutissimum* is affected by the presence of bacteria and that the quantity of EPS increased when the diatom was cocultured with bacterial communities. Berthold et al. (2019) further found that coculturing various algal species with bacterial colonies also increased algal biomass and lipid production. The increase in algal biomass was potentially due to a mutualistic relationship with bacterial colonies that make vitamins and dissolved inorganic carbon available to algal species and promoted growth while algal cells that produce EPS create an organic matter base necessary for bacterial decomposition (Croft et al. 2006; Kim et al. 2014; Berthold et al. 2019). The antibiotic may also have had an effect on other species in the mixed cultures, such as cyanobacteria. Prior studies have found conflicting evidence regarding this relationship. Numerous studies have found that certain species of freshwater cyanobacteria may contain an antibiotic resistant gene or naturally be non-susceptible to antibiotics (Dias et al. 2015; Stoichev et al. 2011) while others have found

that antibiotics had a harmful effect on the survival and growth of freshwater cyanobacteria (Pan et al. 2008; van der Grinten et al. 2010). In addition to our findings on the effect of an antibiotic on EPS area and cell mortality, cell size between the treated and untreated diatom cultures was different between Weeks 1 and 2 but not in Weeks 3 and 4. This may be due to a lack of cellular growth in the antibiotic treated cultures, which had cell sizes similar to those at the beginning of colony growth and the cell size regenerated between Weeks 3 and 4. Bruckner et al. (2008) cultured numerous species of diatoms with and without bacterial communities to determine the relationship between diatoms and bacteria. Many of the diatom species demonstrated increased cellular growth and amplified EPS formation when bacteria were present (Bruckner et al. 2008). The effect of bacterial colonies on the growth of diatom colonies and EPS production may be variable among species, but the evidence provided by our culture samples indicates that there may be a relationship between bacteria and *M. calcarea* communities that lead to EPS production and mat cohesion.

EPS production occurs in many species of diatoms, and results from this study furthers our understanding of why this occurs. From the culturing study, I found that sexual reproduction may trigger the secretion of EPS in *M. calcarea* cells, but the environmental cues in the field study such as phosphorus scarcity trigger sexual reproduction in cells that are at or below the threshold size. The production of EPS in Everglades periphyton as well as other MeCoCos is critical for the biological, chemical, and physical maintenance of these communities (Flemming et al. 2016). Many of these communities form the base of ecosystem food webs and their formation and maintenance are critical to the persistence and success of these ecosystems (Weitere et al. 2018).

Understanding how changes in environmental conditions play a role in triggering sexual reproduction leading to increased EPS production gives insight into how periphyton communities are formed and sustained.

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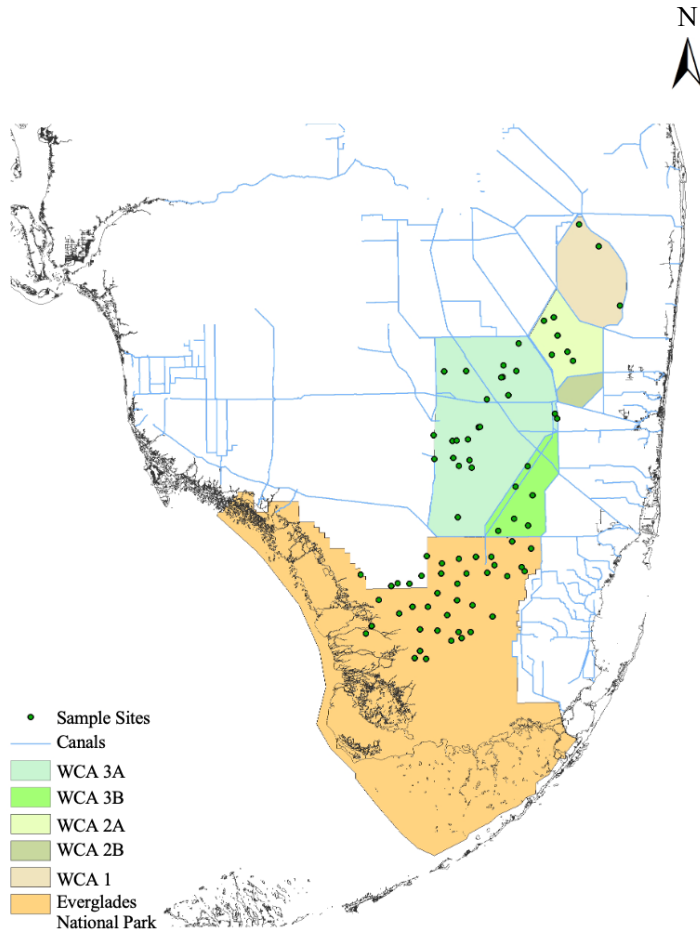
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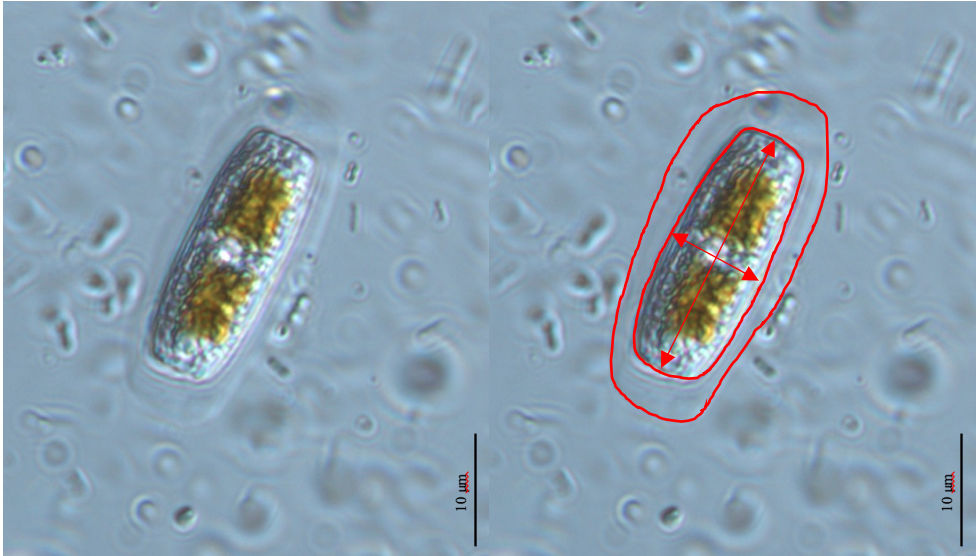
**FIGURES AND TABLES**



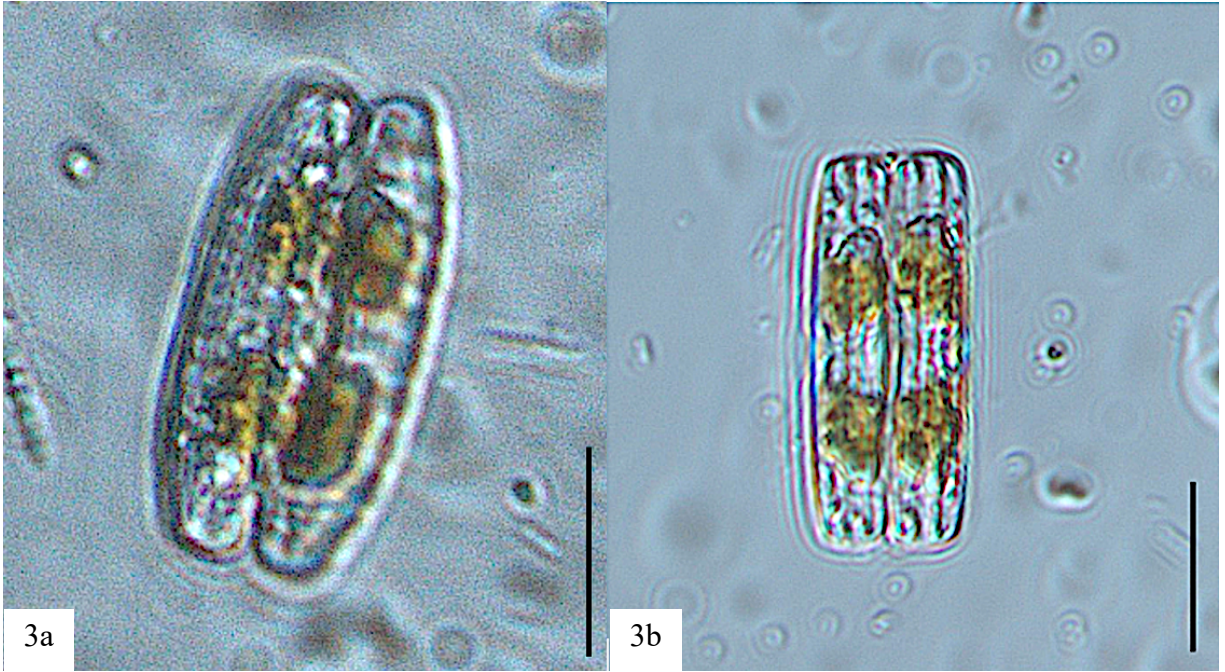
**Figure 1:** Site map of field survey sample sites. Sample sites cover environmental gradient to capture range of environmental conditions in the Everglades ecosystem.

**Table 1:** Environmental variables range and mean from sample sites considered for the generalized additive model.

Environmental Variable	Range	Mean
Periphyton TP ( $\mu\text{g g}^{-1}$ )	18.41 – 1457	210.9
Ionic Conductivity ( $\mu\text{S cm}^{-1}$ )	98.3 – 1769	542.1
pH	6.16 – 7.64	7.07
Days since last dry down	65 – 3426	464.2
Proportion Organic Content	0.1861 – 0.8831	0.5505



**Figure 2:** Measurements of *Mastogloia calcarea* cells. Light micrograph of a *M. calcarea* cell from the Everglades showing mucilage halo. Red lines indicate how cell length, width, and area and halo area were measured.

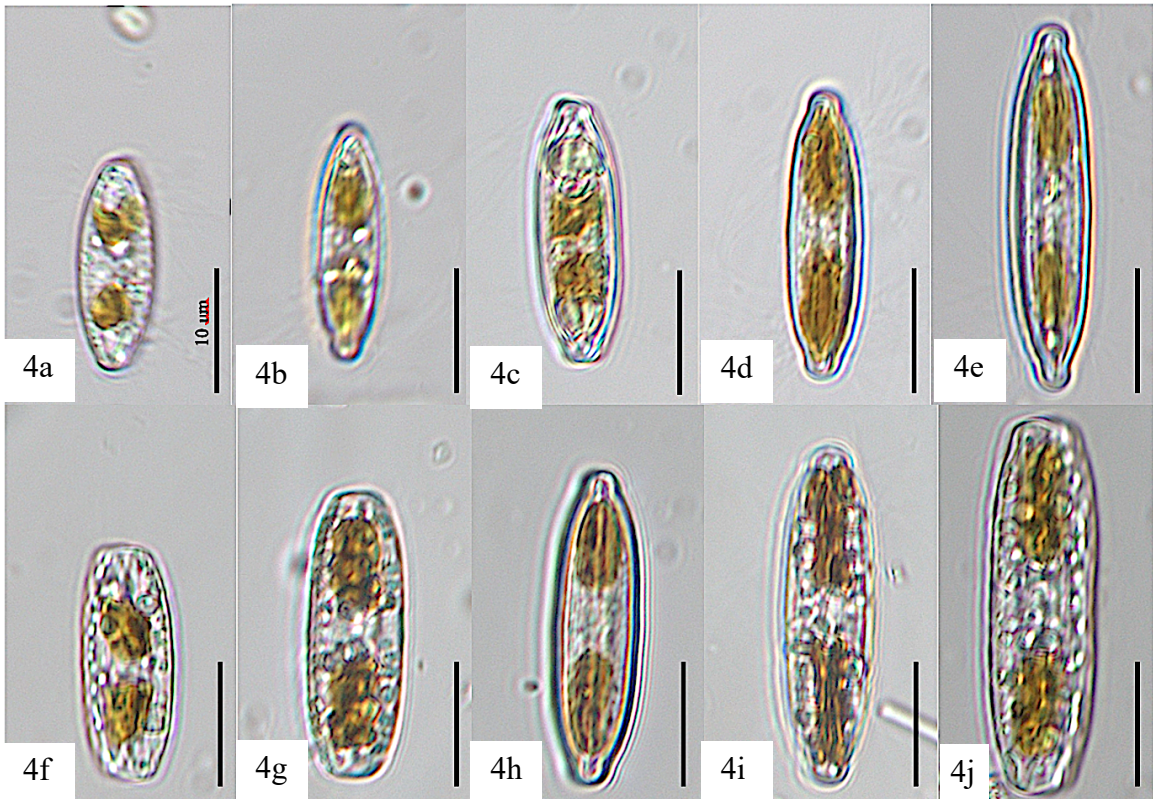


**Figure 3:** Paired and single cells (a) with and (b) without EPS halo.

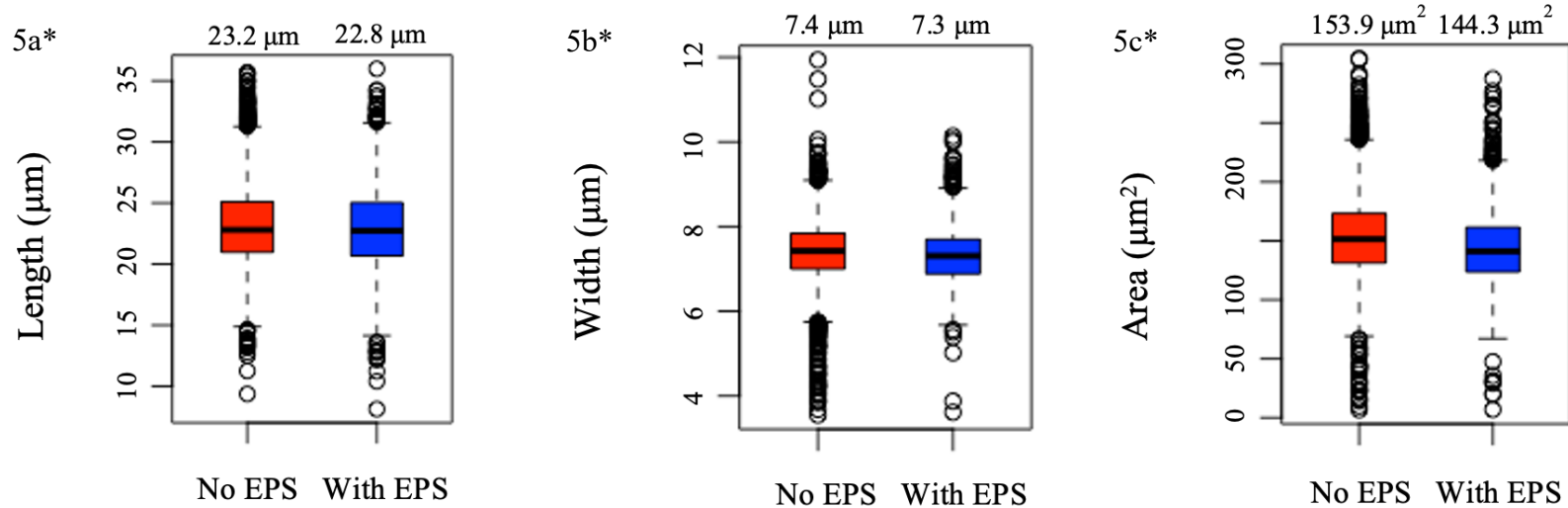
**Table 2:** Pearson correlation coefficients for the environmental variables tested in the GAM to explain EPS halo area and percentage of cells producing EPS in each PSU. Asterisk denotes significance ( $P < 0.05$ ).

	Periphyton TP	Ionic Conductivity	pH	Days since last dry down	Proportion Organic Content
Periphyton TP	1.000				
Ionic Conductivity	0.0539	1.000			
pH	-0.2382*	0.04045	1.000		
Days since last dry down	0.0347	0.01546	-0.2133*	1.000	
Proportion Organic Content	0.5384*	-0.04362	-0.2767*	0.1245	1.000





**Figure 4:** Size declination of *Mastogloia calcarea*. Example size declination series of *M. calcarea* cells with (4a-4e) and without (4f-4j) EPS halos.

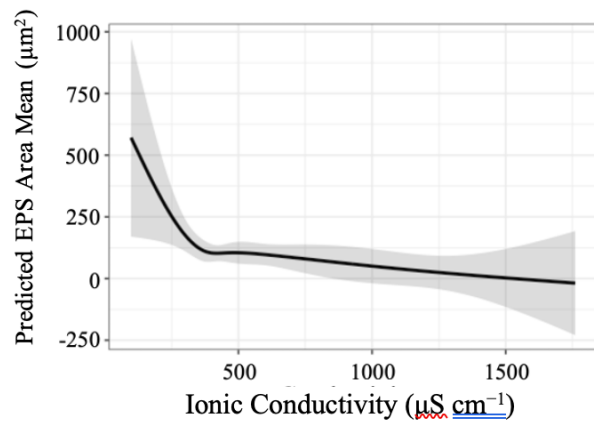


**Figure 5:** Distribution of measurements in cells from field samples. Length (5a), width (5b), and area (5c) were measured in all *Mastogloia calcarea*. A total of 5,042 cells were measured with no EPS and 1,918 cells with EPS. Mean length, width, and area for cells with EPS and with no EPS are given above each plot. Asterisks denotes significance.

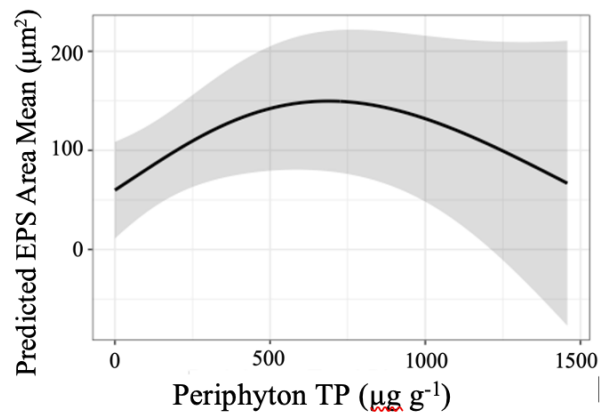
**Table 3:** Generalized additive model output for environmental variables and the area of EPS produced in field samples. Ionic conductivity was kept with the model due to a large increase in the percentage of deviance explained by the model. Deviance explained is 45.2%. Asterisk denotes significance.

Parameter	df	F	p-value
Interaction (pH, Ionic Conductivity)	15.1	2.3	0.0093*
Periphyton Total Phosphorus	2.0	3.2	0.0725
Ionic Conductivity	1.8	2.0	0.1468

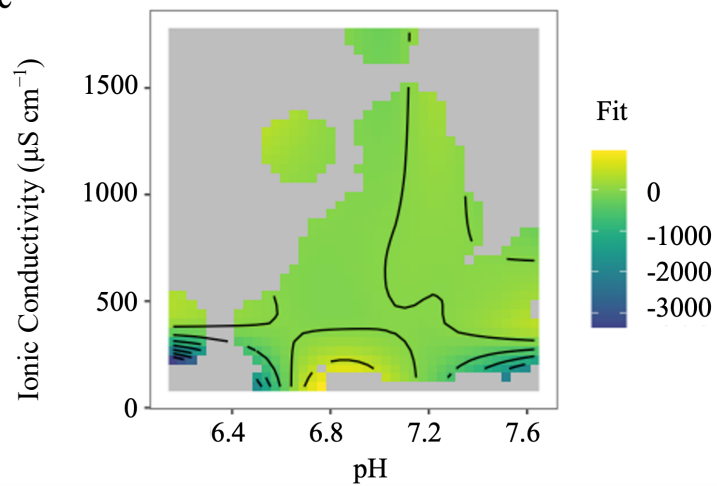
6a



6b



6c

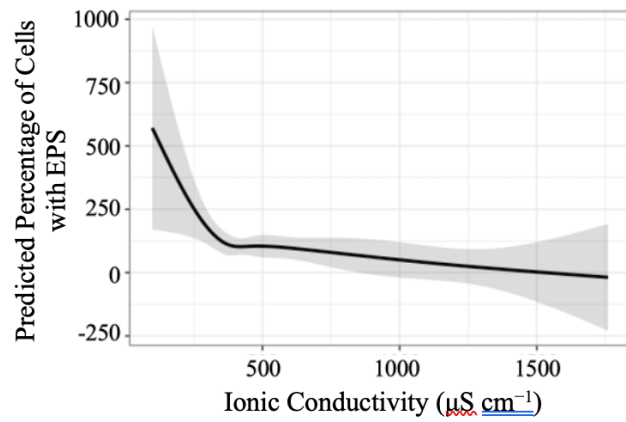


**Figure 6:** Interaction plot and predicted values for EPS area from GAM. (6a) Predicted EPS area mean based on ionic conductivity. (6b) Predicted EPS area mean based on periphyton total phosphorus concentrations within the mat. (6c) Interaction plot in EPS production GAM model with pH and ionic conductivity.

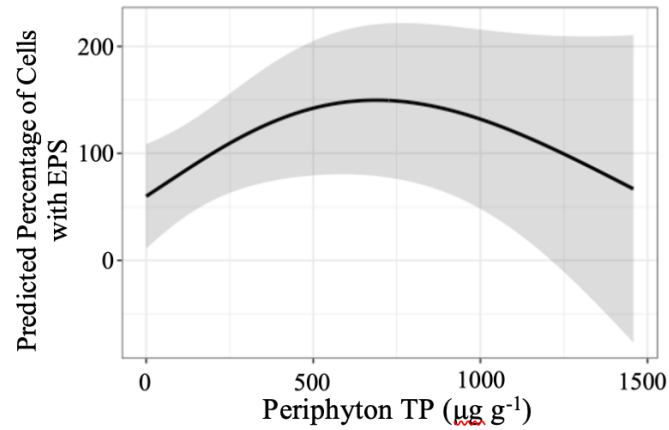
**Table 4:** Generalized additive model output for environmental variables and the percentage of cells producing EPS in field samples. Deviance explained is 42.8%. Asterisks denotes significance.

Parameter	df	F	p-value
Interaction (pH, Ionic Conductivity)	14.8	1.8*	0.04185*
Periphyton Total Phosphorus	2.0	5.6*	0.00827*
Ionic Conductivity	1	5.0*	0.02949*

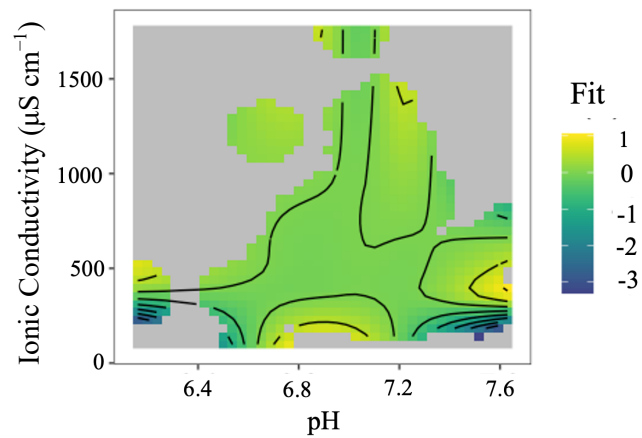
7a



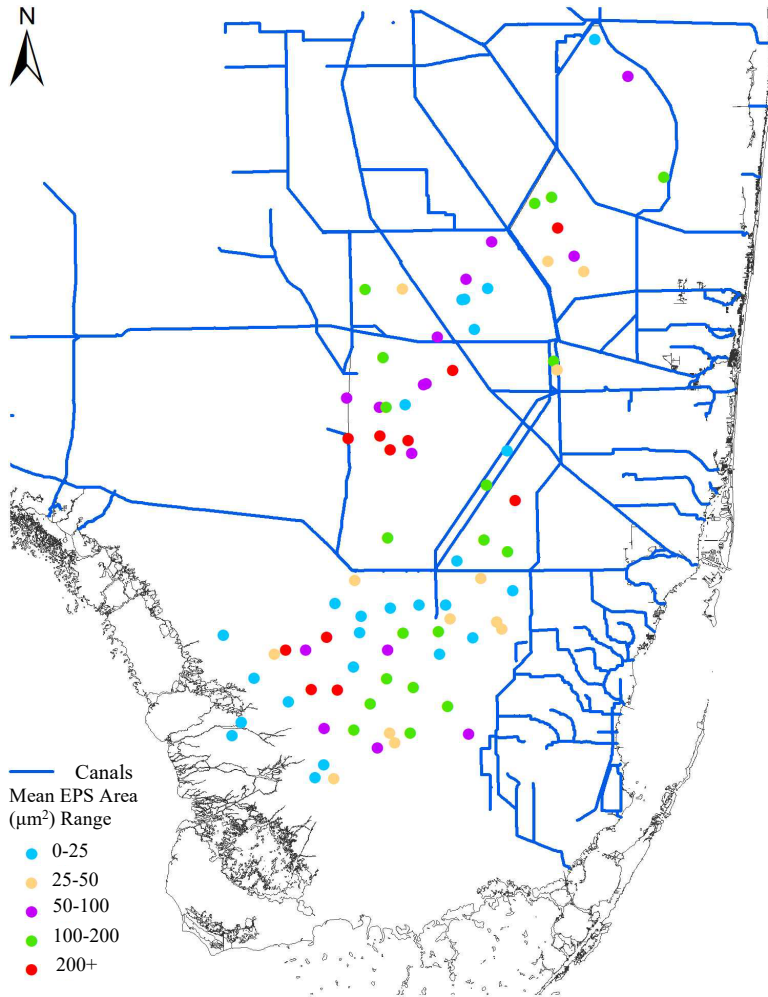
7b



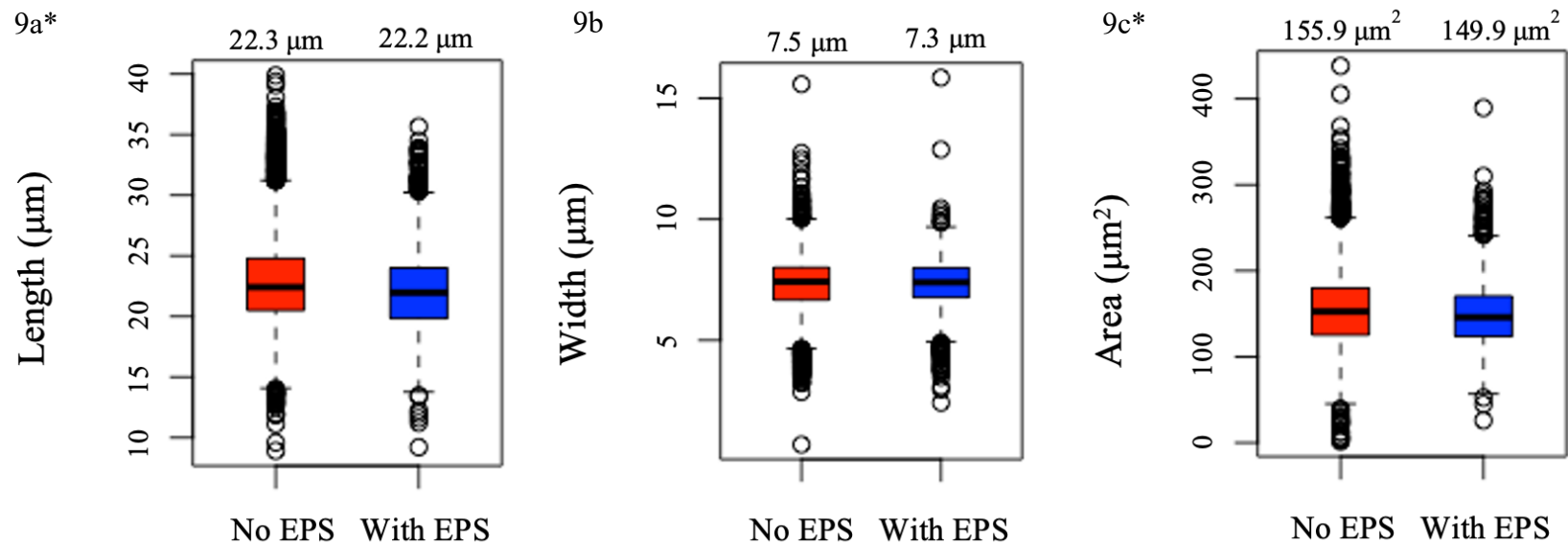
7c



**Figure 7:** Interaction plot and predicted values for percentage of EPS cells from GAM. (7a) Predicted percent of *M. calcarea* cells with EPS based on ionic conductivity. (7b) Predicted percent of *M. calcarea* cells with EPS based on periphyton total phosphorus concentrations. (7c) Interaction plot in percentage of *M. calcarea* cells with EPS GAM model with pH and ionic conductivity.

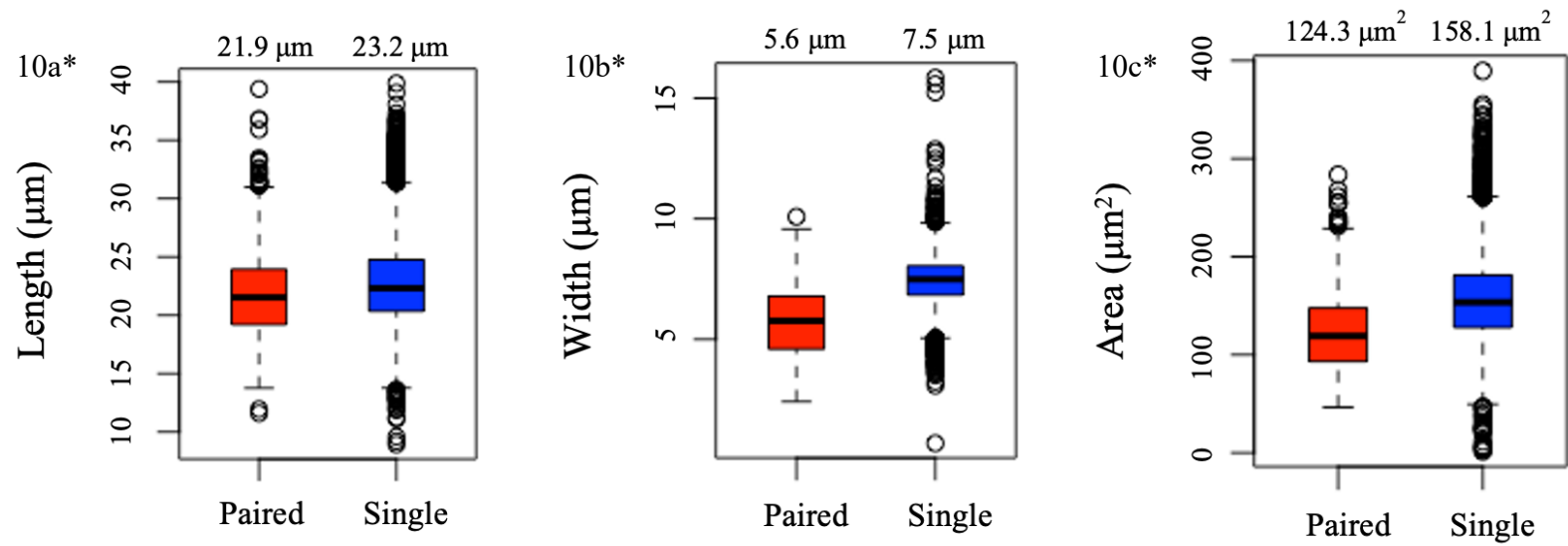


**Figure 8:** Distribution of mean EPS halo area at each field site. No correlation was found between location or proximity to canals and EPS halo area.



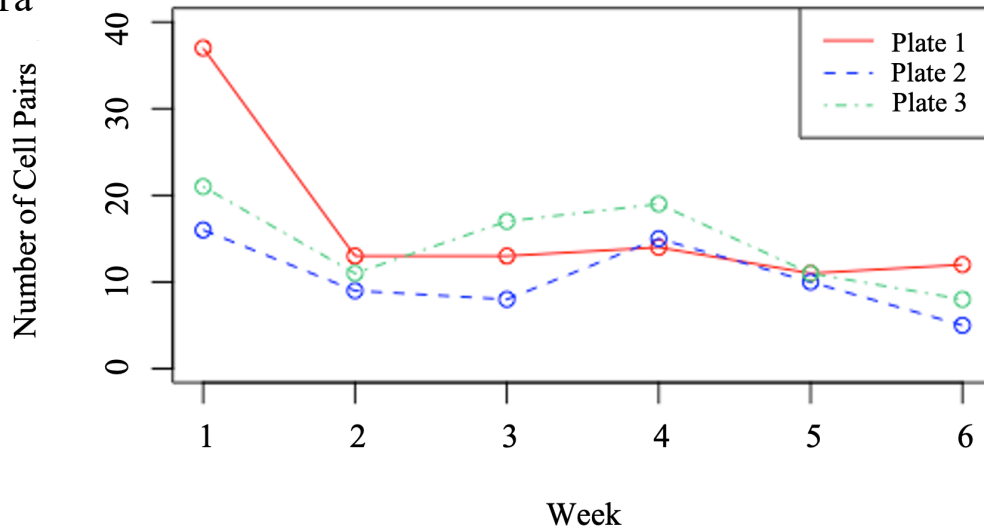
**Figure 9:** Distribution of measurements in cells from culture samples. Length (9a), width (9b), and area (9c) of cultured *Mastogloia calcarea* cells were measured. A total of 1,097 cells were measured with no EPS and 5,308 cells with no EPS. Mean length, width, and area for cells with EPS and with no EPS are given above each plot. Asterisk denotes significance.



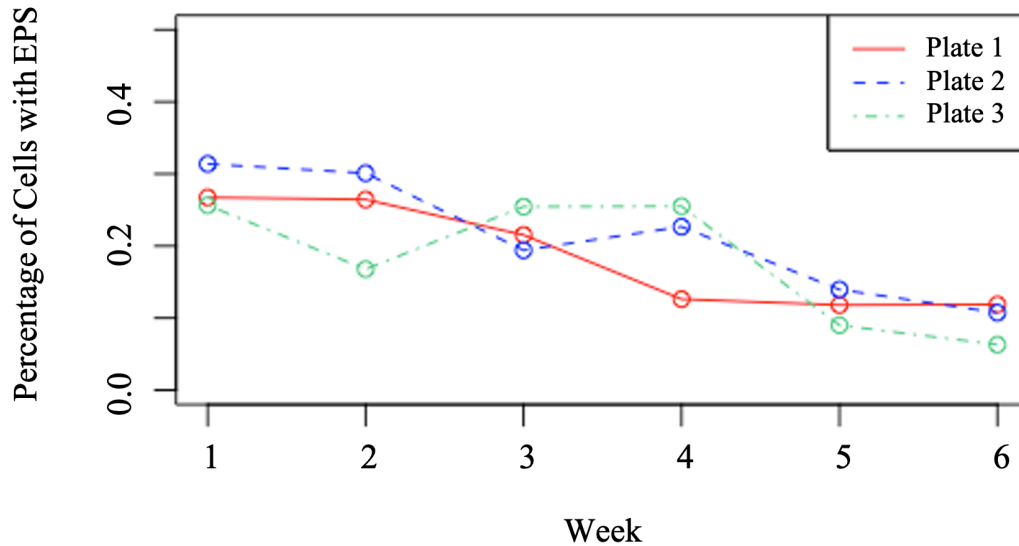


**Figure 10:** Distribution of measurements in paired and single cells. Length (10a), width (10b), and area (10c) of paired and single cells were measured from cultured samples. A total of 501 paired cells and 4,545 single cells were measured and analyzed. Mean length, width, and area for paired cells and single cells are given above each plot. Asterisk denotes significance.

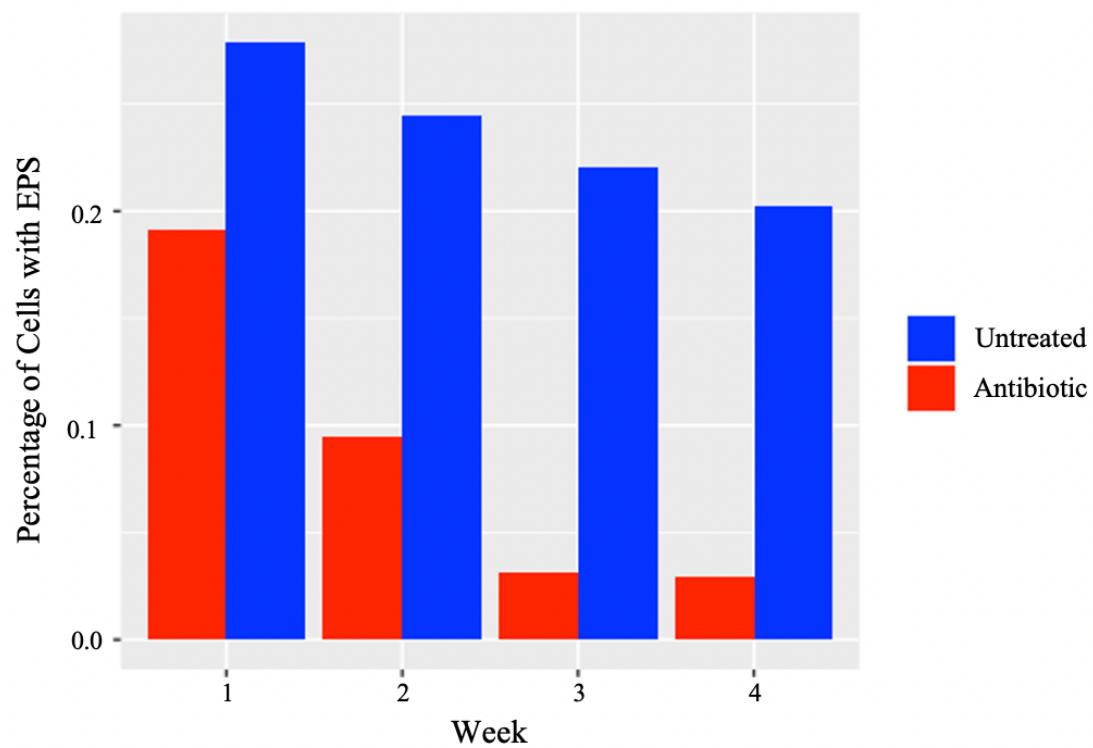
11a



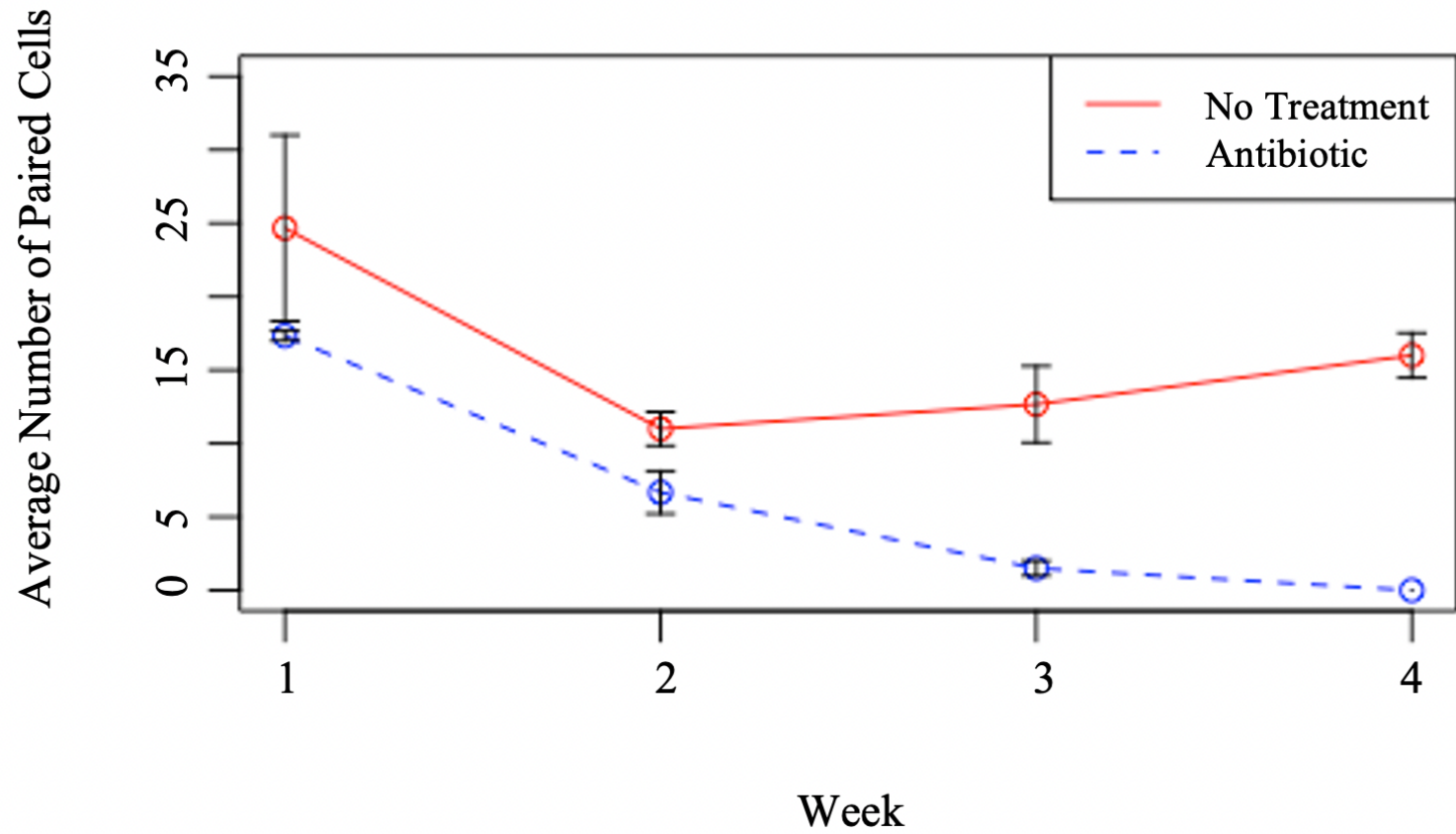
11b



**Figure 11:** Paired cell count and percentage of cells with EPS over 6 weeks. (11a) Number of paired cells in the three untreated cultures over the 6 week observation period. There is an increase in the number of pairs around week 4 for 2 of the 3 cultures indicating the restoration of colony size. (11b) The percentage of cells with EPS present in the three untreated cultures over a 6 week observation period. There is an increase in the percentage of cells producing EPS around week 4 in 2 of the 3 cultures indicating that, along with an increase in the number of cell pairs increasing, colony restoration may be linked to EPS production. Number of cell pairs appeared to be correlated with the percentage of cells with an EPS halo in Plates 1, 2, and 3 (0.5829, 0.6491, and 0.9310 respectively).



**Figure 12:** The Percentage of Cells with EPS in Untreated and Antibiotic Treated Cultures.



**Figure 13:** Paired cell count comparison between culture treatments. Mean number of paired cells with standard error for cultures with no treatment and cultures treated with the antibiotic.