

ABSTRACT

Title of Dissertation: IMPACT OF PLANT-DERIVED
 ALLELOCHEMICALS ON HARMFUL
 ALGAL BLOOMS

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Philosophy, 2023

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Harmful algal blooms (HABs) are a global concern in both freshwater and coastal systems; creating dire consequences for public health, water resources, and local economies. Thus, there is a focus among scientists and environmental managers on HAB prediction, prevention, and mitigation. Current chemical mitigation methods include algicides such as copper sulphate, chlorination, and hydrogen peroxide, which can have high financial costs and secondary pollution associated with them. The use of natural allelochemicals produced by plants and bacteria has received considerable attention as an alternative to synthetic algicides, as they can have negligible toxins, be highly selective, and easily degraded in the environment. This dissertation is a coalition of research looking into new sources of plant allelochemicals and whether natural levels of allelochemicals in the water column, can impact phytoplankton communities and the presence of toxin-producing algal species. The first objective focused on the use of the waste product: brewer's spent grain (BSG), as a new control mechanism to inhibit the growth of toxic algae. BSG extract of doses higher than 250mg/L inhibited the growth of freshwater and marine toxin-producing cyanobacteria and dinoflagellate species (*Microcystis*

aeruginosa and *Karenia brevis*), while not impacting the diatom and chlorophyte tested (*Scenedesmus obliquus* and *Prorocentrum tricornutum*). This same dosage of BSG caused cyanobacteria abundance in lake water to decline by 90% within 4 days and chlorophytes to dominate the community by day 6 during a microcosm study. However, an experiment controlling bacteria levels demonstrated that the decline of *K. brevis* growth was likely due to the increase in abundance or presence of certain types of bacteria growing with exposure to BSG extract rather than due to chemicals released from the BSG. The second and third objectives shifted focus to the New Jersey Pinelands and whether the chemicals released into the water from terrestrial and marine plants in these waters, like phenolic compounds, impact the phytoplankton community and toxin-producing species. The second objective focused on the spatial and temporal distribution of phycotoxins along two New Jersey estuaries using passive samplers and whether the utility of passive samplers was impacted by the excess phenolic compounds in the water. By utilizing passive samplers in New Jersey, phycotoxins not previously reported in the area were described, such as azaspiracids, goniodomin-A and yessotoxins. However, this objective also showed some of the caveats of passive samplers, especially at sites with high phenolic compounds. The third objective focused on identifying the primary environmental drivers of chlorophyll a concentration and phytoplankton community along the freshwater – marine continuum of two New Jersey Estuaries with varying levels of disturbance. This dissertation explored BSG as a novel control method of HABs, and provided new information for monitoring, managing, and modeling HABs based on phenolic content measured in the water.

IMPACT OF PLANT-DERIVED ALLELOCHEMICALS ON HARMFUL ALGAL
BLOOMS

by

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Foreword

Contained within this dissertation are three unique research chapters, of which I am the principal author of each. Chapter 1 introduces the research chapters presented in Chapters 2-4. Chapter 5 summarizes the key findings of Chapters 2-4 and notes future directions of research. All three research chapters will be submitted for publication after the completion and defense of this dissertation.

Dedication

I would like to dedicate this dissertation to my husband, David Walters, and parents, Lou and Cindy Armstrong. Without their support, this PhD journey would not have been possible. To my dog, Addie, who pushed me to go for walks to clear my mind during the writing process. To our daughter Eleanor, for giving me a good kick in the stomach to remind me to keep writing and for waiting to be born until after 37 weeks and 3 days, so I could defend this dissertation before their arrival.

Acknowledgements

Chapter 2. Impact of the waste product brewer's spent grain on the growth of common freshwater and marine harmful algal bloom species

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Chapter 3. Phycotoxins along two New Jersey estuaries, a look at the use of passive samplers in areas with high phenolic content. And Chapter 4. Environmental Drivers of Harmful Algal Bloom Species in two New Jersey Estuaries

I would like to acknowledge and thank Gregg Sakowicz, Dr. Thomas Grothues and Patricia McHugh at Jacques Cousteau National Estuarine Research Reserve for their valuable field and laboratory contributions. Thank you to Marta Sanderson and Juliette Smith at Virginia Institute of Marine Science for teaching me how to use the TQMS and allowing me to run my samples each year. Many thanks to Dr. Kim Reece and Drs. Thomas and Constance Harris for providing purified Goniodomin A used in this study. Thank you to Mara Coughlin for help with the lab work. Thank you to Sweetwater Marina and Riverdeck, Chestnut Neck Boat Basin, Water Street Bar and Grill, Ocean Gate Yacht Basin, Seaside Yacht Club, and Lighthouse Marina for allowing the authors to deploy equipment and collect water

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Table of Contents

Foreword.....	ii
Dedication.....	iii
Acknowledgements.....	iv
Table of Contents.....	vi
List of Tables.....	ix
List of Figures.....	x
List of Abbreviations.....	xiv
Chapter 1: Introduction.....	1
1.1 Management of Harmful Algal Blooms.....	1
1.2 Allelochemicals from plants.....	3
1.3 Terrestrial and Aquatic Plant Chemicals on Phytoplankton Communities.....	3
1.4 Aims and Objectives.....	4
Chapter 2: Impact of the waste product brewer's spent grain on the growth of common freshwater and marine harmful algal bloom species.....	6
Abstract.....	6
2.1 Introduction.....	7
2.2 Methods.....	9
2.2.1 Preparation of plant extracts.....	10
2.2.2 Exposure of microalgal cultures to plant extracts.....	11
2.2.3 Exposure of <i>Karenia brevis</i> to brewer's spent grain with and without antibiotics.....	12
2.2.4 Heterotrophic bacteria community analysis.....	13
2.2.5 Total Phenolic Content analysis.....	14
2.2.6 Photosynthetic efficiency analysis.....	14
2.2.7 Exposure of <i>Cylindrospermopsis raciborskii</i> bloom water to brewer's spent grain extract.....	15
2.2.8 Statistics.....	15
2.3 Results.....	16
2.3.1 Exposure of microalgae to brewer's spent grain, sargassum, and barley extract.....	16
2.3.2 Exposure of <i>Karenia brevis</i> to brewer's spent grain with and without antibiotics.....	19
2.3.3 Exposure of <i>Cylindrospermopsis raciborskii</i> bloom water to brewer's spent grain extract.....	21
2.4 Discussion.....	23
2.4.1 Exposure of microalgae to brewer's spent grain, sargassum, and barley extract.....	23
2.4.2 Exposure of <i>Karenia brevis</i> to brewer's spent grain with and without antibiotics.....	27
2.4.3 Exposure of <i>Cylindrospermopsis raciborskii</i> bloom water to brewer's spent grain extract.....	29
2.5 Conclusion.....	31
2.6 Tables.....	32

2.7 Figures.....	33
Chapter 3: Phycotoxins along two New Jersey estuaries, a look at the use of passive samplers in complex water systems with high phenolic content	47
Abstract	47
3.1 Introduction.....	48
3.2 Materials and Methods.....	51
3.2.1 Materials	51
3.2.2 SPATT construction.....	51
3.2.3 Study site.....	51
3.2.4 Pre- and Post- loaded toxin SPATT experiment.....	52
3.2.5 SPATT extraction for toxins and phenolics.....	53
3.2.6 Toxin analysis	54
3.2.7 Total phenolic content analysis.....	56
3.2.8 Statistics	56
3.3 Results.....	57
3.3.1 Spatial and temporal distribution of phycotoxins	57
3.3.2 Replicates and repeated collections over one month	60
3.3.3 Pre- and post- loaded toxin SPATT experiment	60
3.4 Discussion	62
3.4.1 Spatial and temporal distribution of phycotoxins	65
3.4.2. Pre- and post- loaded toxin SPATT experiment	71
3.5 Conclusion	74
3.6 Tables.....	74
3.7 Figures.....	78
Chapter 4: Environmental Drivers of Phytoplankton Communities in Two New Jersey Estuaries	83
Abstract	83
4.1. Introduction.....	84
4.2 Materials and methods	87
4.2.1 Study location	87
4.2.2 Field sampling.....	89
4.2.3 Nutrient analysis	90
4.2.4 Phytoplankton classification	90
4.2.5 Total phenolic content.....	91
4.2.6 Chlorophyll a analysis.....	92
4.2.7 Statistics	93
4.3 Results.....	95
4.3.1 Abiotic characteristics of the two estuaries	95
4.3.2 Environmental drivers of Chlorophyll a	98
4.3.3 Phytoplankton community composition	100
4.4 Discussion	101
4.4.1 Trends in Total Phenolic Content and Dissolved Organic Carbon.....	101
4.4.2 Environmental drivers of Chlorophyll a	103
4.4.3 Phytoplankton community composition	105
4.5 Conclusion	107
4.6 Tables.....	108

4.7 Figures.....	109
Chapter 5: Summary	121
5.1 General findings and expansion of previous knowledge	121
5.1.1 Brewer's Spent Grain impact on phytoplankton species	122
5.1.2 Spatial and temporal trends of phycotoxins through two estuaries in NJ	123
5.1.3 Impact of phenolic content on SPATT performance for phycotoxin tracking	125
5.1.4 How Total Phenolic Content in the water varies with seasons and other abiotic factors.....	127
5.1.5 Impact of total phenolic content on chlorophyll concentrations and phytoplankton community composition	128
Appendices.....	130
Appendix A: Adsorption of flavonoids onto Diaion HP-20 resin	130
A.1 Introduction.....	130
A.2 Methods.....	131
A.3 Results and Discussion.....	132
A.4 Tables	133
A.5 Figures.....	134
Appendix B: Fractionation of algicidal compounds from brewers spent grain	134
B.1 Introduction	134
B.2 Methods.....	135
B.3 Results and Discussion.....	137
B.4 Figures.....	138
References.....	139

List of Tables

- 2.1 Bacterial reads from 16S Miseq Illumina in *Karenia brevis* (strain New Pass) at day 4 of cultures exposed to brewer's spent grain extract and luteolin dissolved in DMSO, with and without the use of antibiotics.
- 2.2 Concentration of nutrients in controls and treatments after six days of exposure to filtrates from brewer's spent grain (BSG).
- 2.3 Some of the phenolic compounds found in *Sargassum* and brewer's spent grain.
- 3.1 The logarithm of the partition coefficient between *n*-octanol and water (Log P), calculated using XLogP3 3.0 (PubChem release 2021.05.07).
- 3.2 Measured differences in toxin levels (ng/g resin $\pm$ standard deviation) between replicate SPATT bags deployed each month in two New Jersey estuaries in 2022.
- 3.3 Percent of toxin $\pm$ standard deviation, recovered from SPATT in 100% ACN fraction versus 100% MeOH fraction in 2022.
- 3.4 Toxin concentrations from SPATT deployed at two sites on September 19, 2022 and collected over a 1-month period.
- 3.5 Recovery of toxins from 1 g HP-20 resin either pre-loaded or post-loaded with 25 ng okadaic acid, microcystin-RR, microcystin-LR, and microcystin-YR, during a 1 day or 1 month deployment at 2 sites along Mullica River – Great Bay.
- 3.6 Recovery of toxin $\pm$ standard deviation, observed in spiked extracts from SPATTs deployed in Mullica River for one day or one month.
- 3.7 Recovery of toxins $\pm$ standard deviation, observed in spiked 100% MeOH extracts from SPATTs deployed in Mullica River-Great Bay and Toms River-Barneget Bay.
- 4.1 Potential toxin producing species identified, salinity range, and month seen during microscopic cell counts in New Jersey in 2022.
- A.1 Percent recovery of flavonoids, apigenin and luteolin, from SPATT bags in saltwater and freshwater and eluted with varying percent MeOH

List of Figures

- 2.1 Growth effects of (A) brewer's spent grain extract, (B) barley extract, (C) sargassum extract, and (D) luteolin dissolved in DMSO, on *Phaeodactylum tricornutum* (strain UTEX 646).
- 2.2 Growth effects of (A) brewer's spent grain extract, (B) barley extract, (C) sargassum extract, and (D) luteolin dissolved in DMSO, on *Scenedesmus obliquus* (strain HTB1).
- 2.3 Photosynthetic efficiency (F_v/F_m) of (A) *Scenedesmus obliquus*, (B) *Phaeodactylum tricornutum*, (C) *Microcystis aeruginosa*, exposed to various doses of plant extracts after 48 hours.
- 2.4 Growth effects of (A) brewer's spent grain extract, (B) barley extract, and (C) sargassum extract, on *Microcystis aeruginosa* (strain LE3).
- 2.5 Growth-inhibition effects of (A) brewer's spent grain extract, (B) barley extract, and (C) sargassum extract, on *Karenia brevis* (strain new pass).
- 2.6 Bacteria count in *Karenia brevis* cultures exposed to various doses of brewer's spent grain, barley, and sargassum.
- 2.7 Growth of *Karenia brevis* (strain new pass) on (A) brewer's spent grain extract, and (B) luteolin dissolved in DMSO, with and without the use of antibiotics.
- 2.8 Bacteria concentration in *Karenia brevis* (strain new pass) cultures exposed to brewer's spent grain extract and luteolin dissolved in DMSO, with and without the use of antibiotics
- 2.9 Class-level (A) and genus-level (B) bacterial relative abundance (%) in *Karenia brevis* (strain New Pass) at day 4 of cultures exposed to brewer's spent grain extract and luteolin dissolved in DMSO, with and without the use of antibiotics.
- 2.10 Non-metric multidimensional scaling (nMDS) based on Bray-Curtis similarities of bacteria community composition in *Karenia brevis* (strain New Pass) at day 4 of cultures exposed to brewer's spent grain extract and luteolin dissolved in DMSO, with and without the use of antibiotics.
- 2.11 Total cells per mL and contributions of different phytoplankton groups versus day in flasks of control treatment, 25 mg/L brewer's spent grain extract, and 250 mg/L brewer's spent grain extract.

- 2.12 Non-metric multidimensional scaling (nMDS) based on Bray-Curtis similarities of phytoplankton community composition between the 3 groups of flasks: control, 0.1% spent grain, and 1% spent grain on days 0,2,4, and 6.
- 2.13 Relative contribution of accessory pigments to total pigments in a natural bloom from Lake Williston, Denton, MD on day 0 and 6 when exposed to 25mg/L or 250mg/L brewer's spent grain (BSG) extract.
- 3.1 Map of the 11 field sampling sites in two New Jersey estuaries.
- 3.2 SPATT toxin data (ng / g resin) for total okadaic acid (OA + esterified forms) along 11 sites in two New Jersey estuaries from September 2020 – November 2022.
- 3.3 SPATT toxin data (ng / g resin) for total dinophysistoxin-1 (DTX1 + esterified forms) along 11 sites in two New Jersey estuaries from September 2020 – November 2022.
- 3.4 SPATT toxin data (ng / g resin) for pectenotoxin-2 (PTX2) along 11 sites in two New Jersey estuaries from September 2020 – November 2022.
- 3.5 SPATT toxin data (ng / g resin) for azaspiracid-1 (AZA1) along 11 sites in two New Jersey estuaries from September 2020 – November 2022.
- 3.6 SPATT toxin data (ng / g resin) for azaspiracid-2 (AZA2) along 11 sites in two New Jersey estuaries from September 2020 – November 2022.
- 3.7 SPATT toxin data (ng / g resin) for Goniiodomin-A (GDA) along 11 sites in two New Jersey estuaries from September 2020 – November 2022.
- 3.8 SPATT toxin data (ng / g resin) for Yessotoxin (YTX) along 11 sites in two New Jersey estuaries from September 2020 – November 2022.
- 3.9 SPATT toxin data (ng / g resin YTX eq) for Homo-Yessotoxin (Homo-YTX) along 11 sites in two New Jersey estuaries from September 2020 – November 2022, quantified using YTX standard curve.
- 4.1 Map of the 12 field sampling sites (circles) in two New Jersey estuaries.

- 4.2 Salinity (ppt) measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.
- 4.3 Temperature (°C) measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.
- 4.4 Mixing diagrams for (A) dissolved organic carbon (DOC) mg/L, (B) total phenolic content (TPC) µg/L GAE eq, over salinity in Toms River-Barnegat Bay (red▲) and Mullica River-Great Bay (blue◆) estuaries from April to November 2022.
- 4.5 Dissolved Oxygen (DO mg/L) measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.
- 4.6 pH measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.
- 4.7 Turbidity (FNU) measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.
- 4.8 Nutrients and Dissolved Organic Carbon (DOC) measured along two New Jersey estuaries during sampling in 2022.
- 4.9 Daily mean discharge (feet³/sec) in lines and monthly precipitation (in) in bars near Toms River-Barnegat Bay (black) and Mullica River-Great Bay (blue) during the sampling period in 2021 and 2022.
- 4.10 Correlation matrix between environmental parameters collected along two New Jersey estuaries during sampling in 2022.
- 4.11 Total Phenolic Content (µg/L) measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.
- 4.12 Chlorophyll a (µg/L) measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.
- 4.13 Observed vs predicted chlorophyll a concentrations (µg/L) by multivariate regression models for samples collected in 2022.
- 4.14 Phytoplankton species richness, diversity, and evenness along two New Jersey estuaries during sampling in 2022.
- 4.15 Relative abundance of different phytoplankton groups (colored bars) and measured Chlorophyll a (dots with connecting line) for each sampling month in the different sites.

- 4.16 Redundancy analysis (RDA) of phytoplankton communities and environmental drivers in the surface water along (A) freshwater sites (<10 ppt), (B) mid-salinity sites (10<x<25 ppt), and (C) saltwater sites (>25 ppt), of two New Jersey estuaries in 2022.
- A.1 Measured flavonoids, luteolin (DAD array 325 nm) and apigenin (DAD array 350 nm), in seawater and freshwater over time with and without an Diaion HP-20 resin SPATT bag.
- B.1 Growth of *Microcystis aeruginosa* (strain LE3) when exposed to diluted 10%, 30%, 60%, 80%, and 100% methanol fractions eluted from Diaion HP-20 resin SPATT bag exposed to brewer's spent grain extract.
- B.2 HPLC DAD 350 nm peaks A, B, and C, collected from brewer's spent grain extract for analysis of algicides.
- B.3 Growth of *Microcystis aeruginosa* (strain LE3) when exposed to DAD 350nm peak fractions from brewer's spent grain extract.

List of Abbreviations

ACN	Acetonitrile
ANOVA	Analysis of variance
BBP	Barnegat Bay Partnership
BSG	Brewer's spent grain
Chl-a	Chlorophyll a
CDOM	Colored dissolved organic matter
DAD	Diode-Array Detection
DMSO	Dimethyl sulfoxide
DOC	Dissolved organic carbon
EEM	Excitation and emission matrix
F-C	Folin-Ciocalteu
FDOM	Fluorescent DOM
F_m	Maximum fluorescence yield
F₀	Initial fluorescence yield
F_s	Steady-state fluorescence yield
F_v	Maximum - initial fluorescence yield
GAE	Gallic acid equivalents
HAB	Harmful algal bloom
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
JCNERR	Jacques Cousteau National Estuarine Research Reserve
LC-MS/MS	Liquid chromatography-mass spectrometer/mass spectrometer
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass to charge ratio
MeOH	Methanol
NMDS	Non-metric multidimensional scaling
NH₄⁺	Ammonium
NO₂⁻	Nitrite
NO₃⁻	Nitrate
NJDEP	New Jersey Department of Environmental Protection
PARAFAC	Parallel Factor Analysis
PO₄³⁻	Phosphate
SPE	Solid phase extraction
Si	Silica
SWMP	System Wide Monitoring Program
TPC	Total phenolic content
TQMS	triple quadrupole mass spectrometer

Chapter 1: Introduction

1.1 Management of Harmful Algal Blooms

Harmful algal blooms (HABs) are a global concern in both freshwater and coastal systems; creating dire consequences for public health, water resources, and local economies (Anderson et al., 2008). HABs can refer to excess primary production that leads to negative shifts in the ecosystem, or phytoplankton that produce toxic secondary metabolites which cause harm to humans and/or other organisms. The economic loss due to HAB events is estimated to exceed millions of dollars in the United States each year, although economic studies are dated and likely underestimate the costs (Anderson et al., 2000; Hoagland and Scatasta, 2006).

Harmful algal blooms have increased in frequency, intensity, and geographical range over the past 50 years due primarily to anthropogenic eutrophication and climate change (Anderson et al., 2008; Paerl et al., 2018; Wells et al., 2015). Thus, there is a focus among scientists and managers on HAB prediction, prevention, and mitigation. The current mitigation options include physical, chemical, and biological approaches that can be adapted to manage different HAB species. However, each of these strategies have limitations, and no remedial technique demonstrates a sustainable solution to HABs on a global scale (Gallardo-Rodríguez et al., 2019; Nwankwegu et al., 2019; Prasath et al., 2022).

- **Physical approaches** include ultrasonic treatment which disrupts algae and leads to algal inactivation, vertical mixing which can reduce stratification and hypoxia, nanobubblers which emits small bubbles that disrupt algal membranes, and dredging which can remove nutrient rich soils and algal

cysts. These technologies tend to be costly, and thus the focus for physical approaches is on lakes and not applicable to large aquatic ecosystems.

- **Biological approaches** focus on maximizing predation and competition of HAB species. However, many times the algal growth rates exceed the predation rate and hypoxia and/or toxicity kill the predators. The use of algicidal microbial species can also have negative consequences as some produce active compounds that can accumulate in the environment. Additionally, food web dynamics can shift for the worse with human introductions and interference.
- **Chemical approaches** include the use of clay to flocculate the algal cells and chemicals such as hydrogen peroxide and copper sulfate which result in cell lysis. Chemical algicides are usually used in emergencies due to ease of deployment and rapidness of results. Chemical treatments have shown massive success in small systems. However chemical treatment can result in adverse effects to other organisms, and residual effects on the system. In addition, the massive cell lysis from chemical treatment can cause a high spike of toxin released from the algae into the water column, compromising water quality.

Each mitigation approach is ultimately system-specific and the solutions are rarely applicable and effective (Kidwell, 2015; Nwankwegu et al., 2019). Even when effective, mitigation strategies without the deployment of preventative measures are often short-term solutions. Preventative measures, such as reduction of nutrients from point and non-point sources, must be taken with different mitigation techniques in an

integrated management intervention in order to achieve effective management of HABs (Anderson, 2009; Nwankwegu et al., 2019). In addition, improvements to mitigation techniques are necessary to reduce negative impacts on the ecosystem (Nwankwegu et al., 2019).

1.2 Allelochemicals from plants

Allelochemicals are the secondary metabolites released into the environment by plants that either inhibit or stimulate another plant or organism. The concept of allelopathy can be dated back to B.C. but the term was first coined in 1937 by Australian plant physiologist Hans Molisch (Willis, 2007). Farmers have long recognized that certain crops can contaminate the soil or release beneficial nitrogen-rich organic compounds for other crops. In addition, gardeners are careful to avoid neighboring certain plants and engage in “companion planting” where both plants benefit from being near each other. Plants are rich with a diversity of secondary metabolites and the controlled release of these chemicals or planting certain aquatic and/or terrestrial vegetation near waterbodies could be effective in mitigating HABs (Hu and Hong, 2008; Li et al., 2020; Sun et al., 2019a).

1.3 Terrestrial and Aquatic Plant Chemicals on Phytoplankton Communities

Scientists have acknowledged the impact terrestrial and aquatic plants can have on phytoplankton communities since the early 20th century. One of the earliest papers in 1949, Hasler and Jones published findings on the negative correlation between phytoplankton populations and density of the aquatic plants *Anacharis canadensis* and *Potamogeton foliosus* (Hasler and Jones, 1949). Freshwater algae-inhibiting allelochemicals then began being extracted and isolated from plants in the

1980s (Zhu et al., 2021). Extraction and identification of plant allelochemicals rapidly progressed with the adoption of high-performance instruments such as gas chromatography-mass spectrometer and liquid chromatography-mass spectrometer. However, the research has often focused on grinded plant extracts in petri dishes, which does not indicate interactions that occur in the field (Willis, 2007). Even for research focusing on terrestrial plants and crops, allelochemical interactions have been difficult to prove in the field (Willis, 2007). Piecing apart the relationship between different dissolved organic matter (DOM) released from terrestrial and aquatic plants and phytoplankton community dynamics will result in better bloom prevention and mitigation measures.

1.4 Aims and Objectives

This dissertation looks at the effects of allelochemicals in purified plant material and natural allelochemicals found in estuarine systems, on phytoplankton communities and harmful algal bloom species. This work will help better identify and understand different HAB control and prevention techniques using plant allelochemicals. The objectives of this study are individually addressed as chapters of the dissertation and are as follows:

1. Assess the ability of the waste product: brewer's spent grain, to inhibit the growth of freshwater and marine harmful algal bloom species.
2. Explore the spatial and temporal trends in phycotoxins throughout two estuaries in NJ using SPATTs and Understand how phenolic content affects SPATT performance for phycotoxin tracking.

3. Understand how total phenolic content in the water varies with seasons and other abiotic factors and how total phenolic content impacts chlorophyll concentrations and phytoplankton community composition.

Chapter 2: Impact of the waste product brewer's spent grain on the growth of common freshwater and marine harmful algal bloom species

Abstract

Synthetic chemical control methods are considered effective in dealing with harmful algal blooms in small systems but can be costly and cause adverse consequences to ecosystems. Scientists have started looking at the diverse array of allelochemicals produced by terrestrial and aquatic plants as a safe and effective way to inhibit the growth of harmful algal blooms. For instance, barley straw bales are regularly used to control the growth of cyanobacteria in lakes. However, the process can be time consuming, labor intensive, and its effectiveness depends on deploying bales ~12 weeks prior to the bloom formation. This study examined brewer's spent grain composed of barley husks as an alternative source of allelopathic compounds. Brewer's spent grain is the grain waste from the brewing process that is still full of phenolic compounds, allelopathic chemicals shown to negatively impact toxic microalgae. Experiments were performed comparing the effectiveness of brewer's spent grain, barley straw, and *Sargassum natans*, on inhibiting the growth of toxin-producing species: *Microcystis aeruginosa* and *Karenia brevis*, and non-toxic species: *Scenedesmus obliquus* and *Phaeodactylum tricornutum*. A microcosm study was also performed exposing a natural lake bloom of cyanobacteria, *Cylindrospermopsis raciborskii*, to various doses of brewer's spent grain. The study found that brewer's spent grain extract of doses higher than 250mg/L can be used to inhibit the growth of freshwater and marine toxin-producing species (*M. aeruginosa* and *K. brevis*), while not impacting the diatom and chlorophyte tested (*S. obliquus* and *P. tricornutum*). An

additional experiment using antibiotics on *K. brevis* revealed that the likely cause of inhibition is due to increased bacteria growth or changes in the microbial community in *K. brevis*, although the mode of death in *M. aeruginosa* cultures is still likely due to allelopathic compounds. When 250 mg/L of brewer's spent grain extract was used on the lake water, the cyanobacteria declined after 4 days and was dominated by chlorophytes by day 6. These findings demonstrate the potential use for a brewery's waste product to control harmful algal blooms, though the concentration needed, nutrients in the waste product, and potential impact on bacteria communities suggest it will not be an effective solution in large systems.

2.1 Introduction

Harmful algal blooms (HABs) are a global concern in both freshwater and coastal systems; creating dire consequences for public health, water resources, and local economies (Anderson et al., 2008). Thus, there is a focus among scientists and managers on HAB prediction, prevention, and mitigation. The current mitigation options include physical, chemical, and biological approaches that can be adapted to manage different HAB species (Gallardo-Rodríguez et al., 2019; Nwankwegu et al., 2019; Paerl et al., 2016). Chemical control methods include algicides such as copper sulphate, chlorination, and hydrogen peroxide (Gibson, 1972; Lusty and Gobler, 2020; McKnight et al., 1983; Rodríguez et al., 2007). However, these chemicals cannot be applied on a large scale as the solutions can have a high financial cost and secondary pollution associated with them. Alternatives to chemical synthetic algicides are necessary to avoid the indirect negative consequences on other organisms and ecosystems. The use of natural allelochemicals produced by plants and bacteria have received considerable attention as an alternative to synthetic algicides. Depending on

the plant, the source of allelochemicals varies from phenolic acids, flavonoids, tannins, or fatty acids (Gao et al., 2020; Lv et al., 2021; Mecina et al., 2019, 2017; Xie et al., 2023). Plant allelochemicals can have negligible toxins, be highly selective, and easily degrade in the environment (Sun et al., 2019b; Zhu et al., 2021). All these characteristics lead to their potential safe use in the environment.

A variety of plants such as aquatic macroalgae, seaweed, sargassum, and eucalyptus have been studied for their effectiveness against toxin-producing microalgae (Tang and Gobler, 2011; Wang et al., 2007; Zerrifi et al., 2018; Zhao et al., 2019). One of the most well studied and commonly used methods is barley straw bales, which have been regularly deployed for control of cyanobacteria in lakes since the 1970s (Barrett et al., 1999; Gibson et al., 1990; Iredale et al., 2012; Sellner et al., 2014; Waybright et al., 2009). However, barley straw dispersal can be time consuming, labor intensive, and its effectiveness is dependent on deploying bales ~12 weeks prior to the bloom formation. Thus, this widespread management technique is better as a preventative measure than as a remediation technique.

Scientists have expanded the research on algicidal compounds to include plant waste generated by humans, including pomegranate peels (Chen and Wang, 2019), watermelon peels (Zhang et al., 2020), and rice and wheat husks (Ma et al., 2018). A potentially viable control solution that has yet to be studied is brewer's spent grain (BSG). BSG is the insoluble part of barley grain husks that constitutes as much as 85% of breweries total by-products. At present, BSG is primarily donated for animal feed but with the increased cost of solid fraction disposal, breweries and scientists are actively looking for alternative uses for this high antioxidant material.

BSG is readily available at low or no cost throughout the year and is produced in large quantities by both large and small breweries. Since the husks contain much of the phenolic components of barley grain, the phenolic acids and flavonoids are an estimated fivefold concentration higher in BSG compared to that of barley straw (Stefanello et al., 2018). The beer making process heats and mashes the barley husks before extracting the sugar-rich liquid from the barley. Consequently, the BSG is already decomposing and ready for use in natural algicide application, with likely no time between application and cell death, unlike barley straw which requires microbial degradation. Therefore, it is reasonable to postulate that BSG could be an economical source of algicides, and a more effective management strategy compared to barley straw.

The primary objective of this study was to assess the ability of the waste product: brewer's spent grain, to inhibit the growth of freshwater and marine harmful algal bloom species. Specifically, the goals were to: (1) research the growth-inhibiting effects of BSG extract compared to barley straw and *Sargassum natans*, on the cyanobacteria *Microcystis aeruginosa*, chlorophyte *Scenedesmus obliquus*, dinoflagellate *Karenia Brevis*, and diatom *Phaeodactylum tricornutum*, (2) identify whether any inhibitory effects from BSG are due to chemicals or bacteria overgrowth, and (3) investigate the effects of BSG on phytoplankton community composition during a natural bloom of *Cylindrospermopsis raciborskii*.

2.2 Methods

To address these objectives, species of microalgae were exposed to various doses of plant extract as well as a purified flavonoid, luteolin, as a positive control. *K. Brevis* was then exposed to various doses of brewer's spent grain (BSG), with

controlling for bacteria, to identify if the effects were due to biotic or abiotic factors. Samples were collected for growth counts and photosynthetic efficiency during the course of the experiments. To understand how BSG affects phytoplankton community composition, 10 liters of lake water was collected during a bloom of *Cylindrospermopsis raciborskii* in Williston Lake, Denton, Maryland and exposed to various doses of BSG.

2.2.1 Preparation of plant extracts

Brewer's spent grain (BSG) was received from Diamondback Brewing Company (Baltimore, MD) following the brewing of a pilsner with 100% barley husks as the grain source in July 2019. *Sargassum natans* was collected on the ship side using a handheld net in the Sargassum Sea in July 2016. Barley straw (*Hordeum vulgare*) was collected from barley bales July 2019. Sargassum and barley were rinsed three times with DI water, then sargassum, barley, and BSG were dried at 70°C for 48hrs and grinded at 25,000 rpm for 10 minutes or until the plants were in powder form. 15 grams of the powdered plants were then transferred to 250 mL autoclaved glass bottles with 200 mL ultrapure H₂O (75 g/L). The bottles were placed on a shaker table for 24hrs and then centrifuged at 2,000xg at 20°C for 10 min. The supernatant was recovered and filtered through a 25mm GF/F filter and then 0.2 µm polycarbonate filters into autoclaved glassware and kept at -20°C until use in further analysis. Three mL from each bottle was dried to get the weight of the organic matter, which were 13.5 mg/mL in the BSG and sargassum extract, and 3 mg/mL in the barley extract. Cultures were dosed based on equal dry weights of the organic matter from the various plant extracts.

2.2.2 Exposure of microalgal cultures to plant extracts

Microalgae were exposed to 50mg/L, 100mg/L, 250mg/L dry weight of BSG, sargassum, and barley extracts (n=3), using 250 mL Erlenmeyer flasks (autoclaved at 121°C for 20 min), with 115 ml medium, 25 mL culture and respective amount of 10 mL plant extract: ultrapure H₂O depending on the specific concentration. The controls had 10 mL ultrapure H₂O added with no plant extract. At the same time, additional flasks of cultures were exposed to 6 mg/L purified luteolin dissolved in DMSO (10mg/mL stock solution) (n=3). Since DMSO is known to impact microalgal growth, the luteolin treatment was compared to a DMSO control of the same addition, 0.12% v/v (n = 3). 50mg/L, 100mg/L, 250mg/L dry weights were equal to extract doses of 0.2%, 0.4%, 1% of BSG and sargassum, and 1%, 2%, and 5% barley.

2.2.2.1 Exposure of freshwater microalgae

Microcystis aeruginosa (strain LE3) and *Scenedesmus obliquus* (strain HTB1) were cultured in BG11 medium, 20°C, and 50 $\mu\text{mol photons/m}^2/\text{s}$ using fluorescent lamps with a 12:12 light/dark cycle. Cultures in exponential growth were inoculated at 1×10^6 cells mL^{-1} . Growth cell counts were performed on days 0, 1, 2, 4, 6, and 8 during the experiment with *S. obliquus* and days 0, 1, 2, 4, and 6 during the experiment with *M. aeruginosa*. Photosynthetic efficiency was measured on days 1 and 2. The growth and percent mortality of *M. aeruginosa* were determined by counting 25 μL on medium speed using a flow cytometer (BD AccuriTM C6), where events with fluorescence above 10^5 measured under FL4-H wavelength 640nm were considered alive. The growth and percent mortality of *S. obliquus* were determined using a hemocytometer.

2.2.2.2 Exposure of *Phaeodactylum tricornutum*

Phaeodactylum tricornutum (strain UTEX 646) was cultured in 20 ppt F/2 medium, 20°C, and 100 $\mu\text{mol photons/m}^2/\text{s}$ using fluorescent lamps with a 12/12 light/dark cycle. *P. tricornutum* in exponential growth was inoculated at 5×10^5 cells mL^{-1} . One mL samples were collected from each flask at days 0, 1, 2, 4, 6, and 8, preserved in 10 μL Lugol's iodide solution, and counted using a hemocytometer.

2.2.2.3 Exposure of *Karenia brevis*

Karenia brevis (strain New Pass) was cultured in 32 ppt modified F-Si medium (f/2 medium of nutrients but f medium of trace metals and vitamins), 24°C, and 100 $\mu\text{mol photons/m}^2/\text{s}$ using fluorescent lamps with a 14/10 light/dark cycle. *K. brevis* in exponential growth was inoculated at 3,000 cells mL^{-1} . Bacteria counts and cell growth counts were performed on days 0, 1, 2, 4, 6, and 8. Bacteria counts were performed by adding 100 μL sample to 100 μL of 1 μM SYBR green I Nucleic Acid Gel stain, incubating in the dark for 10 min, and counting 25 μL on medium speed using flow cytometer (BD AccuriTM C6). Events with fluorescence above 10^4 measured under FL1-H wavelength 550nm were considered bacteria. Flow cytometer measurements were multiplied by two for total bacteria per μL . Five mL samples were collected from each flask for cell counts and counted using a Multisizer 4e Coulter Counter equipped with a 70 μm aperture. pH measurements were taken on days 0, 4, 6, and 8.

2.2.3 Exposure of *Karenia brevis* to brewer's spent grain with and without antibiotics

Karenia brevis (strain New Pass) was cultured in modified F-Si medium (f/2 medium of nutrients but f medium of trace metals and vitamins), 24°C, and 100 $\mu\text{mol photons/m}^2/\text{s}$ using fluorescent lamps with a 14/10 light/dark cycle. *K. brevis* in

exponential growth was inoculated at 500 cells/mL and exposed to either the control of 10 mL ultrapure H₂O, 0.12% DMSO, 6 mg/L purified luteolin dissolved in DMSO, 50 mg/L BSG extract, or 200 mg/L BSG extract, using 250 mL Erlenmeyer flasks (autoclaved at 121°C for 20 min), with 100 mL medium (n=6). Three flasks of each treatment were dosed with 200 µL of each 50 µg/mL Kanamycin, 100 µg/mL Carbenicillin, and 50 µg/mL Spectinomycin to inhibit bacteria growth (n=3). Respective amounts of ultrapure H₂O were added to flasks for a total volume of 200 mL. Bacteria counts and cell growth counts were performed on days 0, 1, 2, 4, 6, and 8, using flow cytometer and culture counter the same way as stated in section 2.2.3.3. 25 mL samples were collected from each flask on day 4 to identify bacteria community composition.

2.2.4 Heterotrophic bacteria community analysis

DNA barcoding analysis was performed to assess the changes in community composition of heterotrophic bacteria in the *K. brevis* cultures with and without the use of antibiotics. At day 4 of the experiment described in 2.2.3, 25 mL was collected from each flask and filtered onto a 0.2 µm polycarbonate filter. The filters were stored at -20°C until analysis. Filters were aseptically cut in half (using sterile scissors and tweezers) and each placed into a DNeasy PowerLyzer Powersoil Kit PowerBead Tube (Qiagen, Valencia, CA, USA). From this point, the kit protocol was followed, and the isolated DNA was eluted into 100 µL of the C6 solution. DNA from the variable V4 regions of the 16S rRNA gene was then amplified using the modified 515F and 806R primers and sequenced via a MiSeq 16S amplicon run (Apprill et al., 2015; Parada et al., 2016).

2.2.5 Total Phenolic Content analysis

Total Phenolic Content (TPC) was estimated for the plant extracts using the Folin-Ciocalteu method of Singleton et al (1974), with some modifications (Singleton et al., 1974). In brief, 100 μ L of the plant extracts were added to 900 μ L ultrapure H₂O and vortexed (n=3). 100 μ L of 1 M NaOH was added, followed by 120 μ L of Folin-Ciocalteu reagent, vortexed, and put in the dark for five minutes. 300 μ L of 200g/L Na₂CO₃ was added, vortexed, and samples were put into the dark for one hour. A 250 μ L aliquot was transferred to a flat bottom 96 well plate and adsorption at 765 nm was measured on a SpectraMax i3x multi-mode microplate reader. The samples were compared to a gallic acid standard curve from 1 to 50 μ g/mL and presented in gallic acid equivalents (GAEs).

2.2.6 Photosynthetic efficiency analysis

Photosynthetic efficiency were measured at 24hrs and 48hrs using a pulse amplitude modulated spectrofluorometer (PAM-2500, WALZ, Germany), integrated with a computer with data acquisition software (PamWin 3.2) using the methods of Srivastava et al 2021 (Srivastava et al., 2021). 1×10^7 cells from each flask were centrifuged at 2,000xg 10min at 20°C, resuspended in 400 μ L of culture media and transferred to a KS-2500 suspension cuvette (WALZ, Germany). After 15 min of dark acclimation, the minimal fluorescence yield (F_0) was determined with $<1 \mu\text{mol m}^{-2}\text{s}^{-1}$. The maximum fluorescence yield (F_m) was measured after a 0.3s pulse of a saturating light (630 nm, intensity 3000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). The ratio of F_v/F_m was used to determine the maximal PSII photochemical efficiency, where F_v is the difference between the maximum (F_m) and initial (F_0) fluorescence after dark acclimation. Only *M. aeruginosa*, *P. tricornutum*, and *S. obliquus*, were analyzed for photosynthetic

efficiency due to the high cell volumes needed for analysis and the difficulty in concentrating *K. brevis* cells.

2.2.7 Exposure of *Cylindrospermopsis raciborskii* bloom water to brewer's spent grain extract

Ten liters of water was collected from the surface of Williston Lake in Denton, Maryland during a bloom of *Cylindrospermopsis raciborskii* on Sept. 4, 2019 (38°49'29.9"N 75°50'19.4"W). The water was immediately filtered through 150 µm filter to remove large zooplankton and debris before transportation. Water was homogenized and 500 mL was transferred to 600 mL culture flasks. The flasks were dosed with either five mL ultrapure H₂O (control), five mL BSG extract (250mg/L; BSG 1%), or 0.5 mL BSG extract/4.5 mL ultrapure H₂O (25mg/L; BSG 0.1%) (n=3). Flasks were placed in an incubator at 20°C, 12:12 light: dark cycle, and 100 µEm⁻²s⁻¹ of PAR.

The phytoplankton community composition was sampled at day 0, 2, 4, and 6 by homogenizing flask and taking 4 mL preserved using 1% Lugol's solution. The preserved phytoplankton samples were analyzed by identifying plankton to the lowest possible taxa in x100 magnification until at least 300 algal cells were counted.

Pigment samples were collected at day 0 and 6 by filtering 5 ml through 25 mm GF/F and freezing at -80°C. The pigment analysis was performed via HPLC at Horn Point laboratory (Cambridge, MD). Water quality data including pH, total alkalinity, NH₄⁺, NO₂⁻, PO₄, and NO₃⁻ were analyzed by Zooquatics (Baltimore, MD) in the inoculum, spent grain extract, and day 6 flasks.

2.2.8 Statistics

Results are expressed as mean values ± standard deviation of the mean. Significant differences among mean response of microalgae and bacteria during

exposure of extracts was determined by one-way analysis of variance (ANOVA). An ANOVA was also performed to test differences in total cells/mL and nutrients. Where significant differences were detected, the Tukey's hsd post hoc test was used for separation of means. Data were assessed for normality (Shapiro-Wilk test) and for homogeneity of variance (Levene test) at the 0.05 significance level. When non-normality or non-homogeneity of variance was found, the data was logarithmically transformed and if tests still failed, non-parametric Kruskal-Wallis test was conducted followed by a Dunn's test. Non-metric multidimensional scaling (NMDS) plot based on Bray–Curtis similarity matrix was performed to visualize differences in phytoplankton and bacteria composition between treatments. A permutational multivariate ANOVA (PERMANOVA) was performed to compare the phytoplankton community composition between groups when lake water was exposed to BSG and between bacterial communities when *K. brevis* was exposed to concentrations of BSG with and without antibiotics. This analysis was performed using 'vegan' package in R. Differences were considered significant at $p < 0.05$. Statistical analyses were carried out with R studio (Version 4.2.2 R script). Algal growth inhibition was calculated as follows: Percent Inhibition = $100 \times ((\text{Control Cell Density} - \text{Treatment Cell Density}) / \text{Control Cell Density})$.

2.3 Results

2.3.1 Exposure of microalgae to brewer's spent grain, sargassum, and barley extract

To determine whether brewer's spent grain (BSG), sargassum, or barley straw impacts microalgae, extracts from each source were added to cultures of *K. brevis*, *P. tricornutum*, *S. obliquus*, and *M. aeruginosa* in varying concentrations. The effect of the extracts on growth and photosynthetic efficiency of the microalgae was monitored

over the course of the experiments and compared to the controls. The cultures were also exposed to a purified flavonoid, luteolin, as a positive control, to understand if the microalgae were affected by a known flavonoid since these are likely in the group of compounds responsible for growth inhibition of *M. aeruginosa* from barley straw (Mecina et al., 2017; Xiao et al., 2014). In addition, luteolin is known to inhibit the growth of *M. aeruginosa* by disruption of the photosynthetic system (Huang et al., 2015). To normalize the plant extract doses, the same amount of dry organic matter was added to flasks (final concentration of 50 mg/L, 100 mg/L, 250 mg/L). However, more material dissolved from the BSG and sargassum during the 24-hour H₂O extraction compared to the barley straw. This meant that five times the extract volume of barley was added to cultures (1, 2, and 5% v/v) compared to BSG and sargassum (0.2, 0.4, 1% v/v). On top of different extract additions, total phenolic content (TPC) additions were not consistent between plant extracts. In BSG treatments, the final TPC concentrations were 0.53, 1.07, and 2.67 mg/L GAE in 50, 100, and 250 mg/L extract treatments; In sargassum treatments, the final TPC concentrations were 0.49, 0.99, and 2.48 mg/L GAE in 50, 100, and 250 mg/L extract treatments; In barley treatments, the final TPC concentrations were 3.22, 6.43, and 16.08 mg/L GAE in 50, 100, and 250 mg/L extract treatments.

After transferring cultures to flasks at the start of the experiment, all microalgae experienced a lag phase lasting one to two days and were then in exponential growth phase through the end of the experiment. For all three plant extracts and at all concentrations tested, inhibition was not evident in the diatom *P. tricornutum* nor the chlorophyte *S. obliquus* (Fig. 2.1.A-C & 2.2.A-C). There was

also no observed growth inhibition of these two microalgae when exposed to 6mg/L luteolin compared to the DMSO control (Fig. 2.1.D & 2.2.D). Additionally, the mean F_v/F_m in *S. obliquus* and *P. tricornutum* at 24 hours was not significantly different from the controls (ANOVA). However, the mean photosynthetic efficiency of photosystem II (F_v/F_m) in *S. obliquus* at 48 hours was significantly higher in BSG 50mg/L and barley 100mg/L compared to the control cultures (ANOVA post-hoc Tukey. $F=13.47$, $p<0.001$. Fig. 2.3.A). The mean F_v/F_m in *P. tricornutum* at 48 hours was significantly higher in BSG 250mg/L, barley 100mg/L and 250mg/L, and sargassum 250 mg/L compared to the control cultures (ANOVA post-hoc Tukey; $F=6.03$ $p<0.001$; Fig. 2.3.B).

The growth patterns of the non-axenic cyanobacteria *M. aeruginosa* (strain LE3) following exposure to plant extracts are presented in Fig. 2.4. BSG and barley extract reduced the growth of *M. aeruginosa* at concentrations ≥ 100 mg/L, exhibiting typical concentration-dependent response curves. Compared to the untreated controls, there was noticeable growth inhibition after two days exposure, and by day six, cell densities of cyanobacteria were reduced by 61% and 89% when treated with 100mg/L and 250 mg/L BSG extract and reduced by 33% and 89% when treated with 100mg/L and 250mg/L barley extract. Growth inhibition by sargassum extract was not observed in the cyanobacteria at the concentrations tested. The mean F_v/F_m in *M. aeruginosa* at 24 hours was significantly lower with exposure to 100mg/L barley (0.15 ± 0.03) and 250mg/L barley (0.10 ± 0.05), and BSG 250mg/L (0.12 ± 0.03), compared to the controls (0.32 ± 0.12) (ANOVA post-hoc Tukey; $F=12.54$ $p<0.001$). The mean F_v/F_m in *M. aeruginosa* at 48 hours was significantly higher in BSG

50mg/L, barley 50mg/L, and sargassum 100 mg/L, and significantly lower in BSG 100mg/L and 250mg/L, barley 250mg/L, and sargassum 250mg/L, compared to the control cultures (ANOVA post-hoc Tukey; $F=60.3$ $p<0.001$; Fig. 2.3.C).

The growth patterns of the toxin producing dinoflagellate *K. brevis* following exposure to plant extracts are presented in Fig. 2.5. Compared to the untreated control, noticeable declines in cell concentrations occurred by day two in 250mg/L plant treatments and by day six the dinoflagellates cell densities were reduced by 90% and 98% when treated with 100mg/L and 250 mg/L BSG extract, 99.7% when treated with 250mg/L barley extract, and 99.4% when treated with 250mg/L sargassum extract. All other plant extract concentrations did not significantly decrease growth of *K. brevis*. Bacteria, counted using SYBR green I Nucleic Acid Gel stain, increased significantly within 24 hours of exposure to all plant extracts and all concentrations compared to the control cultures, and remained significantly higher during the experiment (ANOVA post-hoc Tukey; $p<0.001$; Fig. 2.6).

2.3.2 Exposure of *Karenia brevis* to brewer's spent grain with and without antibiotics

To determine whether the reduced growth of *K. brevis* was primarily due to chemicals or the growth of bacteria, cultures of *K. brevis* with and without the addition of antibiotics were exposed to BSG extracts in varying concentrations. The growth patterns of the toxin producing dinoflagellate *K. brevis* following exposure to BSG extracts with and without the addition of antibiotics are presented in Fig. 2.7. The use of antibiotics did not significantly impact the growth of *K. brevis* in controls during the 8-day experiment (Paired T-Test; $t=-1.42$ $p=0.17$; Fig. 2.7.A). However, the use of antibiotics significantly reduced bacteria counts in the control flasks

compared to the control flasks without antibiotics through day 6 of the experiment (ANOVA; $F=34.36$ $p<0.01$ [day 1], $F=132.7$, $p<0.001$ [day 2]; $F=25.84$ $p<0.01$ [day 4], $F=38.58$ $p<0.01$ [day 6], $F=6.22$ $p=0.067$ [day 8]). Bacteria counts were also significantly lower in BSG extract cultures with antibiotics compared to the BSG extract cultures without antibiotics for two days in the 50mg/L treatments (ANOVA; $F=643.5$ $p<0.001$ [day 1], $F=478.7$ $p<0.001$ [day 2]) and for four days in the 200mg/L treatments (ANOVA; $F=379.7$ $p<0.001$ [day 1], $F=124.7$ $p<0.001$ [day 2], $F=8.208$ $p<0.05$ [day 4]) (Fig. 2.8).

K. brevis cell densities treated with 6 mg/L luteolin were reduced by 90% by day six. These reduced cell densities occurred in luteolin flasks with and without antibiotics (Fig. 2.7.B). 200 mg/L of BSG extract without antibiotics caused cell densities to decline by 93% compared to the control flasks by day six. While 200 mg/L of BSG extract with antibiotics did not impact *K. brevis* growth through day four, but cell densities were reduced by 70% by day eight. 50 mg/L of BSG extract with antibiotics did not significantly impact growth through the entire experiment, while 50 mg/L of BSG extract without antibiotics had large differences in cell densities, as indicated by the standard deviations (Fig. 2.7.A). Some of the 50mg/L BSG extract without antibiotic flasks had similar growth rates to the control flasks while there was loss in other flasks, even though relatively the same number of bacteria were found within the treatment (Fig. 2.7.A & Fig. 2.8).

When comparing the bacterial community between treatments and the use of antibiotics, the bacteria community composition at the genus level was significantly different between treatments and with/without antibiotics (PERMANOVA;

Treatment: $R^2 = 0.26$ $F = 3.43$ $p < 0.001$, Antibiotics: $R^2 = 0.12$ $F = 6.63$ $p < 0.001$, Treatment*Antibiotics: $R^2 = 0.27$ $F = 3.56$ $p < 0.001$; Fig. 2.12). The Shannon Species Diversity of bacteria was significantly lower in flasks exposed to 50mg/L BSG extract without antibiotics compared to 50mg/L BSG extract with antibiotics (ANOVA; $F = 5.11$ $p < 0.001$; Table 2.1). The BSG 50mg/L and 200mg/L treatments were dominated by *Alteromonas* sp. when no antibiotics were added ($81.65 \pm 10.53\%$ compared to $7.89 \pm 4.57\%$ with antibiotics) but shifted to a more diverse bacterial community when antibiotics were added, with higher percentages of *Blastopirellula* sp. (from 0.01% to $55.71 \pm 22.56\%$) and *Bacillus* sp. (from $0.07 \pm 0.07\%$ to $6.57 \pm 2.5\%$) (Fig. 2.9B). In the control, DMSO, and 6mg/L luteolin treatments, the percentage of *Marivita* sp. declined when antibiotics were added to the treatments (from $49.76 \pm 6.4\%$ to $16.6 \pm 11.77\%$) (Fig. 2.9B).

2.3.3 Exposure of *Cylindrospermopsis raciborskii* bloom water to brewer's spent grain extract

To determine what impact BSG extracts had on non-targeted species in the phytoplankton community, water from Williston Lake was exposed to varying concentrations of BSG extract in lab microcosms. At the start of the experiment, the Williston Lake water had an average cell density of 420,000 cells/mL with the dominant group Cyanophyta of the species *Cylindrospermopsis raciborskii* (88%), *Microcystis aeruginosa* (2%), *Microcystis wesenbergii* (1%), and *Aphanocapsa* sp (5%). Less than 5% of the phytoplankton were composed of Chrysophyta, Cryptophyta, Chlorophyta, and unidentified flagellates (<10 μ l) (Fig. 2.11). When exposing lake water to 250mg/L of BSG, it caused significantly lower total cells/mL on days two and four compared to the control flasks (log transformed. $p < 0.01$ [day2],

$p < 0.001$ [day 4]; Fig. 2.11). In addition, the phytoplankton community composition was significantly different between the 250mg/L BSG extract treatment and controls (PERMANOVA; $F=6.88$ $p < 0.05$; Fig. 2.12). By day two, four, and six the concentration of *C. raciborskii* in the 250mg/L flasks were 22%, 10%, and 7% that of the controls (Fig. 2.11). The phytoplankton community in the flasks shifted from Cyanophyta dominated to Chlorophyta dominated by day six (Fig. 2.11). Alternatively, the addition of 25mg/L or 0.1% spent grain extract caused slightly higher overall cell counts compared to the control throughout the experiment, with relatively the same proportion of Cyanophyta. However, these differences in cell counts were not statistically significant (Fig. 2.11).

The pigment analysis of day six cultures showed a similar trend to the microscopic phytoplankton identification (Fig. 2.13). In the 250mg/L treatment, there was higher chlorophyll b, which is associated with chlorophytes, (ANOVA; $F=595$ $p < 0.001$) and a smaller ratio of zeaxanthin, pigment associated with cyanobacteria, compared to the control treatments. Total chlorophyll-a declined in the control treatment over the six days, going from an average of 68 $\mu\text{g/L}$ to 14 $\mu\text{g/L}$ (Fig. 2.13).

When analyzing the nutrients in BSG extract, there was high PO_4^{3-} (0.361 mg/L), high NH_4^+ (9.61 mg/L) levels, and a low pH (5.31) (Table 2.2). By day 6, NO_3^- and PO_4^{3-} concentrations were significantly higher in the 1% spent grain extract flasks at 1 mg/L and 0.6 ± 0.07 mg/L, compared to < 0.2 and 0.04 ± 0.01 mg/L in the control flasks (PO_4^{3-} and NO_3^- : $p < 0.01$; Table 2.2).

2.4 Discussion

2.4.1 Exposure of microalgae to brewer's spent grain, sargassum, and barley extract

These experiments demonstrate that brewer's spent grain (BSG) could be used as a selective control substance, capable of restricting growth of the toxin-producing cyanobacteria and dinoflagellate, *M. aeruginosa* and *K. brevis* (Fig. 2.4.A & 2.5.A), while not impacting the growth of the diatoms and chlorophytes, *P. tricornutum* and *S. obliquus* (Fig. 2.1.A & 2.2.A). This study also demonstrates that the impact of the flavonoid, luteolin, is selective, impacting the growth of the dinoflagellate and cyanobacteria, but not the diatom nor the chlorophyte (Fig. 2.1.D, 2.2.D, & 2.7.B). To our knowledge, this is the first study to demonstrate luteolin does not impact a chlorophyte nor diatom. In addition, luteolin did not have a strong impact on the growth of bacteria concentrations like BSG extract did in *K. brevis* cultures (Fig. 2.8).

There was a clear dose-response curve between concentrations of BSG and barley straw extract and *M. aeruginosa* cells over the course of the experiment. BSG at concentrations of 100 mg/L acted as an algistatic agent, inhibiting the growth of *M. aeruginosa* during the experiment, but 250mg/L of BSG acted as an algicide, killing the algae (Fig. 2.4.A). Similar growth patterns were seen in the 250 mg/L barley straw treatments (Fig. 2.4.B). However, even the high sargassum treatments had no impact on the growth of *M. aeruginosa* (Fig. 2.4.C). Alternatively, 250mg/L of all plant extracts along with 100 mg/L BSG caused *K. brevis* cultures to die (Fig. 2.5.A-C).

Bacteria levels in the *K. brevis* cultures increased with the addition of all the plant extracts, with the highest bacteria concentration in the 250mg/L sargassum treatment on day two (Fig 2.8). The increase of bacteria with all plant doses is

expected since f/2 medium does not contain a carbon source that would allow for the overgrowth of bacteria in normal conditions (Sun et al., 2022). Although bacteria levels seemed to increase/correlate with the dose of the plant extract, the response of the *K. brevis* did not (Fig 2.7.A & 2.8). The *K. brevis* cultures either grew the same as the control cultures or declined by around 90% with the exposure to plant extracts. This suggests that the response of *K. brevis* to all three plant extracts was unlikely due to allelochemicals, but rather the type and/or concentration of bacteria grown with these extracts. However, based on the different responses of dose-dependent cell death in *M. aeruginosa* and not in *K. brevis*, and the fact no cell death for *M. aeruginosa* when exposed to sargassum extract, the mode of death from the plant extracts seems to be different between the two species of algae.

Previous studies have reported on similar allelopathic effects of plant extracts on controlling cyanobacteria and other harmful algae, with polyphenols being an important category (Chen and Wang, 2019; Gao et al., 2020; Nakai et al., 2000; Tang and Gobler, 2011; Wang et al., 2007; Xiao et al., 2014; Zerrifi et al., 2018; Zhao et al., 2019). In this study, total phenolic content (TPC) was measured in the plant extracts, with barley straw extracts having the highest TPC per mg/L organic matter, and BSG and sargassum having relatively the same concentrations of TPC per mg/L organic matter. However, the growth responses of *M. aeruginosa* and *K. brevis* were not correlated to the doses of TPC. If it is phenolic content in barley and BSG inhibiting the growth of *M. aeruginosa*, the specific compounds matter or there are additional inhibitory compounds other than phenolic compounds, as the higher TPC in the doses of barley resulted in similar growth inhibition to BSG (Fig. 2.4.A-B). In

addition, the TPC was the same for BSG and sargassum doses, but sargassum had no impact on the growth of *M. aeruginosa* (Fig. 2.4.C). Evidence supporting this idea has previously been published, where some phenolic acids inhibited the growth of *M. aeruginosa*, while others had no impact or required higher doses to have the same impact (Huang et al., 2015; Li et al., 2023; Verni et al., 2020). One of the dominant group of polyphenols in brown algae like sargassum, is phlorotannins (Table 2.3) (Catarino et al., 2023; Leandro et al., 2020; Powers et al., 2019). Phlorotannins have been shown to impact the growth of red tide microalgae like *Karenia mikimotoi*, however, no study was found looking at the impact of phlorotannins on *Microcystis* spp. (Nagayama et al., 2003). The differences in the types of polyphenols between the plant extracts could be the reason for the variable responses (Table 2.3).

Many allelochemicals, including polyphenols, have been well documented to impact photosystem II (PSII) (Chen and Wang, 2019; Huang et al., 2015; Zhao et al., 2019). F_v/F_m represents the maximum efficiency of PSII photochemistry in cells and a decrease in F_v/F_m has been associated with cellular stress when exposed to biotic and abiotic factors. 250mg/L BSG extract along with 250mg/L barley extract, showed cellular stress through a significantly reduced PSII efficiency in *M. aeruginosa* at days one and two (Fig. 2.3.A-B). This suggests that the cells are stressed in these treatments within 24 hours. 250 mg/L sargassum also seemed to significantly decrease the efficiency of PSII in *M. aeruginosa* after 48 hours exposure, however this was not associated with a significant decrease in cellular growth over the eight days (Fig. 2.4.C). This suggests that even though high doses of sargassum extract

may have caused some initial stress to *M. aeruginosa*, the cells were able to recover and it was not a high enough dose to lead to apoptosis.

It is difficult to compare doses used in this study to other plant extracts previously tested, as many studies had different ways of creating extracts. Most studies estimated g/L doses based on the mixture of dried powder mass/volume of DI water, even though the studies agitate the mixture under different conditions and for various amounts of time ranging from one hour to one week. As shown in this study, various plant powders in H₂O release different amounts of organic matter and TPC, and it is reasonable to assume this amount would differ based on extraction time and conditions. In addition, this study used H₂O as the extraction solvent which has been shown to not be a good method for extracting phenolic compounds from plants. For instance, studies using rice husks, argued similar concentrations were needed to that of barley straw to inhibit growth of *M. aeruginosa* using H₂O extracts (2-10 g/L), but significantly lower concentrations were needed when rice husks were extracted in MeOH (0.01-10 mg/L) (Park et al., 2006; Su et al., 2014). Future studies could use MeOH, ethanol, or acetone, to extract much higher volumes of phenolic compounds from BSG (Socaci et al., 2018). Overall, the doses of 0.4-1% BSG H₂O extract (equaling 100-250mg/L organic matter) are too high to be reasonably added to a lake for controlling HABs and thus a higher density of allelochemicals per mL from BSG would need to be achieved.

The dose-dependent impact of BSG on growth and efficiency of PSII cultures in *M. aeruginosa* cultures, and the lack of impact of sargassum extract on growth, which caused similar changes to the bacteria concentration, pH, and other abiotic

factors in *K. brevis*, suggest the restricted growth in *M. aeruginosa* is primarily due to the allelochemicals in BSG (Fig. 2.4.A). However, the high bacterial abundance in BSG exposed *K. brevis* cultures and the lack of a dose-dependent response, suggest that the growth inhibition in *K. brevis* is primarily due to biotic factors rather than allelochemicals in the BSG extracts (Fig. 2.5.A & 2.7.A). These results led to the second set of experiments comparing the response of the *K. brevis* to BSG with and without the addition of antibiotics and exposing an axenic strain of *M. aeruginosa* to the same plant extracts.

2.4.2 Exposure of *Karenia brevis* to brewer's spent grain with and without antibiotics

To further understand whether BSG extract impacts *K. brevis* cultures through released chemicals or bacterial concentrations, bacteria growth in *K. brevis* cultures were controlled using antibiotics. Similar growth rates of *K. brevis* were achieved between the control treatments with and without the antibiotics (Fig. 2.7). In addition, 6mg/L luteolin caused the same growth inhibition of *K. brevis* with and without the use of antibiotics, suggesting that antibiotics did not prevent the flavonoid from interacting with the cells and inhibiting growth (Fig. 2.7B). The antibiotics were not as good at keeping bacterial abundance low in BSG treatments, and likely multiple doses of antibiotics should be used in experiments that extend longer than 48 hours. The antibiotics kept bacterial abundance the same as the controls for one day in the 200mg/L treatment and two days in the 50mg/L BSG treatment (Fig. 2.8). After these first few days, bacteria counts were significantly higher in the BSG treatments compared to the controls. Even with only a few days of controlling bacteria growth, the use of antibiotics had a large impact on whether BSG extract impacted the growth

of *K. brevis*. 200 mg/L BSG without antibiotics inhibited growth of *K. brevis* through the experiment but 200 mg/L BSG with antibiotics had no impact on growth compared to the control treatments (Fig. 2.7.A). However, there was large variation in bacteria concentrations and cell numbers between flasks of *K. brevis* within the same BSG treatments, with no clear trends. Some flasks had high bacteria levels but *K. brevis* still grew while others had lower bacteria levels and the *K. brevis* growth was inhibited in both 50mg/L and 200mg/L BSG extract treatments. These results suggest that the growth inhibition of *K. brevis* caused by BSG extract is not fully chemical but could be due to either increased bacteria in the cultures or fueling of a specific type of bacteria. These results are interesting as other laboratory experiments have shown growth inhibition of sargassum extracts on axenic cultures of various dinoflagellate species (Wang et al., 2012, 2007). The differences could be because insufficient doses were used in this study, the type of phenolics in sargassum a different from that in brewer's spent grain, or *K. brevis* could be more resistant to the allelochemicals compared to the other dinoflagellate species tested (Table 2.3). It is clear from these results that the flavonoid, luteolin, is capable of inhibiting growth in *K. brevis* and more research is needed to look at the impact of other flavonoids as well as why high bacterial abundance led to inconsistent inhibition of *K. brevis* growth.

To further understand if the bacteria communities were changing between treatments and antibiotics use, 16S rRNA sequencing was used to analyze the bacterial communities in flasks on day four of the experiment. The microbial community changed significantly based on the treatment and with the use of antibiotics. The overall Shannon diversity index of bacteria was not impacted using

antibiotics, except in 50 mg/L BSG extract without antibiotics where it was significantly lower (Table 2.1). These findings are different from other studies which found bacteria diversity associated with phytoplankton significantly decreased with the use of antibiotics (Cao et al., 2019).

Sun et al 2022 found that *Alteromonas* sp. and *Halomonas* sp. dominated at the same time as high mortality of *Karenia mikimotoi* occurred after carbon nutrient stimulation in a controlled laboratory study (Sun et al., 2022). These results are similar to this study where *Alteromonas* sp. were highest in flasks with 50mg/L and 200mg/L BSG extract without antibiotics where high mortality of *K. brevis* occurred. BSG extract is a clear source of organic carbon that can fuel microbial growth (Table 2.2). This experiment along with the Sun et al. 2022 study suggest *Alteromonas* sp. have a competitive advantage with the addition of carbon, although the antibiotics used in this study seem to reduce this advantage (Fig. 2.9B). Additionally, studies have found species of *Alteromonas* to inhibit the growth of *K. mikimotoi* (Zheng et al., 2018). These results further suggest that the response of *K. brevis* to BSG extracts was unlikely due to allelochemicals, but rather the type and/or concentration of bacteria grown with the addition of a carbon source. Since the microbial community can shift based on growth stage and physiological state of the dinoflagellate, further experiments should analyze the bacterial community over more than one time point (Li et al., 2019; Sun et al., 2022).

2.4.3 Exposure of *Cylindrospermopsis raciborskii* bloom water to brewer's spent grain extract

A dosage of 250 mg/L BSG extract had a significant impact on phytoplankton community composition and pigments in the Williston Lake water microcosms over

the six-day experiment (Fig. 2.11 & Fig. 2.13). In particular, the concentration of *C. raciborskii* and the pigment zeaxanthin ratio declined, while chlorophytes and the pigment chlorophyll b concentration increased over the six days compared to the control treatment (Fig. 2.13). This is similar to what was found to happen with exposure of lake water to barley straw, where studies have found decomposing barley straw to affect the cyanophyceae class the most rather than diatoms and green algae (El-Moneem et al., 2012; Ferrier et al., 2005). Alternatively, a dosage of 25 mg/L BSG extract had no significant impact on phytoplankton community composition nor pigment composition compared to the controls over the six-day experiment. These results are interesting as Lusty and Gobler (2020) found *Cylindrospermopsis* to be most resistant to hydrogen peroxide treatment (Lusty and Gobler, 2020). Since barley straw and flavonoids are supposed to have a similar mode of action against cyanobacteria through increased reactive oxygen species and causing oxidative stress, there is a need to replicate this experiment during a bloom of *Microcystis* or another common cyanobacteria HAB species, as lower doses could still be effective against other species (Mecina et al., 2019, 2017; Xiao et al., 2014). After 2 days, the 25 mg/L BSG treatment had slightly more cyanobacteria than the control treatment, although the difference was not significant. The extra nutrients added in the BSG extract likely fueled the algal bloom, and 25 mg/L was not a high enough dose of allelochemicals to cause a reduction in *Cylindrospermopsis* (Table 2.2). These results emphasize the importance of dosage in using BSG extract as a mitigation technique in the field, as a small dose could have the opposite effect of the goal, and the added nutrients with this control technique is not ideal for longer term HAB prevention.

2.5 Conclusion

This study demonstrated the potential use of brewer's spent grain (BSG) for controlling harmful algal blooms (HABs). The results revealed that BSG extract can reduce the growth of toxin-producing dinoflagellate and cyanobacteria species, *Karenia brevis* and *Microcystis aeruginosa*, in controlled laboratory experiments, without impacting the growth of the diatom, *Phaeodactylum tricornutum*, and the chlorophyte, *Scenedesmus obliquus* (Fig. 2.1.A, 2.2.A, 2.4.A, & 2.5.A). However, the growth of bacteria or a specific bacterial strain seemed to be the cause of reduced growth in the *K. brevis* cultures rather than the presence of growth-inhibiting chemicals (Fig. 2.8 & 2.9). In addition, exposing *Cylindrospermopsis raciborskii* bloom water from a Maryland lake to 250mg/l of BSG extract, caused the bloom to crash and allowed chlorophytes to dominate. Unfortunately, BSG extract had high PO_4^{3-} and NH_4^+ levels, and low pH, all of which are expected from the mashing of barley husks to achieve optimal brewing liquor. However, these would not be ideal when adding the extracts to water systems (Table 2.2). In addition, dosing concentrations of 250 mg/L BSG extract are logistically too high to be scaled up for field use. Therefore, the allelochemicals would need to be isolated from the BSG extract before it could be considered as a realistic control strategy for cyanobacteria, which could be achieved through using a high organic solvent for extraction, but this would add to the cost of this management strategy. More research is needed looking into the cost of isolating the inhibitory compounds from BSG using different extraction solvents and continued exploration into alternative uses of this waste product. In addition, further research is needed to understand if using luteolin would

be a good control mechanism and how best to disperse the compound into a HAB impacted waterbody.

2.6 Tables

Table 2.1. Bacterial reads from 16S Miseq Illumina in *Karenia brevis* (strain New Pass) at day 4 of cultures exposed to brewer's spent grain extract and luteolin dissolved in DMSO, with and without the use of antibiotics.

Sample	Number Reads	% Reads Classified to Genus	Shannon Species Diversity	Number of Species Identified
Control with antibiotics	1265248	91.65	0.712	998
	150779	81.96	0.974	383
	78449	83.72	1.032	292
Control without antibiotics	159766	89.22	0.708	349
	263652	90.34	0.705	523
	282534	88.57	0.896	645
DMSO with antibiotics	136540	85.22	0.948	397
	146554	96.14	0.542	279
	135817	87.38	1.178	433
DMSO without antibiotics	201255	88.44	0.978	365
	200948	89.49	0.965	370
	35637	89.95	0.948	226
Luteolin with antibiotics	74231	86.34	1.24	346
	407962	97.76	0.412	388
	44382	84.72	1.194	308
Luteolin without antibiotics	235912	90.82	1.164	580
	187943	90.49	1.262	449
	245537	91.2	1.445	487
Brewers Spent Grain 50mg/L with antibiotics	1288014	86.45	1.551	1198
	317658	86.26	1.544	469
	107673	92.6	1.107	294
Brewers Spent Grain 50mg/L without antibiotics	2379086	98.76	0.272	679
	251288	98.5	0.293	269
	215497	98.33	0.283	262
Brewers Spent Grain 200mg/L with antibiotics	258226	93.17	1.088	465
	148879	96.17	0.84	307
Brewers Spent Grain 200mg/L without antibiotics	130402	97.53	0.875	296
	333593	97.71	0.981	419
	148100	98.34	0.586	253

Table 2.2. Concentration of nutrients in controls and treatments after six days of exposure to filtrates from brewer's spent grain (BSG).

Day	n	Treatment	pH	NH ₄ ⁺ (mg/L)	NO ₃ ⁻ (mg/L)	NO ₂ ⁻ (mg/L)	PO ₄ ³⁻ (mg/L)	CO ₃ ²⁻ (mg/L)
0	1	Lake water	7.34	0.24	1	0.01	0.091	NA
	1	BSG extract	5.31	9.61	0.2	<0.01	0.361	NA
	2	Control	8.37 ± 0.28	0.03 ± 0.02	< 1	< 0.01	0.04 ± 0.01	55.47 ± 3.28
6	2	25mg/L BSG extract	8.23 ± 0.19	0 ± 0.01	< 1	< 0.01	0.07 ± 0.02	54.97 ± 1.03
	2	250mg/L BSG extract	7.76 ± 0.11	0.02 ± 0.02	1	< 0.01	0.5 ± 0.14	64.4 ± 5.89

Table 2.3. Some of the phenolic compounds found in Sargassum and brewer's spent grain. Data for the table was collected from Catarino et al 2023, Verni et al 2020, Klejdus et al 2017, Stefanello et al 2018.

Polyphenol Class	Brewer's Spent Grain	Sargassum
Phlorotannin		Phlorethol
		Tetrafuhalol
		Fucol
		Fucophlorethol
		Fuhalol
		Eckol
		Carmalol
		Catechin
		Epicatechin
		Liquiritigenin
Flavonoids	Catechin	Catechin
	Epicatechin	Epicatechin
	5,7 - Dihydroxychromone	Liquiritigenin
	Quercetin	Caylcosin
Phenolic Acid	Benzoic acid derivatives	Benzoic acid derivatives
	Cinnamic acid derivatives	Cinnamic acid derivatives
Tannin	Hydroxybenzoate derivatives	

2.7 Figures

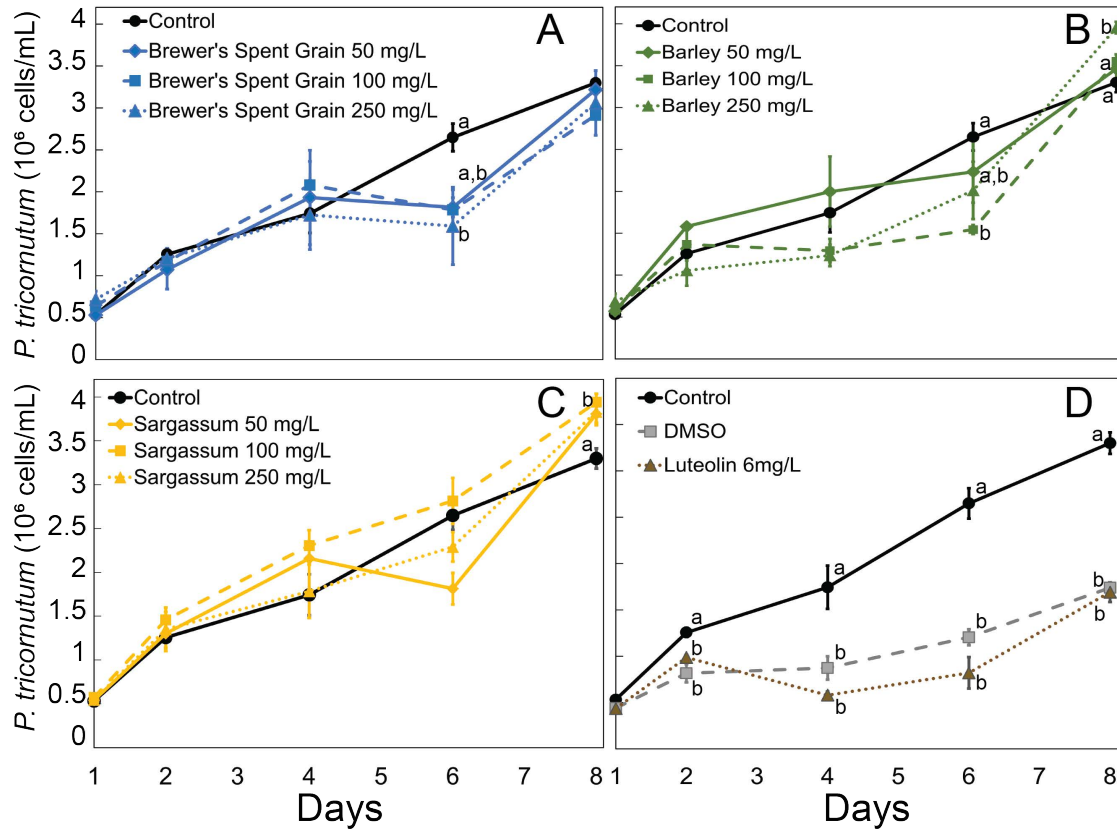


Figure 2.1. Growth effects of (A) brewer's spent grain extract, (B) barley extract, (C) sargassum extract, and (D) luteolin dissolved in DMSO, on *Phaeodactylum tricornutum* (strain UTEX 646). Data points are means $\pm$ standard deviation ($n = 3$). Letters represent significantly different groups using Tukey HSD following ANOVA ($p < 0.05$). No letters at a time point indicates no significant differences at that time. $n = 3$.

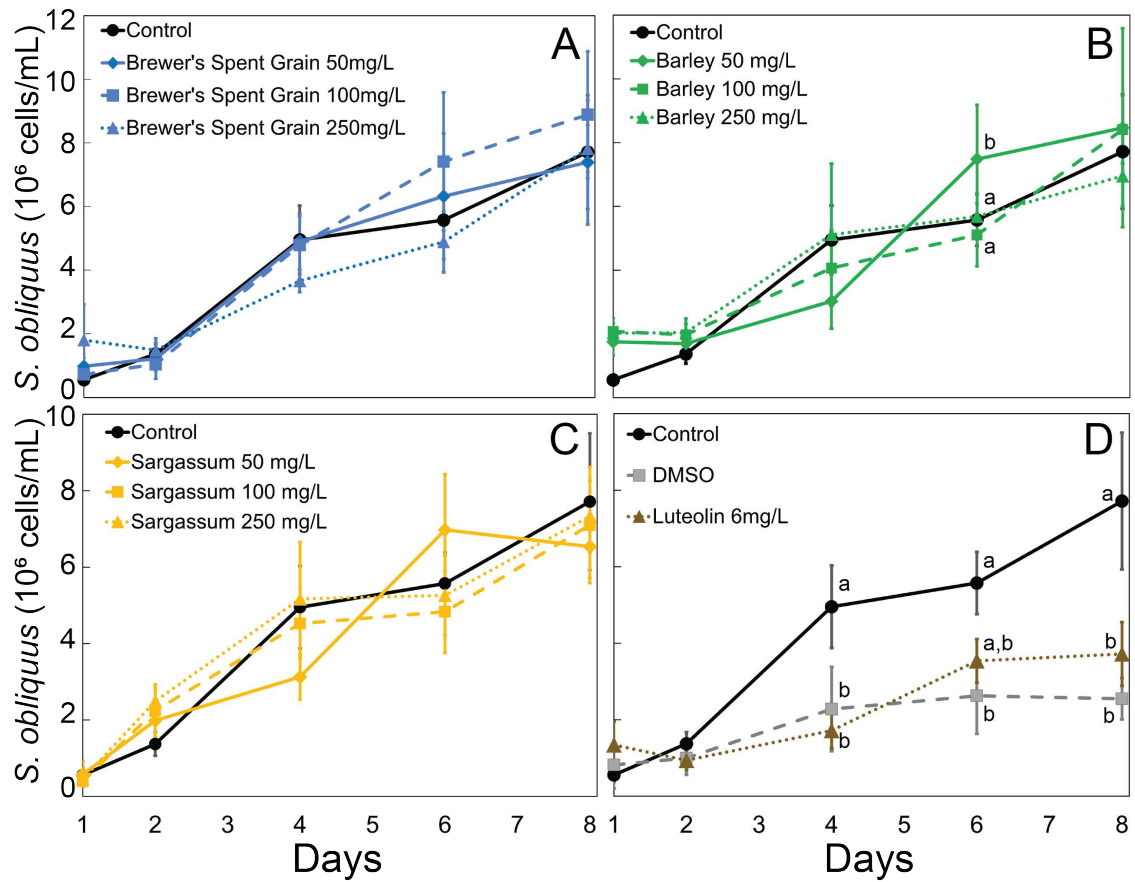


Figure 2.2. Growth effects of (A) brewer's spent grain extract, (B) barley extract, (C) sargassum extract, and (D) luteolin dissolved in DMSO, on *Scenedesmus obliquus* (strain HTB1). Data points are means $\pm$ standard deviation ($n = 3$). Letters represent significantly different groups using Tukey HSD following ANOVA ($p < 0.05$). No letters at a time point indicates no significant differences at that time. $n = 3$.

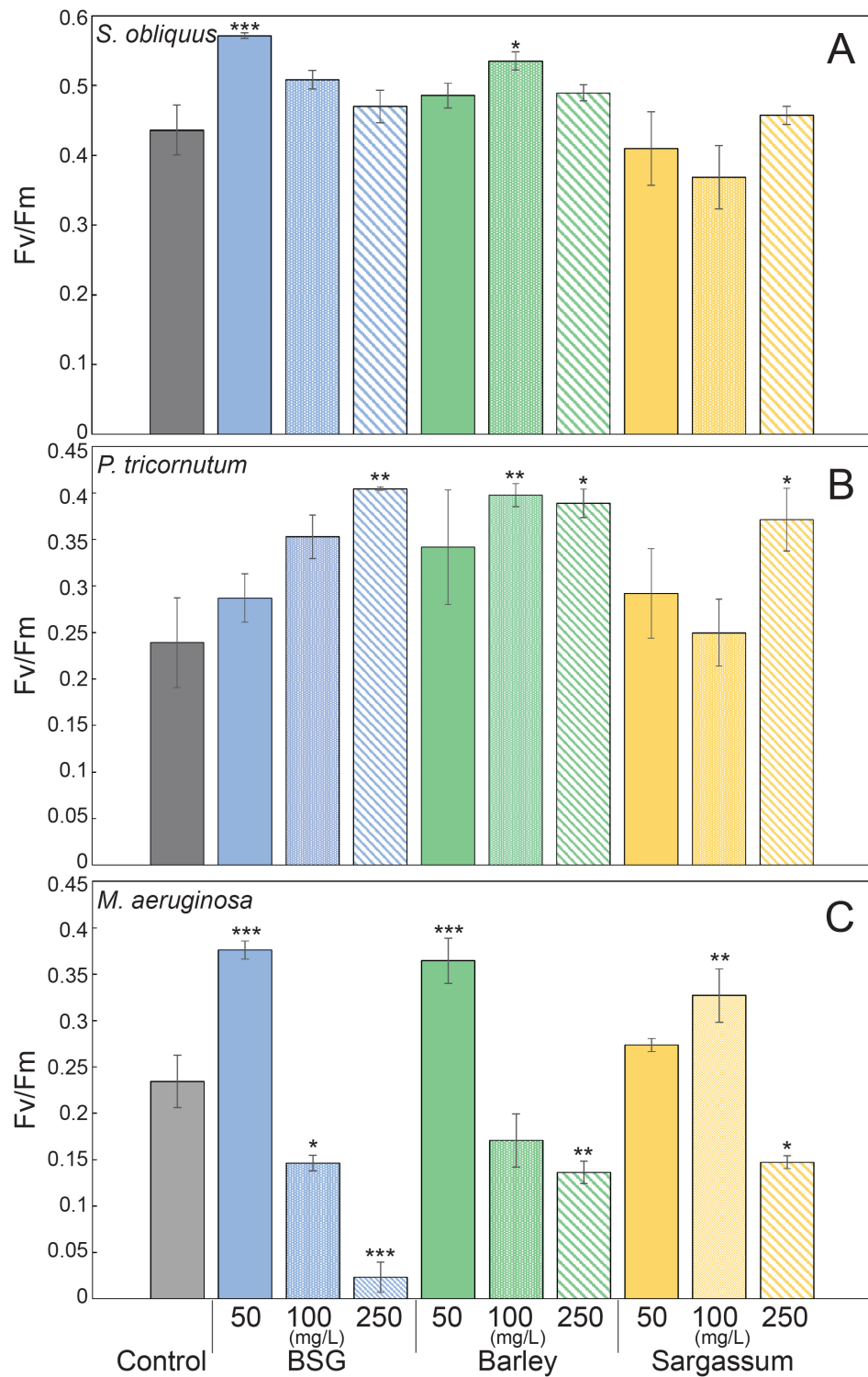


Figure 2.3. Photosynthetic efficiency (F_v/F_m) of (A) *Scenedesmus obliquus*, (B) *Phaeodactylum tricornutum*, (C) *Microcystis aeruginosa*, exposed to various doses of plant extracts after 48 hours. Significant differences between treatments and control are indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). $n=3$.

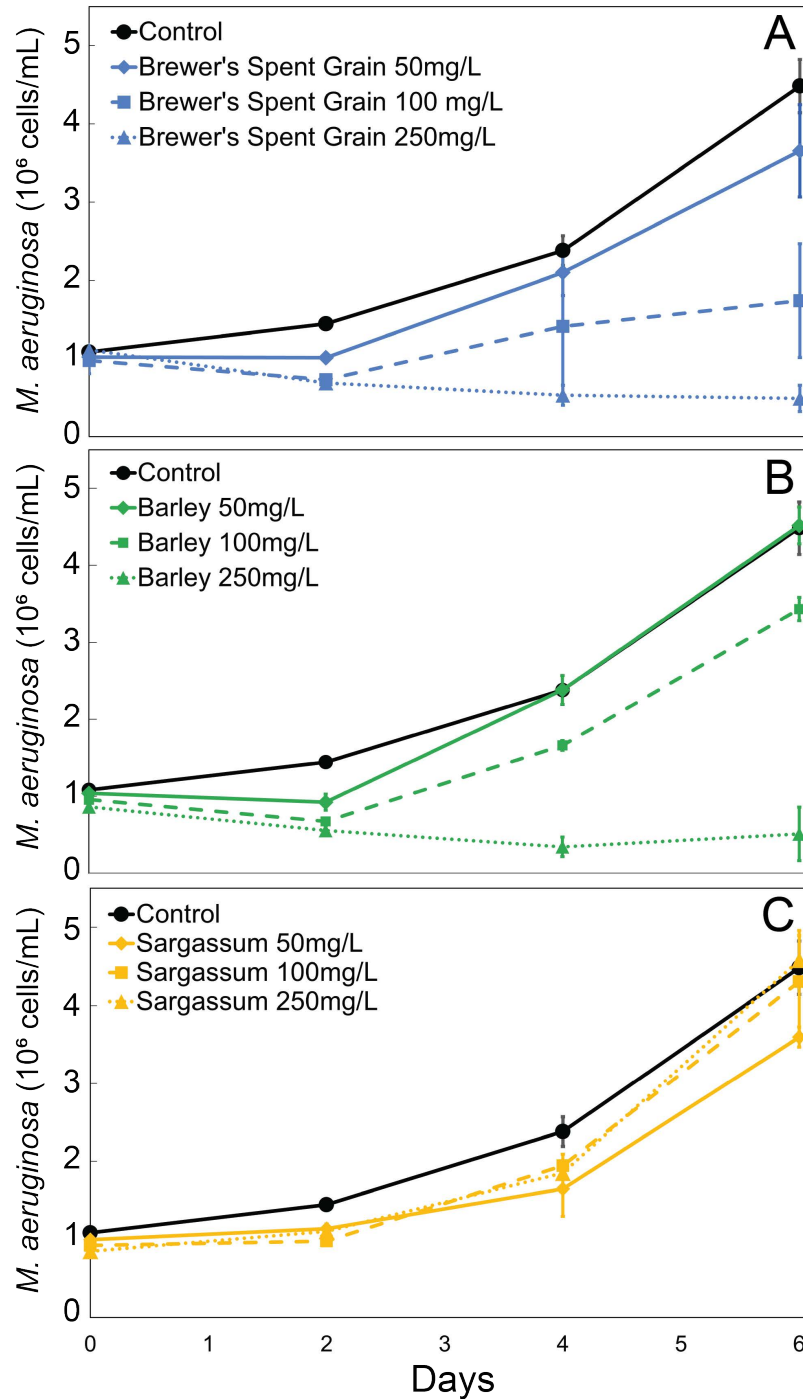


Figure 2.4. Growth effects of (A) brewer's spent grain extract, (B) barley extract, and (C) sargassum extract, on *Microcystis aeruginosa* (strain LE3). Data points are means $\pm$ standard deviation. Letters represent significantly different groups using Tukey HSD following ANOVA ($p < 0.05$). No letters at a time point indicates no significant differences at that time. n=3.

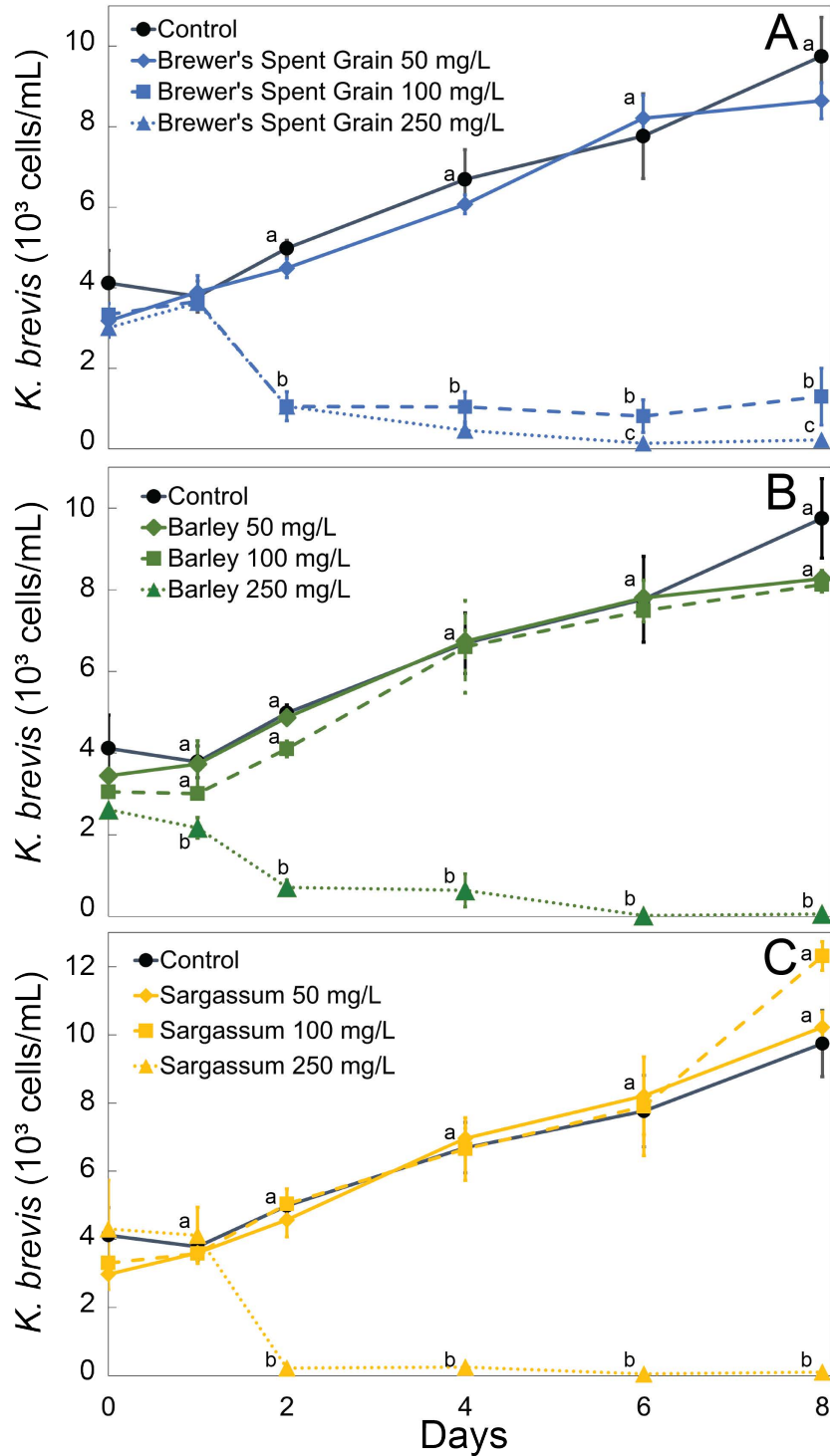


Figure 2.5. Growth-inhibition effects of (A) brewer's spent grain extract, (B) barley extract, and (C) sargassum extract, on *Karenia brevis* (strain new pass). Data points are means $\pm$ standard. Letters represent significantly different groups using Tukey HSD following ANOVA ($p < 0.05$). No letters at a time point indicates no significant differences at that time. $n = 3$.

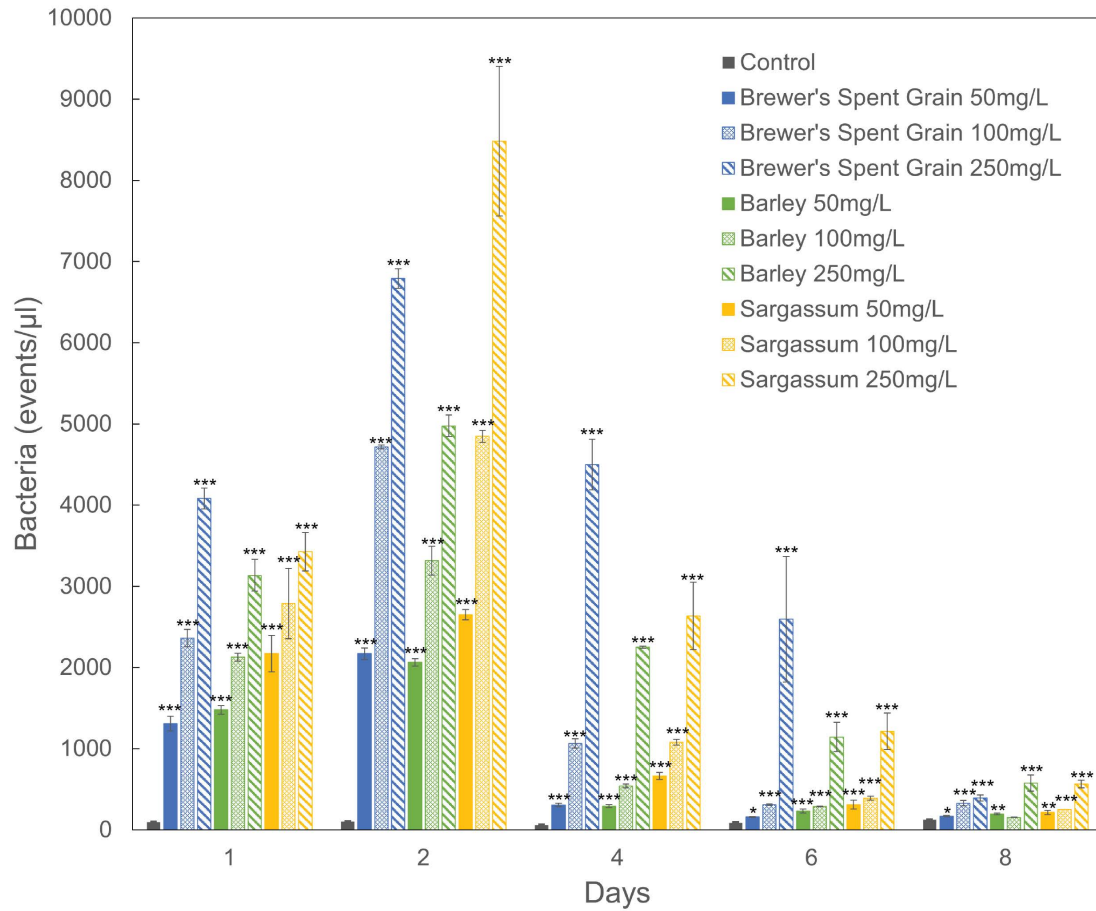


Figure 2.6. Bacteria count in *Karenia brevis* cultures exposed to various doses of brewer's spent grain, barley, and sargassum. Significant differences between treatments and control are indicated with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). $n=3$.

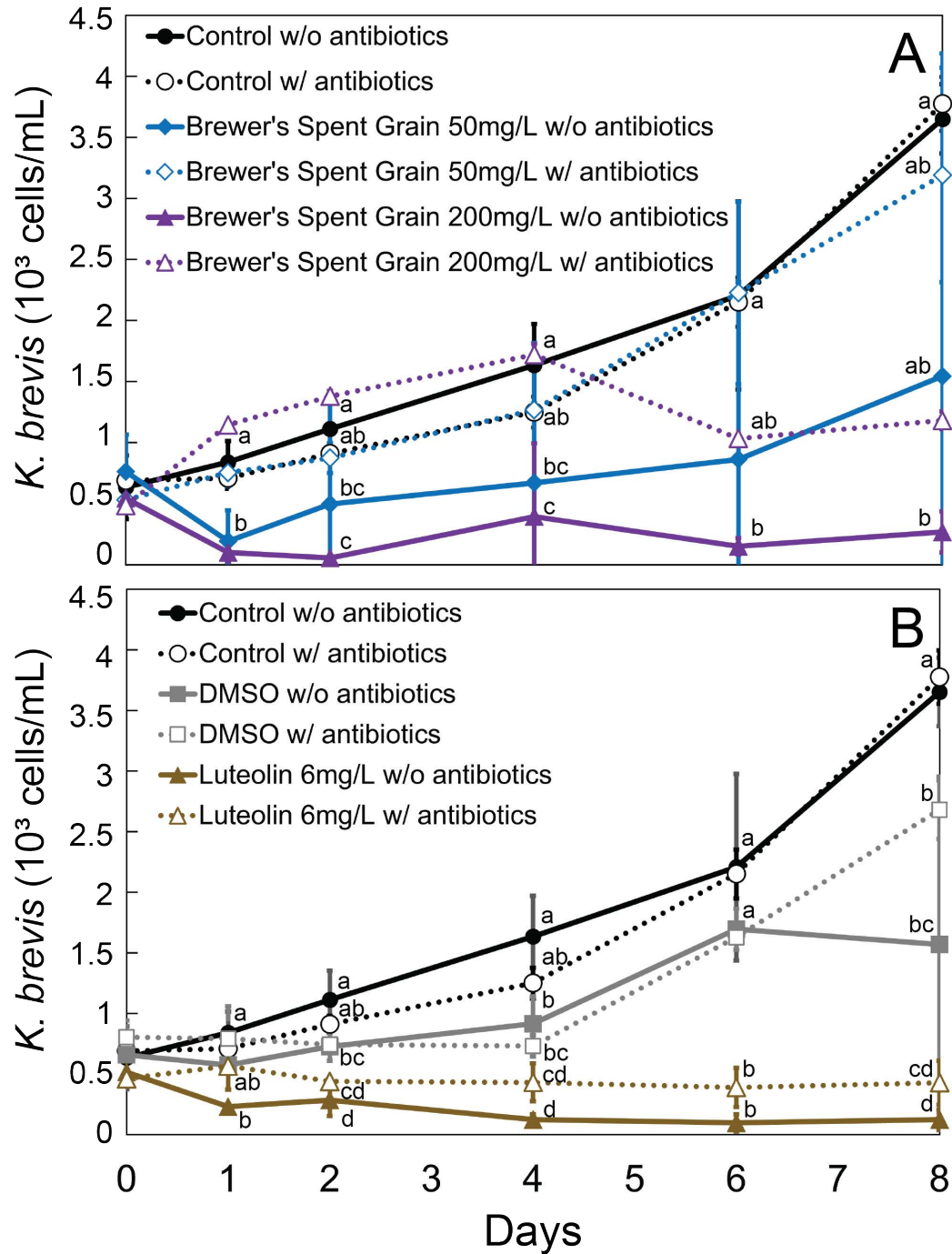


Figure 2.7. Growth of *Karenia brevis* (strain new pass) on (A) brewer's spent grain extract, and (B) luteolin dissolved in DMSO, with and without the use of antibiotics (n=3). Letters represent significantly different groups using Tukey HSD following ANOVA ($p < 0.05$). No letters at a time point indicates no significant differences at that time. n=3.

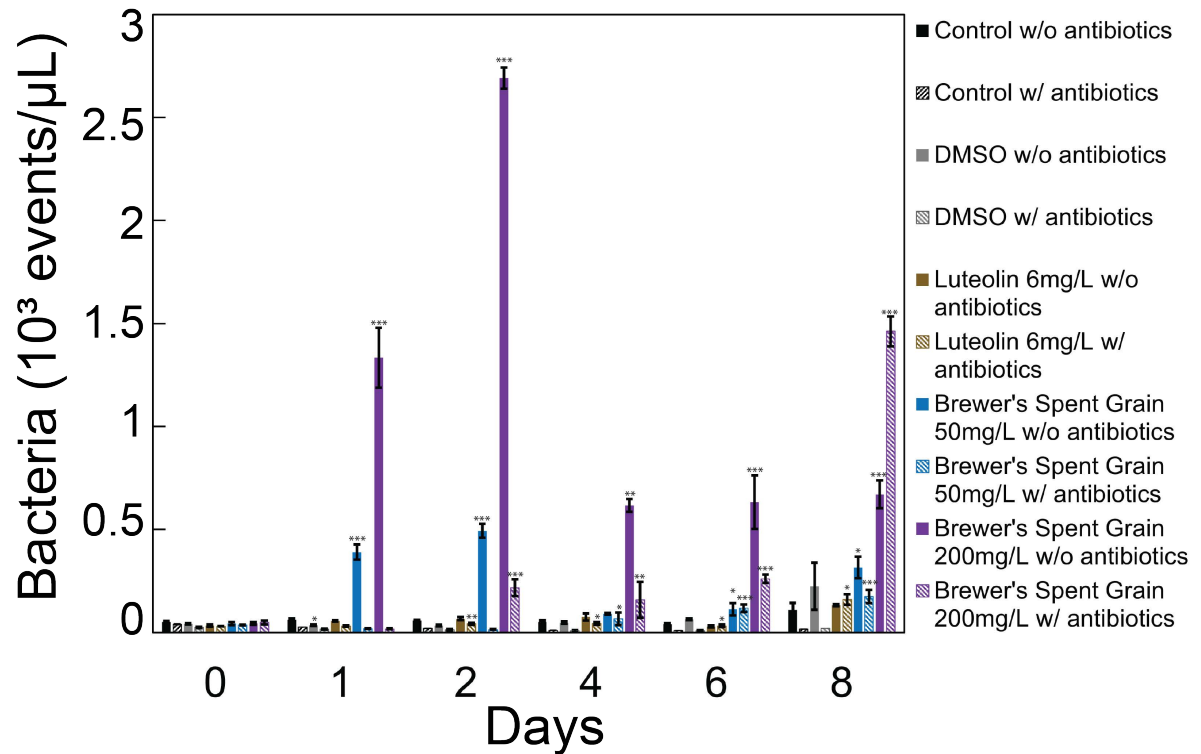


Figure 2.8. Bacteria concentration in *Karenia brevis* (strain new pass) cultures exposed to brewer's spent grain extract and luteolin dissolved in DMSO, with and without the use of antibiotics (n=3). Letters represent significantly different groups using Tukey HSD following ANOVA ($p < 0.05$). Treatments with antibiotics were compared to controls with antibiotics and treatments without antibiotics were compared to controls without antibiotics. No letters at a time point indicates no significant differences at that time. n=3.

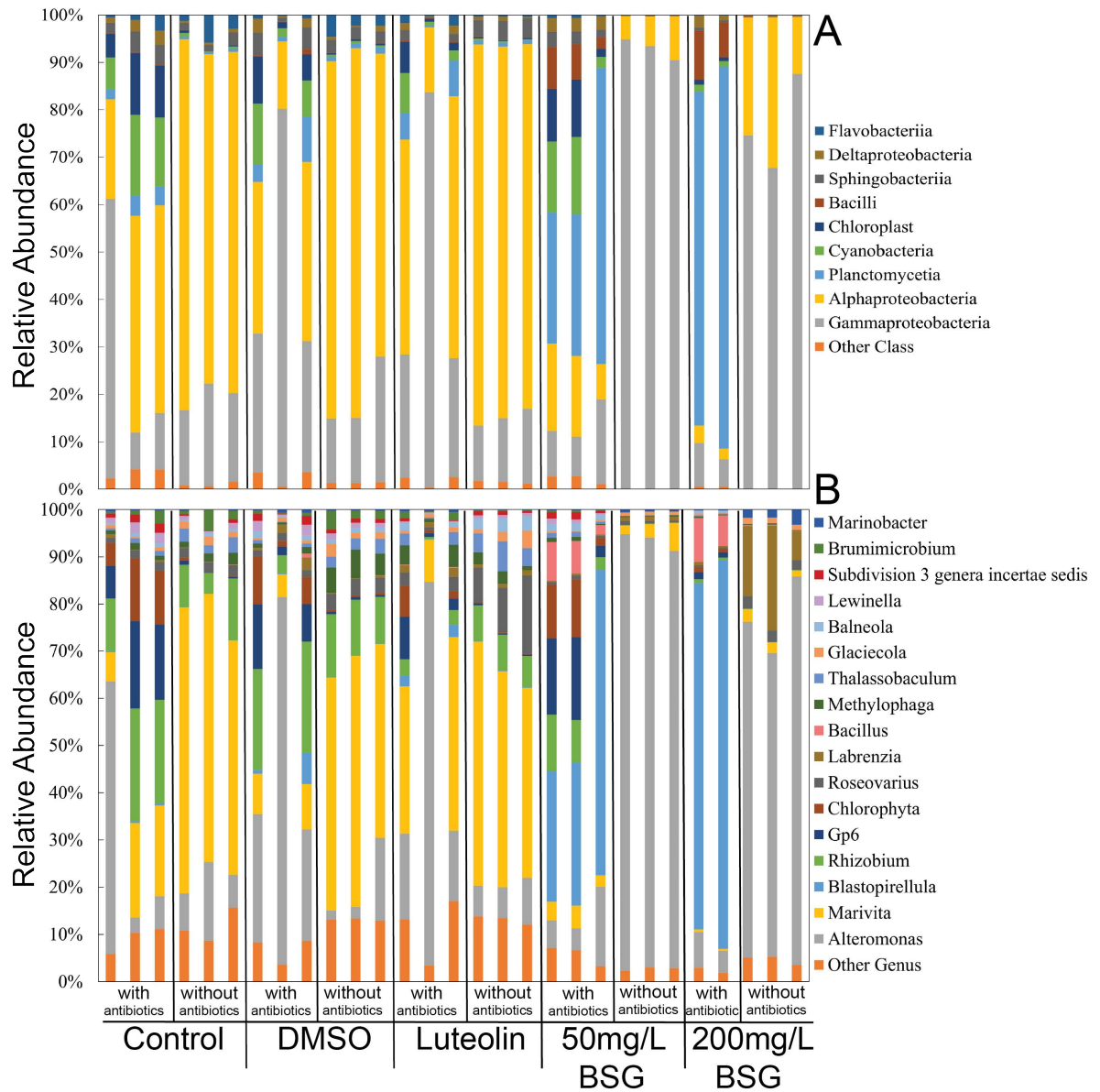


Figure 2.9. Class-level (A) and genus-level (B) bacterial relative abundance (%) in *Karenia brevis* (strain New Pass) at day 4 of cultures exposed to brewer's spent grain extract and luteolin dissolved in DMSO, with and without the use of antibiotics.

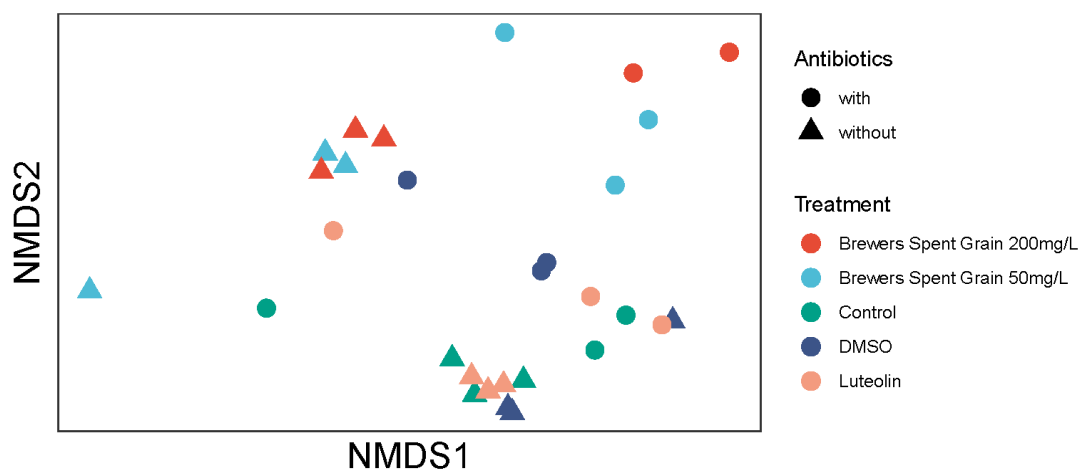


Figure 2.10. Non-metric multidimensional scaling (nMDS) based on Bray-Curtis similarities of bacteria community composition at genus level in *Karenia brevis* (strain New Pass) at day 4 of cultures exposed to brewer's spent grain extract and luteolin dissolved in DMSO, with and without the use of antibiotics. Stress = 0.14.

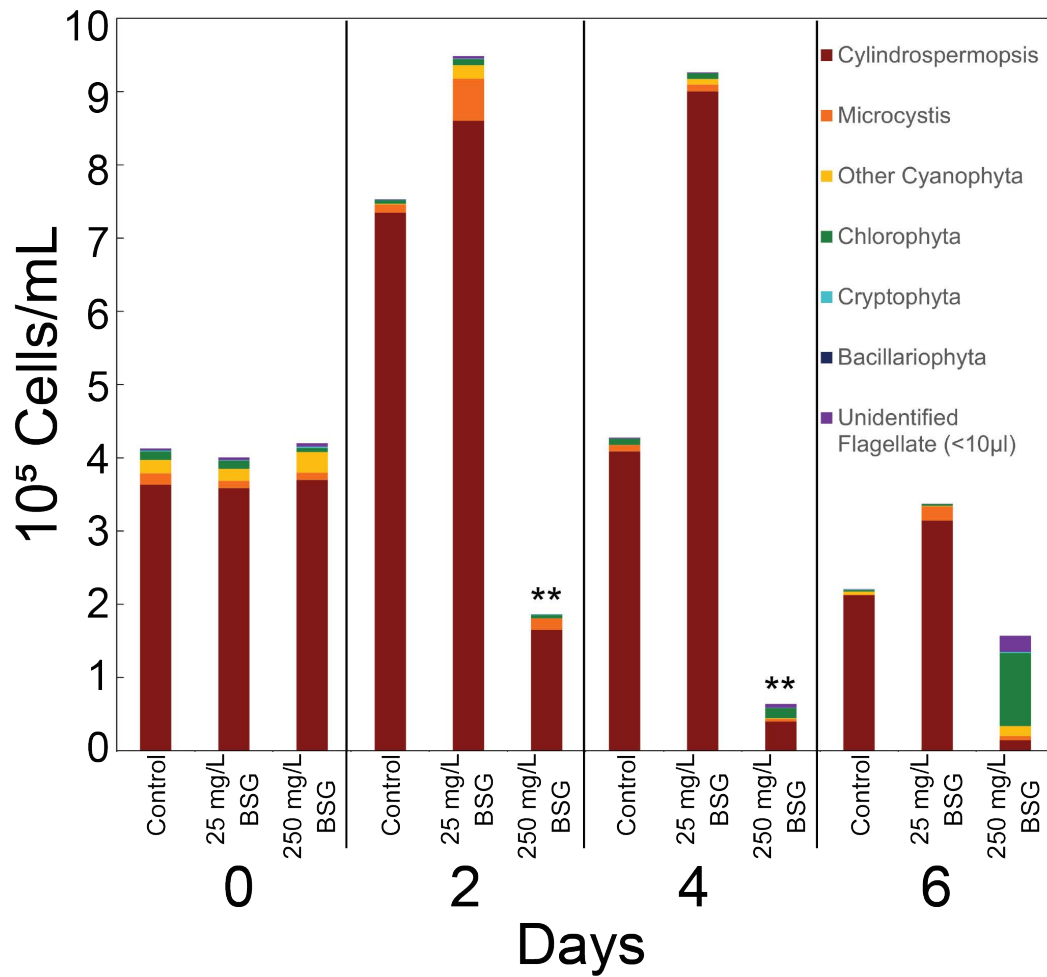


Figure 2.11. Total cells per mL and contributions of different phytoplankton groups versus day in flasks of control treatment, 25 mg/L brewer's spent grain extract, and 250 mg/L brewer's spent grain extract. Significant differences in total cells per mL between groups and control are indicated with * (p<0.05), ** (p<0.01), *** (p<0.001). n=3.

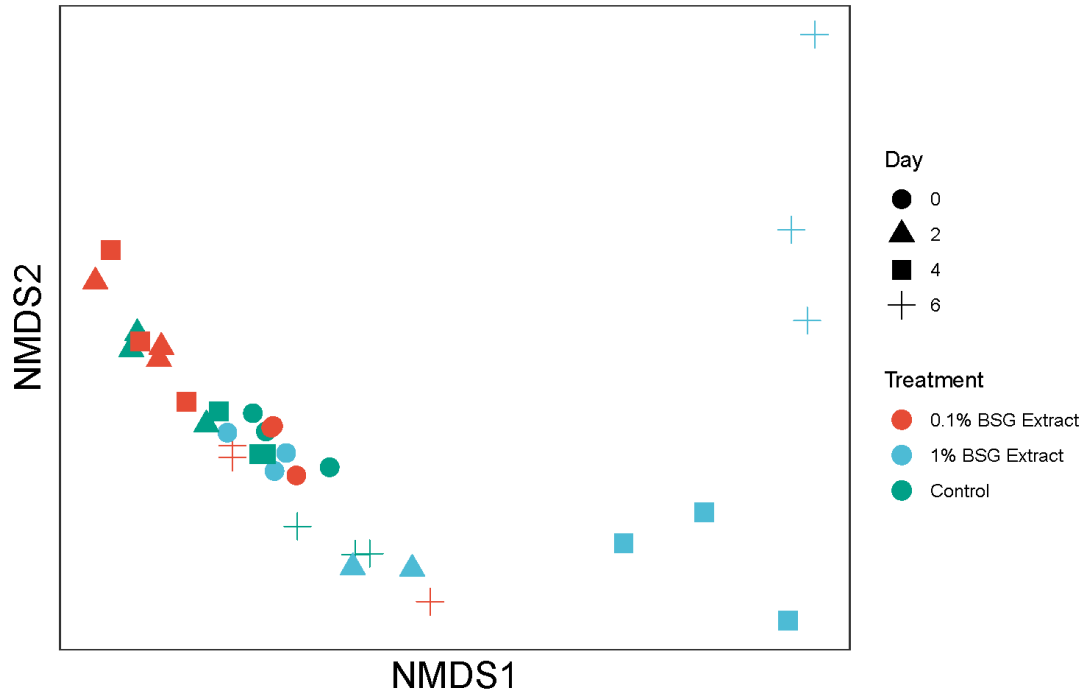


Figure 2.12. Non-metric multidimensional scaling (nMDS) based on Bray-Curtis similarities of phytoplankton community composition between the 3 groups of flasks: control, 0.1% spent grain, and 1% spent grain on days 0,2,4, and 6. Stress = 0.032.

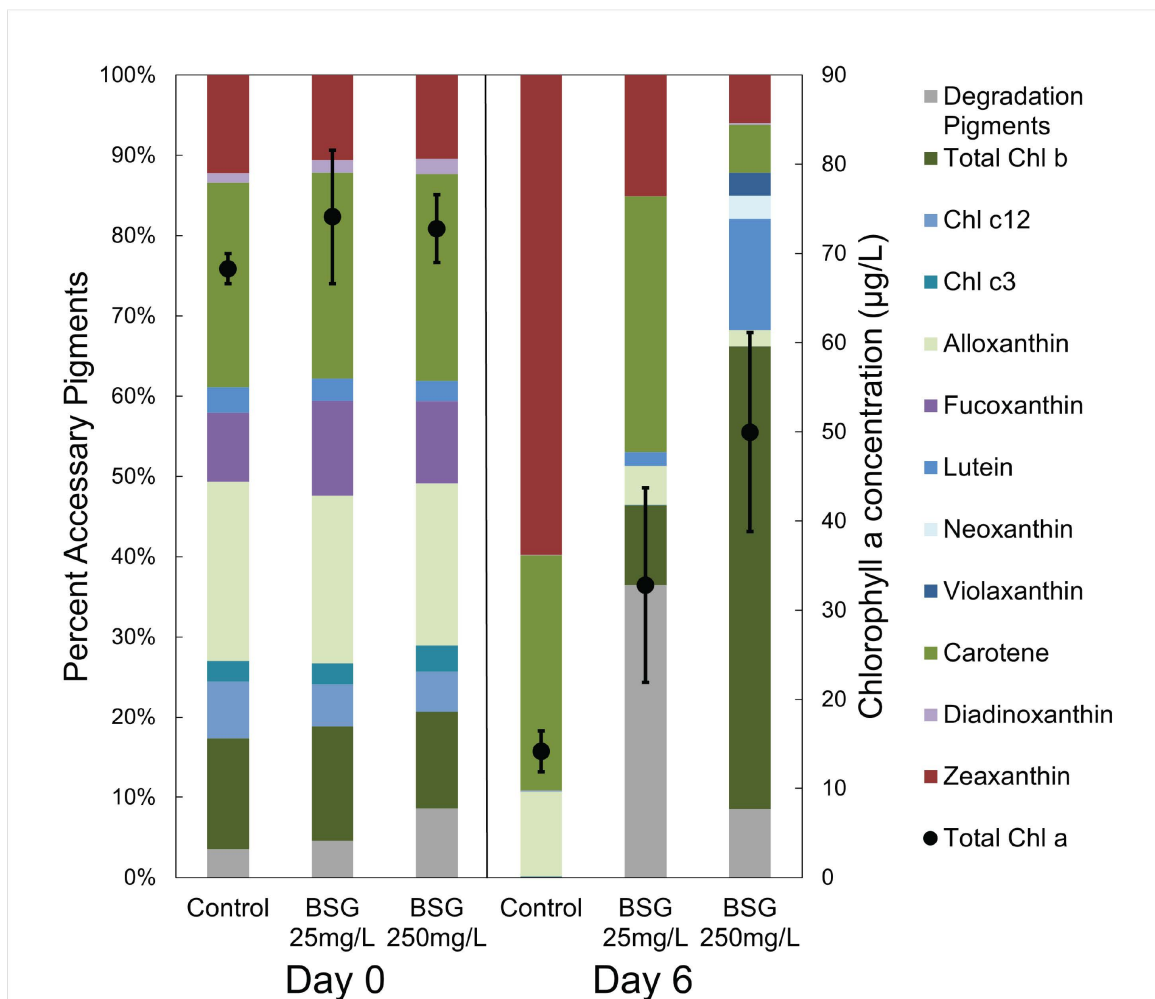


Figure 2.13. Relative contribution of accessory pigments to total pigments in a natural bloom from Lake Williston, Denton, MD on day 0 and 6 when exposed to 25mg/L or 250mg/L brewer's spent grain (BSG) extract. The black circles represent the chlorophyll *a* concentration.

Chapter 3: Phycotoxins along two New Jersey estuaries, a look at the use of passive samplers in complex water systems with high phenolic content

Abstract

As the leading state in many shellfisheries, it is imperative that New Jersey managers are knowledgeable regarding the occurrence of phycotoxins, their temporal patterns, and how to monitor and assess their threats to ecological and public health. However, to date, there have been no publications on phycotoxin presence nor concentrations in New Jersey estuarine systems. Regular phytoplankton identification and toxin testing in grab samples requires expert personnel and important events can be missed as phytoplankton move in the water column. Passive samplers can be a low-tech method in identifying emerging threats and long-term trends in phycotoxins, which can be used to help narrow the sampling window for grab samples and shellfish testing, but not without caveats. The New Jersey Pinelands release high levels of phenolic content into the water, which readily binds to the resin used in biotoxin research and could limit its efficacy. The goals of this study were to: 1. Explore the spatial/temporal trends in phycotoxins along two estuaries in New Jersey from 2020-2022, and 2. Understand if passive samplers can be employed to generate beneficial information in areas with high phenolic content. Over the course of the study, seven phycotoxins were detected in SPATT extracts: azaspiracid-1 (AZA1), azaspiracid-2 (AZA2), okadaic acid (OA), dinophysistoxin-1 (DTX1), pectenotoxin-2 (PTX2), yessotoxin (YTX), and goniodomin A (GDA). This is the first publication of YTX on the East Coast and the farthest north that GDA, AZA1, and AZA2 has been quantified on the East Coast of the United States. However, matrix effects caused by phenolic

compounds among others, caused high background noise and suppressed the signal of the toxins on the TQ-MS. It is clear from the study that phycotoxins interact with the resin differentially based on the properties of the toxin and environmental factors, and if a toxin is “below detected”, it does not necessarily mean it is “not present”. This study demonstrates both the clear benefits and limitations of using passive samplers to study phycotoxins in complex water systems, such as those with high phenolic content.

3.1 Introduction

New Jersey (NJ) is a leading state in many commercial shellfisheries including the surf clam, quahog, and sea scallop (*Fisheries of the United States*, 2019, 2021). Since shellfish can consume harmful algae from the water column and assimilate their toxins, it is important that the state knows for what phycotoxins to monitor and when to monitor for them in order to protect the health of humans and the viability of the industry (Neves et al., 2021; Sanderson et al., 2022). Changing climate is causing shifts in the geographical range of harmful algal species, and some species and phycotoxins may become a problem for areas in which they have not previously been seen (Anderson et al., 2008; Paerl et al., 2018; Wells et al., 2015). Thus, there is a need among scientists and managers to regularly assess problematic algal species as well as monitor for other phycotoxins that could emerge as threats to the area.

Solid Phase Adsorption Toxin Tracking (SPATT) is a passive sampling technology, developed as an early warning detector for HABs and to measure low concentration phycotoxins (Lane et al., 2010). SPATTs are small mesh bags filled with porous synthetic resin that can be suspended in the water column for a duration

of one day to approximately one month. SPATTs can be employed to alert managers to the presence of low-level toxic species before they are detected in phytoplankton samples and/or before phycotoxins accumulate in shellfish, although post extraction and analysis of samples can be time consuming. In addition, SPATTs can be used to sample water over a longer period of time compared to active sampling techniques (e.g. phytoplankton grab samples), which only provide a snapshot of the algal composition at a given time and place. SPATT bags also help eliminate matrix problems that occur during analysis of shellfish meat and uncertainties involved with biotransformation of phycotoxins in shellfish tissues (MacKenzie et al., 2004). Although SPATTs can neither fully replace shellfish testing nor phytoplankton monitoring, the technology can provide a holistic, long-term assessment of phycotoxins in the water column.

SPATT bags filled with Diaion™ HP20 resin has been shown effective in binding a multitude of lipophilic phycotoxins including okadaic acid, pectenotoxins, yessotoxins, azaspiracids, microcystins, and brevetoxins, among others (Onofrio et al., 2020). However, Diaion™ HP20 resin is also regularly used in laboratory experiments to bind a wide range of other compounds of varying degrees of polarity and hydrophobicity, including trace metals, phenols, and flavonoids (Armagan et al., 2002; Soto et al., 2012; Wu et al., 2017). For instance, Diaion™ HP20 filled columns are regularly used to separate phenols and flavonoids from sugars and other contaminants for chemical analyses (Kim et al., 2014; Soto et al., 2012). This suggests that SPATT bags are capable of binding more than just the desired phycotoxins in field environments, which may have implication towards accurate

toxin quantification in some complex water systems due to the limited binding capacity of the resins. For example, the NJ Pinelands release high levels of phenolic content into the water that cause streams to be tea-colored (Garnett et al., 2004). However, to date, no research has looked at how other compounds in the water impact the ability of the resin to bind phycotoxins, which further complicates an incomplete understanding of the uptake and accumulation mechanisms of different phycotoxins on the resin (Zabiegała et al., 2010).

The goals of this study were to 1. explore the spatial and temporal trends in phycotoxins throughout two estuaries in NJ using SPATTs, and 2. understand how phenolic content affects SPATT performance for phycotoxin tracking. To understand accumulation of different phycotoxins on the resin and understand how phenolic content impacts SPATT bag performance, two experiments were designed. The first was a weekly SPATT bag collection over a one-month period to understand the desorption/adsorption of certain phycotoxins and at what timepoint accumulation equilibrium is reached. The second was a “loaded toxin SPATT experiment” to help further understand whether high phenolic content impacts the adsorption/desorption of microcystins (MCs) and okadaic acid (OA) from SPATT bags after one day or one month. These phycotoxins were chosen for this experiment for two reasons: 1. The octanol water coefficients vary widely between the two groups from around -0.2 (MCs) to 3.4 (OA) (Table 3.1), and 2. The presence in samples varied widely as OA was present in almost every sample while MCs were expected to be seen due to their regular presence in NJ lakes, but were not identified in any samples.

3.2 Materials and Methods

3.2.1 Materials

All acetonitrile (ACN) and methanol (MeOH) used were LC-MS grade. The following standards were purchased from the National Research Council Canada: CRM-AZA1-b, CRM-AZA2-b, CRM-DA, CRM-DTX1-b, CRM-DTX2-b, CRM-OA-d, CRM-PTX2-b, CRM-YTX-c. Brevetoxin-2 (PbTx2) was purchased from Abcam. Microcystin-RR, -YR, and -LR mixed solution was purchased from Sigma Aldrich (33568-1ML). Goniiodomin A was purified from *Alexandrium monilatum* by Drs. Thomas and Constance Harris (Harris et al., 2020).

3.2.2 SPATT construction

SPATTs were constructed with Diaion™ HP20 and activated following the methods described by Lane et al. (2010) with some modifications. SPATT bags were constructed by putting 3 g resin in between two pieces of 100 µm Nitex bolting cloth (Wildlife Supply Company, Product No. 24-C34) and sealed with a manual heat sealer in 2020-2021 and an embroidery hoop in 2022. For activation, SPATT bags were soaked in 100% MeOH for 24 hours, then rinsed with ultrapure H₂O and transferred to individual sealed plastic sandwich bags with enough ultrapure H₂O to cover the SPATT bag. The plastic bags were stored at 4°C and used within one week of construction.

3.2.3 Study site

Field sampling was conducted monthly between September-November in year one (2020) and between April-November for the next two years (2021-2022). Sampling occurred at six sites along Toms River-Barnegat Bay, and five sites along Mullica River-Great Bay (Fig 3.1). The stations were along a clear salinity gradient: two in the upper estuary, one or two in the mid-estuary, and two in the lower estuary,

with one inlet site. Sampling sites along Mullica River-Great Bay were co-located with four permanent water-quality monitoring stations through the Jacques Cousteau NERR System Wide Monitoring Program (SWMP) (Kennish et al., 2016). Dissolved organic carbon (DOC) levels can range from 200-1000 $\mu\text{M C}$ in Mullica River-Great Bay, with the general trends of the highest DOC occurring in June-July and the lowest occurring January-April (Flynn, 2008). Less is known about the DOC levels in Toms River-Barnegat Bay.

A 3g SPATT bag was deployed near the surface at each sampling site and submerged via a weighted rope. The bags were replaced with a new SPATT bag during each monthly sampling event and salinity was measured with a YSI probe during replacement at each side. To ensure bags remained submerged, they were deployed at varying depths based on the tidal range. For each month in 2022, one site was randomly assigned a replicate SPATT to confirm repeatability (Table 3.2). Between September and October 2022, the number of SPATT bags deployed increased to four at sites C-Chestnut Neck and H-Seaside. Each week, one of the four SPATT bags was collected from the site and stored at -20°C until extraction.

3.2.4 Pre- and Post- loaded toxin SPATT experiment

One gram SPATT bags were prepared and activated as described above, except that the mesh bags were sewn together. A portion of the bags were preloaded with phycotoxins by submersion in 50 mL of 500 ng/L microcystin RR/LR/YR (MC-RR/LR/YR) standard and 500 ng/L okadaic acid (OA) standard in ultrapure water for one hour at 20°C in the dark, then removed and stored at 4°C (“Pre-Loaded Field” SPATTs). The one hour SPATT adsorption time was based on the findings of Kudela (2011) and Li et al. (2011) (Kudela, 2011; Li et al., 2011). On Aug. 22, 2022, six of

the “Pre-Loaded Field” SPATT bags were placed at the surface along with 12 activated fresh SPATT bags at two sites: C-Chestnut Neck and E-Sweetwater. After one day, three of the “Pre-Loaded Field” SPATT bags along with six of the non-loaded SPATT bags were collected from each site. The three “Pre-Loaded Field” SPATT bags and three control SPATT bags (“1 Day Field Controls”) from each site, along with three “Pre-Loaded Lab Controls” SPATT bags that remained in 4°C, were immediately stored at -20°C. Three non-loaded SPATT bags from each site along with three fresh SPATT bags still at 4°C were transferred to separate amber glass jars with 50 mL of 500 ng/L microcystin RR/LR/YR standard and 500 ng/L okadaic acid standard in ultrapure H₂O (“Post-Loaded Field” SPATTs). The collection and creation of “Post-Loaded Field” SPATT bags was repeated for one-month, on Sept. 20, 2022. The extracts were analyzed for phycotoxins and total phenolic content as described below. Percent Recovery data is presented using equation: *Percent Recovery (%) = 100 x (ng per g resin measured in sample / (ng per g resin field control + ng per g resin lab control))*. One sample from each set of extracts was spiked with known concentrations of OA and MC-RR/YR/LR and compared with the non-spiked samples to analyze analyte suppression or enhancement due to matrix effects.

3.2.5 SPATT extraction for toxins and phenolics

Toxins from the field study were extracted from SPATT resin following the methods of Onofrio (2020) (Onofrio, 2020). In brief, resin was transferred to 50-mL centrifuge tubes with a removable PVDF 0.45-µm spin filter cup. Four sequential extractions were performed using 1) 10 mL 35% MeOH wash that was discarded, 2) 10 mL 100% MeOH, 3) 10 mL 100% MeOH, and 4) 10 mL 100% ACN. The two 10-

mL 100% MeOH extractions were pooled together, and all extracts were stored at -20°C in amber glass vials until toxin analysis. For the “Loaded Toxin” experiment, toxins and phenolic content were extracted from the one-gram SPATT bags using 2 x 3 mL MeOH in disposable chromatography columns (Econo-Pac Chromatography columns, 1.5x12cm, polypropylene) and filtered through 0.22-µm syringe filter (13-mm, Millex PVDF, Durapore) (See Appendix A for phenolic content extraction from resin).

3.2.6 Toxin analysis

Quantification of phycotoxins was carried out using ultra-performance liquid chromatography-tandem mass spectrometry with a trapping dimension and at-column dilution (Waters UPLC-MS/MS with trap/ACD) (Onofrio et al., 2020). The phycotoxins analyzed included pectenotoxin-2 (PTX2), okadaic acid (OA), dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2), yessotoxin (YTX), microcystin-RR (MC-RR), microcystin-YR (MC-YR), microcystin-LR (MC-LR), domoic acid (DA), brevetoxin-2 (PbTx-2), goniiodomin A (GDA), azaspiracid-1 (AZA1) and azaspiracid-2 (AZA2). The UPLC-MS/MS methods, conditions, and parent > daughter transitions are described in Onofrio et al (2020; 2021). All phycotoxins were extracted through 0.22-µm syringe filter (13-mm, Millex PVDF, Durapore) within a week prior to analysis on a Waters Xevo TQMS attached to Waters Acquity UPLC system. An injection volume of 100 µL was used. Standard curves were prepared in the same solvent as the extracts using a series of nine dilutions from 0.1 to 50 µg/L, except for AZA1 and AZA2 which used a series of nine dilutions from 0.004 to 2 µg/L. Anything below the calibration curves and/or below a signal-to-noise of 10 was considered “below detection”. Alkaline hydrolysis

was used to convert DSP ester toxin derivatives into the parent, “free” toxins OA, dihydroDTX1, DTX1 and DTX1 in the 100% MeOH fractions following the methods of Villar-Gonzalez et al. (2008) (Villar-González et al., 2008). These results are presented as “Total Okadaic acid” and “Total Dinophysistoxin-1”. Results of the amount phycotoxins extracted from the pooled 100% MeOH fractions are presented in figures 2-9, calculated in ng phycotoxin/g resin.

In 2020 and 2021, the 100% ACN fractions were analyzed using a simplified MRM method that focused solely on quantification and confirmation of PbTX-2. However, in 2022 the same MRM targets used for the 100% MeOH fraction were used to analyze the 100%ACN fractions.

Samples that were within the calibration curve for YTX, were confirmed in a separate analysis using an additional MRM transition of 570.2 m/z > 396.4 m/z, as well as analyzed for homo-YTX (577.4 > 474.4; 577.4 > 403.4), 45OH YTX (578.4 > 396.4; 578.4 > 467.4), and COOH YTX (586.4 > 396.4; 586.4 > 467.4) (Gerssen et al., 2013). In addition, these same samples were spiked with YTX standard to confirm the identity of the detected analyte.

A random subsample of fifteen extracts was spiked with a multi-toxin mixture over different sites and years and compared with the non-spiked samples to analyze analyte suppression or enhancement due to matrix effects. Endogenous toxins were quantified and accounted for in the recovery calculation used. Percent suppression or enhancement was calculated as *Matrix Effect (%) = 100 x (μg measured in spiked sample - μg endogenous toxin)/ μg toxin added*).

3.2.7 Total phenolic content analysis

Total Phenolic Content (TPC) was estimated using the Folin-Ciocalteu method of Singleton et al (1974), with some modifications (Singleton et al., 1974). In brief, 500 μL of the 100% MeOH SPATT fraction was dried down via a rotary SpeedVac concentrator without light and resuspended in 100 μL MeOH. 1.8 mL ultrapure H_2O and 100 μL of 0.5 M NaOH was added and mixed by vortex. An aliquot of 120 μL of Folin-Ciocalteu reagent was added to each sample, vortexed, and put in the dark for five minutes. Then, 300 μL of 200g/L Na_2CO_3 was added, vortexed, and samples were put into the dark for one hour. A 250 μL aliquot was transferred to a flat bottom 96 well plate and adsorption at 765 nm was measured on a SpectraMax i3x multi-mode microplate reader. The samples were compared to a gallic acid standard curve from 1 to 50 $\mu\text{g/mL}$ and presented in gallic acid equivalents (GAEs).

3.2.8 Statistics

Results are expressed as mean values in ng toxin/ g resin. Significant differences among mean phycotoxins between the two estuaries were determined by unpaired two sample t-test. Correlation between phycotoxin means and salinity were calculated using Spearman's rank correlation rho. For the pre- and post- loaded toxin SPATT experiment, significant differences in Total Phenolic Content between sites, time points and before/after sitting in water for one hr was determined by unpaired two sample t-test. Significant differences in phycotoxin measured after storage was determined by unpaired two sample t-test. Data were assessed for normality (Shapiro-Wilk test) and for homogeneity of variance (Levene test) at the 0.05 significance

level. Differences were considered significant at $p < 0.05$. Statistical analyses and generation of figures were carried out with R studio (Version 4.2.2 R script).

3.3 Results

3.3.1 Spatial and temporal distribution of phycotoxins

3.3.1.1 DSP toxins and pectenotoxin-2

Okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxin-2 (PTX2) were detected in both estuaries during every month that sampling was conducted (April-November 2020-2022; Figs. 3.2-3.4). All three phycotoxins had clear spatial variation with a positive association with salinity (Spearman's rank correlation rho; DTX1: $p < 0.001$, $\rho = 0.855$; OA: $p < 0.001$, $\rho = 0.827$; PTX2: $p < 0.001$, $\rho = 0.758$). These phycotoxins were also detectable at freshwater sites during some months. Blanks of 100% MeOH dispersed throughout the instrumental runs confirmed that positive identification of these three phycotoxins was not due to carryover between samples. DTX2 and dihydro-DTX1 were below detection in all field samples. OA was found in the greatest quantities compared to DTX1 and PTX2 in the 100% MeOH fraction, with the highest recorded amount at site A on the SPATT put in the water between Sept. 13, 2021 and Oct. 7, 2021 (1281 ng/g resin or 58 ng/g resin/day). However, OA, DTX1, and PTX2 were all detected in the 100% ACN SPATT fraction during the MRM run methods in 2022 (Table 3.3). OA and DTX1 had around 15% of the toxin detected while PTX2 had 200-300% toxin in its 100% ACN extract compared to the 100% MeOH extract (Table 3.3).

Since 100% ACN extracts were not run in 2021 for DSP toxins and only a select number were run in 2022, the figures, observations, and analyses below were performed on just the 100% MeOH fractions. When looking at temporal variations,

the three phycotoxins showed slight differences in 2021 compared to 2022. Total OA was high in saltwater sites in both estuaries throughout the sampling months in 2021, with the highest concentrations in spring and summer (April-August) in 2022 (Fig. 3.2). Total DTX1 loads were highest May to July in both 2021 and 2022 (Fig. 3.3). PTX2 loads were relatively low throughout the sampling dates in 2020 and 2021, and highest between May-July in 2022 in both estuaries (Fig. 3.4). Overall, the trends were consistent between the 2 estuaries, but the overall concentrations were statistically higher in Mullica River-Great Bay estuary compared to Toms River-Barnegat Bay (Unpaired two sample t-test; DTX1: $p < 0.05$; OA: $p < 0.05$; PTX2: $p < 0.05$).

3.3.1.2 Azaspiracids

Azaspiracid 1 and 2 (AZA1 & AZA2) were detected in mid and high salinity sites between 2020-2022. However, they occurred at concentrations around three orders of magnitude lower than the other phycotoxins detected in this study (Figs. 3.5&3.6). AZA1 and AZA2 differed spatially and temporally when comparing 2020, 2021, and 2022 (Figs. 3.5&3.6). In 2020, both AZA1 and AZA2 were detected in both estuaries during the sampling period of September-November, in the high to mid salinity sites. In 2021, AZA1 was detected from April-May in Great Bay and July-September in Barnegat Bay. That same year, AZA2 was detected from April-June and September-November in Great Bay and August-November in Barnegat Bay. In 2022, AZA1 was only detected in one sample, the September-October sample at the highest salinity site in Great Bay. AZA2 was higher in 2022 compared to 2021, with detection in all months except June in Great Bay and detected from July-Nov in Barnegat Bay (Figs. 3.5&3.6). AZA2 was highest in Barnegat Bay at mid-salinity

sites. The highest recorded amount of AZA1 occurred at site F between Sept. 13 to Oct. 7, 2021 (0.70 ng/g resin or 0.032 ng/g resin/day). The highest recorded amount of AZA2 occurred at site I between Aug. 22 to Sept. 19, 2022 (1.34 ng/g resin or 0.048 ng/g resin/day).

3.3.1.3 Goniiodomin A

Goniiodomin A (GDA) was detected at mid to high salinities in 2021 and 2022 (Fig. 3.7). Peak concentrations occurred at different times between 2021, July-August, and 2022, August-October. GDA had higher concentrations within Toms River-Barnegat Bay compared to Mullica River-Great Bay (Unpaired two sample t-test; $p < 0.01$), with the highest recorded amount occurring at site H in a SPATT put in the water between Aug. 22 to Sept. 19, 2022 (406.03 ng/g resin or 14.5 ng/g resin/day). However, the highest per day recorded amount of GDA occurred during the 1-week SPATT collections with 55.17 ng/g resin/day collected on a SPATT deployed between Sept. 19-28, 2022 (Table 3.4).

3.3.1.4 Yessotoxin

Yessotoxin (YTX) was below the calibration curve in all SPATT samples from 2020 and 2021. However, YTX was detected at high salinity sites in both estuaries from June-November, 2022 (Fig. 3.8). The highest amount of YTX measured was at site F in a SPATT put in the water between Aug. 22 to Sept. 19, 2022 (44.29 ng/g resin or 1.58 ng/g resin/day). Homo-YTX (Fig. 3.9) was detected in all samples that had detectable quantities of YTX and was not detected in the YTX standard. 45OH YTX and COOH YTX levels were below the limit of detection in samples with detectable quantities of YTX.

3.3.1.5 Domoic acid, Brevetoxin-2 and Microcystins

Domoic acid, brevetoxin-2 microcystin RR, LR, and YR were not detected in any of the SPATT bags from the field.

3.3.2 Replicates and repeated collections over one month

To understand at what timepoint accumulation equilibrium is reached for phycotoxins in New Jersey waters, SPATT bags were collected from the water each week over a one-month period at two mid-salinity sites. SPATT bags deployed for two weeks showed decreased toxin levels (ng/g resin) when compared to bags deployed for one week, for all observed phycotoxins except for PTX2 at site H (Table 3.4).

3.3.3 Pre- and post- loaded toxin SPATT experiment

To understand whether high phenolic content impacts the adsorption/desorption of microcystins (MCs) and okadaic acid (OA) from SPATT bags after one day or one month, an experiment was designed where the phycotoxins were loaded onto resin prior to field deployment or post-field deployment to understand if binding capacity was reached after those time periods. At both sites C and E, the total phenolic content (TPC in GAE eq/g resin) that was released from the SPATT resin with 100% MeOH elution was higher in SPATT deployed for one month compared to one day (Unpaired two sample t-test; $p < 0.01$). TPC was significantly higher at site C compared to site E (Unpaired two sample t-test; one-day: $p < 0.01$, one-month: $p < 0.01$). There was no significant difference in TPC between the resin that was pre-loaded with phycotoxins and those that were rinsed for one hour in ultrapure H₂O + phycotoxins after field deployment (Unpaired two sample t-test; $p = 0.9661$).

MC-RR/LR/YR were below the calibration curve in all SPATT bags at both locations after a one-month deployment where toxin was pre-loaded or post-loaded onto bags (Table 3.5). MC-RR was only detected in SPATTs at site E where phycotoxins were loaded post-field deployment with a percent recovery of ~31% (Table 3.5). One day SPATT percent recovery of MC-LR were higher at site E compared to site C. MC-YR was not detected at site C but was at site E after one day with a percent recovery of ~41% when the toxin adsorbed to resin before field deployment, and 98% recovery when the toxin was exposed to resin after field deployment for one day (Table 3.5). Between 86-101% recovery of OA was achieved at site C after one day and one month deployment.

LC-MS/MS analysis of the pre-loaded control bags, three of which sat at 4°C and were transferred to -20°C when the one-day SPATT samples were collected, and three of which sat at 4°C and were transferred to -20°C when the one-month SPATT samples were collected, showed significantly different OA concentrations based on how long the SPATT was stored at 4°C (Unpaired two sample t-test; $p < 0.01$). The pre-loaded control SPATTs stored at 4°C, went from 12.3 ± 0.85 ng OA/g resin to 5.98 ± 1.01 ng OA /g resin after one-month of storage. Alternatively, MC-RR/LR/YR concentrations remained the same after one-month storage at 4°C (Unpaired two sample t-test; MCRR: $p = 0.34$, MCYR: $p = 0.06$, MCLR: $p = 0.36$).

When comparing extracts spiked with known concentrations of the phycotoxins, MC-RR was suppressed in all samples, even SPATT put out for 1 day (Table 3.6). MC-LR and MC-YR were enhanced in one-day SPATT resin at site E.

Matrix effects suppressed OA by 40-50% in SPATT deployed for one month (Table 3.6).

3.4 Discussion

This study is the first to use solid phase adsorption toxin tracking (SPATT) to analyze the spatial and temporal variation of phycotoxins in New Jersey (NJ) and the first to study the use of SPATTs in areas with high phenolic content. Of the 13 phycotoxins screened via UPLC-MS/MS in the SPATT extracts, 7 were detected: OA, DTX1, PTX2, GDA, AZA1, AZA2, and YTX. Multiple phycotoxins were detected in SPATTs deployed in Great Bay and Barnegat Bay that had not previously been seen in NJ, including: AZA1, AZA2, GDA, and YTX. In addition, many of the SPATT extracts tested positive for multiple phycotoxins during the same period (Fig. 3.2-3.9).

The phycotoxin recovery experiment and the repeated weekly collections over a one-month period demonstrated the clear limitations of using SPATT sampling. Caution should be used when analyzing SPATT data from areas with high total phenolic content (TPC) and when analyzing phycotoxins with lower octanol-water coefficients. SPATT bags collected weekly over a one month period demonstrated that many lipophilic toxins transition from kinetic uptake to an equilibrium accumulation regime within a few weeks, a considerably shorter timeframe than the month-long deployment periods used in this study (Zabiegała et al., 2010). The variable adsorption/desorption patterns of phycotoxins creates a situation in which the use of SPATTs filled with Diaion™ HP20 resin is not a good technique for quantifying phycotoxins in the water column over long deployment periods nor estimating toxicity of shellfish in an area, especially since levels can vary based on

the environmental properties of the waterbody and the octanol-water coefficients of the phycotoxins of interest (Lin et al., 2014; Mergia et al., 2022). In addition, it is recommended that data be reported as “ng/g resin” versus “ng/g resin/day” depending on the length of deployment, as reporting in “ng/g resin/day” assumes linear kinetic uptake of the phycotoxins by the passive sampler throughout the entire deployment. It is likely that the resin data shows what toxins were present in the water the few days before collection versus the entire deployment period for many of the lipophilic toxins (Table 3.4).

Current SPATT extraction protocol follows similar methods to this study, with 2 x 10 ml of 100% MeOH used to extract the phycotoxins from Diaion™ HP20 resin. However, this study clearly demonstrates this method yields incomplete extraction of multiple phycotoxins, particularly PTX2, GDA, and YTX (Table 3.3). Some of the differences between 100% ACN and 100% MeOH could be due to reduced matrix effects since the 100% ACN extracts have reduced background noise compared to the 100% MeOH extracts; the lower background signal would result in a larger peak area in the 100% ACN extracts. Still, the quantified difference between the two fractions were not consistent based on site, salinity, or sampling month and had a large standard deviation. Thus, the total phycotoxin concentrations are difficult to calculate unless every 100% ACN extract is also run which significantly increases the cost of analyzing SPATT extracts via LC-MS/MS for routine analysis.

The phycotoxin recovery experiment demonstrated that SPATT bags cannot be used to show which phycotoxins are not present as even when known amounts of MC-RR/YR/LR were added to samples, the results were “below detection” (Table

3.5). The study also demonstrated how substantial an impact matrix-effects can have on measuring phycotoxins in SPATT samples. Although levels of MC-RR/YR/LR were always below the limit of detection in the SPATTs collected in NJ, microcystins could have still been present in these estuaries and could still be a future concern, as it is of concern in NJ lakes. This research suggests that standard practice when analyzing SPATTs using LC-MS/MS should include internal standards; although there were clear differences in suppression/enhancement rates of the various phycotoxins and all these differences would be difficult to control for using one or even a few internal standards (Table 3.7). In addition, it is recommended that SPATT bags not be deployed for an entire month as they were in this study due to the matrix effects previously mentioned and the potential for oversaturation of resin from other compounds in the water (Table 3.4 & 3.5). Although this study did not find oversaturation of Diaion™ HP20 resin to fully inhibit phycotoxin binding, resin deployed for one month did seem to have reduced binding capacity compared to resin deployed for one day; this could be a more significant issue in other systems.

Although this study does not correlate SPATT extracts to toxin levels in shellfish, the results suggest that SPATTs cannot be used as an early warning system from many phycotoxins, including microcystins, for the shellfish industry, and may be more useful in larger systems compared to smaller water bodies with high inputs of phenolic content from the terrestrial environment and aquatic plants. However, this study still demonstrates the importance of SPATT technology in detecting potential emerging phycotoxins to the area and identifying when and where monitoring should take place to protect human health and aquaculture operations.

3.4.1 Spatial and temporal distribution of phycotoxins

3.4.1.1. DSP toxins and pectenotoxin-2

The SPATT results show that OA, DTX1, and PTX2 are of greater concern in mid and high salinity sites than in freshwater sites. The infrequent detections of these phycotoxins in the NJ freshwater sites are likely due to either high boating activity in the region or strong tidal cycles, and not likely due to DSP producing algae at these sites. OA and DTX1 are a concern throughout April-November in both estuaries and could be a concern in winter months as well, although sampling stopped during these months (Fig. 3.2-3.3; sites A, B, C, F, G, H, & I). Conversely, PTX2 showed pronounced annual trends, with increased levels in 2022 compared to 2021. SPATT bags needed to capture seasonal trends were lost in 2021 due to issues with heat sealing the bags. However, in 2022 all three phycotoxins peaked in SPATT extracts deployed between May-July in both estuaries. This is consistent with phytoplankton data collected by NJ Department of Environmental Protection, where major producers of these 3 phycotoxins, *Dinophysis* spp., were primarily seen in June and July in Great Bay and Barnegat Bay (<https://www.nj.gov/dep/bmw/phytoplankton.htm>).

Since the proportions of the three phycotoxins did not remain consistent, it is likely that more than one species and/or strain are producing different toxin profiles in these estuaries (Fux et al., 2009; Onofrio et al., 2021). There are historical observations of *Prorocentrum lima* and *Dinophysis* sp. in NJ, and more recent studies where *P.lima*, *D. acuminata*, *D. acuta*, *D.norvegica* and “small *Dinophysis* sp.” were observed in Barnegat Bay and Great Bay (Olsen and Mahoney, 2001; Ren et al., 2017; Wolny et al., 2020). These are all potential producers of these phycotoxins and are likely the causative species of OA, DTX1, and PTX2 in NJ as these species have

been associated with these phycotoxins in water and shellfish samples in the Northeast and Mid-Atlantic region (Ayache et al., 2023; Onofrio et al., 2021; Wolny et al., 2020). In addition, *P. lima* was associated with a blue crab kill west of Barnegat Bay inlet in 1987, although no toxin analyses were performed at the time (Gastrich, 2000). To our knowledge there is no data available on the toxin profiles of these algal species from NJ. *Dinophysis* spp. are considered a bloom in NJ at 5 cells/mL and since this is not enough to “discolor water”, it can make monitoring for DSP toxins difficult and expensive.

OA levels in the 100% MeOH SPATT extracts were primarily the highest concentration of phycotoxins found coming off the resin. However, caution should be taken when comparing amounts of the various phycotoxins, as lipophilic phycotoxins can interact differentially with the resin. Upon analysis of the 100% ACN extracts in 2022, it was clear that not all the phycotoxins were eluted with the two, 100% MeOH rinses, and in the case of PTX2, only 30-40% of the bound toxin was eluted off with the 100% MeOH rinses (Table 3.3). In addition, the total OA and total DTX1 concentrations presented in this paper are from the pooled 100% MeOH extracts after base hydrolyzation to convert esterified derivatives back into the parent toxin forms for quantification. This provides a more realistic reflection of environmental toxin loads for managers and health officials, since the esterified form can be present in high amounts and contributes to shellfish toxicity (Fux et al., 2009; Onofrio et al., 2021; Villar-González et al., 2008). During alkaline hydrolysis, many other compounds are either destroyed or converted, allowing for lower background noise on the UPLC-MS/MS and reduced ion suppression, increasing the signal of OA and

DTX1. Thus, it is difficult to compare these phycotoxins to others, like PTX2, that was run under conditions with higher matrix effects. Nonetheless, the consistent detection of these DSP toxins and PTX2 should warrant frequent shellfish monitoring for these phycotoxins in the state of NJ, especially from April-August.

3.4.1.2. Azaspiracids

This study marks the first report of Azaspiracids (AZAs; AZA1 and AZA2) in NJ (Fig. 3.5 & 3.6). In 2021, Onofrio et al. published the first report of these phycotoxins on the east coast of the U.S. with detection on SPATT extracts from the Chesapeake Bay and Virginia (VA) coastal bays (Onofrio et al., 2021). Similar to their findings, only low levels of AZA1 and AZA2 were detected in NJ throughout the study period. The seasonal trend of AZA2 was similar between VA and NJ with the highest amounts detected in the fall; it should be noted that while their study occurred year-round, this study ended every November (Fig. 3.6). While AZA1 was only detected in trace, below limit of quantification in VA, NJ had quantifiable amounts with the highest levels in saltwater and mid-salinity sites of Barnegat Bay in September 2021 (Fig. 3.5). However, these differences in AZA1 levels between NJ and VA could be due large annual variability rather than differences between the two states as there was only one sample with a quantifiable concentration of AZA1 in 2022 in NJ. In addition, AZA1 and AZA2 levels were not consistently proportional to one another; AZA2 was highest in Barnegat Bay in 2022 when AZA1 was not detected (Fig. 3.5 & 3.6). This suggests that there may be multiple algal producers of AZAs in NJ or multiple strains of the same species that produce various toxin profiles, as toxin profiles do not usually change within a strain.

The AZA spatial trends differed between the two estuaries studied. For instance, in 2022, the production of AZA2 appeared to occur within Barnegat Bay since the highest concentrations were at mid-salinity sites. However, in Great Bay, the production of AZA2 appeared to be coastal, as the toxin was primarily detected at the inlet site. Alternatively, these differences could be due to lower water residence time in Great Bay compared to Barnegat Bay and so the AZA producer could be transported and dying closer to the inlet in the Great Bay estuary (Defne et al., 2020; Defne and Ganju, 2015). More research is needed to fully explore the temporal and spatial differences between the two estuaries as there were large annual trends between 2020-2022, and it is recommended that studies be extended throughout the year as the highest concentrations of AZA could be occurring in the winter, as it does in VA, which this study would have missed (Onofrio et al., 2021).

NJ DEP currently has a biotoxin contingency plan with an azaspiracid shellfish poisoning (AZP) closure concentration threshold of 0.16 mg/kg AZA1 equivalent in the edible portion of raw shellfish. Known AZA producers are *Azadinium spinosum* and *Azadinium poporum*, although research is ongoing for additional species (Kim et al., 2017; Tillmann et al., 2009). It is not currently known what species produce AZAs in this region, which makes monitoring difficult without constant testing. Therefore, more research is needed looking into the producer of AZAs on the East Coast and increasing states' monitoring windows beyond the summer as these phycotoxins seem to be more of an issue in the fall and winter.

3.4.1.3. Goniiodomin A

This study is the farthest north that GDA has been documented in the United States. The nearest records for GDA are in the Southern Chesapeake Bay, where

Alexandrium monilatum blooms produce large concentrations of GDA between August-October. Similarly, the peak GDA levels observed occurred in July in 2021 and between August-October in 2022 in Barnegat Bay (Fig. 3.7). However, *A. monilatum* is primarily a tropical species and more research is needed to identify the species producing GDA in NJ as GDA is also known to be produced by other species including *A. hiranoi*, *A. pseudogonyaulax*, and *A. taylorii* (Harris et al., 2020; Tillmann et al., 2020).

GDA concentrations were clearly different between the two estuaries with higher concentrations occurring in the Toms River area of Barnegat Bay, the relatively urban portion of this estuary (Unpaired two sample t-test; $p < 0.01$). This suggests that Toms River-Barnegat Bay is more conducive for growth of GDA-producing organisms. This could be due to the longer residence times at Toms River-Barnegat Bay compared to Mullica River-Great Bay, which can be optimal abiotic conditions for many *Alexandrium* species (Fauchot et al., 2008). Additionally, it is suspected that GDA is produced by an estuarine species as the GDA levels were highest at mid-salinity sites in Toms River-Barnegat Bay.

GDA levels were relatively low compared to concentrations found on SPATT bags deployed in Chesapeake Bay (max 102,050 ng/g resin/day in VA vs. max 55.17 ng/g resin/day in NJ) (Onofrio et al., 2021). However, these values cannot provide a direct comparison as SPATT bags were deployed for a month (compared to one week by Onofrio et al.) and clearly GDA on SPATT is not a time-integrated measurement over the entire month (Table 3.4). Further work should investigate the identity of

GDA producers in NJ and the reason for the shift in the timing of annual peak GDA concentrations (Fig. 3.7).

3.4.1.4. Yessotoxin

This study marks the first report of YTX along the east coast of the United States, although it has previously been identified in the dinoflagellate *Protoceratium reticulatum* in Nova Scotia, Canada (Shartau et al., 2023; Stobo et al., 2003). YTXs are not regulated in shellfish in the United States as they are not considered a threat to human health. However, the toxin has caused mass mortalities of shellfish throughout the world, including Washington state in 2018 and 2019 and is lethal in mice at intraperitoneal injections of around 100 µg/Kg (King et al., 2021; Paz, 2008). Comparing to one of the few studies that used SPATT bags to quantify YTX, the concentration of YTX found in this study are low compared to those found in Ireland (max 347 ng/g resin in Ireland vs. max 44.29 ng/g resin in NJ) (Fux et al., 2009). However, due to the same reasons listed in previous sections, it is difficult to compare levels.

Potential YTX-producing organism *Gonyaulax spinifera* has historically been documented in this area (Olsen and Mahoney, 2001), and other species such as *Lingulodinium* sp. and *Protoceratium reticulatum* are potential YTX producers as well. A study by Howard et al (2008) analyzed a *G. spinifera* strain (CCMP409) from Maine and found that it did not produce YTX (Howard et al., 2008). However, the *G. spinifera* strain(s) in NJ could produce YTX or the source of YTX could be due to a different species. Since YTX was found primarily in SPATT extracts from the two inlets, it is more likely that the YTX is from a coastal species rather than a species that cysts and blooms in the estuaries. In addition, since YTX was only above levels

of detection in 2022 samples, currents could have transported unidentified YTX producing species into the area during that one year (Fig. 3.8&3.9). Further research is needed to identify the producer of YTX on the east coast and if NJ continues to have measurable levels in the future.

3.4.1.5. Domoic acid, microcystins, and brevetoxin-2

Although *Pseudonitzschia* spp. are regularly seen in NJ waters, including in 2020-2022 grab samples (Gastrich, 2000), all SPATT resin were below the limit of detection for Domoic acid (DA) in this study. This could be due to DA not adsorbing onto SPATTs, DA coming off primarily in the 35% MeOH fraction not run in this study, or due to the species of *Pseudonitzschia* not producing DA in NJ. Additionally, PbTx2 was never above levels of detection in the study, and this could be due to either no species producing PbTx-2 during the course of the experiment, species producing different PbTx analogs, or only low levels of these phycotoxins being present from 2020-2022. In addition, MC-RR/LR/YR were below detection in all monthly SPATT samples during the study. However, the spike recovery experiment demonstrates this could be due to matrix effects and binding of MCs onto resin rather than absence of these MC analogs in the system.

3.4.2. Pre- and post- loaded toxin SPATT experiment

This study further demonstrates that phenolic content from the water readily binds to HP20 resin and at least some is extracted with a 100% MeOH rinse (Appendix A and Table 3.5). It is known that high levels of humic and/or fulvic material can cause signal enhancement or suppression on an LC-MS/MS. This is an important factor that future studies should consider when quantifying phycotoxins extracted from Diaion™ HP20 resin. In addition, this study demonstrated that the

phenolic content that binds to the resin does not readily desorb when the resin is rinsed in ultrapure H₂O for one hour (Table 3.5).

Phenolic content in the water seemed to strongly impact suppression and enhancement of the phycotoxin signals on the UPLC-MS/MS (Table 3.5). Some chemicals at site E seemed to enhance the signal of MC-LR and MC-YR on the UPLC-MS/MS, but MC-RR was suppressed even after one day at both sites (Table 3.6). Alternatively, OA signal was around 100% after one day at both sites but dropped to around 40-50% suppression in one-month extracts, likely due to the extra phenolics and other chemicals that are extracted from the resin after that length of deployment (Table 3.6). These differences between suppression/enhancement based on site, length of time, and phycotoxin studied, show how important it is to spike at least a subset of SPATT samples with known quantities of the phycotoxins being studied. This underscores the caution that should be taken when comparing quantities of different phycotoxins in extracts and even the same phycotoxin between SPATT bags, as the length of deployment and time of year can impact the matrix effects in the extracts.

Although some SPATT bags were deployed for a month, the resin did not seem to reach binding capacity as OA was still able to bind to the resin after being pulled from the water (Table 3.6). However, at site E, only around 50% of the OA adsorbed onto the resin after deployment, compared to the controls; it is possible the SPATT resin could have been near saturation reaching at one month, but this could also be due to signal suppression. OA was also relatively stable on SPATT bags in the water and did not desorb from the resin during the month (Table 3.5). This data

suggests that compounds with higher octanol-water coefficients, like OA, are not as affected by water with high phenolic content as they did not seem to be released nor stopped from binding onto resin.

The interaction between OA and resin differed from the results found for the group of MCs. After just one day at both sites, almost all the MC-RR was either desorbed off of the SPATT or suppressed when running on the LCMS (Table 3.5). Even considering the results of high signal suppression for this toxin, a peak area of around 2 ng/g resin of MC-RR would still be expected based on the percent suppression found by spiking the samples. Additionally, some MC-RR was able to bind onto resin after it was at Site E for one day. Thus, at least some of the toxin was removed from the resin after one day in the field. After one month, all MCs on the resin was either removed or suppressed and MCs did not seem to be able to bind to SPATT after it had been deployed for one month (Table 3.5). This may be partially explained by the phenolic content in the water, since some MC-YR was retained on the resin during deployment, and MC-YR was also able to bind onto resin deployed for one day at site E, which had lower phenolic content, but not at site C, with higher phenolic content. Although the Pre-field deployment adsorption results could be due to the differences in salinity between these two sites as well as phenolic content, loading of phycotoxins post-field deployment was always performed with ultrapure H₂O. Since this group of phycotoxins are found primarily in lakes, where higher phenolic content is common as opposed to coastal systems, these results suggest that Diaion™ HP20 resin should not be used in passive samplers for MCs.

Although not the goal of the study, this experiment also showed that storing SPATT bags at 4°C for even one month could lead to substantial losses of OA. However, this is not the case for all phycotoxins as MC-RR/LR/YR concentrations remained consistent over this time. More research is needed studying the best practices for use and storage of SPATT bags when studying different phycotoxins.

3.5 Conclusion

Solid phase adsorption toxin tracking (SPATT) is a low-tech sampling method that can provide beneficial information on the spatial/temporal trends of phycotoxins and the presence of new/emerging threats to an area. By utilizing SPATTs in New Jersey, phycotoxins not previously reported in the area were described, such as AZA1, AZA2, GDA, and YTX. Additionally, New Jersey managers can now know better when and where to monitor certain phycotoxins and phytoplankton species within these two estuaries, to protect human and organism health. However, there are caveats to the use of passive samplers in biotoxin monitoring that this study has only begun to explore. It is clear that phycotoxins interact with the resin differentially and that if a toxin is “below detection”, it does not necessarily mean it is “not present”. More research into the chemical and environmental factors that can impact adsorption rates of phycotoxins to SPATTs is needed.

3.6 Tables

Table 3.1. The logarithm of the partition coefficient between *n*-octanol and water (Log P), calculated using XLogP3 3.0 (PubChem release 2021.05.07).

Toxin	Log P
DA	-1.3
MC-RR	-0.2
MC-YR	2.2
MC-LR	2.3
GDA	2.8
YTX	3.4
OA	3.4
DTX2	3.4
PbTX-2	3.7
PTX2	3.8
DTX1	3.8
AZA2	3.8
AZA1	3.9

Table 3.2. Measured differences in toxin levels (ng/g resin $\pm$ standard deviation) between replicate SPATT bags deployed each month in two New Jersey estuaries in 2022 (n=2).

Sample	Toxin						
	AZA1	AZA2	DTX1	GDA	OA	PTX2	YTX
Site B June-July 22	BD ^a	BD ^a	333.17 $\pm$ 41.44	BD ^a	698.59 $\pm$ 86.54	396.49 $\pm$ 60.32	BD ^a
Site I July-Aug 22	BD ^a	0.59 $\pm$ 0.16	47.18 $\pm$ 4.61	11.35 $\pm$ 0.32	224.68 $\pm$ 13.38	5.70 $\pm$ 1.32	BD ^a
Site B Aug-Sept 22	BD ^a	BD ^a	70.67 $\pm$ 0.71	14.94 $\pm$ 0.72	256.34 $\pm$ 20.2	68.85 $\pm$ 9.96	5.29 $\pm$ 0.44
Site H Aug-Sept 22	BD ^a	0.78 $\pm$ 0.1	18.99 $\pm$ 3.49	434.12 $\pm$ 28.08	113.49 $\pm$ 1.93	2.07 $\pm$ 0.08	BD ^a
Site A Oct-Nov 22	0.18 $\pm$ 0.03	0.42 $\pm$ 0.05	48.91 $\pm$ 15.93	BD ^a	223.36 $\pm$ 31.92	105.92 $\pm$ 25.95	15.91 $\pm$ 0.97

^a Below Detected

Table 3.3. Percent of toxin $\pm$ standard deviation, recovered from SPATT in 100% ACN fraction versus 100% MeOH fraction in 2022. Only samples with >0.5 $\mu\text{g/L}$ of toxin were used to quantify percent recoveries. If the toxin is not present in the table, then it was not detected in the 100% ACN fraction.

Toxin	Number of Samples	Percent Recovery in 100% ACN vs 100% MeOH fractions
DTX1	46	14.24 ± 10.05%
OA	60	16.24 ± 12.83%
PTX2	46	195.08 ± 89.61%
GDA	24	58.69 ± 33.04%
YTX	4	76.72 ± 19.54%

Table 3.4. Toxin concentrations from SPATT deployed at two sites on September 19, 2022 and collected over a 1-month period.

Site	Collection Date	Toxin (ng/g resin)				
		AZA2	GDA	DTX1	OA	PTX2
C	9/29/2022			2.77	39.73	14.91
	10/5/2022	0	0	1.59	28.38	9.8
	10/10/2022			9.17	44.53	14.81
	10/20/2022			8.91	45.93	16.64
H	9/29/2022	0.92	500.75	17.52	85.67	3.7
	10/5/2022	0.44	197.63	7.15	65.99	4.07
	10/11/2022	0.57	310.85	11.99	81.37	4.65
	10/20/2022	0.84	502.92	21.62	99.62	4.58

Table 3.5. Recovery of toxins from 1 g HP-20 resin either pre-loaded or post-loaded with 25 ng okadaic acid, microcystin-RR, microcystin-LR, and microcystin-YR, during a 1 day or 1 month deployment at 2 sites along Mullica River – Great Bay. Total Phenolic Content data is presented in µg/g resin GAE eq. Percent Recovery data is presented using equation: *Percent Recovery (%) = 100 x (ng per g resin measured in sample / (ng per g resin field control + ng per g resin lab control))*. n=3.

Site	Time Deploye	Toxin Exposure Time	Total Phenolic Content	Percent Recovery (% ± std)			
				MC-RR	MC-LR	MC-YR	OA
C	1 Day	Pre-Field	249.82 ± 23.47	BD ^a	16.71 ± 4.22	BD ^a	86.89 ± 5.97
		Post-Field	289.35 ± 37.01	BD ^a	17.36 ± 1.57	BD ^a	101.85 ± 11.68
	1 Month	Pre-Field	2274.6 ± 136.7	BD ^a	BD ^a	BD ^a	101.08 ± 0.68
		Post-Field	1964.22 ± 560.99	BD ^a	BD ^a	BD ^a	90.07 ± 10.73
E	1 Day	Pre-Field	120.43 ± 48.98	BD ^a	86.42 ± 33.64	41.39 ± 19.35	64.31 ± 17.81
		Post-Field	111.53 ± 7.39	31.72 ± 9.86	209.59 ± 40.08	98.25 ± 17.77	120.77 ± 37.76
	1 Month	Pre-Field	341.85 ± 23.02	BD ^a	BD ^a	BD ^a	110.36 ± 11.44
		Post-Field	548.69 ± 45.36	BD ^a	BD ^a	BD ^a	44.89 ± 15.28

^a Below Detected

Table 3.6. Recovery of toxin ± standard deviation, observed in spiked extracts from SPATTs deployed in Mullica River for one day or one month. Signal suppression indicated by (<100%) or enhancement (>100%). n=2.

Site	Time Deployed	Suppression or Enhancement (%)			
		Toxin			
		MC-RR	MC-LR	MC-YR	OA
C	1 Day	13.9±1.86	105.18±8.71	59.03±5.21	83.92±14.17
	1 Month	9.85±0.1	24.05±0.47	7.02±0.13	37.11±0.14
E	1 Day	39.47±20.78	207.71±5.3	163.99±10.94	105.64±18.89
	1 Month	15.23±1.45	90.97±11.2	41.05±8.35	47.35±1.96

Table 3.7. Recovery of toxins ± standard deviation, observed in spiked 100% MeOH extracts from SPATTs deployed in Mullica River-Great Bay and Toms River-Barnegat Bay (n=15). Signal suppression indicated by (<100%) or enhancement (>100%).

Toxin	Suppression or Enhancement (%)
MC-RR	11.27±14.56
MC-LR	8.77±13.04
MC-YR	16.82±14.61
DA	78.2±19.18
AZA1	83.23±14.76
AZA2	95.63±18.24
DTX1	57.58±22.34
GDA	90.56±28.85
OA	78.42±23.88
PTX2	56.74±23.65
YTX	42.30±26.49
PbTX2	25.26±35.67

3.7 Figures

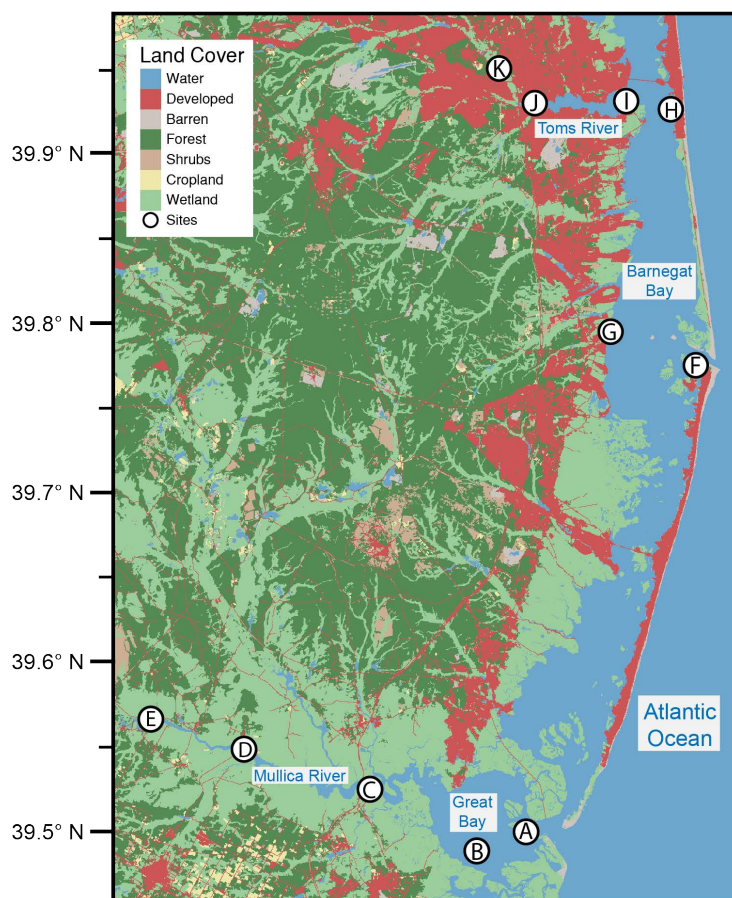


Fig.3.1. Map of the 11 field sampling sites (circles) in two New Jersey estuaries. Land Cover data is from National Land Cover Database (2016).

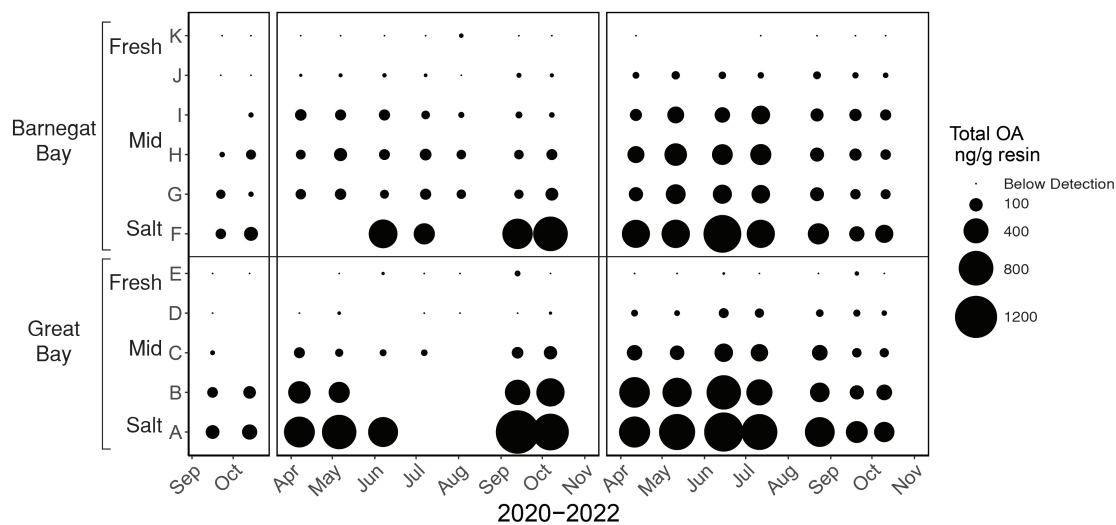


Fig. 3.2. SPATT toxin data (ng / g resin) for total okadaic acid (OA + esterified forms) along 11 sites in two New Jersey estuaries from September 2020 – November 2022. No dot means sample was lost.

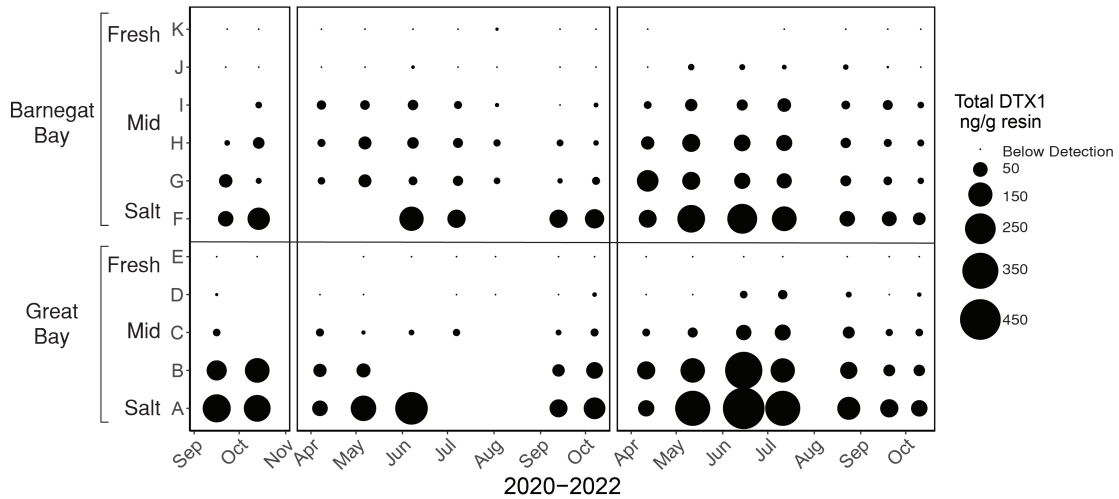


Fig. 3.3. SPATT toxin data (ng / g resin) for total dinophysistoxin-1 (DTX1 + esterified forms) along 11 sites in two New Jersey estuaries from September 2020 – November 2022. No dot means sample was lost.

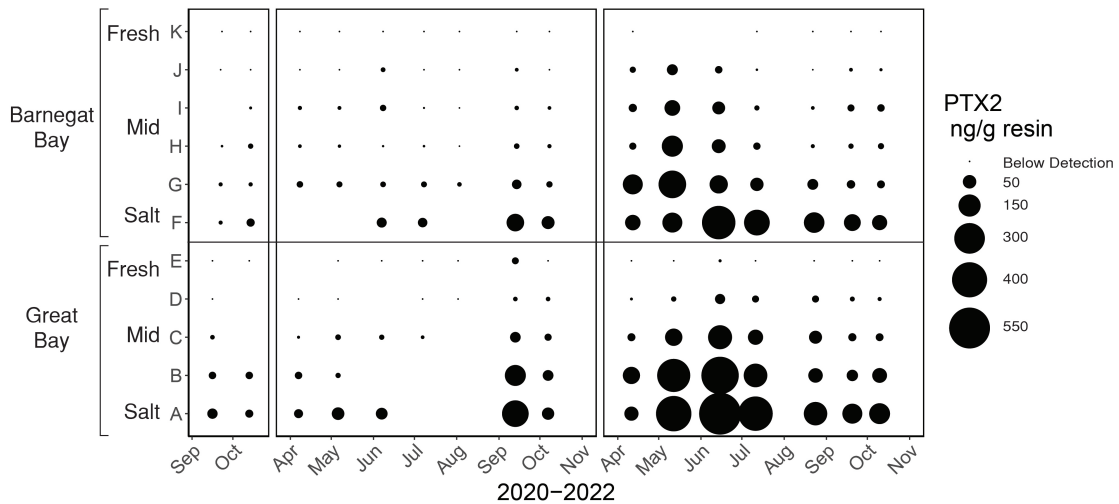


Fig. 3.4. SPATT toxin data (ng / g resin) for pectenotoxin-2 (PTX2) along 11 sites in two New Jersey estuaries from September 2020 – November 2022. No dot means sample was lost.

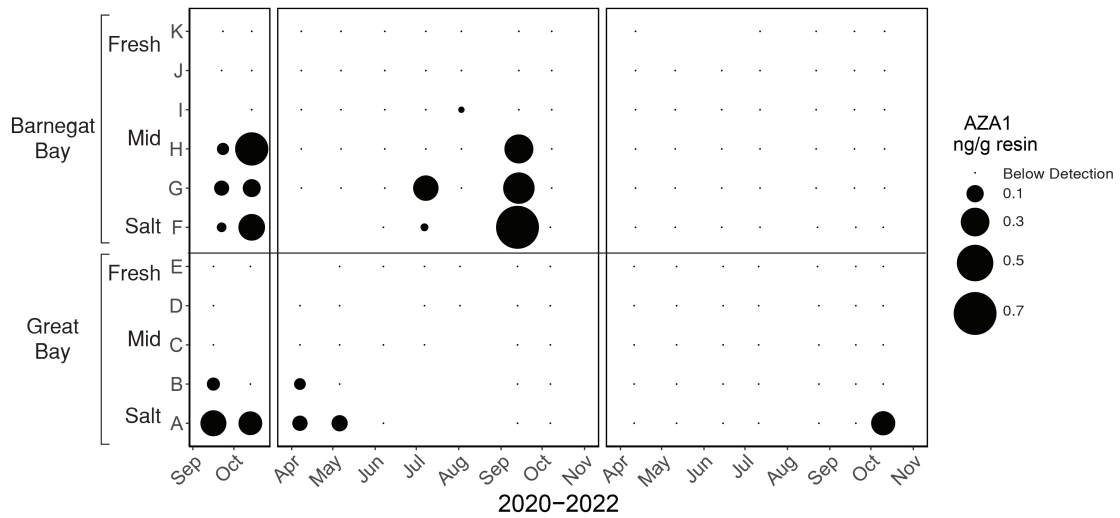


Fig. 3.5. SPATT toxin data (ng / g resin) for azaspiracid-1 (AZA1) along 11 sites in two New Jersey estuaries from September 2020 – November 2022. No dot means sample was lost

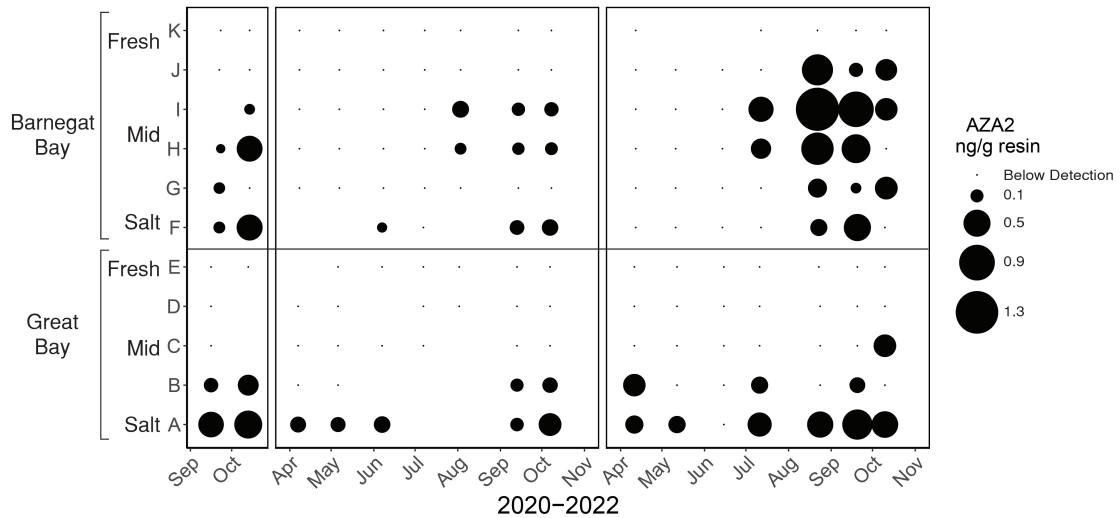


Fig. 3.6. SPATT toxin data (ng / g resin) for azaspiracid-2 (AZA2) along 11 sites in two New Jersey estuaries from September 2020 – November 2022. No dot means sample was lost.

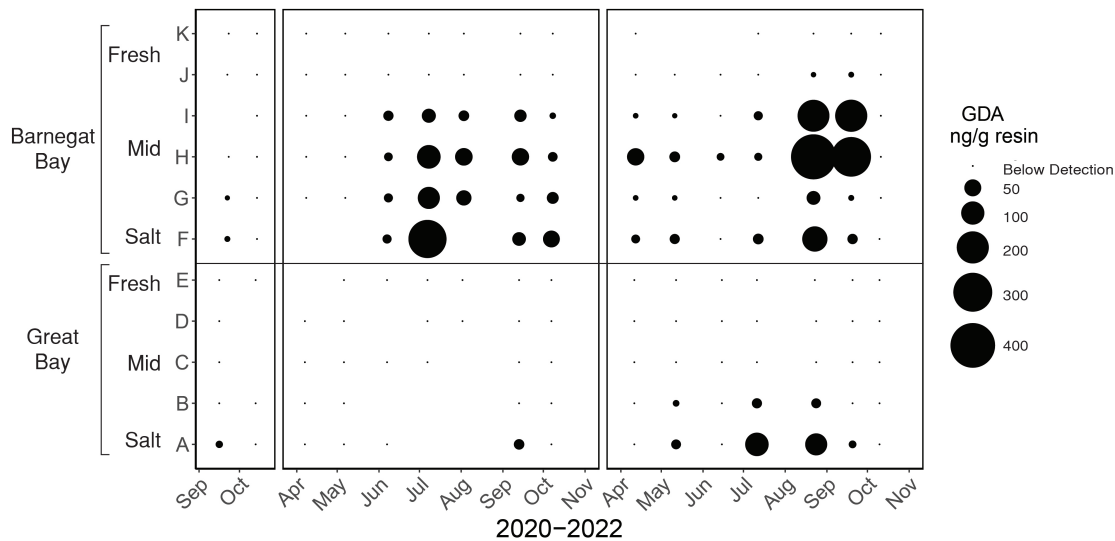


Fig. 3.7. SPATT toxin data (ng / g resin) for Goniodomin-A (GDA) along 11 sites in two New Jersey estuaries from September 2020 – November 2022. No dot means sample was lost.

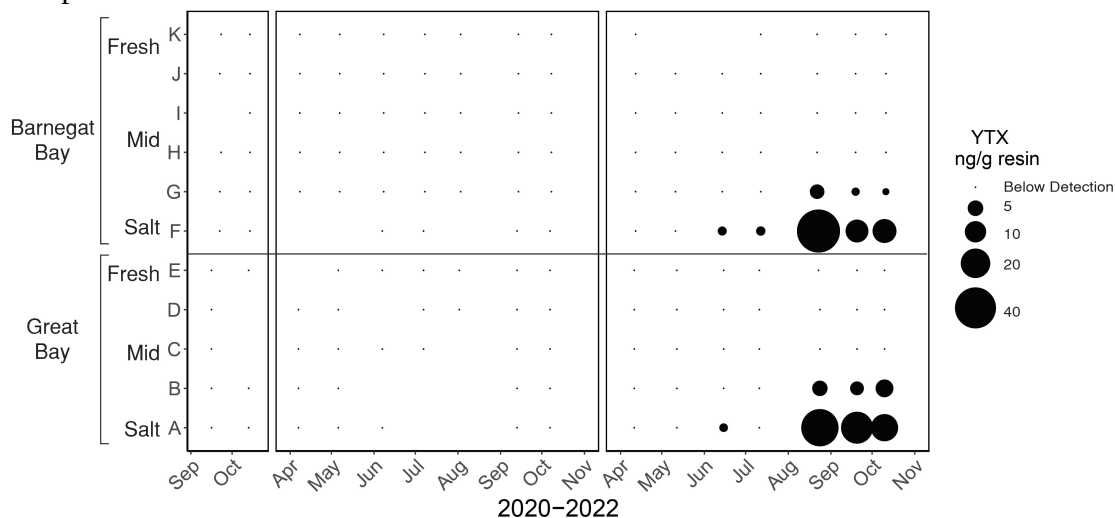


Fig. 3.8. SPATT toxin data (ng / g resin) for Yessotoxin (YTX) along 11 sites in two New Jersey estuaries from September 2020 – November 2022. No dot means sample was lost.

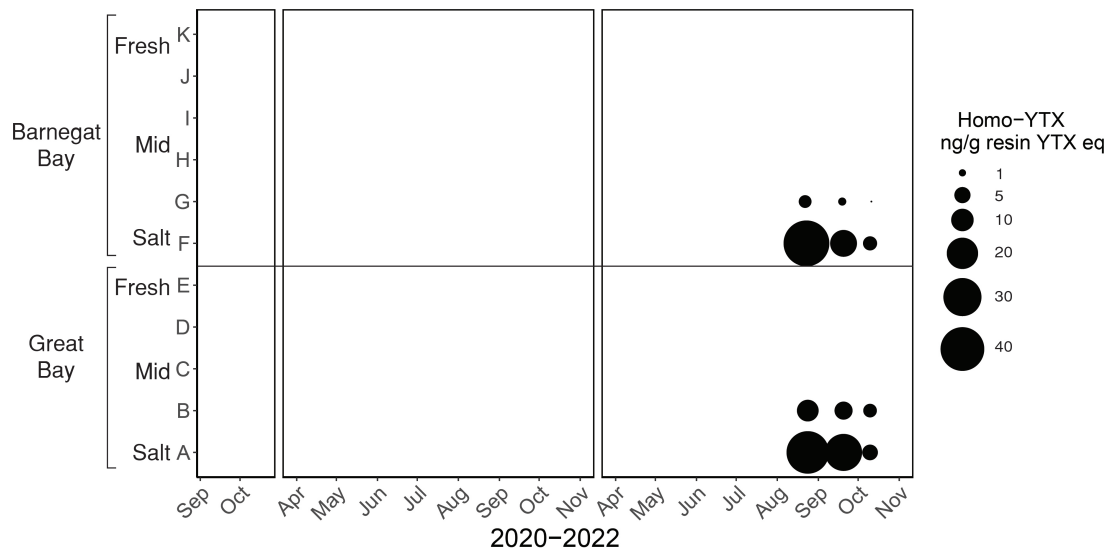


Fig. 3.9. SPATT toxin data (ng / g resin YTX eq) for Homo-Yessotoxin (Homo-YTX) along 11 sites in two New Jersey estuaries from September 2020 – November 2022, quantified using YTX standard curve. No dot means sample was not run.

Chapter 4: Environmental Drivers of Phytoplankton Communities in Two New Jersey Estuaries

Abstract

Land cover type changes organic carbon composition and river discharge to downstream aquatic environments, which in turn impacts other water quality variables including pH, nutrients, light levels, and the presence of algicides.

There is literature on laboratory experiments using natural algicides released by terrestrial plants and aquatic vegetation, like phenolics and flavonoids, that can change phytoplankton composition and inhibit the growth of toxic microalgae. The streams along the New Jersey pinelands are stained brown from decaying vegetation, leading to high phenolic content, low pH, and low light attenuation which could protect estuaries in this area from harmful algal blooms. In this study, phytoplankton data and abiotic factors, including total phenolic content, were collected from 12 stations along the salinity gradient of two New Jersey estuaries with varying levels of disturbance, in order to understand how total phenolic content affects chlorophyll a and HAB species. Total phenolic content (TPC) in both estuaries was positively associated with discharge and highest in the spring season, with peak concentrations of 1924 $\mu\text{g/L}$ GAE eq in the surface waters. TPC and chlorophyll a were negatively correlated, however the relationship was weak. Redundancy analysis models showed the phytoplankton class Cyanophyceae was positively correlated to inorganic nitrogen levels ($\text{NO}_2^- + \text{NO}_3^- + \text{NH}_4^+$), and negatively correlated to TPC at freshwater sites. However, at mid-salinity sites Cyanophyceae was positively correlated to both inorganic nitrogen and TPC. Additional controlled laboratory studies and field studies with estuaries of varying TPC concentrations would further our knowledge of how

phytoplankton communities are impacted by river runoff and TPC input. This project provides new information for monitoring, managing, and modeling phytoplankton communities, based on dissolved organic carbon (DOC) and TPC.

4.1. Introduction

Estuaries are among the most productive and dynamic aquatic ecosystems in the world, with unique chemical and physical dynamics caused by the freshwater outflow meeting the tidal saltwater intrusions from the coast. Phytoplankton composition is often used as a biotic indicator of the state of an estuary as they can reflect both internal fluctuations and external inputs to a system (Ren et al., 2017). Diverse phytoplankton communities can be an indicator of good water quality and lead to productive food webs. Studying the impact of environmental variables on phytoplankton abundance and composition is important in management plans to preserve or restore the health of an estuary (Paudel et al., 2016).

Around 60% of the world's human population lives near the coast, causing large impacts to waterways through development and coinciding deforestation, agricultural and aquacultural activities. These activities can cause nutrient pollution through point and non-point sources, causing shifts in phytoplankton composition, with increased frequency of harmful algal blooms (HABs) and loss of phytoplankton diversity. In freshwater systems, low forest cover tend be associated with increased cyanobacteria blooms, increased microcystin concentrations (toxins produced by species of cyanobacteria), and a reduction in overall water quality (Beaver et al., 2018; Clément et al., 2017; Marion et al., 2017). Forest coverage exceeding 47% is estimated to prevent eutrophication in streams (Clément et al., 2017). Low forest cover can also change phytoplankton communities and the occurrence of HABs

further down the estuary and along coasts with high nutrient transport. Some of the other physical and chemical variables impacted by land cover type include pH, light levels, discharge, and organic carbon composition, all of which can impact phytoplankton communities (Canuel et al., 2012; Dow and Zampella, 2000; Hounshell et al., 2022; Paudel et al., 2016; Reitsema et al., 2018).

Rivers are not only responsible for transporting nutrients but can transport large amounts of organic matter as well. Thus, organic matter sources, composition, and amount can shift based on land cover and these are all expected to change under future climate conditions, particularly with shifts in precipitation (Canuel et al., 2012; Hounshell et al., 2022). Terrestrially derived dissolved organic carbon (tDOC) can reduce pH, light levels, and bind nutrients like phosphorus and iron, all of which can impact phytoplankton community composition (Dow and Zampella, 2000; Havens et al., 2016; Lenard and Ejankowski, 2017). Plant allelochemicals from terrestrial and submerged aquatic vegetation can also be composed of compounds like phenolic acid, tannins, and/or flavones that can inhibit the growth of toxic phytoplankton (Chen and Wang, 2019; Huang et al., 2015; Wang et al., 2007; Xiao et al., 2014). For instance, the spreading of deciduous leaf litter has been shown as an effective algicide in laboratory studies and medium-sized ponds (Neilen et al., 2017; Ridge et al., 1999). Peng et al. 2022 found that cyanobacteria were inhibited and fluorescence parameters were affected by total phenolic content from the high coverage and biomass of submerged aquatic vegetation in lakes (Peng et al., 2022).

For this study, two New Jersey estuaries were chosen to explore the relationship between physicochemical parameters, including total phenolic content

(TPC), and phytoplankton communities. Within the New Jersey Pinelands ecosystem, Mullica River-Great Bay estuary consists of a variety of terrestrial, marsh, and aquatic habitats. The Jacques Cousteau National Estuarine Research Reserve (JC-NERR) helps to maintain this healthy ecosystem and provides long-term research and monitoring of the system. It is considered one of the least disturbed estuaries in the Northeast United States with less than one percent human development. The high flowing water from the Mullica River is stained brown from the Pineland National Reserve's decaying vegetation (pine needles/oak leaves) and iron content in the soils. This leads the water to be acidic (pH as low as 4.4), be nutrient poor, have low light attenuation, and have a high content of tannins/phenolic acids (potential algal inhibitors). Few harmful algal species have been found and/or researched in Mullica River-Great Bay (Heddendorf, 2008).

Next door to Mullica River-Great Bay, Toms River-Barnegat Bay estuary is a shallow, poorly flushed estuary classified by NOAA as eutrophic as it is bordered by developed areas (Baker et al., 2014). Over the past few decades, Barnegat Bay has experienced increased occurrence of HABs, which causes negative ecological impacts through the produced toxins, and the loss of seagrass habitat through shading (Kennish et al., 2007; Lacey, 2018). A 2011-2013 survey of phytoplankton in Barnegat Bay basin found HAB species *Heterocapsa rotundata*, *Synechococcus*, *Aureococcus*, *Prorocentrum*, *Dinophysis*, and *Pseudo-nitzschia* species (Olsen and Mahoney, 2001; Ren, 2015; Ren et al., 2017). *Aureococcus anophagefferens*, which causes brown tides, is particularly detrimental to the coast, with concentrations exceeding those that cause negative impacts to shellfish and seagrasses (Bricelj et al.,

2012; Gastrich et al., 2004). According to monitoring data from the last decade, the mouth of Toms River (NJDEP site BB04a, near station 10 on Fig 4.1) has the highest total nitrogen and chlorophyll concentrations in the Barnegat Bay watershed (Fantasia et al., 2017; Pang et al., 2017).

These two New Jersey estuaries offer ideal comparison sites to look at how tDOC and future changes in land cover could impact phytoplankton community composition. With the continuing increase in human populations along coasts and climate related stressors, land cover will persist in changing freshwater and coastal water quality. There is a need to study how land cover and corresponding changes in DOC composition will impact both freshwater and marine HABs.

In this study, phytoplankton community composition data and abiotic factors, including DOC and TPC, were collected from the freshwater-marine continuum of the two New Jersey estuaries which varied in surrounding land cover. The aims of this research were to 1. Understand how TPC in the water varies with seasons and other abiotic factors, 2. Identify the main driving factors affecting the seasonal variations in phytoplankton community characteristics along the salinity gradient, 3. Understand if TPC in the water column is a driving factor affecting chlorophyll a and HAB species.

4.2 Materials and methods

4.2.1 Study location

The study took place along two freshwater-to-marine continuums: Toms River-Barnegat Bay estuary and Mullica River-Great Bay estuary. Great Bay estuary is in the Pinelands National Reserve near Tuckerton, NJ about 15 kilometers north-northeast of Atlantic City. The dominant tree in the Pinelands is pitch pine, and the

Mullica River headwaters contain cedar swamps, Sphagnum bogs, and cranberry bogs. Freshwater enters the estuary primarily through surface flow from Mullica River and groundwater inputs. Tidal salt marshes form islands and are an extensive margin between the pinelands and Great Bay. Here, there is little development and low industrial pollution, and it is considered one of the least disturbed estuaries in the northeast United States (Jacques Cousteau National Estuarine Research Reserve, 2016; Kennish et al., 2016). The area has occasional seasonal beds of macroalgae, fast moving tidal currents with tidal ranges of approximately 0.85-1.22 m and short residence times of 0-3 days (Defne et al., 2020).

The Barnegat Bay watershed is around 660-mi², and has gone through dramatic population growth in the last 70 years, shifting from forest and wetland land cover to suburban development. This change in land use has caused eutrophic conditions in the estuary and there have been documented loss of submerged aquatic vegetation and hard clams (Bricelj et al., 2017; Lacey, 2018). This study focused on central Barnegat Bay from Toms River to Barnegat Inlet, where the max depth is 3-4 m and the residence time is relatively long with about 30-50 days along Toms River (Defne and Ganju, 2015). The tide is semidiurnal and has a tidal range of less than 0.4 m on average (Ganju et al., 2014). Toms River contributes about one-third of the freshwater flow into Barnegat Bay, with a mean flow of 214.3 cfs (from site 12; Fig. 4.1) and its headwaters are within Pinelands National Reserve (Goodrow et al., 2017; Pang et al., 2017). Barnegat Bay at site 9 has a mean tidal range of one ft (waterdata.usgs.gov).

4.2.2 Field sampling

Field sampling was conducted monthly between April-November in 2021-2022. Sampling occurred at six sites along Toms River-Barnegat Bay, and six sites along Mullica River-Great Bay (Fig. 4.1). The stations were along a clear salinity gradient: two in the upper estuary, two in the mid-estuary, and two in the lower estuary, with one inlet site (Fig. 4.2). Sampling sites along Mullica River-Great Bay included five permanent water-quality monitoring stations through the Jacques Cousteau NERR SWMP (Fig. 4.1 Sites 1-5) (Kennish et al., 2016). Sampling sites along Toms River-Barnegat Bay included one permanent water-quality monitoring station through Barnegat Bay Partnership (BBP) (Site 9), two sites close to buoys deployed every summer by New Jersey Department of Environmental Protection (NJDEP) (Sites 8 & 10), five sites regularly sampled by NJDEP for nutrients and phytoplankton microscopy in the summer months (Sites 8-11), and one site with a USGS discharge monitor that is also regularly monitored and used in field studies by NJDEP, USGS, and BBP (Site 12).

During each monthly sampling event, a series of water quality measurements were collected. Temperature (°C), salinity (ppt), optical dissolved oxygen (mg/L & percent saturation), pH, and turbidity (FNU), were collected using a YSI multiparameter sonde (EXO2). Water clarity was collected with a secchi disk and if the secchi disk reached the bottom, maximum depth was recorded. Two surface grab samples were taken in acid-washed 500 mL amber Nalgene bottles and stored on ice to measure dissolved organic carbon (DOC), $\text{NO}_3^-/\text{NO}_2^-$, NH_4^+ , PO_4^{3-} , Total Phenolic Content (TPC), and chlorophyll a (Chl a). In 2022, surface water was filtered through a 200 μm mesh and 10 mL transferred to a 15 mL falcon tube with 1% lugols solution

to identify phytoplankton community composition. A phytoplankton net sample (Forest suppliers) was also taken, filtered through a 200 μm mesh, and transferred to an acid-washed Nalgene bottle. Mullica River-Great Bay sites were always collected during ebb tide, while tidal cycle varied for sampling of Toms River-Barnegat Bay and was recorded after every collection. Rainfall data for the week prior to sampling was retrieved from NOAA ([ncdc.noaa.gov](https://www.ncdc.noaa.gov)) using Mullica River site #US1NJAT0021 and Toms River site #US1NJOC0009. Discharge (ft^3/sec) data was retrieved from USGS (waterdata.usgs.gov) using Toms River site #01408500 and Mullica River site #01409400.

Within 48 hours of collection, both grab samples were filtered through 47mm pre-combusted GF/F filter into muffled glassware. The filtered water was stored at -20°C and the filters were stored at -80°C .

4.2.3 Nutrient analysis

During 2021 sampling, half the samples run for DOC and nutrients were unreliable and therefore, all DOC and nutrient data for 2021 were not used in the analyses. During 2022 sampling, 60 mL filtered samples were run for DOC (mg/L) each month by Chesapeake Bay Laboratory Nutrient Analytical Services (Solomons, MD). Two replicate filtered samples were transferred to 50 mL falcon tubes each month and sent to Virginia Institute of Marine Science Analytical Services Center (Gloucester Point, VA) for analysis of $\text{NO}_3^-/\text{NO}_2^-$, NH_4^+ , and PO_4^{3-} .

4.2.4 Phytoplankton classification

Two replicates of grab samples preserved in 1% Lugol's solution were analyzed via Sedgwick-rafter in 2022. The preserved phytoplankton samples were analyzed by identifying plankton to the lowest possible taxa in x100 magnification

until at least 300 algal cells were counted. Taxonomic confirmation were checked against the website on Diatoms of the North America (<https://diatoms.org>) and AlgaeBase (<http://www.algaebase.org>).

Data analysis of species richness, phytoplankton diversity (H), and phytoplankton evenness (J) were calculated using the following equations:

$$\text{Shannon-Wiener diversity index } (H) = - \sum_{i=1}^S \frac{n_i}{N} \ln \left(\frac{n_i}{N} \right)$$

$$\text{Pielou's evenness index } (J) = \frac{H}{\ln S}$$

where n_i is the number of individuals of species i within a given area, N is the number of total individuals of all species, and S is the total species number. Species richness, Shannon–Wiener diversity index, and Pielou's evenness index were calculated and graphed using 'ggplot2' and 'vegan' packages in R (v.4.1.2).

4.2.5 Total phenolic content

To extract phenolic content from the water sample, solid phase extraction (SPE) was used following the methods of Dittmar et al. 2008, Takeda et al. 2013, and Powers et al. 2019 (Dittmar et al., 2008; Powers et al., 2019; Takeda et al., 2013). Samples were thawed, and acidified using concentrated HCl (Sigma-Aldrich 32%, pura) to a pH of 2.5. Agilent Bond Elut PPL (200 mg, 3mL) cartridges were activated with 10mL LC-MS grade MeOH and rinsed with 10mL ultrapure H₂O acidified to a pH of 2.5. 100 mL of the water samples were gravity filtered through the cartridge and rinsed with 20 mL ultrapure H₂O acidified to a pH of 2.5, to remove salt residue. The bound compounds were eluted with 5mL LC-MS grade MeOH into a 20mL pre-combusted amber glass vial with a Teflon-lined silicon lid and stored at -20°C until analysis.

Total phenolic content (TPC) was determined following modifications of the Folin-Ciocalteu method for the determination of TPC in SPE-DOM samples (Powers et al., 2019; Takeda et al., 2013). One mL of the SPE extracted sample was dried via speedvac system without the use of light, resuspended in 100 μ L LCMS grade MeOH, vortexed, and 900 μ L ultrapure H₂O added. 50 μ L of 0.5 M NaOH was added followed by 60 μ L of Folin-Ciocalteu reagent, vortexed, and put into the dark for five minutes. 150 μ L of 20% sodium carbonate solution was then added and the vials were put back into the dark for one hour. 250 μ L was transferred to a 96 well plate and adsorption at 765 nm was measured on a SpectraMax i3x multi-mode microplate reader. The samples were compared to a gallic acid standard curve from 1 to 30 μ g/mL with the same proportion of H₂O:MeOH as the samples and presented in gallic acid equivalents (GAEs).

4.2.6 Chlorophyll a analysis

The filters from the grab samples were removed from -80°C and placed into 15mL falcon tubes. Three mL of 90% acetone was added to each tube, samples were sonicated for 1 minute of ice, and stored for 24 hours at 4°C. After 24 hours, samples were centrifuged at 675xg for 15 minutes. Supernatant was extracted and filtered through 0.22 μ m PVDF filter into new falcon tubes. 400 μ L sample was transferred to a cold glass 96 well plate to reduce sample loss and measured on a SpectraMax i3x multi-mode microplate reader with a fluorescent emission of 680 nm and excitation of 436 nm. 1% HCl was added to each well and it was read a second time. All Chl a extraction and analysis was performed under subdued light. Chl a was calculated using the following formula:

$$\text{Chlorophyll } a = K * \frac{F_m}{F_m - 1} * V_e * \frac{F_o - F_a}{V_f}$$

Where:

K = Calibration coefficient = µg Chl *a* per ml 90 % acetone per instrument fluorescence units.

F_m = maximum acid ratio (F_o/F_a) of pure chlorophyll *a* standard.

F_o = sample fluorescence before acidification.

F_a = sample fluorescence after acidification.

V_e = extraction volume in milliliters.

V_f = filtered volume in liter

4.2.7 Statistics

Abiotic environmental variables were compared between basins using a Welch two sample t-test. Environmental variables and phytoplankton were compared between seasons (Spring: April-May, Summer: June-September, Fall: October-November) and between salinity categories (Freshwater: <5 ppt, Mid-Salinity: 5<x<20 ppt, High-Salinity: x>20 ppt) using one-way analysis of variance (ANOVA). The seasons were chosen based on temperature data (Fig. 4.3). Where significant differences were detected, the Tukey's hsd post hoc test was used for separation of means. Data were assessed for normality (Shapiro-Wilk test) and for homogeneity of variance (Levenes test) at the 0.05 significance level. When non-normality or non-homogeneity of variance was found, the data was logarithmically transformed and if tests still failed, non-parametric Kruskal-Wallis test was conducted followed by a Dunn's test.

Conservative mixing lines (Fig. 4.4) were constructed using the linear line between the two samples of highest and lowest salinity for the 2022 data of TPC and DOC separated by season. The percent deviation of each sample from the theoretical conservative mixing line was then calculated and averaged based on the following calculations:

$$E_{\text{expected}} = m * (S_{\text{sample}} - S_{\text{fresh}}) + E_{\text{fresh}} \quad (1)$$

$$\text{Percent deviation} = \left(\frac{E_{\text{sample}} - E_{\text{expected}}}{E_{\text{expected}}} \right) * 100 \quad (2)$$

Where m is the slope of the conservative mixing line, S_{sample} is the salinity value of the sample, S_{fresh} is the salinity of the freshwater endpoint, E_{fresh} is the environmental parameter value at the freshwater endpoint, and E_{sample} is the environmental parameter value for the sample. A high positive value represents high addition of the environmental variable in the estuary and a high negative value represents high removal of the environmental variable in the estuary.

Redundancy analysis (RDA) is a combination of PCA and multiple linear regression where an explanatory dataset (i.e., environmental parameters) is used to explain the variation in a response data matrix (i.e., phytoplankton community composition). RDAs were performed to identify environmental factors influencing phytoplankton community class separated into three salinity groups: freshwater (<10 ppt), mid-salinity (10< x <25), and high salinity (>25 ppt) samples. Environmental variables were standardized before use in the analysis to remove the impact of different size units and one environmental variable in highly collinear variables were removed from the data set. Nitrite+nitrate and ammonium (NO_x + NH₄) were combined into one environmental parameter for the analysis. Final environmental

variables included in the analysis were turbidity, pH, phosphate (PO₄), nitrite+nitrate+ammonium (NO_x + NH₄), total phenolic content (TPC), and temperature (Temp). Phytoplankton community classes were Hellinger data transformed (all values in a row are divided by the row sum and then the square root of the values are taken), in order to remove the strong impact of zeros in the dataset.

In order to understand which environmental variables best predict chlorophyll a concentrations, a multiple regression model was run with all sites combined, and separated by salinity into three categories: <10 ppt, 10<x<25 ppt, and >25 ppt. The variables tested were: turbidity, temperature, salinity, pH, dissolved oxygen, NO₂⁻ + NO₃⁻, NH₄⁺, Ortho-PO₄³⁻, DOC, and TPC. There were 96 observations with all variables analyzed. Colinear variables were removed by running a correlation matrix and using the Variance Inflation Factor (VIF) with a cutoff value of 5. The colinear variables changed based on the three salinity categories and so variable removal varied between the models. A step-wise AIC analysis was run to calculate a model with reduced number of variables. The reduced model was then compared to the full model using an ANOVA test to see which model was best for predicting chlorophyll a, with the null hypothesis that the reduced model is adequate. The mean absolute error was calculated for each reduced model.

4.3 Results

4.3.1 Abiotic characteristics of the two estuaries

4.3.1.1 Temperature, pH, Dissolved Oxygen, and Turbidity

Temperature varied from 9-28°C during the sampling period and was hottest between June and September in both 2021 and 2022 (Fig. 4.3). Alternatively, dissolved oxygen (mg/L) was significantly lower in the summer season compared to the fall and spring (Fig. 4.5; ANOVA Tukey's post hoc; F=85.81 p<0.001). The mean

temperatures and mean dissolved oxygen levels were similar between estuaries, years, and salinity categories. pH varied from 4.74 – 8.41 during the sampling period and was significantly lower when salinity was below 5 ppt (Fig. 4.6; ANOVA Tukey's post hoc; $F=362.7$ $p<0.001$). The pH was similar between estuaries, years, and seasons. Mean turbidity was higher in Mullica River-Great Bay compared to Toms River-Barnegat Bay (6.83 FNU vs 3.49 FNU; Fig. 4.7; T-Test; $t=5.4$ $p<0.001$). Turbidity was similar between years, salinity, and seasons.

4.3.1.2 Nutrients

2021 nutrient data was lost due to unreliable results and facilities were switched in 2022. Mean NH_4^+ (mg/L) and mean $\text{NO}_2^- + \text{NO}_3^-$ (mg/L) was higher in Toms River-Barnegat Bay compared to Mullica River-Great Bay during sampling in 2022 (Fig. 4.8A & B; NH_4^+ : 0.039 vs 0.025 mg/L, T-Test $t=-2.331$ $p<0.05$; $\text{NO}_2^- + \text{NO}_3^-$: 0.2 vs 0.04 mg/L, T-Test $t=-3.99$ $p<0.001$). However, Ortho- PO_4^{3-} (mg/L) was not significantly different between estuaries (Fig. 4.8C). The highest NH_4^+ levels also occurred in different areas of the two estuaries. In the Mullica River-Great Bay estuary, the high salinity sites had higher NH_4^+ levels compared to the freshwater sites (ANOVA Tukey's post hoc; $F=7.62$ $p<0.001$), while in Toms River-Barnegat Bay, there was no statistical difference between salinity categories. Alternatively, the mean $\text{NO}_2^- + \text{NO}_3^-$ levels in the Toms River-Barnegat Bay estuary were higher in the freshwater sites compared to the high and mid salinity categories (ANOVA Tukey's post hoc; $F=27.35$ $p<0.001$), while in Mullica River-Great Bay estuary, there was no statistical difference between salinity categories. For Ortho- PO_4^{3-} (mg/L), the high salinity category had the highest mean compared to the mid salinity and freshwater categories in Toms River-Barnegat Bay estuary (ANOVA Tukey's post hoc; $F=22.53$

$p < 0.001$) and Mullica River-Great Bay estuary (ANOVA Tukey's post hoc; $F = 34.47$ $p < 0.001$). Mean Ortho- PO_4^{3-} was also significantly lower in the spring compared to the fall and summer seasons (ANOVA Tukey's post hoc; $F = 5.36$ $p < 0.01$). Mean NH_4^+ and $\text{NO}_2^- + \text{NO}_3^-$ levels were similar between seasons.

4.3.1.3 Rainfall and discharge

Although weekly rainfall was relatively the same in both areas of the two estuaries, mean discharge was higher in Toms River compared to Mullica River (Fig. 4.9; 76.65 vs 165.19 feet^3/sec , T-Test $t = -10.14$ $p < 0.001$). Discharge at times of collection were significantly higher in 2021 compared to 2022 (136.18 vs 105.65 feet^3/sec , T-Test $t = 2.88$ $p < 0.01$), although rainfall was relatively the same between years. Sampling occurred during statistically higher times of discharge in the spring season of 2022 compared to the summer and fall of 2022 (Fig. 4.9; ANOVA Tukey's post hoc; $F = 40.51$ $p < 0.001$). Rainfall was statistically lower in summer 2022 compared to the spring and fall seasons but there was no difference between seasons in 2021 (Fig. 4.9; ANOVA Tukey's post hoc; $F = 11.33$ $p < 0.001$).

4.3.1.4 Trends in Total Phenolic Content and Dissolved Organic Carbon

TPC and salinity had a strong linear relationship and was negatively associated (Fig. 4.10; Linear regression $F = 196.1$ $R^2 = 0.55$, $p < 0.001$; -68.89% correlation), while TPC and rainfall the week before sampling were positively associated (Linear regression $F = 11.21$ $R^2 = 0.15$, $p < 0.01$; 41.78% correlation). Overall, there was no difference in mean TPC concentrations between the two estuaries even within salinity categories but there was higher TPC in 2022 compared to 2021 (583.01 vs 369.71 $\mu\text{g/L}$ GAE eq, Fig. 4.11; Log transformed, T-Test $t = -4$ $p < 0.001$). Comparing the 2022 data, there was a strong positive correlation between

DOC and TPC (Fig. 4.8D & 11; Log transformed; Spearman's rank correlation ρ ; $p < 0.001$, $\rho=0.893$). There were similar trends with the two variables, DOC and TPC, along the freshwater-marine continuum of the two estuaries as well. Analyzing the conservative mixing lines and the calculated average deviations from samples in 2022, summer months in Mullica River-Great Bay had the lower DOC and TPC from the freshwater endmember compared to the spring season (Log transformed; ANOVA Tukey's post hoc; $F=4.46$ $p < 0.05$) but had net additions of DOC and TPC within the estuary (Fig. 4.4). Toms River-Barnegat Bay also had net additions of DOC and TPC between July to September, but it wasn't as high as the other estuary. During the spring season (April-May), there was the highest TPC and DOC coming from the freshwater endmember (Log transformed; ANOVA Tukey's post hoc; $F=10.9$ $p < 0.001$), with little addition/removal of the compounds within the estuary (Fig. 4.4). The fall season in both estuaries also had little addition/removal of the compounds within the estuary. TPC and Chl a had a linear relationship and was negatively associated (Linear regression; $F = 8.833$, $R^2=0.046$, $p=0.003$; -23.01% correlation). However, the R^2 value is very small and therefore it does not seem that TPC explains very much of the variance in chlorophyll a.

4.3.2 Environmental drivers of Chlorophyll a

Overall, chlorophyll a (Chl a) was relatively low in both estuaries during the monthly surface samples in 2021 and 2022. The mean was higher in Toms River-Barnegat Bay compared to Mullica River-Great Bay (Fig. 4.12; T-Test: $t=-1.97$ $p < 0.05$). The highest Chl a value was 24 $\mu\text{g/L}$ in September at site 10 in 2021 and 23 $\mu\text{g/L}$ in July at site 11 in Toms River in 2022. Chlorophyll a was consistently lower in samples below five ppt, compared to sites at higher salinities (ANOVA tukey post-

hoc: $F=46.43$ $p<0.001$). When comparing seasons, the mean Chl *a* was highest in the summer months (June-September), compared to the spring (April-May) and fall (October-November) seasons salinities (ANOVA Tukey post-hoc: $F=15.7$ $p<0.001$).

Spearman correlations between environmental factors and Chl *a* showed that Chl *a* was significantly and positively correlated with temperature, turbidity, pH, and salinity, and was significantly and negatively correlated with NO_x , TPC, Rainfall, DO, discharge, and NH_4^+ (Fig 4.10). In order to understand which environmental variables most predict Chl *a* concentrations, a multiple regression model was run with all sites combined (96 observations total), and separated by salinity into three categories: <10 ppt (30 observations), $10<x<25$ ppt (29 observations), and >25 ppt (37 observations). The observed versus the predicted Chl *a* values are presented in Fig. 4.13. The following reduced models were found to be the best for predicting Chl *a* in each of these environments with the mean absolute error (MAE) presented.

All 2022 data (MAE=1.82):

$$Chl\ a = -22.62 + 0.0017 \times TPC + 0.37 \times Temp + 2.87 \times pH - 16.22 \times NH_4^+ - 116.47 \times PO_4^{3-}$$

Freshwater data (MAE=1.12):

$$Chl\ a = 8.76 + 1.34 \times pH - 2.03 \times NO_x$$

Mid-Salinity data (MAE=1.72):

$$Chl\ a = 5.07 - 0.013 \times TPC - 3.98 \times NO_x - 55.12 \times NH_4^+ + 1.94 \times DOC + 0.59 \times Turb$$

High-Salinity data (MAE=1.17):

$$Chl\ a = -4.28 - 0.006 \times TPC + 0.24 \times Temp - 23.58 \times NH_4^+ + 1.77 \times DOC + 67.6 \times PO_4^{3-} + 0.13 \times Turb$$

Where: Chl a = chlorophyll a ($\mu\text{g/L}$); TPC = total phenolic content ($\mu\text{g/L}$ GAE eq);
Temp = temperature ($^{\circ}\text{C}$); $\text{NO}_x = \text{NO}_2^- + \text{NO}_3^-$ (mg/L); DO = dissolved oxygen (mg/L);
DOC = dissolved organic carbon (mg/L); Turb = turbidity (FNU); PO_4^{3-} = Ortho- PO_4^{3-}
(mg/L); NH_4^+ (mg/L).

4.3.3 Phytoplankton community composition

Phytoplankton species richness, Shannon Wiener diversity index, and Pielou's evenness index were calculated for each sample (Fig. 4.14). While the species richness and evenness (J), were not significantly different between estuaries, the mean diversity (H) was higher in Mullica River-Great Bay compared to Toms River-Barnegat Bay (mean 2.41 compared to 2.12; T-Test: $t=2.45$ $p<0.05$). The species richness, evenness (J), and diversity (H) did not differ based on salinity and the species richness and diversity (H) did not differ between seasons. However, species evenness (J), was higher in the spring (April-May) compared to summer (June-September) (ANOVA Tukey's post-hoc; $F=5.75$ $p<0.01$).

Over the study period, the most common species of algae were diatoms (Bacillariophyceae), dinoflagellates (Dinophyceae), cryptophytes (Cryptophyceae), chlorophytes (Chlorophyceae), euglenoids (Euglenophyceae), and cyanobacteria (Cyanophyceae). Bacillariophyceae was almost always the dominant class of algae, although this varied between sites and seasons (Fig. 4.15). The highest percent Cyanophyceae occurred in the fall at the freshwater sites of Toms River-Barnegat Bay. Many different harmful algal bloom species occurred during the 2022 sampling period and were shown to be present within the two estuaries but never dominated the community and remained at low quantities during sampling periods (Table 1).

The influence of environmental variables on the abundance of different phytoplankton classes was explored using Redundancy analyses (RDA). In samples at freshwater sites (Salinity < 10 ppt; Fig. 4.16A), the first two RDA axes explained 30.17% of the variation in the phytoplankton community composition across samples and months, and the model was statistically significant (RDA; $n=29$ $F=1.58$ $p = 0.032$). RDA1 was mainly influenced by total phenolic content, nitrogen levels ($\text{NO}_2^- + \text{NO}_3^- + \text{NH}_4^+$), and temperature, and RDA2 had high scores for pH and nitrogen levels ($\text{NO}_2^- + \text{NO}_3^- + \text{NH}_4^+$) (Fig. 4.16A).

In samples at mid-salinity sites (Salinity $10 < x < 25$ ppt; Fig. 4.16B), the first two RDA axes explained 38.06% of the variation in the phytoplankton community composition across samples and months, and the model was statistically significant (RDA; $n=29$ $F=2.25$ $p = 0.002$). RDA1 was mainly influenced by temperature, pH, and PO_4^{3-} levels, and RDA2 had high scores for temperature, turbidity, and nitrogen levels ($\text{NO}_2^- + \text{NO}_3^- + \text{NH}_4^+$) (Fig. 4.16B).

In samples at high-salinity sites (Salinity $x > 25$ ppt; Fig. 4.16C), the first two RDA axes explained 23.21% of the variation in the phytoplankton community composition across samples and months. RDA1 was mainly influenced by nitrogen levels ($\text{NO}_2^- + \text{NO}_3^- + \text{NH}_4^+$) and turbidity, and RDA2 had high scores for temperature, and nitrogen levels ($\text{NO}_2^- + \text{NO}_3^- + \text{NH}_4^+$) (Fig. 4.16C). However, this model was not statistically significant (RDA; $n=37$ $F=1.51$ $p = 0.072$).

4.4 Discussion

4.4.1 Trends in Total Phenolic Content and Dissolved Organic Carbon

Few studies have measured total phenolic content in the water column (Aké-Castillo and Vázquez, 2008; Peng et al., 2022; Takeda et al., 2013; Thoss et al.,

2002). Of those few studies, the concentrations found throughout the two estuaries in New Jersey were an order of magnitude higher at times compared to those. For instance, Peng et al. 2022 measured between 25 µg/L-115 µg/L GAE eq total phenolic content in a lake site with low submerged aquatic vegetation compared to one with high biomass coverage (Peng et al., 2022). In contrast, the highest total phenolic content found in this study was 1924 µg/L GAE eq at site 12 in April 2022, while the lowest was 16 µg/L GAE eq at site 7 in October 2021 (Fig. 4.11). These differences in TPC concentrations could be what is expected in lakes versus estuaries or indicative of the unique ecosystem near the New Jersey Pinelands. The ratio of TPC to DOC ($100 \times (\text{total phenol content})_{\text{mg/L}} / (\text{DOC})_{\text{mg/L}}$) found in this study, 5-27%, was similar to that measured in controlled laboratory studies using tanks with artificial seawater and degrading *Sargassum natans*, 5-18%, by Powers et al. 2019 (Powers et al., 2019). These measurements suggest that the amount and ratio of TPC in the waterways of the NJ Pineland is high compared to other regions.

One of the hypotheses of this study was that TPC would consistently be higher in Mullica River-Great Bay compared to Toms River-Barneget Bay due to the difference in land cover along these two estuaries. This was not the case within the years sampled, as the two estuaries did not significantly differ in TPC concentrations even when considering location along the freshwater-marine continuum and season. This is likely due to both estuarine rivers located within the New Jersey Pinelands and the high discharge but low flushing times along Toms River-Barneget Bay (Defne and Ganju, 2015). The strong negative relationship between TPC and salinity suggests that most phenolic compounds along these two estuaries come from

upstream and the positive correlation to weekly rainfall suggests most of the TPC is flushed into the rivers from terrestrial sources (Fig. 4.10). However, the conservative mixing lines showed there are higher additions at mid-salinities during the summer at Mullica River-Great Bay. These are likely due to higher inputs of organic matter from marshes and submerged vegetation during this time (Fig. 4.4). The input of organic matter from these sources is likely higher in Mullica River-Great Bay compared to Toms River-Barnegat Bay due to the salt marsh islands in the pristine estuary compared to Barnegat Bay (Fig. 4.1).

While Peng et al. 2022 found that TPC was highest in autumn, this study found the highest TPC in the spring. The high TPC concentrations in the spring are likely due at least partially to high discharge as there was a significant positive correlation between the two variables (Fig. 4.10) and Sherrell & Ross 1999, also found the spring season to have the highest trace metals in Mullica River due to the high discharge during the winter/spring (Sherrell and Ross, 1999). Other studies have found higher terrestrial dissolved organic matter in the spring and lower in the summer due to differences in discharge (Paczkowska et al., 2020, 2019). However, there were also higher overall TPC concentrations in 2022 compared to 2021 even though the discharge was lower in 2022 compared to 2021 and so there are more variables impacting TPC than discharge alone (Fig. 4.9). Further research is needed to fully understand the seasonal differences in TPC concentrations along these two estuaries.

4.4.2 Environmental drivers of Chlorophyll a

The mean chlorophyll a (Chl a) was higher in Toms River-Barnegat Bay compared to Mullica River-Great Bay and highest in the summer months when

temperature was at its peak. These results are consistent with what was expected as higher urban land cover usually leads to higher nutrient runoff which increases algae production, and the higher temperatures in summer tend to be ideal conditions for more species of algae. In addition, the high flushing rates and low residence times in Mullica River-Great Bay could lead to lower phytoplankton biomass and less harmful algal blooms compared to Toms River-Barnegat Bay as calm waters usually lead to higher phytoplankton abundance (Paudel et al., 2016).

The upper estuary was characterized by low Chl a which differs from other estuaries throughout the world where the upper estuary can have the highest phytoplankton cell abundance and Chl a (Santos et al., 2022). This could be due to the high TPC, low light attenuation, and low pH in these rivers. TPC and Chl a were negatively correlated, although the statistics suggested that TPC did not explain much of the variance in Chl a (Fig. 4.10). Paudel et al. 2016 studied the direct and indirect effects of water quality parameters on spatial distribution of phytoplankton in the northern and southern Barnegat Bay (Paudel et al., 2016). The authors noted Toms River transports high amounts of organic carbon from the watershed to Barnegat Bay and suggested higher inflow in the northern Barnegat Bay from Toms River likely caused high riverine nitrogen input, and resulted in higher Chl a in this area. In this study, high levels of NO_x did seem to be transported through Toms River to Barnegat Bay and were positively correlated to discharge, rainfall, and TPC (Fig. 4.10). Alternatively, NO_x was relatively low throughout Mullica River despite similar levels of TPC to Toms River. This is likely due to the differences in land cover and sources of nitrogen (Fig. 4.8).

The environmental factors that most predicted Chl a varied based on the estuarine region. For samples taken at sites below 10 ppt, observed Chl a was most predicted by pH and NO_x . For samples taken at sites between 10 and 25 ppt, observed Chl a was most predicted by TPC, NO_x , NH_4^+ , DOC, and turbidity. For samples taken at sites greater than 25 ppt, observed Chl a was most predicted by TPC, temperature, NH_4^+ , DOC, PO_4^{3-} , and turbidity. Although these three models demonstrated strong correlations between observed and predicted Chl a (Fig. 4.13), assumptions should not be made that these variables cause increases/decreases in Chl a. For instance, nutrient concentrations may be low during higher concentrations of Chl a due to nutrient uptake by the phytoplankton.

4.4.3 Phytoplankton community composition

Upstream discharge is well known to influence phytoplankton production and species composition in estuarine waters. In addition, phytoplankton community characteristics such as species richness, diversity, and evenness can be impacted by abiotic factors. Species diversity takes into account both the number of taxa at a site (species richness) and how much of each taxa is present (species evenness).

Phytoplankton diversity can be viewed as a proxy of estuarine health and has been shown to be impacted by plant cover. According to a national dataset, deciduous forests are the type of land cover associated with the lowest frequency of lake algal blooms (Marion et al., 2017). For instance, Aké-Castillo and Vázquez (2008) found phytoplankton diversity to decrease with increasing mangrove disturbance (Aké-Castillo and Vázquez, 2008). Similarly, Phytoplankton diversity was higher in Mullica River-Great Bay compared to Toms River-Barneget Bay and this could be

due to land cover and increased wetland coverage in Mullica River-Great Bay estuary.

The environmental drivers that best explained the phytoplankton community class differed based on the estuarine region (Fig. 4.16). At the freshwater sites, Cyanophyceae was positively correlated to inorganic nitrogen levels ($\text{NO}_2^- + \text{NO}_3^- + \text{NH}_4^+$), Cryptophyceae was positively correlated to pH and temperature, and Bacillariophyceae and Prymnesiaceae were positively correlated to TPC (Fig. 4.17A). Cyanophyceae was positively correlated to inorganic nitrogen levels ($\text{NO}_2^- + \text{NO}_3^- + \text{NH}_4^+$) at mid-salinity samples as well. Ren (2015) also found high cyanobacteria in the summer in Barnegat Bay to be related to total nitrogen and discharge. It is interesting that Cyanophyceae was positively correlated to inorganic nitrogen but negatively correlated to TPC at freshwater sites. This demonstrates the benefits of having both estuaries in the RDA plots as Toms River and Mullica River had the same amount of TPC, but Toms River had more nitrogen allowing for separation between correlated environmental variables. Still, further controlled laboratory studies and additional field studies with additional estuaries of varying TPC concentrations would further our understanding of how phytoplankton communities are impacted by river runoff and TPC input, especially downstream of the river.

At mid-salinity sites, Dinophyceae was positively correlated to PO_4^{3-} levels and temperature, and pennate Bacillariophyceae were positively correlated to turbidity and negatively correlated to nitrogen levels (Fig. 4.16A). Like at mid-salinity sites, Dinophyceae were positively correlated to temperature and Bacillariophyceae were positively correlated to turbidity at high salinity sites. These

results are like other studies demonstrating diatoms benefit from movement and dinoflagellates peak when temperatures are high. In addition, the phytoplankton community composition found at the mid and high salinity sites were similar to those found by Ren et al. 2017 (Ren et al., 2017).

The low concentrations of HAB species found during this study limited the ability to model how total phenolic content and other environmental factors impact HABs. Although the abundance of HAB species was relatively low through 2022, this study showed their presence in both estuaries, demonstrating toxin-producing blooms could occur in the future (Table 1). Due to the choice of phytoplankton identification and enumeration, only cells above 10 μm in size were counted, removing the ability to identify harmful algal species like *Synechococcus* and *Aureococcus anophagefferens*, which commonly cause blooms in Barnegat Bay. Studies over multiple years in these two estuaries and expanding to include smaller phytoplankton species, could lead to more conclusions on environmental factors that trigger a bloom and what effect TPC concentrations have on toxin-producing algae. In addition, concluding the impact of environmental drivers on phytoplankton community composition over one year of samples may inevitably exhibit uncertainty and this study should be expanded to confirm conclusions.

4.5 Conclusion

Reports from federal agencies recognize that estuaries are threatened by eutrophication and the resultant HABs, with a key influencing factor being land cover and practices. Land cover type changes the organic carbon composition in nearby aquatic environments, which in turn impacts other variables including pH, nutrients, light levels, and the presence of natural vegetative algicides, like phenolic

compounds. This study focused on whether phenolic content released into the water from terrestrial and aquatic plants impacts phytoplankton community composition and the occurrence of HABs. The high concentrations of TPC along both estuaries in this study made comparisons looking at the impact of this environmental variable on phytoplankton communities and HABs difficult. However, Chl a was found to be negatively correlated to TPC. In addition, RDA plots showed that the major toxin-producing algal class, Cyanophyceae, was positively correlated with nitrogen loads and negatively correlated with TPC at freshwater sites. These conclusions suggest that land cover that causes high TPC in estuaries could reduce the occurrence of HABs. Future work should include controlled laboratory studies looking at the growth of toxic algal species in high TPC water from New Jersey. This research will contribute to our knowledge of whether the type of plants and foliage matter when considering how land cover impacts harmful algal blooms. Some geographical regions will clearly be more affected by HABs compared to others. Continued research is needed to help understand the areas and habitats that are most susceptible or resilient to HABs based on water quality variables and how this will change in the future.

4.6 Tables

Table 4.1. Potential toxin producing species identified, salinity range, and month seen during microscopic cell counts in New Jersey in 2022. ATX = anatoxin-a; PbTx=brevetoxin; CYN=cylindrospermopsin; DA=domoic acid; DST=diarrhetic shellfish toxins (dinophysistoxins, okadaic acid, pectenotoxins); ICX=ichthyotoxin; KmTX=karlotoxin; MC=microcystin; NOD=nodularin; STX=saxitoxin. Genus with “?” require genetic sequencing confirmation.

Genus	Group	Potential Toxins/Effects	Salinity Range			Month							
			Low	Moderate	High	April	May	June	July	August	September	October	November
Oscillatoria	Cyanobacteria	ATX, CYN, MC, STX	X								X		
Snowella	Cyanobacteria	MC	X	X					X				
Microcystis	Cyanobacteria	MC	X			X		X	X	X			
Nodularia	Cyanobacteria	NOD	X										X
Cylindrospermopsis	Cyanobacteria	CYN	X					X	X	X		X	X
Planktothrix	Cyanobacteria	ATX, MC	X	X						X			
Aphanizomenon	Cyanobacteria	ATX, CYN, STX	X	X	X				X				
Anabaena	Cyanobacteria	ATX,CYN, MC, STX	X	X						X		X	X
Azadinium?	Dinoflagellate	AZT	X	X	X			X	X	X	X	X	X
Gonyaulax spinifera	Dinoflagellate	STX, YTX			X					X	X	X	
Karlodinium?	Dinoflagellate	KmTx		X	X	X	X	X	X	X	X	X	X
Prorocentrum	Dinoflagellate	DST		X	X	X	X	X	X	X	X	X	X
Lingulodinium	Dinoflagellate	YTX			X	X	X	X	X	X			X
Alexandrium	Dinoflagellate	STX		X	X	X	X	X	X	X	X	X	X
Dinophysis	Dinoflagellate	DST		X	X			X					
Karenia	Dinoflagellate	PbTx		X	X	X			X	X	X	X	
Pseudonitzschia	Diatom	DA			X		X			X	X	X	X
Heterosigma	Raphidophyte	ICX			X					X			

4.7 Figures

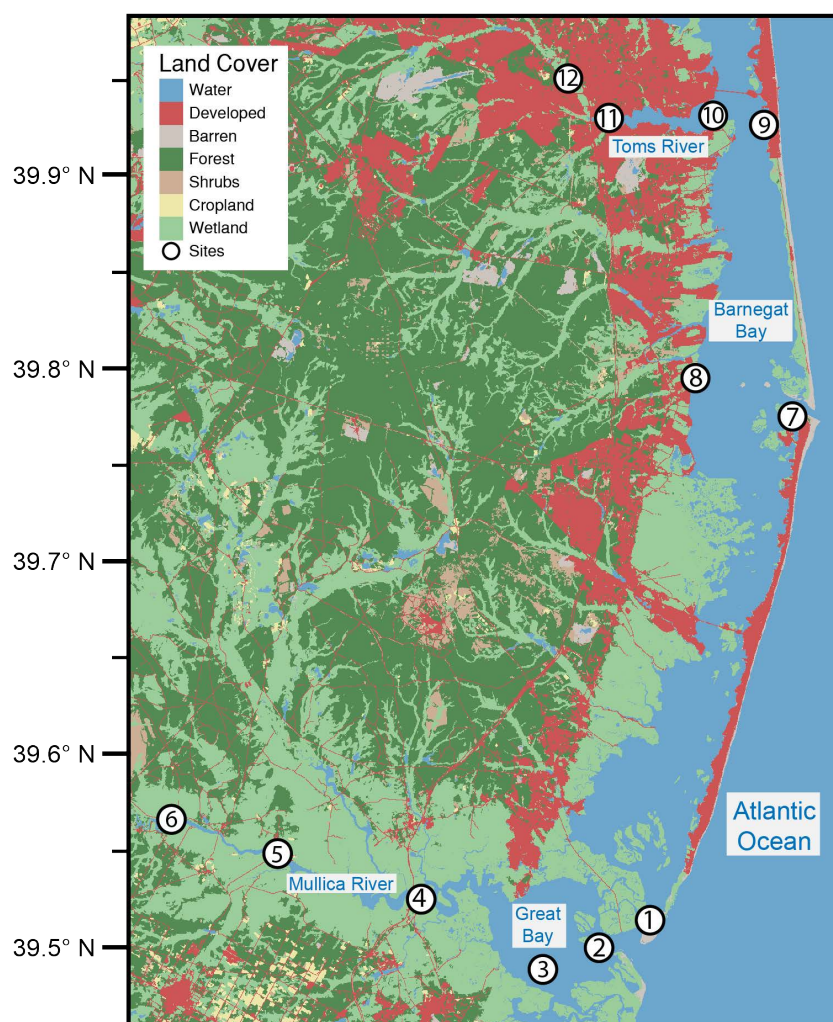


Fig. 4.1. Map of the 12 field sampling sites (circles) in two New Jersey estuaries. Land Cover data is from National Land Cover Database (2016).

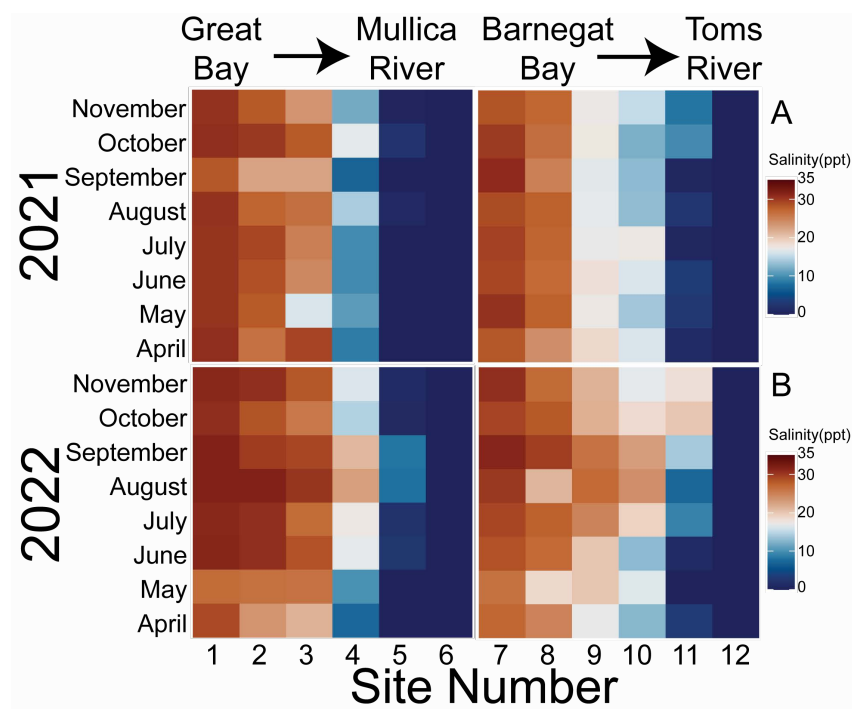


Fig. 4.2. Salinity (ppt) measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.

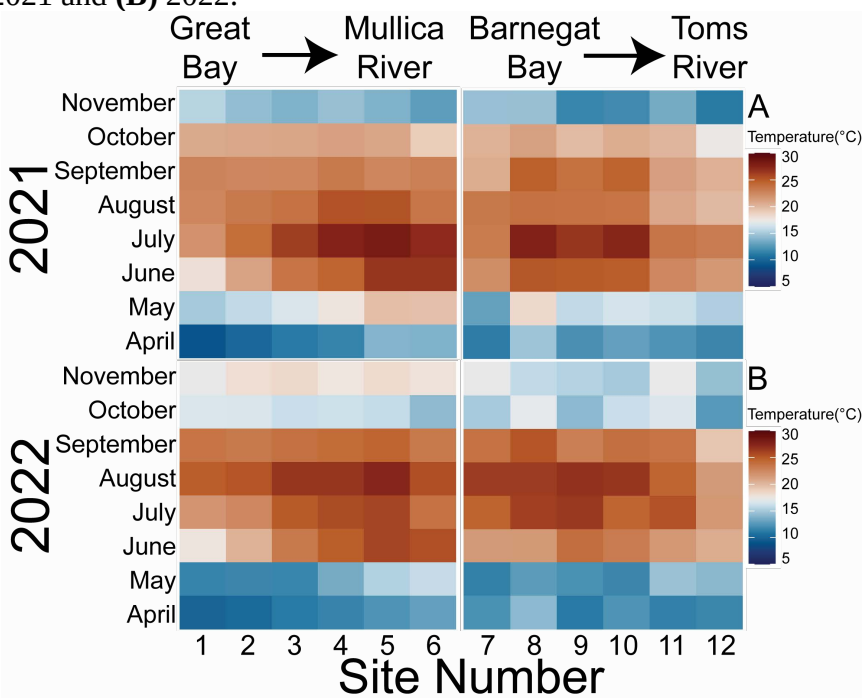


Fig. 4.3. Temperature (°C) measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.

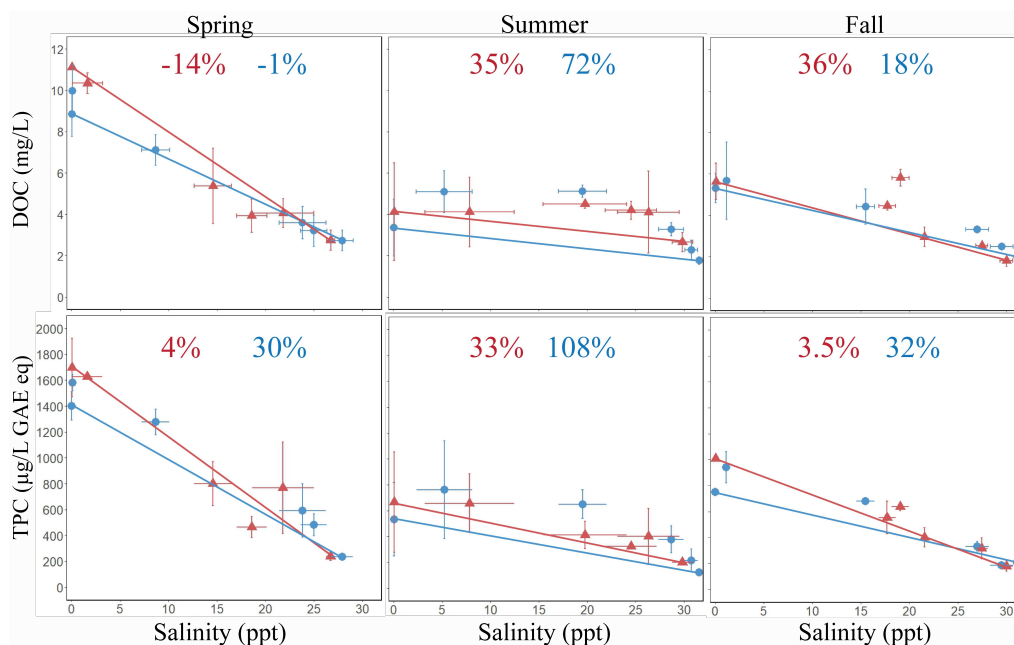


Fig. 4.4. Mixing diagrams for (A) dissolved organic carbon (DOC) mg/L, (B) total phenolic content (TPC) µg/L GAE eq, over salinity in Toms River-Barnegat Bay (red▲) and Mullica River-Great Bay (blue◆) estuaries from Spring (April-May), Summer (June-September), and Fall (October-November), 2022. The straight lines denote the theoretical conservative mixing line between endmembers. Numbers above lines denote average percent deviation of measured values from the conservative mixing line (see Methods sections 4.2.8).

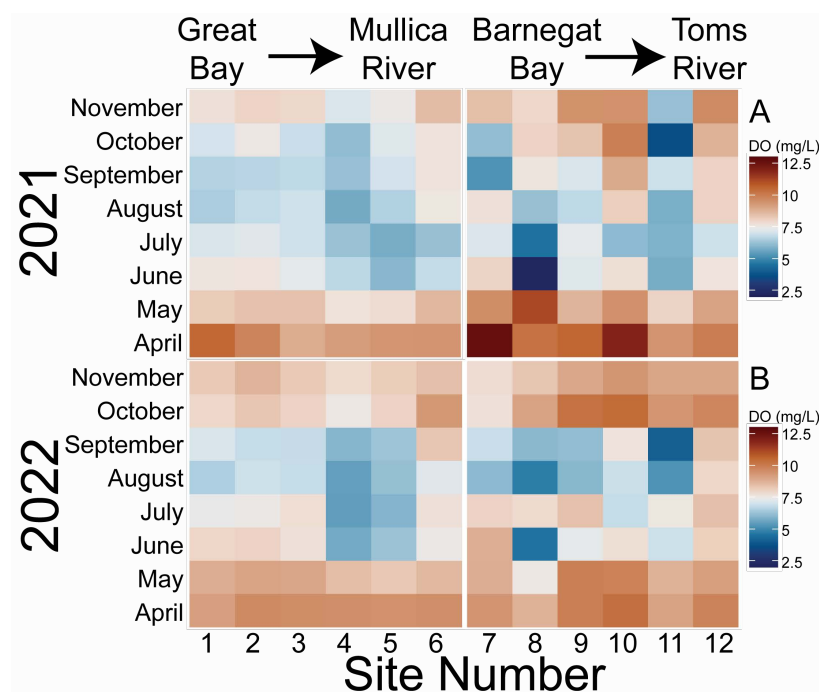


Fig. 4.5. Dissolved Oxygen (DO mg/L) measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.

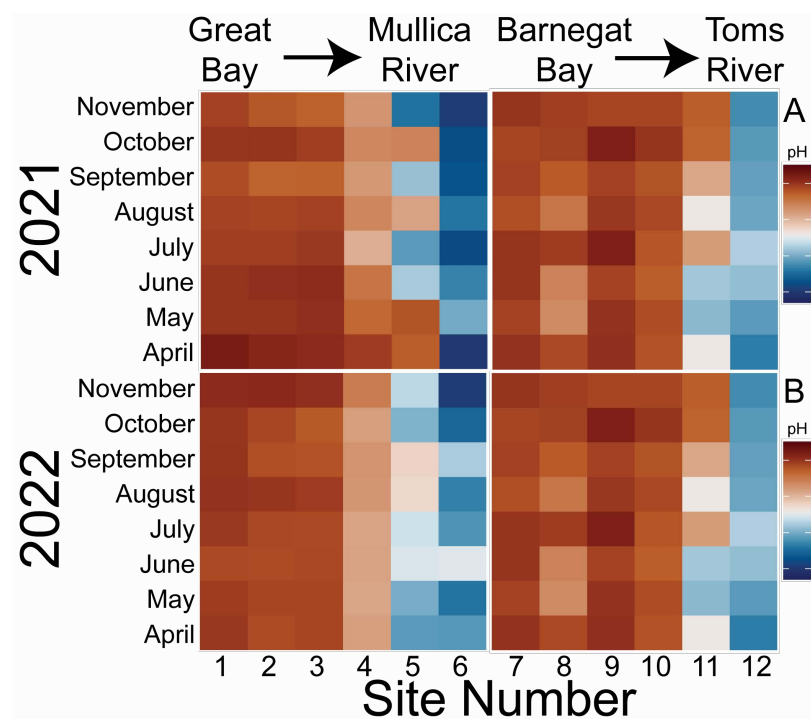


Fig. 4.6. pH measured along two New Jersey estuaries during sampling in **(A)** 2021 and **(B)** 2022.

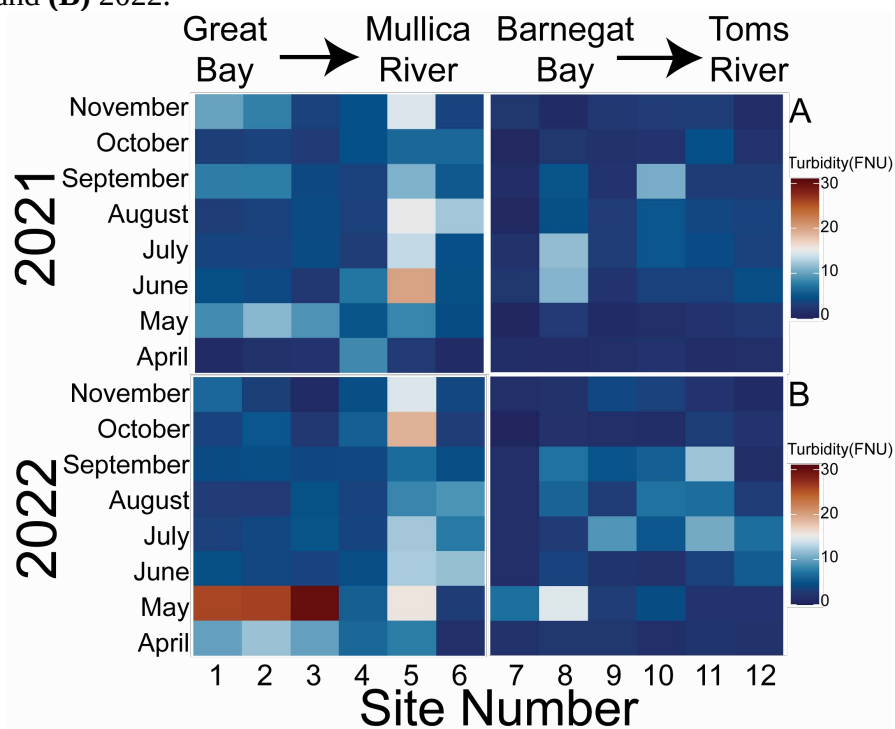


Fig. 4.7. Turbidity (FNU) measured along two New Jersey estuaries during sampling in **(A)** 2021 and **(B)** 2022.

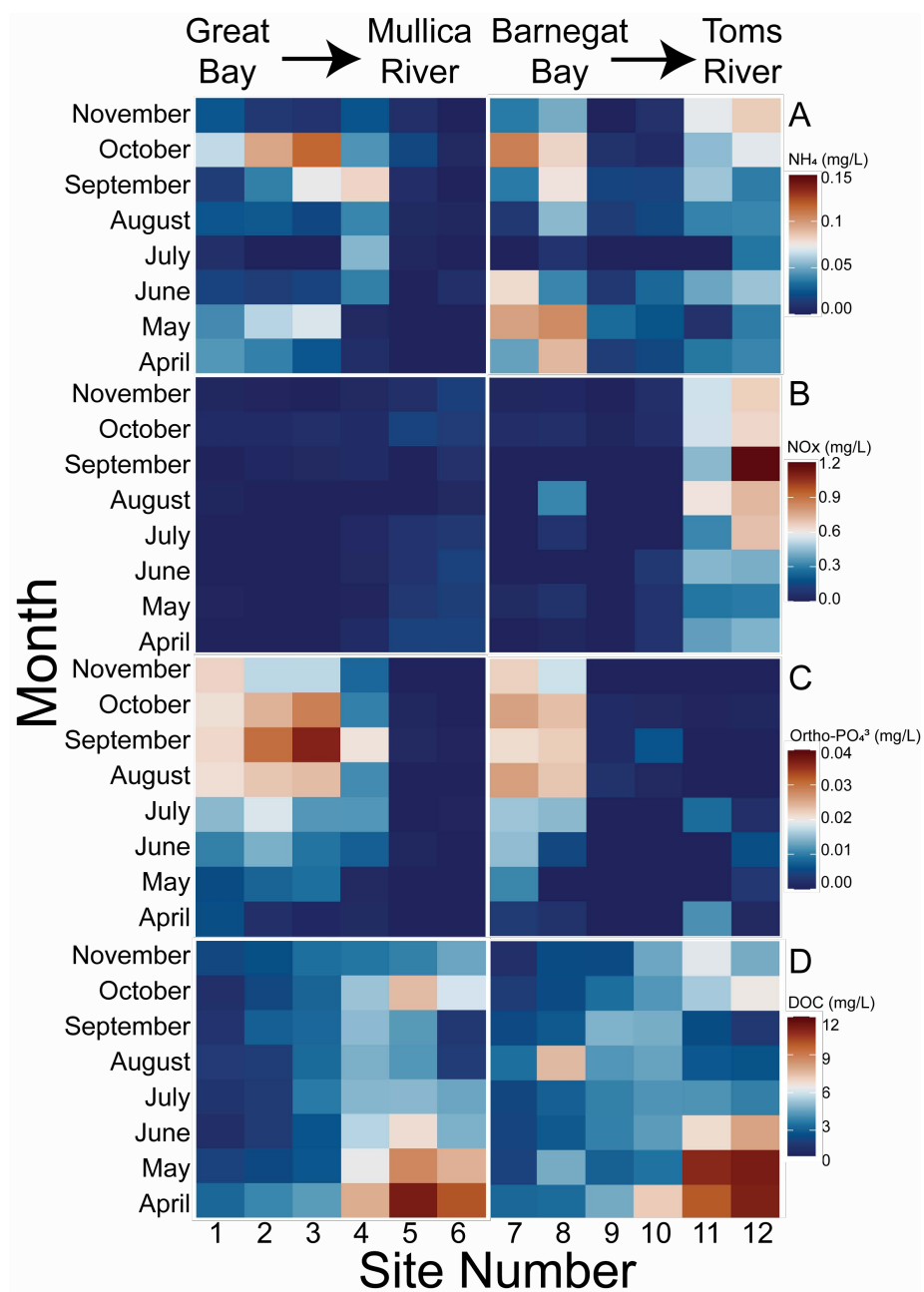


Fig. 4.8. Nutrients and Dissolved Organic Carbon (DOC) measured along two New Jersey estuaries during sampling in 2022. (A) NH_4^+ (mg/L); (B) NO_x (mg/L); (C) Ortho- PO_4^{3-} (mg/L); (D) DOC (mg/L).

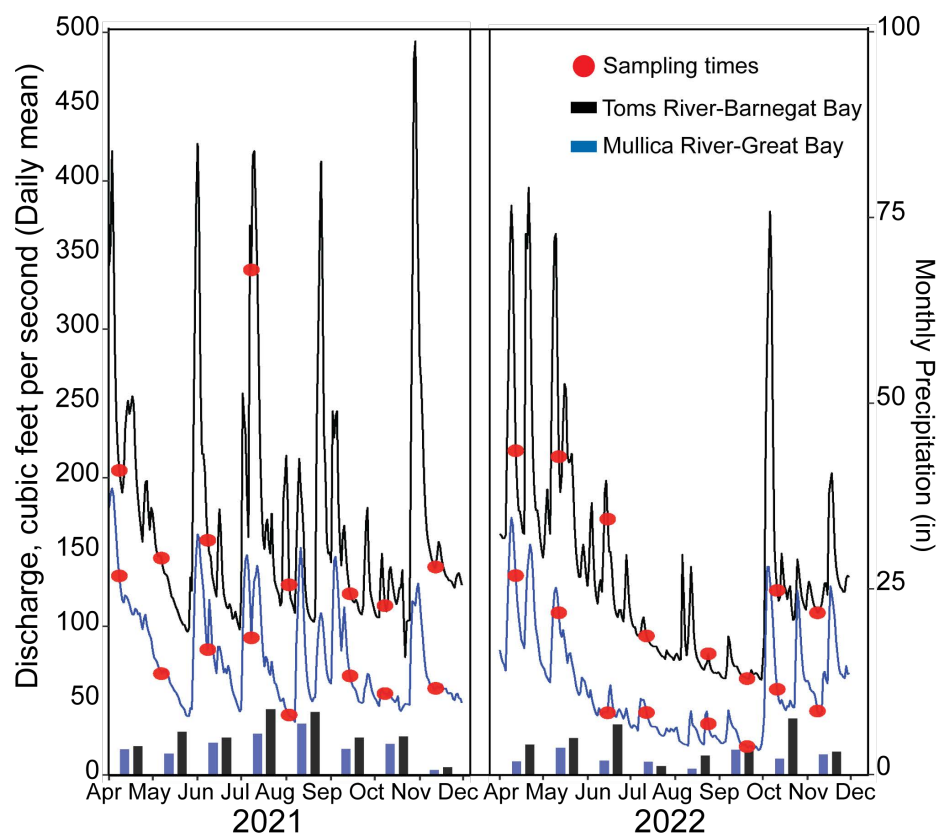


Fig. 4.9. Daily mean discharge (feet³/sec) in lines and monthly precipitation (in) in bars near Toms River-Barnegat Bay (black) and Mullica River-Great Bay (blue) during the sampling period in 2021 and 2022.

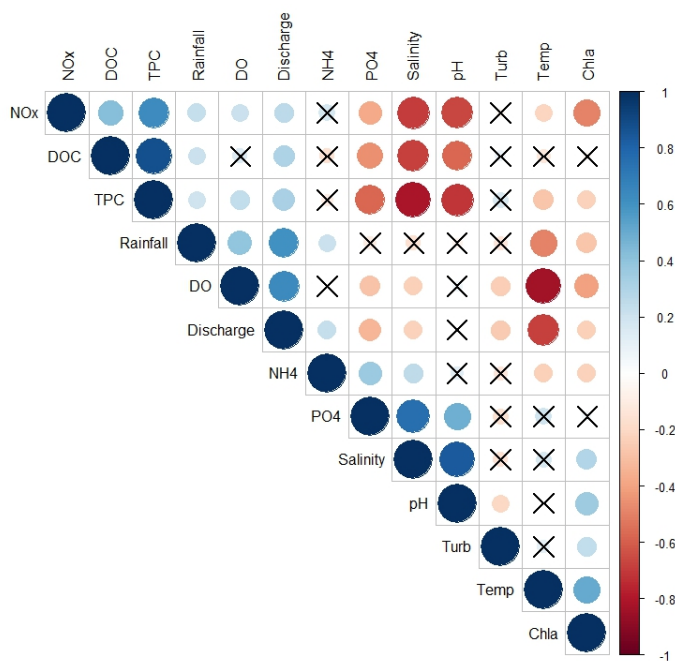


Fig. 4.10. Correlation matrix between environmental parameters collected along two New Jersey estuaries during sampling in 2022. Environmental parameters

include: Ortho-phosphate (PO₄), Salinity, pH, Temperature (Temp), Chlorophyll a (Chl a), Dissolved oxygen (DO), Discharge, Nitrite+Nitrate (NO_x), Dissolved organic carbon (DOC), Total phenolic content (TPC), Turbidity (Turb), Ammonium (NH₄), and rainfall the week before collection (Rainfall). Xs are over insignificant correlations according to spearman's rank correlation test.

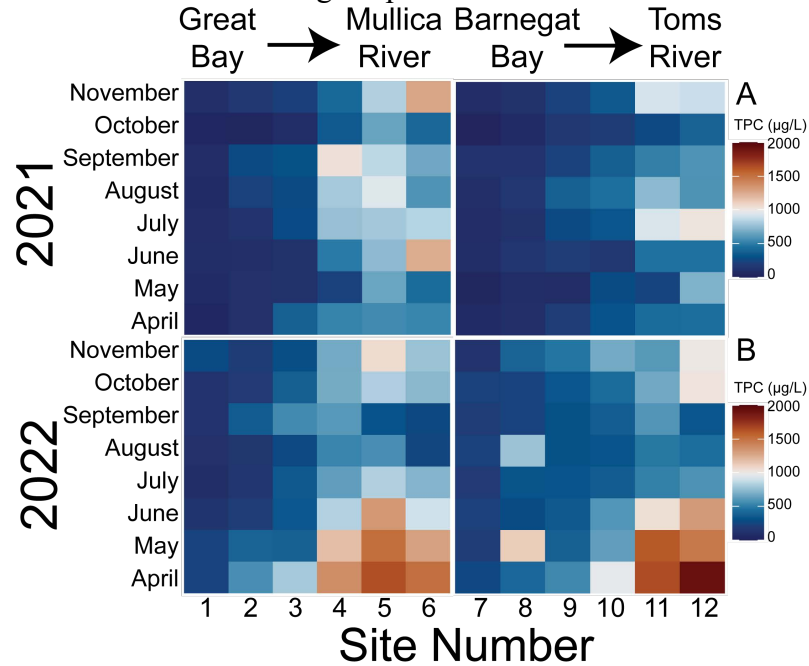


Fig. 4.11. Total Phenolic Content (µg/L) measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.

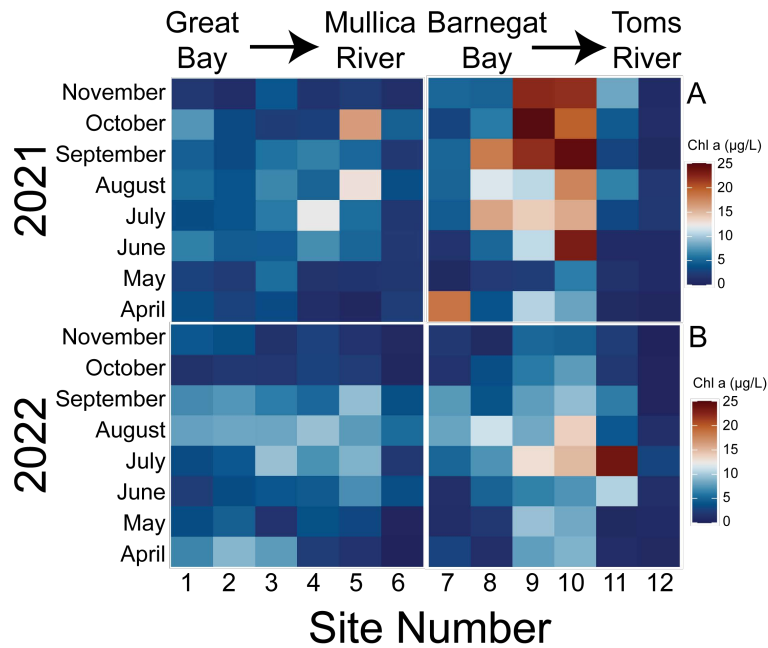


Fig. 4.12. Chlorophyll a (µg/L) measured along two New Jersey estuaries during sampling in (A) 2021 and (B) 2022.

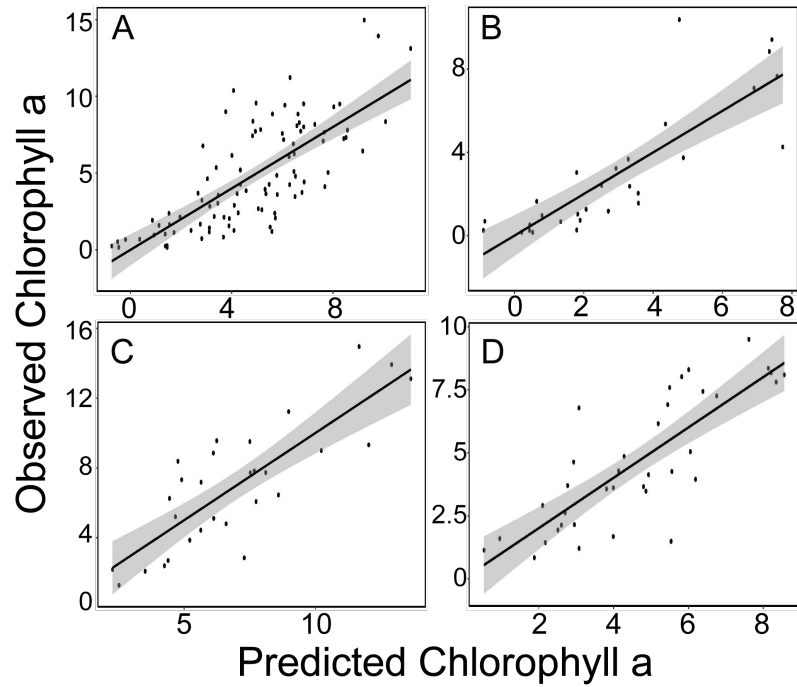


Fig. 4.13. Observed vs predicted chlorophyll a concentrations ($\mu\text{g/L}$) by multivariate regression models for samples collected in 2022. **(A)** All samples, **(B)** freshwater <10 ppt sample model, **(C)** mid-salinity $10 < x < 25$ ppt sample model, **(D)** high-salinity >25 ppt sample model. Straight line represents the ideal model prediction with standard error in gray.

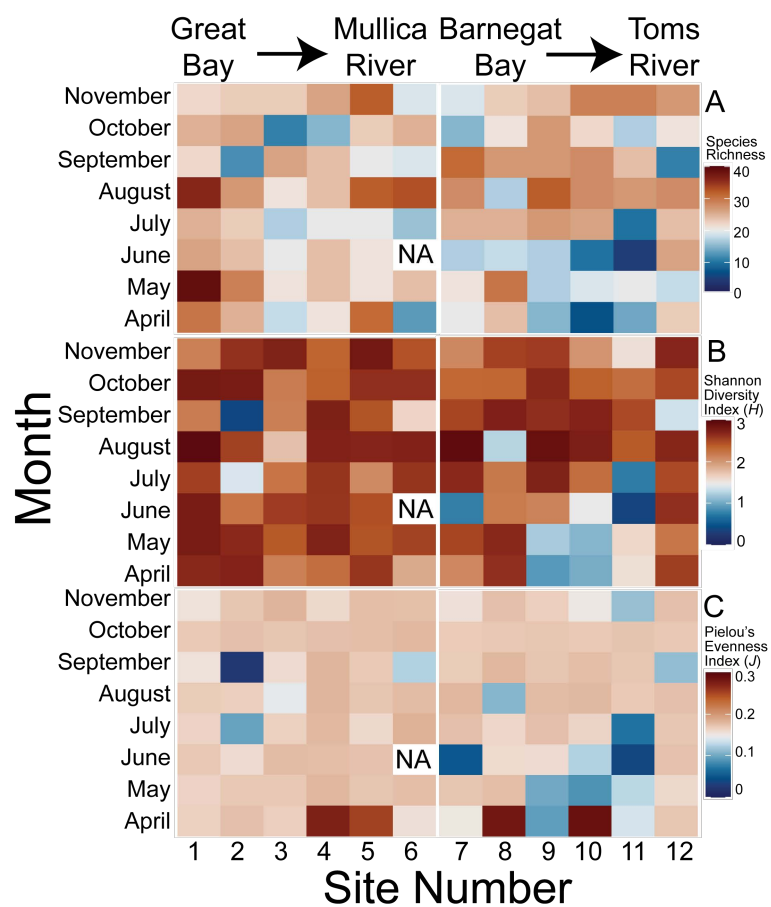


Fig. 4.14. Phytoplankton species richness, diversity, and evenness along two New Jersey estuaries during sampling in 2022. **(A)** Species Richness; **(B)** Shannon Diversity Index (H); **(C)** Pielou's Evenness Index (J). No data available in June 2022 at site 6.

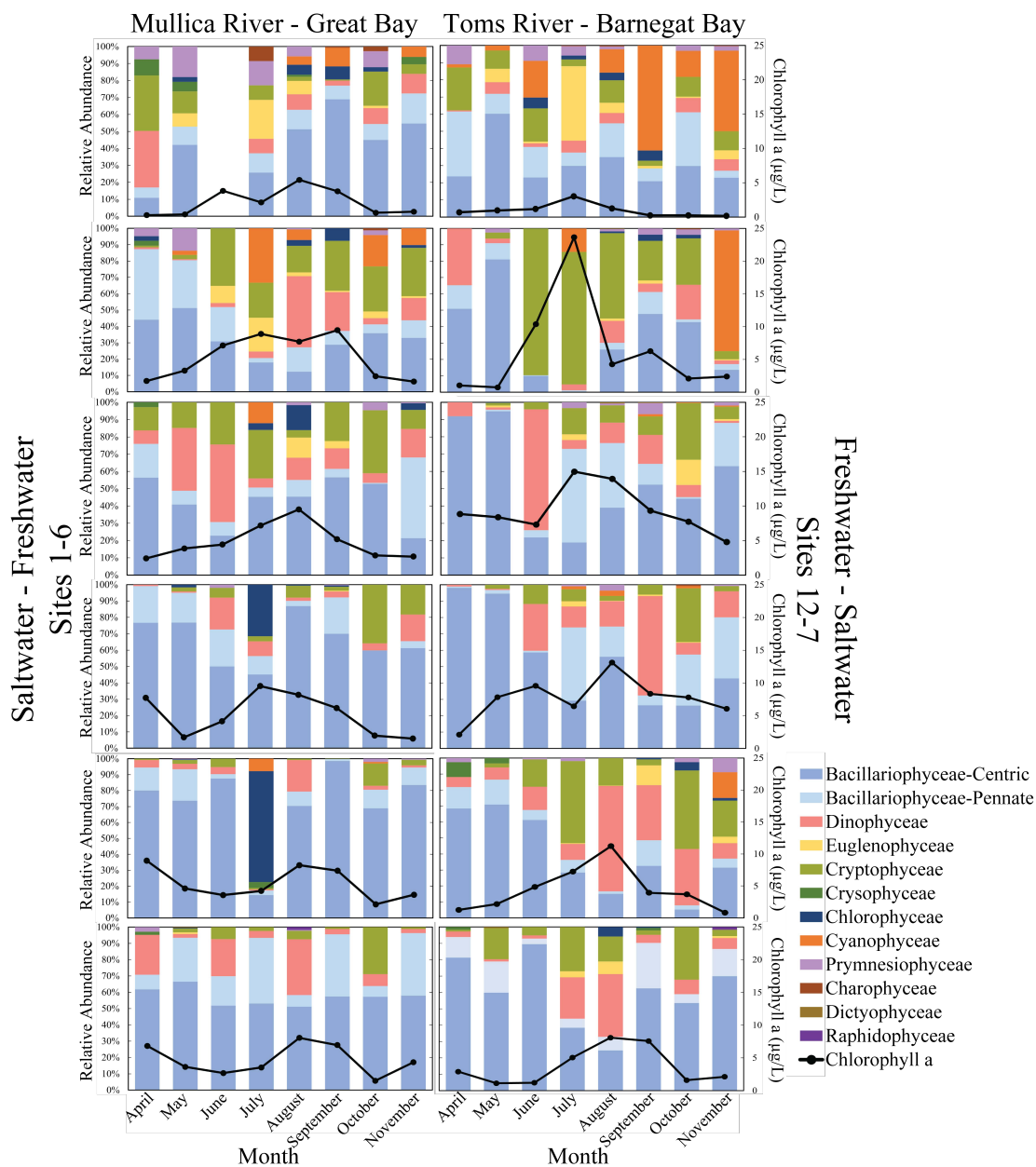


Fig. 4.15. Relative abundance of different phytoplankton groups (colored bars) and measured Chlorophyll a (dots with connecting line) for each sampling month in the different sites. No data available in June 2022 at site 6.

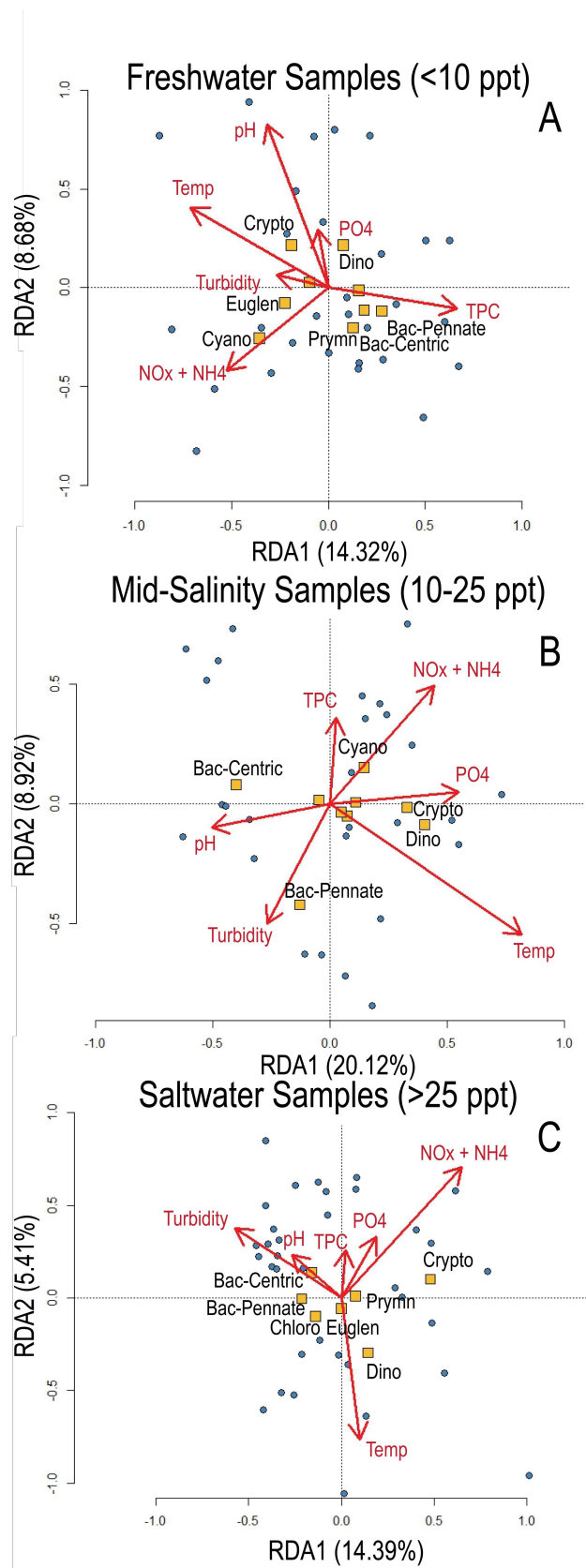


Fig. 4.16. Redundancy analysis (RDA) of phytoplankton communities and environmental drivers in the surface water along **(A)** freshwater sites (<10 ppt), **(B)** mid-salinity sites (10<x<25 ppt), and **(C)** saltwater sites (>25 ppt), of two New Jersey estuaries in 2022. Each sample is represented by a blue circle. Phytoplankton communities are separated into class represented in yellow squares (Bac-Centric: centric Bacillariophyceae, Bac-Pennate: pennate Bacillariophyceae, Dino: Dinophyceae, Crypto: Cryptophyceae, Chloro: Chlorophyceae, Prymn: prymnesiaceae, Euglen: Euglenophyceae). Environmental variables represented in red include turbidity, pH, phosphate (PO₄), nitrite+nitrate+ammonium (NO_x + NH₄), total phenolic content (TPC), and temperature (Temp).

Chapter 5: Summary

5.1 General findings and expansion of previous knowledge

The research conducted for this dissertation sets out to understand how allelochemicals in purified plant materials and natural allelochemicals in an estuarine system, impact phytoplankton community composition. The research looked at the impact of brewer's spent grain (BSG) on algal species which was based on the previous knowledge and common practice of using barley straw bales to reduce cyanobacteria blooms (Iredale et al., 2012; Murray, 2009; Sellner et al., 2014). Due to the various drawbacks in using barley straw to control Harmful Algal Blooms (HABs), Chapter 2 of this dissertation sought to start answering the question, could the waste product: BSG, be a cheaper, more effective, control method? This is the first study to research the effects of the waste product BSG on algal species for utilization as a management strategy (Chapter 2).

Although there have been many laboratory studies researching the effect of polyphenols from different terrestrial and aquatic plants on phytoplankton, few studies have expanded this area of research to explore the impact of polyphenols in the field (Gross and Sütfield, 1994; Peng et al., 2022; Stoler and Relyea, 2016). Allelochemical interactions, even among terrestrial plants and crops, have been difficult to prove in the field (Willis, 2007). New Jersey is home to the New Jersey pinelands where the unique pitch pine, *Pinus rigida*, grows. *P. rigida* is full of phenolic compounds and the water flowing from the New Jersey Pineland National Reserve is stained brown from decaying vegetation (Kim et al., 2013). Few harmful algal species and blooms have occurred and/or researched along the coasts of New

Jersey (Heddendorf, 2008). This led to the question: Do the pinelands protect New Jersey estuaries from HABs or are there HABs but no one regularly monitoring phytoplankton communities? To our knowledge, these are the first studies to research how phenolic compounds impact phytoplankton communities in estuarine environments (Chapters 3&4).

Below are the major findings from this dissertation. Overall, the work performed in this dissertation will help better identify and understand different HAB control and prevention techniques using plant allelochemicals.

5.1.1 Brewer's Spent Grain impact on phytoplankton species

250 mg/L brewer's spent grain extract inhibits the growth of freshwater and marine toxin-producing species (*M. aeruginosa* and *K. brevis*), while not impacting the diatom and chlorophyte tested (*S. obliquus* and *P. tricornutum*).

These results are consistent with other studies which found barley straw to have a higher impact on cyanobacteria, compared to diatoms and chlorophytes. However, studies have also found that the response to barley straw extract varied within classes and species of algae, and thus generalizations of the impact of BSG on classes of algae cannot be fully concluded from these laboratory experiments (El-Moneem et al., 2012; Ferrier et al., 2005; Terlizzi et al., 2002). These laboratory experiments are also the first to expose these algal strains to Sargassum, which at the doses tested, did not have an impact on the cyanobacteria, diatom, and chlorophyte. The needed dose of 250 mg/L BSG is difficult to compare to other studies as described in Chapter 2. However, the smaller dosage needed compared to the well-studied barley straw

suggests there are a high number of allelochemicals in BSG that could be utilized for the control of cyanobacteria in lakes.

Inhibition of *K. brevis* is likely due to increased bacteria growth or changes in the microbial community rather than allelopathic compounds from the brewer's spent grain. Even with only a few days of controlling bacteria growth, the use of antibiotics had a large impact on whether BSG extract reduced the growth of *K. brevis* (Fig. 2.7A). These results suggest that the growth inhibition of *K. brevis* caused by BSG extract is not fully chemical but could be due to either increased bacteria in the cultures or fueling of a specific type of bacteria.

250 mg/L brewer's spent grain extract reduced a cyanobacteria bloom in lake water and shifted the phytoplankton community to primarily chlorophytes. The concentration of *C. raciborskii* and the pigment zeaxanthin declined during a six-day microcosm study, while chlorophytes and the pigment chlorophyll b increased compared to the control treatment (Fig. 2.11 & 2.13). However, 25 mg/L BSG did not have this effect on the phytoplankton community. These results emphasize the importance of dosage in using BSG extract as a mitigation technique in the field, as a small dose could have the opposite effect of the goal, and the added nutrients with this control technique is not ideal for longer term HAB prevention. This shows the downfalls of using whole BSG in managing lakes due to the high ammonium and phosphate levels in BSG but shows promise in using isolated BSG compounds for mitigating toxin-producing cyanobacteria.

5.1.2 Spatial and temporal trends of phycotoxins through two estuaries in NJ

Seven phycotoxins were detected along Mullica River-Great Bay and Toms River-Barnegat Bay using Solid Phase Adsorption Toxin Tracking (SPATT): AZA1, AZA2, OA, DTX1, PTX2, YTX, and GDA. Many of the SPATT samples tested positive for multiple phycotoxins during the same period.

To date, there have been no publications on phycotoxin presence nor concentrations in NJ estuarine systems. Although *Dinophysis* spp. And *Prorocentrum* spp., potential producers of OA, DTX1, and PTX2, are regularly present in grab samples from these two estuaries (<https://www.nj.gov/dep/bmw/phytoplankton.htm>). As the leading state in many shellfisheries, the presence of these phycotoxins warrants further monitoring, especially as the spatial and temporal trends of many of the phycotoxins found in this study coincide with shellfish collection and distribution.

SPATT bags deployed along Mullica River-Great Bay and Toms River-Barnegat Bay detected multiple phycotoxins with no known producer in NJ, including: AZA1, AZA2, GDA, and YTX. GDA, YTX, AZA1, and AZA2 were all primarily detected from July-November in 2022 (Fig 3.5-3.9). This is the first publication of YTX on the East Coast of the United States and the farthest north that GDA, AZA1, and AZA2 has been detected on the East Coast of the United States. Although, YTX was only detected in 2022 at the two inlets, and so it could be that the YTX producing species was transported with currents from another region and does not regularly bloom in NJ. It is unknown what species of algae produce these phycotoxins in NJ, which causes difficulties in monitoring and managing these toxins. Therefore, more work is needed to identify the producers. In the case of

AZAs, it is likely more than one producer, as the ratio of AZA1 & AZA2 were not consistent over the sampling times.

Diarrhetic Shellfish Poisoning toxins (OA & DTX1) are a concern throughout April-November in both estuaries at mid and high salinities. OA and DTX1 peaked in both estuaries between May-July in 2022 (Fig 3.2&3.3). This is the time when the New Jersey Department of Environmental Protection most often has identified *Dinophysis* spp. in field samples collected from these estuaries (<https://www.nj.gov/dep/bmw/phytoplankton.htm>). These toxins could also be a concern in winter months as well, but these months were not sampled in this study. The consistent detection of these DSP toxins should warrant frequent shellfish monitoring for these phycotoxins in the state of NJ, especially from April-August.

5.1.3 Impact of phenolic content on SPATT performance for phycotoxin tracking

Phenolic compounds in the water column readily bind to Diaion HP-20 resin, a resin regularly used in SPATT bags. Diaion HP20 resin is regularly used in laboratory experiments to bind a wide range of compounds of varying degrees of polarity and hydrophobicity, including phenolic compounds. This is the first study demonstrating the binding of these compounds from water of varying salinity and other abiotic factors. This potentially complicates the uptake and accumulation mechanisms of different phycotoxins to Diaion HP20 resin. However, this study did not find that the phenolic compounds oversaturated resin after a month in the field as OA was still able to adsorb to resin after this amount of time (Table 3.5). In addition, at least some of the phenolic compounds are desorbed from the resin with a 100%

MeOH rinse, the same rinse used to desorb many of the phycotoxins for detection and quantification analyses (Table 3.5).

The octanol-water coefficient of the phycotoxin impacts how water with high phenolic content impacts the adsorption/desorption of the phycotoxin to the SPATT resin. An experiment adsorbing phycotoxins to Diaion HP20 resin before and after field deployment suggests that compounds with higher octanol-water coefficients, like OA, are not as affected by water with high phenolic content compared to phycotoxins with lower octanol-water coefficients, like MCs. Okadaic acid did not desorb from resin in the field and was able to adsorb to resin that had been out in the field for a month. After just one day in the field, all MC-RR desorbed from resin. After one month, all MCs on the resin were either removed or suppressed and MCs were unable to adsorb to resin after it had been deployed for one month (Table 3.5). These results suggest that Diaion HP20 resin should not be used in passive samplers for phycotoxins with low octanol-water coefficients, like MCs.

Many lipophilic phycotoxins transition from kinetic uptake to equilibrium accumulation on the Diaion HP-20 resin within a few weeks. The repeated weekly collections demonstrated that many lipophilic phycotoxins did not continuously increase on resin with longer exposures in the water (Table 3.4). This means that Diaion HP20 resin filled SPATT bags should not be used in quantifying phycotoxins in the water column with deployment periods over a week and cannot estimate phycotoxins in shellfish.

5.1.4 How Total Phenolic Content in the water varies with seasons and other abiotic factors

The amount of total phenolic content was high in the two estuaries and was primarily deposited into the water through the rivers in the New Jersey Pinelands. During the sampling times and sites, the Total Phenolic Compound (TPC) ranged from 16-1924 $\mu\text{g/L}$ GAE eq. This is an order of magnitude higher than concentrations measured in lakes with high submerged aquatic vegetation (Peng et al., 2022). In addition, the ratio of TPC to DOC (5-27%) was similar to that of a laboratory study with degrading *Sargassum natans*, a known major source of phenolic compounds like phlorotannins and phenolic acids (5-18%) (Powers et al., 2019). The comparison of the values found in this study to the few other studies that measured TPC in water, suggest that the amount and ratio of TPC in the waterways of the NJ Pineland are high compared to other regions (Peng et al., 2022; Powers et al., 2019). The TPC was highest at freshwater sites and decreased as water was transported to the coast. In addition, TPC was highest when rainfall the week before sample collection was high and discharge was high. These correlations suggest that most TPC is flushed into the NJ rivers from terrestrial sources in the Pinelands (Fig. 4.10).

Seasonal variations in total phenolic content correlated with water discharge in the two estuaries. When discharge was high in the spring season, the highest concentrations of TPC were observed at the freshwater endmember (Fig. 4.4). During this season, there was also little addition/removal of TPC along the freshwater-marine continuum according to the conservative mixing lines. The fall season also indicated there was little addition/removal of TPC along the freshwater-marine continuum. Alternatively, when discharge was at its lowest during the summer

season, low concentrations of TPC at the freshwater sites were observed, but there were net additions of TPC along the Mullica River-Great Bay estuary. This is likely due to the high production of TPC from salt marsh islands in the mid-estuary during this time. These results are similar to other studies that have found discharge to cause higher deposits of trace metals and dissolved organic matter in the spring and lower in the summer (Cotrim Da Cunha et al., 2001; Paczkowska et al., 2020, 2019; Sherrell and Ross, 1999).

5.1.5 Impact of total phenolic content on chlorophyll concentrations and phytoplankton community composition

Total Phenolic Content and Chlorophyll a concentrations were negatively associated. However, the R^2 value is very small and therefore it does not seem that TPC explains very much of the variance in chlorophyll a. These results are similar to some studies that have found high DOC to be associated with low primary production (Paczkowska et al., 2019), while other studies have found lignin-phenols associated with high chlorophyll a concentrations (Cotrim Da Cunha et al., 2001).

Cyanophyceae, a major class for toxin-producing was positively correlated with nitrogen loads and negatively correlated with TPC at freshwater sites. These results suggest that land cover that deposits high TPC into rivers could reduce the occurrence of cyanobacteria blooms (Fig. 4.17). This is in agreement with a study by Ren (2015), who found high cyanobacteria was related to total nitrogen in Barnegat Bay (Ren, 2015). Additionally, these findings are similar to other field and laboratory studies that have found nitrogen to increase cyanobacteria growth and phenolic content to decrease growth or cause death of cyanobacteria (Aké-Castillo and Vázquez, 2008; Feuchtmayr et al., 2019; Li et al., 2023; Neilen et al., 2017;

Paczkowska et al., 2019; Peng et al., 2022). However, the environmental drivers that best explained the phytoplankton community differed between the fresh-, mid- and high- salinity sites (Fig. 4.17). Additional controlled laboratory studies and field studies with more estuaries of varying TPC concentrations would further our understanding of how phytoplankton communities are impacted by river runoff and TPC input, especially downstream of the rivers.

Appendices

Appendix A: Adsorption of flavonoids onto Diaion HP-20 resin

A.1 Introduction

SPATTs filled with Diaion™ HP20 resin are used by field scientists for biotoxin monitoring. However, Diaion™ HP20 resin is also regularly used in laboratory experiments to bind a wide range of other compounds of varying degrees of polarity and hydrophobicity, including trace metals, phenols, and flavonoids (Armagan et al., 2002; Soto et al., 2012; Wu et al., 2017). For instance, Diaion™ HP20 filled columns are regularly used to separate phenols and flavonoids from sugars and other contaminants for chemical analyses (Kim et al., 2014; Soto et al., 2012). Terrestrial dissolved organic matter (DOM) tend to contain large concentrations of polyphenols compounds like flavonoids and tannins from lignin degradation (Powers et al., 2019). Additionally, phenol derivatives released by agriculture, pharmaceuticals and industry are considered marine pollutants as they can be endocrine disrupters in fish (Anku et al., 2017). The phenolic content in the water could have an impact on using SPATT bags for biotoxin monitoring including the length of deployment as the resin has limited binding capacity and the cleaning of samples to remove the high matrix effects caused by phenolic compounds after deployment.

A preliminary experiment was conducted to identify if flavonoids, luteolin and apigenin, could bind to Diaion HP-20 resin. If the flavonoids were able to bind to Diaion HP-20 resin, the experiment was set up to identify (1) how long it takes the

flavonoids to adsorb onto the resin, (2) what percent methanol is needed to elute the flavonoids from the resin, and (3) whether adsorption is impacted by salinity.

A.2 Methods

Four 250 mL glass beakers were filled with either 200mL saltwater or freshwater. Freshwater was collected from Lake Williston, Denton, MD and 28ppt saltwater was collected from Ocean City, MD and autoclaved. Both were filtered through a GF/F filter prior to the experiment. Total alkalinity and pH were measured in both freshwater and saltwater before the experiment. A SPATT bag with 3 g Diaion HP-20 resin was hung in one saltwater and one freshwater beaker. Two hundred μ l flavonoid mixture was added to each beaker for a final concentration of 10 μ g/mL apigenin and 12 μ g/mL luteolin. Beakers were covered in tinfoil and a cardboard box to reduce potential impact of light. One mL water samples were collected at times 0, 1, 2, 3, and 24 hrs, and stored at -20°C until analysis. SPATT bags were collected at 24 hrs and stored at -20°C until extraction.

SPATT resin was transferred to chromatography column (BioRad, Econo-Pac 1.5x12 cm, polypropylene, 20mL bed) and rinsed with 10 mL ultrapure water. The resin was eluted with (1) 10mL 30% MeOH, (2) 10mL 60% MeOH, and (3) 2x10mL 100% MeOH. Each fraction was collected in autoclaved glassware, dried down using a rotary vacuum, and resuspended in one mL DMSO. The one mL water samples and one mL fractions were filtered through a GF/F filter, transferred to a two mL amber vial, and stored at -20°C. Resin in column was dried and weighed.

Samples were injected onto a C8 (LiChrosphere, 125 mm · 4 mm 5-micron bead size RP-8, Agilent) column and subject to a 0.8 mL/min flow using an Agilent 1100 HPLC. Toxin peaks were detected using an Agilent Diode Array Detector

(Model# G1315B) with a micro high-pressure flow cell (G1315B#020; 6 mm pathlength, 1.7 lK volume) over the wavelength range 190 to 700 nm. The linear gradient of mobile phases (A) water with 1% formic acid and (B) acetonitrile were A/B (90/10) for 3 min, to A/B (40/60) by 7 min, to A/B (10/90) by 17 min, back to A/B (90/10) by 20 min. The photodiode array detector measured 350 nm and 325 nm wavelengths over the 20 min runs. Apigenin and luteolin standards were used for quantification with apigenin standard curve: 1, 5, 10, 20, 30, 40 µg/mL, and luteolin standard curve: 1, 7.5, 12.5, 25, 38, 50 µg/mL. Apigenin recovery was quantified using 350 nm wavelength and luteolin recovery was quantified using 325 nm wavelength. Percent Recovery was calculated based on theoretical addition of flavonoids to water.

A.3 Results and Discussion

During the preliminary experiment, there was clear disappearance of flavonoids from the water column when Diaion HP-20 resin was added to both freshwater and saltwater (Fig. A.1), and there was high recovery, 70-80%, of apigenin and luteolin eluted from the SPATT bag (Table A.1). There was no clear loss of flavonoids overtime in the water column without a SPATT bag, suggesting apigenin and luteolin are stable in freshwater and saltwater over at least 24 hours (Fig. A.1). Most of the flavonoids desorbed from the resin in 100% MeOH eluent, although 18% of the luteolin came off at 60% MeOH in the saltwater.

There was higher recovery of flavonoids in freshwater compared to saltwater, suggesting there are matrix effects of flavonoids binding to the SPATT bag in saltwater or ion suppression during analysis on the HPLC (Table A.1). Post-spike recoveries of flavonoids during a larger experiment would assist in answering this

question. The saltwater had a higher pH (8.08 compared to 7.46) and total alkalinity (84 mg/L compared to 28 mg/L) compared to the freshwater which could also impact the binding of flavonoids to the SPATT bags.

The results of this preliminary study show that at least some flavonoids are capable of binding to Diaion HP-20 resin and require a high organic solvent to desorb from the resin. The flavonoids tested adsorbed to the resin within three hrs in both the freshwater and saltwater. This preliminary experiment shows that flavonoids in the water column has the potential to bind to SPATT bags deployed in the field at both freshwater and saltwater sites, and could impact the binding capacity of the resin and cause matrix effects in the 100% MeOH eluent when analyzing for phycotoxins.

A.4 Tables

Table A.1. Percent recovery of flavonoids, apigenin and luteolin, from SPATT bags in saltwater and freshwater and eluted with varying percent MeOH (n=1).

Elution Fraction	Freshwater		Saltwater	
	Luteolin	Apigenin	Luteolin	Apigenin
100% MeOH	79.03%	77.83%	58.82%	69.24%
60% MeOH	1.21%	0.44%	18.59%	1.12%
30% MeOH	0.15%	0.03%	0.24%	0.84%
Total	80.39%	78.30%	77.65%	71.20%

A.5 Figures

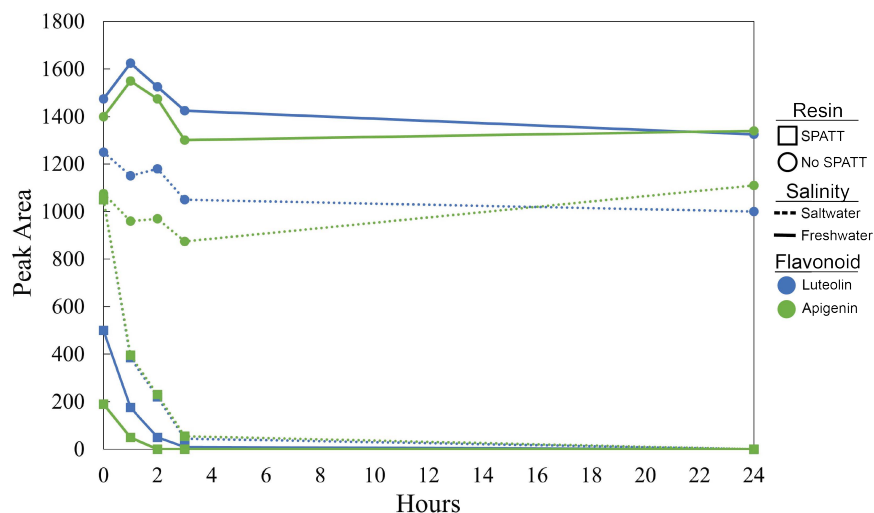


Fig. B.1. Measured flavonoids, luteolin (DAD array 325 nm) and apigenin (DAD array 350 nm), in seawater and freshwater over time with and without an Diaion HP-20 resin SPATT bag.

Appendix B: Fractionation of algicidal compounds from brewers spent grain

B.1 Introduction

When isolating the algicidal compounds in decomposing barley straw, scientist have found the likely inhibitors to be polyphenols, and more specifically flavonoids, released from lignin (Mecina et al., 2017; Murray, 2009; Waybright et al., 2009). Analysis of water in a reservoir treated with barley straw resulted in higher concentrations of tannins, phenolic acids, and flavonoids post treatment (Place 2011, unpublished).

Flavonoids are polyphenolic compounds mainly synthesized by plant roots, stems, and leaves (Harborne and Williams, 2000). They are well investigated classes of allelochemicals and can cause inhibition of two algal processes: 1. Alkaline phosphatase activity and 2. Photosystem II activity (Cao and Li, 2018; Eigemann et al., 2013; Huang et al., 2015; Xiao et al., 2019; Yu et al., 2019). This response seems

to be species specific, with chlorophytes generally being less sensitive compared to cyanobacteria in terms of both growth and photosynthetic inhibition. However, the response is also contingent on type of phenol/flavonoid, which makes predicting the response difficult.

Brewer's spent grain (BSG) likely has the same mode of action on *Microcystis aeruginosa* to barley straw since the compounds in barley straw and barley husks are likely similar. However, due to the high nutrients in BSG (Table 2.1) and the increase in bacterial abundance BSG caused in *K.brevis* cultures (Fig. 2.6), effort should be made to separate the key group of allelochemicals from the BSG before it can be considered in Harmful algal bloom mitigation tactics.

Preliminary experiments were conducted to separate some of the allelochemicals in BSG with anti-cyanobacterial properties and help identify how best to extract the compounds from BSG for HAB mitigation. These experiments worked under the assumption that the compounds were flavonoids based on the previous studies researching barley straw. There were two steps performed for separation: 1. Binding of the material to Diaion HP-20 resin and elution in different percents organic solvent (MeOH), and 2. Running of active fractions on HPLC and collection of separate 350nm DAD peaks.

B.2 Methods

A SPATT bag was made with 100 µm nitex cloth filled with 100 g of Diaion HP-20 resin and sealed. The SPATT bag was activated by submerging in 100% LCMS grade MeOH for 24 hours, then rinsed with ultrapure water and stored in a Ziplock bag with ultrapure water in 4°C until use. Frozen BSG from Diamondback

Brewing (Baltimore, MD) was dried at 60°C for 48 hrs. BSG was ground into powder and 250 g was put into an acid washed/autoclaved Erlenmeyer flask with 5 L ultrapure water. The BSG liquid was mixed with stir bar for 24 hrs. The BSG liquid was then put through a 300 µm mesh into new acid washed/autoclaved Erlenmeyer flask with hanging 100 g SPATT bag and stir bar. The bottle sat stirring for three days and then the SPATT bag was collected and stored at -20°C.

SPATT resin was rinsed with one L ultrapure water, and eluted with one L 10%, 30%, 60%, 80%, 100% MeOH. MeOH extracts were dried with a rotary evaporator, resuspended in five mL of the same percent MeOH extract. The extracts were filtered through a 0.7 µm GF/F filter into an acid washed/autoclaved amber glass vial. One mL extracts were dried via speed evaporation and resuspended in one mL DMSO to expose to *M. aeruginosa*.

A bioassay was conducted in a 24 well plate with 1780 µl BG-11 media, 250 µl *M. aeruginosa* strain LE3, and 20 µl of a 1:10 dilution of MeOH extracts resuspended in DMSO. Cells were counted using flow cytometry via FL4 channel on days 0, 3, 6, and 9. Anything greater than 10⁴ florescence size was considered a viable cell.

The MeOH fraction with the highest impact on *M. aeruginosa*, the 60% MeOH fraction (Fig. B.1), was further separated using HPLC by collecting DAD peaks at 350 nm using the methods described in section A.2. Three peaks were identified between five to ten minutes and the peaks were extracted using a fraction collector (Fig. B.2). Fifty µl of each peak fraction collected was dried and weighed using an aluminum weight boat.

Five hundred μl of each peak fraction collected was dried via speed evaporation and resuspended in 500 μl DMSO to expose to *M. aeruginosa*. A bioassay was conducted in a 24 well plate with 1675 μl BG-11 media, 300 μl *M. aeruginosa* strain LE3, and 25 μl of either H_2O , DMSO, peak A, peak B, or peak C. Cells were counted using flow cytometry via FL4 channel on days 0, 2, 4, 6, 8, and 10 days. Anything greater than 10^4 florescence size was considered a viable cell.

B.3 Results and Discussion

BSG liquid has high levels of NH_4^+ and PO_4^{3-} and therefore cannot be directly added to water bodies (Table 2.1 & A.1). When experiments were conducted to separate the allelochemicals with anti-cyanobacterial properties from BSG, these studies demonstrated that *M. aeruginosa* growth inhibition was due to more than one chemical. Inhibitory material was found in all fractions of MeOH eluted from Diaion HP-20 (Fig. B.1). The 60% MeOH fraction had the largest effect on growth of *M. aeruginosa* and 10% MeOH fraction had the least impact on growth. Within 3 days exposure to all but the 10% MeOH fraction, *M. aeruginosa* cell number had either remained the same or declined and continued decreasing during the 9-day experiment (Fig. B.1).

Since the 60% MeOH fraction caused the most algal death after the 9-day experiment, this fraction went through further separation by isolating peaks at 350 nm wavelength on an HPLC-DAD (Fig. B.2). This same method was used to detect and measure flavonoids in appendix A and so it was assumed that these peaks were at least similar to flavonoids. Peak C had algistatic effects on *M. aeruginosa* (Fig. B.3). However, peak C had twice the organic matter compared to peak A and B (34 mg/L

vs 15-16 mg/L) and so peak A and B could also have time same amount of inhibitory material but not as much was added as peak C (Fig. B.3). These preliminary experiments separating the allelochemicals with anti-cyanobacterial properties from BSG demonstrated that the inhibitory effects are 1) due to more than one compound, 2) at least some of the compounds can bind to diaion HP-20 resin, and 3) at least some of the compounds are similar to flavonoids. More work is needed to identify the best methods for separating allelochemicals from BSG.

B.4 Figures

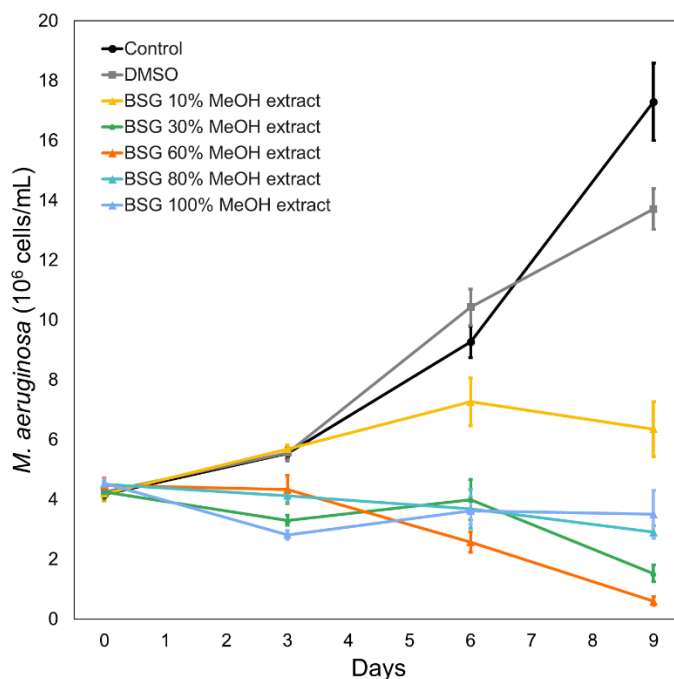


Fig. B.1. Growth of *Microcystis aeruginosa* (strain LE3) when exposed to diluted 10%, 30%, 60%, 80%, and 100% methanol fractions eluted from Diaion HP-20 resin SPATT bag exposed to brewer's spent grain extract. All methanol fractions were dried and resuspended in DMSO (n=3).

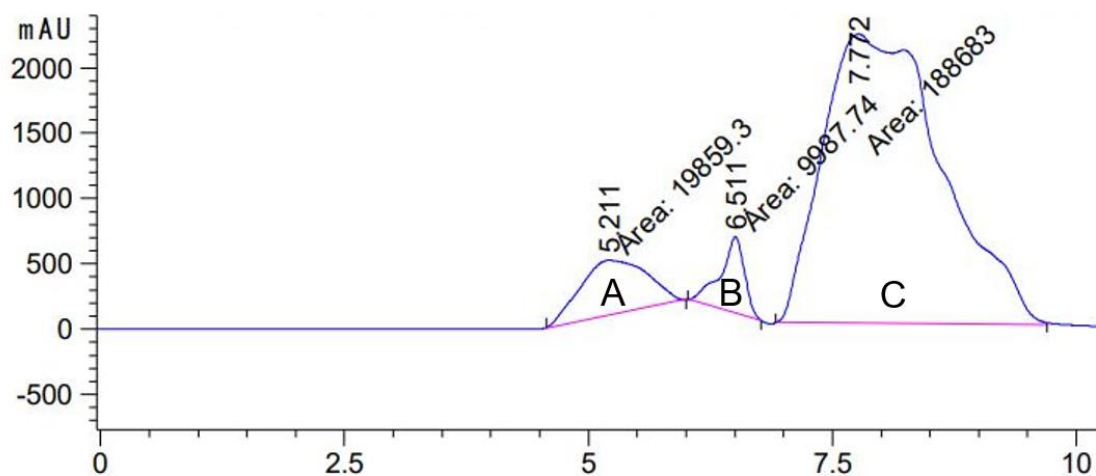


Fig. B.2. HPLC DAD 350 nm peaks A, B, and C, collected from brewer's spent grain extract for analysis of algicides.

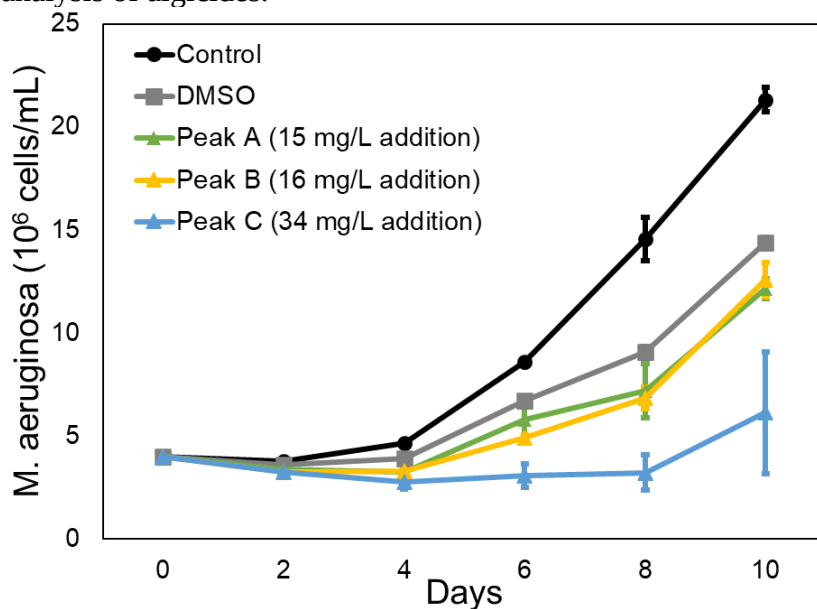


Fig. B.3. Growth of *Microcystis aeruginosa* (strain LE3) when exposed to DAD 350nm peak fractions from brewer's spent grain extract. All fractions were dried and resuspended in DMSO (n=3).

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