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Kristin Hampel
Western Michigan University, hampelk@gmail.com

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THE CHARACTERIZATION OF ALGAE GROWN ON NUTRIENT REMOVAL SYSTEMS AND EVALUATION OF POTENTIAL USES FOR THE RESULTING BIOMASS

by

Kristin Hampel

A dissertation submitted to the Graduate College in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Doctoral Committee:

John B. Miller, Ph.D., Chair
Steven B. Bertman, Ph.D.
Andre R. Venter, Ph.D.
Carla M. Koretsky, Ph.D.
Eutrophication resulting from excess nutrient loads is a major environmental issue that affects surface water quality and causes surplus primary production, thereby reducing dissolved oxygen concentrations. A method for managing nutrients in surface waters involves absorption of excess nutrients by deliberately cultivating benthic algal turf biomass, then harvesting it for a variety of uses, including biofuels, soil amendments, or feed supplements, thus coupling nutrient removal to additional economic drivers.

The goals of this work are to (1) evaluate the composition of algal biomass grown to remove surface water nutrients, (2) compare biomass grown at different geographic locations and in dissimilar water conditions, and (3) investigate uses for the biomass products.

The algal biomasses harvested from a range of locations, were characterized by measuring organic and inorganic carbon, nitrogen, phosphorus, and ash profiles. Algal biomass grown on wastewater effluent has the highest nutrient content and lowest ash (40 wt% C, 7.0 wt% N, >1.0 wt% P, 32 wt% ash) while that grown in brackish water had the lowest nutrient content and highest ash (10 wt% C, 1.0 wt% N, 0.13 wt% P, 79 wt% ash).
The algal turfs were also analyzed for silica and toxic metals. Silica in the algal turf was partitioned into biogenic and terragenic origin. Algae harvested from freshwater locations had ≤ 3.5 wt% biogenic silica while mixed fresh-salt water locations had biogenic silica content ranging from 10-27 wt%. Metals composed 0.045 wt% to 0.075 % of the total dry algal biomass, with relative concentrations of As > Cu ≈Cr > Co ≈ Mo > Cd.

The potential for using algal biomass as bio-ethanol feedstock was investigated by quantifying the monosaccharides in freshwater algal turf, which include glucose, galactose, xylose, mannose, ribose, and arabinose, varying from 2-30 % based on ash free dry mass. The application of biochar made from algal biomass for sorption of pharmaceuticals from water was assessed using model compounds. The order of sorption was 2-[4-(2-methylpropyl)phenyl]propanoic acid ≈ 2,4-dinitroaniline > 2-phenylethanol ≈ 2-phenylethylamine.

Two commonly used analytical methods, the Boehm Titration and molybdenum blue colorimetric method, were investigated for applicability to biomass analysis. Systematic errors inherent in the methods indicate that they are inappropriate for analyzing non-standard materials.
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Kristin Hampel
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CHAPTER 1

INTRODUCTION

1.1 Environmental Concerns

According to the EPA, water nutrient pollution is one of the most costly and ubiquitous problems facing the United States.¹ Nutrient pollution in the form of excess nitrogen and phosphorus can have a far-reaching effect on water quality, health, and the economy. Human activities have increased the flow of nutrients to estuaries and other coastal marine systems over the last half century, ² and the input is likely to increase globally as human use of inorganic fertilizers and fossil fuels, the two dominant sources of nutrients, continues to grow on a global basis.³ Nutrient pollution comes from point sources and non-point sources. A point source is any discernible, discrete source from which pollution is discharged, i.e. any pipe, ditch, well, concentrated animal feeding operation, from which pollutants are or may be emitted.⁴

Non-point sources are defined as pollution that comes from many diffuse sources. Typical non-point sources of nutrients include over-fertilized agriculture lands, urban areas with excessive usage of herbicides and insecticides, and runoff from such areas, which may result in serious environmental concerns for the downstream water quality. (p. 313) ⁵

Worldwide the effects of nutrient pollution can be far-reaching because significant portions of the mobilized nutrients are transported to rivers and streams and end up in coastal zones.⁶ Excess nitrogen and phosphorus cause an over-enrichment of
nutrients in waters leading to excessive primary production and eutrophication. Consequences of eutrophication include: increased levels of primary producers such as phytoplankton, decreased concentrations of dissolved oxygen due to decomposition of biomass, shifts in species composition, blooms of toxic algae, red tides, water discoloration, foaming, and loss of submerged vegetation. In the worst cases eutrophication eventually leads to “dead zones.” The “dead zones” are hypoxic areas that can no longer support aquatic aerobic life, such as fish, crabs, and most vertebrates and invertebrates. Recently, dead zones have developed in continental seas, such as the Baltic, Kattegat, Black Sea, Gulf of Mexico, and East China Sea, all of which are major fishery areas.

Nutrient pollution can have adverse effects on the economy and industry. Potential economic losses can be related to social and ecological impacts associated with eutrophication. Ecological costs are associated with loss of fishing industry (commercial and recreational) due to anoxic environments and dead zones. Eutrophication negatively impacts property values, leads to decreased recreational use, and loss of tourism. Additional economic costs come from the remediation of the polluted water bodies. A recent estimate of the potential cost of eutrophication to the US economy is over 2.2 billion dollars annually.

Worrisome consequences of the increased nutrient loads include possible links to multiple health problems in humans. The health effects of having high nitrate concentrations in water (above 10 ppm) include reproductive problems, methemoglobinemia, and cancer. A series of experimental and correlative studies in Belize have linked increased vector production of malaria in mosquitoes to nutrient
enrichments and wetland vegetation. Evidence also suggests that rapid growth of *Vibrio cholerae* can accompany the growth of marine algae in eutrophic conditions. High nutrient conditions favoring algal growth have been implicated in recent cholera outbreaks.18,15

Additional health problems have been associated with the worldwide increases in harmful algal blooms which can cause harm to humans through the release of biotoxins, cyanotoxins, neurotoxins, and cyclic peptide hepatotoxins.19,20 The mechanisms by which the biotoxins enter humans are by direct ingestion or contact, aerosol transport, or concentrating and vectoring by marine organisms that are then eaten.19 Respiratory problems in humans have been linked to red tides. Additionally, blooms of Cyanobacteria have been known to cause rashes, blisters, cancer, and other chronic diseases.19

Unfortunately, the frequency and duration of algal blooms are predicted to increase due to climate change.21,22 Specifically, climate change likely will increase external nutrient loading by increasing net precipitation in the winter and will cause an elevation in water temperatures.23 Increase in water temperatures has been shown to affect the length of the algae growing season, and data from 2001 indicates blooms now occur up to 4-6 weeks earlier than in 1958.24,25 Climate change scenarios also predict longer-lasting bloom durations.24

The rate at which climate change is occurring appears to be accelerating due to anthropogenic activities like agricultural, industry, and commerce.26,27 These activities release potent greenhouse gas (GHG) emissions such as carbon dioxide, nitrous oxide, and methane into the environment. To curb greenhouse gas emissions, it has been
suggested that we reduce our dependence on fossil fuel and develop alternative energy forms. In 2007 the US Congress passed the Energy Independence and Security Act, which requires the US to increase the quantity of biofuels produced to 36 billion gallons by the year 2022.  

### 1.2 Reducing Nutrient Pollution and Mitigating Climate Change

Reduction of nutrient input and removal of nutrients from polluted waters is required to avoid the problematic conditions associated with eutrophication. The main options for the removal of nitrogen and phosphorus from water involve either physicochemical or biological methods. Some common physicochemical methods involve precipitation using chemical addition, coagulation-flocculation, ion exchange, electrodialysis, and reverse osmosis. Biological methods include the construction of wetlands and the strategic use of bacteria and algae.

In one study by Coats et al. (2011), an environmental life cycle analysis was completed to compare chemical and biological methods of treating eutrophic water, specifically the removal of phosphorus. According to Coats et al. (2011) and Tanwar et al. (2007), best practices for treatment are biological because of their reduced environmental impact compared to chemical methods. Biological treatments also produce fewer biosolids and no chemical sludge. However, chemical processes for removing nutrients tend to be more stable than biological processes. Given that biological treatment can be unreliable and unstable at times, integrating chemical removal with biological approaches might more consistently mitigate the water quality impacts of eutrophication.
Algae are excellent for nutrient removal processes as they exhibit several-fold higher nitrogen and phosphorus concentrations than other plants, about 10% and 1% dry weight, respectively. Because algae produce oxygen as a byproduct of photosynthesis they can increase the dissolved oxygen concentration in water. Algae have also been shown to have an excellent capability to remove heavy metals from water. The implementation of algae for nutrient removal is advantageous over other methods because it is a continuous treatment process and does not physically disrupt the natural ecosystem.

An additional advantage of using algae to alleviate eutrophication is that it has the ability to fix carbon dioxide. Algae absorb CO\(_2\) present in the atmosphere during photosynthesis, capturing it as biomass. The algae assimilate CO\(_2\), nitrogen, and phosphorus into lipids, proteins, and carbohydrates. The production of these co-products could give the biomass value in markets such as the agriculture and biofuel industries.

1.3 Overview of Algae Based Nutrient Removal Technology

W.J. Oswald and C.G. Golueke first pioneered the concept of nutrient removal by algae in 1960. They developed the idea of using large-scale raceway ponds to cultivate microalgae on wastewater nutrients and then anaerobically ferment the algal biomass to produce methane fuel. Since then, a variety of methods have been developed for removing nutrients from water, but the two primary strategies employ the use of either benthic or planktonic algae. Benthic algae are those species that are attached to or closely associated with various substrata or bottom surfaces. They occupy a variety of microhabitats, including stones, sediments, sand grains, logs, and a variety of artificial...
substrata. Planktonic algae are species that are free floating and suspended in the water column. One of the major and practical limitations in developing algal nutrient removal systems is the harvesting or separation of algal biomass from the treated water discharge, as efficient removal of algal biomass is essential for recycling of the wastewater. Because benthic algae are an attached species, they have advantages over planktonic species; benthic algae can withstand high vertical water velocities, such as would be found in streams, they are not diffusion limited, and they are easier to harvest than planktonic species.

Previously, filamentous benthic green algae have been successfully employed in water contaminated with animal manure (swine and cattle) for the removal of nitrogen and phosphorus. Ruiz-Marín et al. (2010) successfully immobilized *Scenedesmus obliquus* algae in artificial wastewater and reported a 97% and 85% removal rate of ammonium and phosphorus, respectively. A study performed by Johnson and Wen (2010) found that the benthic algae system generated more biomass than the suspended systems. Their work also demonstrated the “cleaning” effects of algae on manure wastewater, which showed a dramatic improvement in water clarity and color in 15 days.

Algal Turf Scrubber -ATS™ is a commercial technology, invented by Walter Adey and developed by HydroMentia, Inc., that is used to grow attached, filamentous algae species to capture the energy of sunlight and build an algal biomass “turf” from CO₂ and excess nutrients. The algae can be grown in a variety of water conditions including fresh, brackish, salt, and wastewaters.

A basic description for design for ATSTM system can be found in Adey and Loveland (2006). The set up for the system is pictured in *Figure 1-1*. The design uses a
fine mesh plastic screen to support the growing algae.\textsuperscript{52} The screen is then fit into a shallow trough ("floways") through which the water is pumped. On the opposite end of the trough, water is allowed to return to the main source through gravity drainpipes. The screens require periodic harvesting to ensure new growth. This is accomplished by first elevating the screens from the water in the trough to allow them to drain. Next, the screens can be scrubbed to remove excess growth. The basal filaments that remain intertwined in the mesh of the screen send up new growth, replacing what has been removed. Optimal harvest intervals range from 7 to 20 days.\textsuperscript{52} While the basic design of floways at all sites is the same, there are some specific features at each site that are described in Table 1-1.

![Diagram of a basic ATS™ system.](image)

Figure 1-1. Illustrated representation of a basic ATS™ system.
1.4 Recycling of Algal Biomass

Once algae have been utilized for the removal of excess nutrients, they need to be harvested and disposed. The disposal of the algae represents an opportunity to recycle the biomass for other purposes. The growth rate of algae is high and the overall biomass productivity from the ATS™ system ranges from 40-70 dry g/m²/day in the summer, depending on latitude, to 5-20 dry g/m²/day during the winter months. It has been suggested that algae has a greater overall biomass yield than other terrestrial crops. Additionally, algae, like most biomass, are composed of protein, lipids, carbohydrates, and other constituents. These characteristics give the harvested algal biomass value as a feedstock for biofuel production. The carbohydrate portion of the biomass has potential to be extracted and fermented into bio-alcohol fuels, and the lipid portion can be converted into bio-diesel.

Among renewable biomass energy sources, algal biomass may be one of the most important because it not only has a high growth rate, but it also does not compete with food production. Other traditional biofuel sources such as corn, soybean, and sugar cane are used for both fuel and food production. With the world population continuing to grow and the likelihood of climate change affecting crop production, these feedstocks will need to be reallocated. Apart from the risk of food security, the demand for these biofuels could place substantial additional pressure on the natural resource base, with potentially harmful environmental and social consequence.

The process of producing biofuel from algae requires that either the sugars or lipids be extracted from the biomass. There are three main methods to extract oil from algae: (1) expeller/press, (2) solvent extraction with hexane, and (3) supercritical fluid
The extracted oil is then converted to biofuels through one of four primary ways: direct use and blending of raw oils, micro-emulsions, thermal cracking, and transesterification. Transesterification, the most commonly used process, reacts the extracted lipids with an alcohol in the presence of a catalyst to form esters and glycerol. The esters are then separated from the glycerol and used directly as fuel.

The main processes for extracting sugars from algal biomass include: enzyme hydrolysis, concentrated acid hydrolysis, and dilute acid hydrolysis. Once the sugars are extracted, they are converted to an alcohol through fermentation by yeast or bacteria. After fermentation is completed, the product is separated to remove any excess water from the bio-alcohol.

After oil or sugar extraction the remaining biomass fraction still contains many nutrients and might be utilized for additional purposes. This could give additional value to the algal biomass, reducing wastes, and further recycling nutrients. Possible uses of the residual biomass include food and feed applications, as well as fertilizer and bioactive molecules (essential and non-essential compounds that occur in nature, are part of the food chain and can be shown to have an effect on human health).

1.4.1 Food Applications

Due to their chemical composition, algae are able to enhance the nutritional content of conventional food preparations and hence, to positively affect the health of humans and animals. Many algae species are rich in amino acids and even synthesize essential amino acids. Algal biomass is a source of polyunsaturated fatty acids (PUFAs), which can only be synthesized by plants. Polyunsaturated fatty acids are
important for human development and growth and should be included in the diets of all people. \textsuperscript{65} “Besides being a primary source of PUFAs, the fatty acids from microalgae have further advantages over fish oils, such as the lack of unpleasant odor and reduced risk of chemical contamination.” \textsuperscript{66}

Algae also synthesize valuable carotenoids and vitamin.\textsuperscript{67} Carotenoids, and especially $\beta$-carotene as pigments, can be utilized in food and beverages, such as fruit juices, soft drinks, confectionary, margarine, baked and dairy products, and generally in the formulation of health foods, to enhance their appearance to consumers.\textsuperscript{68} Algae also represent a valuable source of nearly all essential vitamin, e.g., A, B1, B2, B6, B12, C, E, nicotinate, biotin, folic acid, and pantothenic acid.\textsuperscript{69}

The unique and interesting compounds found in algae could provide significant value to human nutrition. However, prior to commercialization, algal material must be analyzed for the presence of toxic compounds to ensure that it is non-toxic.\textsuperscript{63}

\subsection*{1.4.2 Animal Feed Applications}

Algae form the basis of the food chain and play a key role in aquaculture, especially marine culture, as they are the food source for larvae of many species of mollusks, crustaceans, and fish.\textsuperscript{66} Reitan \textit{et al.} (1997) compared fish larva that were fed microalgae, to larva that were not and found that the algae addition enhanced the rearing success of the larva, including survival, growth, and quality.\textsuperscript{70} Additionally, algae appeared to boost the appetite of the larvae and improve the tank and gut microflora.\textsuperscript{70}

Residual algal biomass may also have applications in the animal feed industry. This is because the amino acid profile of many different algae species is comparable to
that of other food proteins, and is sometimes superior to conventionally used plant proteins. The PUFAs, vitamin, and carbohydrates in algae also make it valuable for animal nutrition. However, if the residual biomass is produced after extraction of oils the PUFAs will not be present. Ginzberge et al. (2008) studied algae as a chicken feed supplement and found that it reduced cholesterol levels in the blood of chickens by 28% and cholesterol in the of egg yolk by about 10%. Another study performed by He et al. (2002) found that pigs given an algae feed supplement which contained 5 mg of Iodine/kg feed exhibited a 10 wt % higher daily weight gain than pigs receiving the basic feed, containing 0.22 mg of iodine/kg of feed. The results of the study indicate algae could be used as a feed additive for pigs, which would be beneficial to both the control of iodine deficiency disorder in humans and would improve pig production. Recycling the algal biomass as animal feed could not only improve the nutritional content of animal feed, but it might also reduce costs.

1.4.3 Fertilizer Application

Algal biomass produced from the treatment of eutrophic waters contains nitrogen and phosphorus, which are essential nutrients for plant growth. Currently, fertilizers are used to supplement soils with these nutrients. In 2009 alone, US fertilizer consumption was 109.4 kg per hectare of arable land. The price of fertilizer is expected to continue to climb as high oil prices increase the cost of processing phosphate rock, which provides a key ingredient in fertilizer. Recycling the nutrients found in algal biomass for fertilizer supplementation may help stabilize or reduce prices. Wilkie and Mulbry et al. (2001) evaluated the nitrogen content in benthic algae produced from dairy manure
streams and found it to be comparable to or higher than levels in organic fertilizer products made of composted manure. Mulbry et al. (2005) amended soil with ATS™ dried biomass and found that it enhanced plant growth to the same level as the commercially produced, organic-fertilizer amended soil.

Algal biomass has an advantage as a slow release fertilizer because less of the nitrogen is available as mineral N at the time of application. Applying dried algal biomass to soils therefore results in less NH₃ volatilization, as is the case with manures. Additionally, algal products likely have lower pathogen levels than raw manure. For these reasons, algal biomass might command a higher return than existing organic fertilizer products. (p. 87, 89)

1.4.4 Additional Uses for Algal Biomass

The natural products and nutrient content found in algae give the biomass many possible uses and provide for the possibility that the biomass can be recycled. Alternative uses for the biomass are still being studied and explored. For example, recently Spirulina platensis, a blue–green microalgae, has been studied for its ability to synthesize silver nanoparticles. Additionally, algae have been investigated for use as biochar to provide nutrients as soil amendments.

The potential applications for algal biomass are vast. The goal of this research was to determine the appropriate applications, by determining the chemical composition of algal biomass grown on eutrophic waters.
1.5 Project Overview

Native periphytic algal biomass communities were produced at five different locations (Figure 1-2) using the Algal Turf Scrubber ® -ATS™ system previously described. The algal consortia were established from the surrounding environment by “self seeding”. No attempt was made to control or modify the ecological community growing on the ATSTM systems outside of mechanical harvesting. The ecological community likely consisted of a mixed assemblage of benthic algae, microalgae, bacteria, and anything that would feed on the growing biomass. 79 Harvested biomasses were never filtered or separated.

The project presented here is not concerned with the specific make-up of the biomass grown using it ATSTM system. It is more concerned with evaluating the overall biomass profile produced on the system. Therefore, for the purposes of this project, all future references to the biomass community produced on this system will be denoted as algal biomass.

The water sources from which the algal biomass was produced ranged in regards to nutrient levels, water type designation, and location (see Figures 1-2 and 1-3). A summary of information regarding each location can be found in Table 1-1; Water quality data are provided elsewhere. The ATSTM systems were set up and operated by each institution. Table 1-1 provides all of the data that was available regarding the system conditions, no other information is could be provided. Forthcoming detail is expected in future publications.
Figure 1-2. Map of algae growth locations. Blue balloons mark each ATSTM location. The boxes state the name of the location and give the latitude and longitude.

- **Muskegon Wastewater Treatment Plant, Muskegon, MI**
  - Latitude: 43.56, Longitude: -86.05

- **Goldsworth Pond, Kalamazoo, MI**
  - Latitude: 42.28, Longitude: -85.61

- **Lake Erie, Buffalo, NY**
  - Latitude: 43.15, Longitude: -78.89

- **York River, Virginia**
  - Latitude: 37.24, Longitude: -76.49

- **Great Wicomico River, Virginia**
  - Latitude: 37.84, Longitude: -76.32
A) Great Wicomico River Site

B) York River Site

(C) Lake Erie Site

Figure 1-3. Magnified site locations (A) Great Wicomico River (B) York River (C) Lake Erie
Table 1-1. ATST™ installation site location summary information.

<table>
<thead>
<tr>
<th>Site</th>
<th>Location</th>
<th>Code</th>
<th>Water Description</th>
<th>ATS System</th>
<th>Algae Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great Wicomico River</td>
<td>Reedville, VA., 6km from Chesapeake Bay</td>
<td>AES-F1</td>
<td>Small Tributary, Semi-diurnal Tidal Current (0.1kn) lacks significant freshwater input</td>
<td>Mesohaline: 11-18 ppt</td>
<td>0.61 x 15.2 m²</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AES-F2</td>
<td></td>
<td></td>
<td>0.61 x 15.2 m²</td>
</tr>
<tr>
<td>River/Lagoon</td>
<td>Location</td>
<td>Type</td>
<td>Characteristics</td>
<td>Volume</td>
<td>Flow</td>
</tr>
<tr>
<td>--------------</td>
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<td>------</td>
<td>----------------</td>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>York River</td>
<td>Gloucester Point, VA, 6km from Chesapeake Bay</td>
<td>Microtidal, partially mixed estuary</td>
<td>20-30 ppt</td>
<td>1.2 x 2 m²</td>
<td>120 L min⁻¹</td>
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</tr>
</tbody>
</table>

Muskegon Wastewater Treatment Plant

<table>
<thead>
<tr>
<th>Plant</th>
<th>Location</th>
<th>Type</th>
<th>Characteristics</th>
<th>Volume</th>
<th>Flow</th>
<th>Primary Algae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muskegon, MI</td>
<td></td>
<td></td>
<td>Wastewater, Freshwater</td>
<td>0.19 x 30.5 m²</td>
<td>20 L min⁻¹</td>
<td>Green Algae</td>
</tr>
<tr>
<td>AMS-F1</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AMS-F2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AMS-F3</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Table 1.1-continued
### Table 1.1-continued

<table>
<thead>
<tr>
<th>Location</th>
<th>Urban Runoff, Freshwater</th>
<th>Area</th>
<th>Flow Rate</th>
<th>Pollutant Description</th>
<th>Green Algae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goldsworthy Pond, Kalamazoo, MI</td>
<td>AGH-F1</td>
<td>0.14 x 6.1 m²</td>
<td>16.5 - 20.6 L min⁻¹</td>
<td></td>
<td>Green Algae</td>
</tr>
<tr>
<td></td>
<td>AGH-F2</td>
<td>0.14 x 6.1 m²</td>
<td>16.5 - 20.6 L min⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGH-F3</td>
<td>0.14 x 6.1 m²</td>
<td>16.5 - 20.6 L min⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGH-F4</td>
<td>0.14 x 6.1 m²</td>
<td>16.5 - 20.6 L min⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lake Erie, Black Rock Canal, Buffalo, NY Located on the 3km from Lake Erie</td>
<td>ALE-F1</td>
<td>1 x 1 m²</td>
<td>53 L min⁻¹</td>
<td>Polluted with polychlorinated biphenyl and dioxin</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>ALE-F2</td>
<td>1 x 1 m²</td>
<td>53 L min⁻¹</td>
<td>Polluted with polychlorinated biphenyl and dioxin</td>
<td>n/a</td>
</tr>
</tbody>
</table>
Table 1-2. ATSTM biomass sample coding information

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Information Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>AES101511F1</td>
<td>Great Wicomico River site, Date (MMDDYY), Floway #</td>
</tr>
<tr>
<td>AES101511F2U</td>
<td>Great Wicomico River site, Date (MMDDYY), Floway 2, Upper half</td>
</tr>
<tr>
<td>AES101511F2D</td>
<td>Great Wicomico River site, Date (MMDDYY), Floway 2, Lower half</td>
</tr>
<tr>
<td>AGH090111F1</td>
<td>Goldsworth Pond site, Date (MMDDYY), Floway #, Screen Position #</td>
</tr>
<tr>
<td>AMS71211F1S2</td>
<td>Muskegon Wastewater Treatment Plant site, Date (MMDDYY), Floway #, Screen Position #</td>
</tr>
<tr>
<td>AVI101410YR</td>
<td>York River site, Date (MMDDYY), York River water</td>
</tr>
<tr>
<td>AVI101410BB</td>
<td>York River site, Date (MMDDYY), Boat Basin water</td>
</tr>
<tr>
<td>AVI101410MF</td>
<td>York River site, Date (MMDDYY), in stream floway</td>
</tr>
</tbody>
</table>
1.6 Objectives and Organization of Thesis

The objectives of this dissertation are (1) to evaluate and compare the nutrient values in periphyton biomass communities grown at different locations and in a variety of water compositions and (2) test potential options for recycling the biomass. To evaluate the periphyton biomass, the nutrient contents in the biomasses were measured. To determine feasibility of recycling periphyton biomass, other important biomass parameters, such as ash, heavy metals, and silica contents were measured. Additionally, periphyton biomass was evaluated for biochar production.

Chapter two focuses on determining and comparing the organic carbon, inorganic carbon, nitrogen, hydrogen, and phosphorus content in the periphyton communities. This information is needed to determine possible uses for the biomass, beyond removal of nutrients from eutrophic waters. The fraction of organic carbon assigned to carbohydrate portion of the biomass was also investigated.

Chapter three is concerned with determining the ash content in the periphyton biomass harvested from the five sites. In addition, studies were performed to determine the ash composition, which included arsenic, cadmium, copper, cobalt, chromium, molybdenum, and silica. The fraction of silica that was biogenic or amorphous in form was assessed using an alkaline time extraction method.

The fourth chapter is concerned with determining if periphyton biomass can be recycled as a biochar, similar to activated carbon, for the sorption of organic compounds from water. Properties of the algal biochar were characterized using methods such as SEM, BET, and ratios of H/C and O/C to determine carbonization. Four different organic compounds, with differing chemical properties, were used to test the sorption capacity of
the biochar.

The fifth chapter considers two commonly used methods for analyzing silica and biochar and attempts to evaluate the potential shortcomings of these methods. The colorimetric method for determining silica in solution has interferences in a complex matrix such as algal biomass. A commonly used method, Boehm Titration, for identifying and calculating the concentration of oxygenated functional groups on biochar was also investigated and found to have inherent problems.

In the sixth chapter, the primary results and knowledge gained from this study are summarized. The limitations and implications of this research are assessed, and ideas for future work are suggested.
1.7 Bibliography

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CHAPTER 2
NUTRIENT ANALYSES OF ALGAL BIOMASS

2.1 Introduction

The weight percent of carbon, hydrogen, and nitrogen content in biomass are commonly referred to as the ultimate analysis. The term comes from the object of the analysis, which is the determination of the ultimate constituents, the elements, of the substance.\(^1\) The information gained from the ultimate analysis, in conjunction with the percent ash, can be used to assess the value of the biomass for use as a biofuel, fertilizer, or feed supplement. Since the late 1800’s many mathematical models have been developed to correlate ultimate analysis information with higher heating value (HHV).\(^2,3\) The atomic ratios of C, H, N and O (O is usually determined by difference) are also used to classify the heating value of biofuels. For example, low oxygen-to-carbon (O/C) ratios and low hydrogen-to-carbon (H/C) ratios increase the HHV of the biomass source. High oxygen and hydrogen content results in high volatile and liquid yields, respectively, on use as a fuel.\(^4\) This is because during combustion the oxygen combines with the hydrogen in the biomass, producing less beneficial water and thus, high H/C content does not translate into high gas yield (flue gases or synthesis gases: $\text{H}_2$, $\text{CO}_2$, $\text{CH}_4$ and $\text{CO}_2$).\(^5\)

Ultimate analysis data can be used to estimate the carbohydrate, lipid, and protein composition of algal biomass. A widely accepted method for determining the protein content in algal biomass is to use the nitrogen content calculated from the ultimate analysis and a conversion factor, which will yield the protein content.\(^6,7\) Also, carbohydrate, and lipid contents are directly related to the carbon content and can be
determined by stoichiometric CHN equations developed by Gnaiger et al. (1984). This information is important for determining the applications for which the algal biomass is optimal and those for which it is not. For instance, high protein content in the biomass would make it more useful as a food amendment, and high nitrogen content is good for use as a fertilizer. Elevated lipids or carbohydrates are good indications that the biomass may have biofuel potential.

Although not explicitly included in the ultimate analysis, phosphorus is an important element for determining potential biomass applications. Phosphorus is an essential nutrient for the growth of plants. Although fertilizer application is not required for plant growth, fertilizers are used to enhance growth. Phosphorus is an essential component in cell structures, such as nucleic acids, phospholipids, glucophosphates, RNA, and DNA. It is also critical in establishing the enzymatic machinery, and in energy storage and transfer because of its presence in ATP, ADP, AMP, and pyrophosphate. Nitrogen is a macronutrient that is required in high quantities by plants. Nitrogen is incorporated into amino acids, required for stem and root growth, a component of chloroplasts, and vital for several enzyme reactions. Because nitrogen and phosphorus are vital for plant development, they are often incorporated into fertilizers.

One of the major disadvantages of biomass that is high in phosphorus and nitrogen is that it will be less useful as a biofuel. Phosphorus is noncombustible and remains in the biomass after heat treatment, increasing the percentage of ash produced, and lowering the caloric value of the biomass. Thus, low phosphorus content is advantageous in biofuels. High nitrogen content is also generally seen as negative in potential biofuel feedstocks because upon combustion the nitrogen can be converted to
Nitrous oxide gas. Nitrous oxide gas has a greenhouse effect that is 289 times more potent than carbon dioxide and also contributes to acid rain. In contrast, bio-alcohol production from biomass does not produce nitrous oxide gas, because carbohydrates are extracted from the biomass, leaving the nitrogen, phosphorus, and other possible undesirables behind.

Nitrogen and phosphorus nutrients are available in “recycled” fertilizers such as crop residues and manures. Unfortunately, much of this phosphorus must be supplemented using non-renewable sources because significant quantities of phosphorus are lost in crop harvesting, erosion, and runoff. Algae have the ability to recapture some of this nutrient loss by taking up the nutrients lost to surface waters and incorporating them into biomass. Once harvested, the algal biomass can then be reused as a soil amendment.

2.2 Experimental Details

Materials. Ammonium molybdate tetrahydrate (81-83%) ACS grade and ascorbic acid were all purchased from Mallinckrodt Baker, Inc. Sodium hydroxide (97%) ACS grade was purchased from EM Science. 1-amino-2-naphthol-4-sulfonic acid (98%+) was purchase from Eastman Organic Chemicals. Antimony potassium tartrate trihydrate (99.0-103.0%) ACS grade, sodium sulfate (98+% ACS grade, liquid bromine (99.5%) ACS grade, formic acid (98%) mass spectrometry grade, potassium dihydrogen phosphate (99.99%), glacial acetic acid (≥ 99.7%), acetic anhydride (≥ 99.0%), ammonium hydroxide (≥ 25% in water), 1-methylimidazole (99%) and potassium borohydride (98+% were purchased from Sigma-Aldrich. D-arabinose (99.9%), D-
xylose (99.0%), D-mannose (99.9%), D-glucose (99.5-99.9%), and D-galactose (≥ 99 %) were purchased from Supelco. Myo-inositol (98+) was purchased from Alfa Aeser. Potassium hydroxide (pellets, 87.8%) was purchased from T.J. Baker. An EDTA standard was purchased from LECO Corporation. Sulfuric acid ACS grade and Dichloromethane (99.5 +%) and 2 mL GC autosampler vials with 9mm PTFE septa caps were purchased from VWR international. All solutions were made up using 18 MΩ water from an in-house system purchased from EMD Millipore Corporation.

2.3 Methods

2.3.1 Biomass Harvesting

Biomass samples were harvested by each collaborating ATS™ operation and shipped to Western Michigan University for processing.

Generally, harvesting took place every 7 days during the spring and summer months and every 14 days during the fall and winter months. Prior to harvesting, water flow was halted on the floway and the system drained for 0.5-1 hour. Floways were never allowed to fully dry. A wet/dry vacuum was used to remove the biomass (and any sediment) from the floway screens. Depending on the amount of biomass accumulated the harvests were then either placed in a previously cleaned 20 L plastic bucket or in a 1 L glass food-storage jar and immediately placed in a freezer. Samples from the Great Wicomico River sites were shipped on dry ice and arrived within 48 hours. Samples from the York River were shipped frozen, but not on ice, and arrived within 48 hours.
Once received, frozen samples were thawed in a refrigerator (at 4°C) over night. Biomasses from the Goldsworth Pond and Muskegon Wastewater Treatment Plant sites were collected as previously stated, however harvests were not frozen but immediately dewatered. Samples were dewatered by sieving the biomass through mesh nylon netting. Sample where then homogenized by blending and lyophilized using a Virtis Lyophilizer. The dried samples were stored in labeled (see Table 1-2 for labeling designations), sealed, plastic bags.

Biomass collected from the Lake Erie site was harvested and lyophilized on location.

2.3.2 Phosphorus Analysis by Colorimetry

The percent (w/w) of total phosphorus was measured in each algal biomass sample using the method developed by Dick and Tabatabai *et al.* (1976), for the determination of total phosphorus in soils. The method extracts the phosphorus in the sample as orthophosphate, which can then be measured using the molybdenum blue colorimetric method. This method of digestion is preferred over other methods that use perchloric acid because it is safer to carry out and does not require the boiling of a super acid.

Previously dried algae samples (see Chapter 1 for details) were finely ground using a mortar and pestle and 100 to 200 mg of each biomass sample were weighed into previously cleaned and labeled 20 mL scintillation vials. A sodium hypobromite solution was prepared by adding 1.5 mL of 99%+ liquid bromine into 50 mL of a 2 M NaOH solution. Next 3.0 mL of the solution was added to each sample scintillation vial. The
samples were swirled by hand and allowed to react for ten minutes prior to being placed in a heated sand bath at 300°C, until all the liquid had been boiled off (about 1 hour) and then heated for an additional 30 minutes. The samples vials were removed from the sand bath and allowed to cool under the fume hood (30 minutes). Once cooled, 4.0 mL of 18 MΩ water and 1.0 mL of 90% formic acid were added to the sample vials. Next, 25 mL of 0.5 H₂SO₄ were used to wash the entire sample into a 50 mL PET centrifuge tube. The samples were centrifuged at a speed of 11,000 rpm for 4 min.

A colorimetric solution was prepared by adding 12.0014 g of ammonium molybdate into 250 mL of 18 MΩ water. Then 0.2907 g of antimony-potassium tartrate was dissolved in 100 mL of 18 MΩ water and both solution were added to 2.5 M H₂SO₄ and finally the solutions was diluting to 2 L. The colorimetric solution is light sensitive, so it was stored in an amber glass bottle away from the light. Just before colorimetric analyses were to be performed 0.5 g of ascorbic acid was weighed out into a beaker and 100 mL of the colorimetric solution was added. The solution was stirred until all the ascorbic acid was dissolved, and then 1 mL was added to each sample. The colorimetric method depends on the reaction of the soluble orthophosphate with ammonium molybdate and antimony potassium tartrate under acidic conditions, forming 1,2-phosphomolybdic acid, which is initially a yellow color. The phosphomolybdic acid complex then may be reduced by ascorbic acid to give a phosphomolybdenum blue complex (see Equation 2-1). The reduced blue complex is used because it has a stronger absorbance than the yellow complex. The blue complex forms within 10 minutes, but full color development may take up to 30 minutes. Therefore, samples were analyzed after 30 minutes.
Equation 2-1. Phosphomolydenum blue complex

\[
\begin{align*}
    H_2PO_4^- + 12 \text{MoO}_4^{2-} & \rightarrow (H_2\text{PMo}_{12}\text{O}_{40})^- \\
    \text{PMo}^{\text{VI}}_{12}\text{O}_{40}^{3-} + 4e & \rightleftharpoons \text{PMo}^{\text{V}}_{4}\text{Mo}^{\text{VI}}_{8}\text{O}_{40}^{7-}
\end{align*}
\]

Silicic acid forms a heteropoly molybdate blue complex as well, which can interfere with the analysis of orthophosphate at concentrations of 100 mg L\(^{-1}\) of SiO\(_2\).\(^{19}\)

The addition of the antimony potassium-tartrate reagent to the solution media eliminates this interference, because antimony binds specifically with phosphorus in a 2:1 stoichiometric ratio forming the complex, \(\text{PSb}_2\text{Mo}_{12}\text{O}_{40}\).\(^{20}\)

To insure silicic acid was not creating an interference, a solution containing only silicic acid was prepared together with the colorimetric reagents. In addition, an orthophosphate standard was prepared with 2 mg L\(^{-1}\) of silicic acid added.

The samples were analyzed on a Lambda Spec 20 spectrophotometer at a wavelength of 711 nm. To perform the colorimetric analyses standards were prepared. A standard solution of phosphorus was made by dissolving 0.2195 g of potassium dihydrogen phosphate into 1.0 L of 18 MΩ water in a volumetric flask. This solution had a concentration of 50 µg of orthophosphate L\(^{-1}\). The standard orthophosphate solution was diluted to make up the calibration standards. To prepare these standards a secondary standard was made by diluting 5.0 mL of the primary stock and with 18 MΩ water in a 100 mL volumetric flask. Next, 0, 2, 4, 6, 8, and 10.0 mL of the secondary stock standard was pipetted into six separate 25 mL volumetric flasks. Then, 4.0 mL of the molybdenum blue colorimetric solution was added to each flask and the volumetric flasks
were filled to the 25 mL mark with 18 MΩ water. The samples were mixed and allowed to rest for 10 minutes to let the color develop. Then the samples were run on the spectrophotometer and absorption data was collected at 711 nm. The wavelength was determined by evaluating the maximum absorbance of the standard solutions between 400 and 800 nm.

Phosphate values were reported as a percentage of the total dry biomass on a g g\(^{-1}\) basis. The values were reported this way because phosphorus may constitutes both the organic and inorganic fractions of the biomass.

2.3.3 Carbon, Hydrogen, and Nitrogen Analyses

The total quantities of carbon, hydrogen, and nitrogen in the algal samples were evaluated using a Truspec ® CHN analyzer from LECO Corporation, St. Joseph, MI. The instrument was calibrated using 20-200 mg samples of a pure EDTA standard purchased from LECO. All samples and standards were weighed using a Sartorius balance. Prior to use a check standard consisting of 5 samples of 200 mg EDTA was evaluated along with 10-15 baseline blank samples, containing only room air. These samples were used to calibrate the instrument for moisture content in the ambient air.

Previously lyophilized or ashed algae samples were each placed in a 20 mL glass scintillation vial and heated at 104 °C for 1-2 hours, capped, and allowed to cool in a desiccator prior to analysis. Then 30-200 mg of each sample was accurately massed to the nearest tenth of a microgram into tin foil caps. Each algae sample was analyzed in duplicate or triplicate and the carbon and nitrogen contents were averaged.
Samples previously ashed at 550 °C, to a constant weight, were used to analyze the percentage of inorganic carbon in the biomass. This method assumes that all the organic carbon had been volatilized at this temperature. Any remaining carbon in the biomass is presumed to be bound in the inorganic form of CaCO$_3$, which is not volatile under 825°C. During the combustion process the CHN instrument attained a temperature of 950 °C, which means it volatilized both organic and inorganic carbon fractions. The organic fraction is determined by simply subtracting out the inorganic carbon quantity in the ash (see *Equation 2-2*). All samples were run in triplicate except where noted. CHN values were reported as a percentage of the total dry biomass on a g g$^{-1}$ basis. The values were reported this way because carbon may constitutes both the organic and inorganic fractions of the biomass.

*Equation 2-2*. Organic carbon fraction of biomass

$$C_{	ext{Organic}} = C_{\text{Total (lyophilize biomass)}} - C_{\text{inorganic (ash Moment)}}$$

### 2.3.4 Monosaccharide Analysis

The method used for extracting and analyzing monosaccharides in algae was developed by Thompson *et al.* (2011). The first step in the process was the extraction of the monosaccharides from the biomass. The previously dried algal biomass was finely ground using a mortar and pestle and 300-600 mg of each algae sample was massed into a previously cleaned and labeled 18 x 150 mm glass test tube. Then 3.0 mL of a 12 M H$_2$SO$_4$ solution were added to each test tube. The test tubes were mixed with a glass stir rod and heated at 30°C under sonication for 120 minutes. Then the algal-sulfuric acid
solutions were transferred into 250 mL glass media bottles. The transfer was completed using small aliquots of 18.2 MΩ water totaling 79 mL. Next, each solution was spiked with 5 mL of the 3.5 mg mL⁻¹ myo-inositol internal standard. The media bottles were sealed, labeled, and placed in an autoclave at 121°C for 30 minutes. The bottles were allowed to cool to room temperature in an ice bath. Each solution was then filtered using a Büchner funnel and 20 mL of the hydrolysis solutions were stored in capped scintillation vials under refrigeration until analysis the next day.

A stock standard solution was prepared by dissolving 100 to 200 mg of arabinose, xylose, mannose, glucose, and galactose in 100 mL of 18.2 MΩ water. These solutions were used to prepare the calibration standards. To prepare the calibration standards 0.5, 1, 2 and 5 mL of each stock standard solution was pipetted into separate media bottles. Next, 5 mL of the 3.5 mg mL⁻¹ myo-inositol internal standard and 3.0 mL of 12 M sulfuric acid solution were added to each calibration standard. The solutions were diluted to 87 mL using 18.2 MΩ water.

Monosaccharides required reduction and acetylation prior to analysis by GC/MS. All samples were run in triplicate for this portion of the analysis. To initiate the reduction, a 1.00 mL aliquot of each of the samples or standards was transferred into a labeled test tube together with 250 μL of concentrated ammonium hydroxide. The test tubes were vortex mixed for 5 seconds. Next, 500 μL of a 0.15 g mL⁻¹ potassium borohydride solution, prepared in 3 M NH₄OH, was added and again the samples were vortex mixed. Then the samples were placed in a water bath, held at 40°C, for 90 minutes to allow the reduction reaction to occur. After the time had elapsed, 500 μL of acetic acid was added to stop the reaction. The samples were again vortex mixed for 5
seconds. The samples were then allowed to cool to room temperature and 400 μL portions were transferred into new test tubes for derivatization.

The acetylation procedures were initiated by the addition of 500 μL of 1-methylimidazole to each sample and mixing the samples for 5 seconds. Then 2.0 mL of acetic anhydride was added and the tubes were mixed and allowed to react for 30 minutes at room temperature. To decompose excess acetic anhydride 5.0 mL of water was added to each tube. The test tubes were put in an ice bath to cool. Next, 2.0 mL of dichloromethane was added and vortex mixed for 10 seconds, then allowed to separate and subsequently mixed for 10 seconds. The solution was allowed to separate for 15 minutes and most of the aqueous top layer was removed by pipette. To increase the pH, 5.0 mL of a 3.5 M KOH solution were added to each test tube. Again the test tubes were vortex mixed and the phases were allowed to separate. Then, the aqueous phase was removed and the dichloromethane layer was pipetted into a GC auto sampler vials for analysis.

GC/MS analyses were performed using a HP 6890 Model GC equipped with a 5973 N MSD mass selective detector and an HP 7683 Series auto sampler. MSD Chemstation software was used to evaluate the chromatography. Separation was completed using a 30m x 0.32 mm x 0.25 μm Stabilwax (PEG) column from Restek (Bellefonte, PA, USA). High purity helium was used as the carrier gas. The GC inlet temperature was kept at 200° C with inlet purge of 30 mL minute⁻¹ starting at 0.5 minutes. The column flow was 2.2 mL minute⁻¹ of helium with a split ratio of 10:1. The initial column temperature was held at 120°C for one minute and increased by 10°C minute⁻¹ until reaching 230°C and held for 12 minutes for total run time of 24 minutes.
The transfer line from the GC to the MSD detector was held at 240°C. The mass selective detector was run in scan mode from m/z 50-300 with a solvent delay of 5 minutes. The detector was calibrated according to the autotune parameters.

2.3.5 Quantitative Analyses

All quantitative analyses were performed by first creating calibration curves using a set of four standards to check the linearity of the signal. The peak area of each analysis was integrated by using the system software and double-checked by hand. The monosaccharide quantity in each sample was calculated using myo-inositol as an internal standard. Monosaccharides in the biomass were reported on an ash free dry mass basis because a large portion of the biomass was composed of inorganic material and sediment and monosaccharides are part of the organic fraction.

2.3.6 Floway Position Experiment

Four floways were in operation at the Goldsworth Pond site. Biomasses harvested from all four floways were combined from April 2010 to May 2011. Biomass Samples were not collected during the winter months because only a small quantity of biomass accumulated on the floway. Starting in June 2011, floways were equally sectioned into four different positions, each section was 0.19 x 3.9 m² and biomass harvests were collected separately from each position (one and four) on the individual floways (see Figure 2-1). Floways were positioned adjacent to one another, used the same inlet water, and received equal illumination. This experiment was done to determine if floway
position had an effect on nutrient content in algae grown in the floway as suggested by Adey et al. (2011).

Figure 2-1. Floway positions at the (A) Goldsworth Pond and (B) Muskegon Wastewater Treatment site.
In theory, algae grown at the beginning of the floway will be exposed to water with higher nutrient concentrations than algae grown at a greater distance from the beginning of the floway. As the water in the floway moves over the algae, the algae will take those nutrients up, leaving fewer nutrients in the water for the algae further down in the floway.

The floway position experiment was also conducted at the Muskegon Wastewater Treatment Plant site. During June and July 2010, three floways, equally sectioned into twenty different positions, were operated at this location. Each screen section was 0.19 x 1.525 m$^2$. Biomass was harvested and separated by floway number and position. Floways were positioned adjacent to one another, used the same inlet water, and received equal illumination. All biomass harvested at this location was immediately put into cleaned glass food jars, and processed within two hours of harvesting.

Biomass samples collected for this experiment were given codes that designated the floway number and position from which the biomass was harvested. For example, in sample AGH070811F4S4, F4 stands for floway number four and S4 stands for screen number four. Lower screen numbers indicate that the screen was located closer to the inlet, or beginning of the floway; higher screen numbers indicate that the screen was more distal.

2.3.7 Carbon Dioxide Addition Experiment

Two floways were in operation at the Great Wicomico River location. Floways were positioned adjacent to one another, used the same inlet water, and received equal
illumination. Biomass was harvested from the entire length of each floway and no experimental manipulations were conducted on floways one and two from July to November 2010. Starting in late November 2010, nutrient addition experiments were conducted on floway two. Floway two was split into two portions, an upper (F2U) and a lower portion (F2D). Each section was 0.61 x 7.6 m². The upper portion of floway two received no experimental manipulation. The lower segment of floway two had nutrients introduced at 0.03 mg L⁻¹ TN and 0.004 mg L⁻¹ TP. Furthermore, from August 8 to December 11, 2011 CO₂ injections were introduced into the lower segment that kept the pH between 7.5-8.5 on cloudy days and 8.2-8.3 on sunny days. The average pH was 8.2-8.3.

CO₂ addition experiments were carried out with stainless steel injectors used for delivering CO₂ to carbonated beverages. A pH controller was also installed to measure the pH of the water. The lower pH limit that the controller operated on was 7.0. When the pH of the water was dropped below 8.0 the CO₂ was automatically shut off. CO₂ injection did not take place a night.

2.3.8 Water Analyses

Water samples at each of the site locations were taken to monitor the nutrient concentrations in the water. The Muskegon County Wastewater System monitored water quality at the Muskegon Wastewater Treatment Plant. The Virginia Institute of Marine Science checked York River nutrient concentrations and analyzed water samples collected from the Great Wicomico River sites.

A small number of water samples were taken at the Goldsworth Pond site and
analyzed for total dissolved phosphorus. Samples were collected directly from the inflow of each floway using acid washed 120 mL glass bottles. Samples were stored in a refrigerator at 4°C. The water quality laboratory at the Muskegon County Wastewater System carried out analyses.

2.4 Results and Discussion

2.4.1 CHN Analyses

2.4.1.1 Goldsworth Pond Site

The total nutrient content in the Goldsworth Pond algal biomass comprised anywhere from 16 to 31 wt % of the total dry biomass. Evaluation of the data displayed in Figure 2-2 shows that biomass grown in the late summer months of 2010 contained the highest concentration of nutrients. The organic carbon content ranged from a low of 7.6 wt % in mid April 2010 to a high of 19.92 wt % in early September 2010 (more detail is available in Appendix A). The decrease in organic carbon during October and November is likely due to decreased sunlight.22
Nitrogen and hydrogen contents in the biomasses were positively correlated with organic carbon levels ($p=0.029$ and $p < 0.001$, respectively). Nitrogen levels were also positively correlated with hydrogen levels ($p=0.001$). The percentages of nitrogen and hydrogen in the algal biomass do not follow any apparent trends in relation to time of year (spring, summer, and fall) or carbon content. This indicates that the algal biomass did not producing a larger store of protein macromolecules during any specific time of year. Longer-term studies need to be conducted into the winter months to verify this.

Algal biomass inorganic and organic carbon levels were inversely correlated (see Figure 2-3). Statistical evaluation gave a Pearson’s Product Moment (PPM) correlation
r-value of -0.551, and p < 0.012 (two tailed t-test). The reason for this correlation has not been determined. However,

It has been suggested that green algae, when grown with or adapted to low air levels of CO₂ in the light, exhibit a photosynthetic evolution with low levels of external DIC (dissolved inorganic carbon) by accumulating inorganic carbon inside the cells. This process has been termed a dissolved inorganic carbon concentrating mechanism or DIC pump. When microalgae are grown with elevated levels of CO₂, the light-dependent intracellular accumulation of inorganic carbon is suppressed. (p.630)²³

Other authors have confirmed these changes in organic carbon acquisition and DIC pump activity with changes in CO₂ levels in algae.²⁴,²⁵

Algae may have developed a DIC pump to overcome the low affinity of the main carboxylating enzyme by storing excess CO₂ at the site of carboxylation.²⁶ Because phytoplankton live in an environment where light (energy) is one of the limiting resources, the energy available for active transport of inorganic carbon and other nutrients is often limited.²⁷ Energy-limitation may be one of the reasons why phytoplankton do not rely entirely on the active uptake of inorganic carbon from the pool of HCO₃⁻, but instead obtain a fraction of their carbon from a small pool of CO₂.²⁷

In this work, CO₂ concentration levels were not specifically manipulated to evaluate changes in the inorganic and organic carbon content of the algae. However, future studies should focus on determining the relationship between organic and inorganic carbon by manipulating exposure to high and low levels of CO₂ and evaluating the effect on each carbon component.
2.4.1.2 Floway Position Analyses

*Goldsworth Pond.* Statistical analyses of the ultimate data collected from the floway position harvests indicated that screen four (farther down from the beginning of the floway) had a significantly (p < 0.001) higher organic carbon content than screen one, regardless of floway. The inorganic carbon content was not significantly different. For the June 18 harvest the nitrogen levels were significantly elevated on the later screens. Hydrogen content was higher on screen four than on screen one of floway three. For the July 8 harvests screen four had a greater amount of hydrogen (p < 0.039), yet the nitrogen level was not significantly elevated. July nutrient level comparisons should be viewed with some skepticism, because a comparison is being made with only one sample from the beginning of the floway. However this comparison agrees with results obtained from the June sampling period.

*Muskegon Wastewater Treatment Plant.* All algal biomass samples contained a relatively large proportion of carbon, hydrogen, and nitrogen (see *Appendix A*). The total

![Graph showing organic vs. inorganic carbon content]

Figure 2-3. Goldsworth Pond Algae plot of organic vs. inorganic carbon.
nutrient levels ranged from 45 to 55 wt % of the total dry biomass. The inorganic carbon content was the highest on July 12, 2011 with over 9.67 wt. %; subsequent harvests had much lower percentages (4.96 to 1.67 wt. %). Inorganic and organic carbon levels showed inverse correlation (PPM r-value of -0.765 and a p-value < 0.001). Nitrogen content was negatively correlated with inorganic carbon content (p=0.036), but positively correlated with organic carbon (p = 0.015). This is an expected result because as the proportion of biomass composed of protein increases, indicated by the increase in nitrogen, the amount of organic carbon must also increase. Interestingly, hydrogen showed the opposite trend, being positively correlated with inorganic carbon levels (p < 0.001) and negatively correlated with organic carbon (p = 0.006).

Table 2-1 displays results from the statistical analyses done to compare the nutrient levels from different screens positions on each date. The evaluation demonstrates that algal biomass grown on upstream screens tends to have the greatest percentage of total carbon. Comparisons made between screen position, and organic and inorganic carbon levels, were not statistically different.
Table 2-1. Analysis of variance (ANOVA) results for nutrient percentages in algae grown at the Muskegon Wastewater Treatment Plant on different screens positions. Only statistically significant values are reported. P-values reflect a comparison between two different screen position on the floway.

<table>
<thead>
<tr>
<th>Harvest Code</th>
<th>Nutrient</th>
<th>Screen Position</th>
<th>Screen Position</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMS071211F1</td>
<td>C</td>
<td>2</td>
<td>8</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>2</td>
<td>8</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>0.02</td>
</tr>
<tr>
<td>AMS071211F3</td>
<td>C</td>
<td>10</td>
<td>20</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>2</td>
<td>10</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>0.027</td>
</tr>
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<td></td>
<td>10</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td>AMS072911F3</td>
<td>None</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>AMS081211F1</td>
<td>C</td>
<td>2</td>
<td>20</td>
<td>0.006</td>
</tr>
<tr>
<td>AMS082211F1</td>
<td>C</td>
<td>2</td>
<td>20</td>
<td>0.042</td>
</tr>
</tbody>
</table>

2.4.2 Great Wicomico River Site

2.4.2.1 Floway One and Floway Two CHN Comparisons

Algal biomasses harvested from floway one and two are compared in Figure 2-4. To provide a comparison of nutrient levels on floways one and two, nutrient levels from each season were averaged. A detailed explanation of data from each site can be found in Appendix A. Biomass collected from floway two tended to have a high percentage of
inorganic carbon and lower percentage of organic carbon than biomass collected from floway one. A pair-wise comparison of the floways showed that the percentage of nitrogen on floway two was significantly higher than on floway one, except during the summer 2010 harvests, when nitrogen content was about equal.

In the fall of 2010 and spring of 2011 biomass harvests from floway two showed higher nitrogen accumulation than biomass harvests from floway one. A comparison of nutrient removal rates from the incoming and outgoing water (see Table 2-2) indicate that nitrogen removal from the incoming water was higher on floway two than on floway one. The algal biomass compositions on the two floways are similar in the fall, however, floway one does have a larger proportion of cyanobacteria (see Chapter 1: Table 1-1). In the spring floway one also shows a spike in chlorophyta population.

Figure 2-4. Great Wicomico River site ultimate analyses. Comparisons of floway one and two. (■) inorganic carbon, (■) organic carbon, (■) hydrogen, and (■) nitrogen composition. Error bars represent sample standard deviations of averaged data.
Table 2-2. Water nutrient levels at the Great Wicomico Site. Total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP).

<table>
<thead>
<tr>
<th>Date</th>
<th>Inflow</th>
<th>Outflow</th>
<th>Inflow</th>
<th>Outflow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>River F1</td>
<td>F2</td>
<td>River F1</td>
<td>F2</td>
</tr>
<tr>
<td>Fall 2010</td>
<td>0.3234</td>
<td>0.2783</td>
<td>0.2442</td>
<td>0.0095</td>
</tr>
<tr>
<td></td>
<td>0.007</td>
<td>0.0054</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring 2011</td>
<td>0.3536</td>
<td>0.3493</td>
<td>0.3176</td>
<td>0.0150</td>
</tr>
<tr>
<td></td>
<td>0.009</td>
<td>0.0059</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|            | Six sampling periods of two samples each | Five sampling periods of two samples each |

2.4.2.2 Carbon Dioxide Addition Experiment

The quantity of nutrients in the algae samples harvested on December 11, 2010, February 23, March 12, March 29, April 18, and May 10, 2011 were compared to determine if the addition of carbon dioxide changed the nutrient make-up of the algae (see Figure 2-5). Statistical analysis was performed using a student-paired t-test. Sample harvests were matched by date and flowway location, which made the paired t-test an acceptable form of statistical evaluation over an independent t-test.

The percentage of inorganic carbon in algae grown on the lower segment of flowway two was lower than on the upper segment, although difference was not significantly different. However, the organic carbon content was significantly higher in algae grown on the lower portion of the flowway, with a p-value of 0.011 (two tailed t-test). The percentages of hydrogen and nitrogen in the lower segment of flowway two are also statistically higher with p-values of 0.008 and 0.030, respectively. From this analysis it appears CO$_2$ addition to algae grown in flowway two significantly enhanced the organic carbon and hydrogen levels.
Figure 2-5. CO₂ addition experiment. (■) inorganic carbon, (■) organic carbon, (■) hydrogen, and (■) nitrogen composition. Error bars represent sample standard deviations of averaged data.

2.4.3 York River Site

Algal biomasses harvested from York River and Boat Basin sites are compared in Figure 2-6. To provide a comparison the nutrient levels from June through September 2010 were averaged (additional data is located in Appendix A). The nutrient levels at both sites were much lower than any other sites evaluated (see Figure 2-8). Nutrient levels in biomass samples from the Boat Basin site were always higher than at the York River site. The inorganic carbon proportion of the biomass from the Boat Basin site was 3.95 ± 0.66 wt %, and remained stable throughout the sampling period. Similarly, the percentage inorganic carbon in York River biomass was not significantly different from the percentage in Boat Basin biomass, averaging 3.85 ± 1.68 wt %. The major difference in nutrient content is in the organic carbon levels, which were 10.22 ± 3.49 and 6.21 ± 1.57 wt % in the Boat Basin and York River biomasses, respectively.
Figure 2-6. York River (YR) and Boat Basin (BB) site comparison. (■) Inorganic carbon, (□) organic carbon, (■) hydrogen, and (■) nitrogen composition. Error bars represent sample standard deviations of averaged data.

The percentage hydrogen in the Boat Basin algae was positively correlated with the organic carbon level (p-value = 0.035). At this sampling location no other statistically significant correlations between nutrient levels were found.

Nutrient removal from the incoming and outgoing water at the York River site, provided in Table 2-3, were compared to nitrogen accumulation in the York River biomass samples. Increased nitrogen levels in the biomass coincided with nitrogen removal from the water.
Table 2-3. York River water nutrients. Total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP).  

<table>
<thead>
<tr>
<th>Date</th>
<th>TDN (mg L⁻¹)</th>
<th>TDP (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inflow</td>
<td>Outflow</td>
</tr>
<tr>
<td>6/10</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>7/10</td>
<td>0.50</td>
<td>0.40</td>
</tr>
<tr>
<td>8/10</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>9/10</td>
<td>0.55</td>
<td>0.35</td>
</tr>
</tbody>
</table>

2.4.3.1 Mini-flume Analyses

The York River mini-flume was a temporary in stream floway system that was set-up in July 2010; it was destroyed by a storm following the July 9 harvest. The nutrient percentages obtained before the storm are displayed in Table 2-6. The in-river mini-flume was implemented to test whether allowing the river current to naturally carry water over the algae effected nutrient content in comparison to pumping water over the growing algae. Unfortunately, mechanical difficulties with the mini-flume system made this comparison problematic.

Pair-wise comparison between the nutrient levels in York River and mini-flume biomasses indicated organic carbon, nitrogen, and hydrogen percentages in harvests from each location did not significantly differ. However, algae from the York River floway did contain a significantly higher percentage of inorganic carbon than mini-flume algae (p-value ≤ 0.01, two tailed t-test). For the shot period that the pumping system and the in-water stream system were both operational, it can be concluded that pumping had no effect on CHN accumulation in the algal biomass.
Table 2-4. The ultimate analyses of algal biomass harvested from floways located in the York River, VA. All samples were run in triplicate.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inorganic Carbon</th>
<th>Organic Carbon</th>
<th>Hydrogen</th>
<th>Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVI070110MF</td>
<td>2.39 ±0.18</td>
<td>6.17</td>
<td>1.41</td>
<td>0.91</td>
</tr>
<tr>
<td>AVI070410MF</td>
<td>3.57 ±0.08</td>
<td>8.33</td>
<td>2.07</td>
<td>1.78</td>
</tr>
<tr>
<td>AVI070610MF</td>
<td>3.26 ±0.24</td>
<td>4.71</td>
<td>1.40</td>
<td>0.78</td>
</tr>
<tr>
<td>AVI070910MF</td>
<td>2.78 ±0.21</td>
<td>9.02</td>
<td>2.03</td>
<td>1.64</td>
</tr>
</tbody>
</table>

2.4.4 Lake Erie CHN Algae Analyses

The percentages of hydrogen and nitrogen in algal biomass harvested from the Lake Erie site are extremely low in comparison to other sites (see Figure 2-7), at less than 1.0 wt % and 2.0 wt % by total dry mass, respectively. This may have been a result of low nitrogen concentrations in the growth media. Although nutrient concentrations in this growth media were not measured, studies have found that growing algae in low nitrogen or nitrogen-deficient mediums decreases the protein quantity in the biomass, which decreases the overall nitrogen content.\textsuperscript{29, 30, 31} Additionally, low or deficient nitrogen growth media has been found to increase the carbohydrate content in the algae.\textsuperscript{29, 31}

Lynn \textit{et al.} (2000), found that under nutrient stress, such as phosphorus or silica deficient growth media, nitrogen limited algae decrease protein production and increase lipid production, resulting in increased carbohydrate content.\textsuperscript{32, 33} They do this for two reasons (a) triglycerides are highly reduced and yield energy when oxidized; and (b) triglycerides are hydrophobic and do not carry the weight of extra water.\textsuperscript{34} The strategy
of Si- and P-limited algae is to convert excess carbohydrates to lipids, particularly triglycerides, however N-limited algae amass carbohydrates and forego the high-energy triglycerides reserves. This is because unlike animals that use insulin to control the conversion of carbohydrates to lipid, lipid synthesis in algae is dependent on the activity of acetyl-CoA carboxylase, a key protein enzyme. \textsuperscript{35,36} This may indicate that in N-limited environments algae are biochemically unable to transform excess carbohydrates to lipid stores, due to the reduced protein content.\textsuperscript{31}

![Figure 2-7. Ultimate analyses site comparisons. (■) Inorganic carbon, (■) organic carbon, (■) hydrogen, (■) nitrogen composition, and (■) total carbon. Inorganic and organic carbon was not determined in Lake Erie biomass because the amount of biomass collected was too small. Error bars represent sample standard deviations of averaged data.](image)
2.4.5 Monosaccharide Analyses

Goldsworth Pond. The monosaccharide content of algal biomass collected from the Goldsworth Pond location was analyzed in harvests from the spring and summer of 2011 (Figure 2-8). Harvests from 2010 were also evaluated, but, likely because samples were not analyzed until 14-16 months after harvest, no detectable carbohydrates were found. The biomass analyzed from 2011 harvests was composed of ribose, arabinose, xylose, mannose, glucose and galactose. Ribose, arabinose and xylose are all five-carbon sugars with the formula C_5H_{10}O_5. Mannose, glucose and galactose are all six-carbon sugars with the formula C_6H_{12}O_6. The greatest proportion of the monosaccharides was composed of glucose. Galactose is the second most abundant monosaccharide followed either by mannose, arabinose, or xylose, with ribose being the scarcest. The total monosaccharide content in the biomass ranges from 14 to 31 wt % ash free dry mass (AFDM). The large deviation in total monosaccharide content may be due to fluctuations in the amount of sunlight: higher light intensity is associated with an increase in saccharide content.\(^\text{22}\)
Figure 2-8. Monosaccharide profile of algal biomasses grown at the Goldsworth Pond location. (▼)Ribose, (△)arabinose, (▲) xylose, (□) mannose, (●) glucose, and (◆) galactose, based on ash free dry biomass. Error bars represent the sample standard deviation of triplicates.
Muskegon Wastewater Treatment Plant. The monosaccharide composition was measured in algal biomasses collected on the same date from floway three at different screen positions (Figure 2-9). The total monosaccharide content in the harvested samples was the same (3.47 wt % AFDM) and the content of individual monosaccharides was not significantly different at either screen position. In order of abundance, the monosaccharides in this algal biomasses were: glucose > galactose > mannose > xylose ≈ ribose > arabinose.

Lake Erie. The total monosaccharide content in biomasses harvested from this site ranged from 1.2 to 13.8 wt % AFDM (Figure 2-10; Table 2-6). Glucose was generally the most abundant monosaccharide present in each harvest; except that galactose is most abundant in harvest ALE062811F2. Abundance of monosaccharides is generally: glucose > galactose > mannose > xylose ≈ arabinose > ribose. Interestingly, only four of the harvests contained detectable ribose, although the content was diminutive, ranging from 0.05 ± 0.02 to 0.16 ± 0.03 wt % AFDM.

The overall monosaccharides content in ALE061411F1 and ALE062811F2 was much lower compared to the other samples from this site, consisting of a mere 1.2 wt % AFDM. However, the total percentage of carbon in these harvests was also the lowest of any of the algal biomass harvests evaluated here, at 15 wt % and 13 wt % of total dry weight, respectively. Given this information, the low carbohydrate content is not surprising because carbon content can be used to estimate total carbohydrates.
Figure 2-9 Monosaccharide profile of algal biomass grown at the Muskegon Wastewater Treatment Plant location. (■) Ribose, (■) arabinose, (■) xylose, (■) mannose, (■) glucose, and (■) galactose, based on ash free dry biomass. Error bars represent the sample standard deviation of triplicates.
Figure 2-10 Monosaccharide profile of algal biomass grown at the Lake Erie location. (■) Ribose, (□) arabinose, (▲) xylose, (▲▲) mannose, (▲▲▲) glucose, and (▲▲▲▲) galactose, based on ash free dry biomass. Error bars represent the sample standard deviation of triplicates.
Table 2-5. The percent of organic carbon accounted for by the monosaccharides.

<table>
<thead>
<tr>
<th>Harvest Code</th>
<th>% wt (g g⁻¹) of total carbon accounted for by monosaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGH051311</td>
<td>1.51 ± 0.12</td>
</tr>
<tr>
<td>AGH061811F3F4</td>
<td>2.01 ± 0.41</td>
</tr>
<tr>
<td>AGH070811F4S4</td>
<td>4.80 ± 0.64</td>
</tr>
<tr>
<td>AGH081211F4S4</td>
<td>4.56 ± 0.56</td>
</tr>
<tr>
<td>AMS082211F3S2</td>
<td>0.79 ± 0.06</td>
</tr>
<tr>
<td>AMS082211F3S20</td>
<td>0.97 ± 0.01</td>
</tr>
<tr>
<td>ALE082210</td>
<td>2.11 ± 0.32</td>
</tr>
<tr>
<td>ALE090710</td>
<td>1.23 ± 0.00</td>
</tr>
<tr>
<td>ALE093010</td>
<td>0.67 ± 0.11</td>
</tr>
<tr>
<td>ALE101910</td>
<td>1.17 ± 0.11</td>
</tr>
<tr>
<td>ALE111910</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>ALE112310</td>
<td>0.68 ± 0.09</td>
</tr>
<tr>
<td>ALE061411F1</td>
<td>0.10 ± 0.00</td>
</tr>
<tr>
<td>ALE062811F2</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>ALE071311F2</td>
<td>2.16 ± 0.11</td>
</tr>
<tr>
<td>ALE072811F1</td>
<td>1.66 ± 0.38</td>
</tr>
</tbody>
</table>

Numbers are based on triplicate samples. Mean ± standard deviation.
2.4.6 Phosphorus Analyses

2.4.6.1 Goldsworth Pond

The level of phosphorus, P, in algal biomasses harvest from Goldsworth Pond was expected to be elevated because of P-rich runoff from surrounding urban area (Table 2-7). The highest percentage of phosphorus was found in algal biomass harvested on June 24 and November 12, 2010 (Figure 2-11). The high P levels in the biomass samples harvested on July 30 and November 12 corresponded with total dissolved phosphorus (TDP) concentrations that were 3.5 times higher in the incoming water. Indicating that water P-levels may have influenced P-accumulation in the biomass. Peak phosphorus levels did not coincide with any particular growing season.

Evaluation of harvested biomass from different floway positions showed that downstream screens had elevated P levels (See Appendix A for more detail), although only samples AGH061819F2S4 and AGH080111F1S4 had significantly higher levels of P accumulation compared to upstream screens. Statistical analyses, however demonstrated that floway position trends in P concentration were not significant (Table 2-9).
Table 2-6. Goldsworth Pond inlet concentrations of total dissolved phosphorus (TDP).

<table>
<thead>
<tr>
<th>Date</th>
<th>Floway</th>
<th>TDP (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/10</td>
<td>Floway1</td>
<td>0.0689</td>
</tr>
<tr>
<td></td>
<td>Floway2</td>
<td>0.0714</td>
</tr>
<tr>
<td>8/10</td>
<td>Floway1</td>
<td>0.0539</td>
</tr>
<tr>
<td></td>
<td>Floway2</td>
<td>0.0599</td>
</tr>
<tr>
<td>9/10</td>
<td>Floway1</td>
<td>0.0541</td>
</tr>
<tr>
<td></td>
<td>Floway2</td>
<td>0.0491</td>
</tr>
<tr>
<td>10/10</td>
<td>Floway1</td>
<td>0.0351</td>
</tr>
<tr>
<td></td>
<td>Floway2</td>
<td>0.0353</td>
</tr>
<tr>
<td></td>
<td>Floway3</td>
<td>0.0511</td>
</tr>
<tr>
<td></td>
<td>Floway4</td>
<td>0.0378</td>
</tr>
<tr>
<td>11/10</td>
<td>Floway1</td>
<td>0.144</td>
</tr>
<tr>
<td></td>
<td>Floway2</td>
<td>0.151</td>
</tr>
<tr>
<td></td>
<td>Floway3</td>
<td>0.165</td>
</tr>
<tr>
<td></td>
<td>Floway4</td>
<td>0.182</td>
</tr>
</tbody>
</table>

Figure 2-11. Percent phosphorus in algae harvested from Goldsworth Pond site. Error bars represent the standard deviation of measured phosphorus. Samples were analyzed in triplicate, except for samples from October 15, 2010 to November 21, 2011, which were analyzed in duplicate.
Table 2-7. Screen location student t-test results.

<table>
<thead>
<tr>
<th>Harvest Code</th>
<th>Paired</th>
<th>P-value (t-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGH061811</td>
<td>F2S1&amp; F2S4</td>
<td>0.002*</td>
</tr>
<tr>
<td></td>
<td>F3S1 &amp; F3S4</td>
<td>0.164</td>
</tr>
<tr>
<td>AGH070811</td>
<td>F1S1 &amp; F1S4</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>F2S1 &amp; F2S4</td>
<td>0.107</td>
</tr>
<tr>
<td></td>
<td>F4S1 &amp; F4S4</td>
<td>0.601</td>
</tr>
<tr>
<td>AGH080111</td>
<td>F4S1 &amp; F4S4</td>
<td>0.221</td>
</tr>
<tr>
<td></td>
<td>F4S1 &amp; F1S4</td>
<td>0.036*</td>
</tr>
</tbody>
</table>

*Starred values are statistically significant at the α ≤ .05 level.

2.4.6.2 Muskegon Wastewater Treatment Plant

The quantity of phosphorus in algae grown at the Muskegon Wastewater Treatment plant is the highest amongst any of the sites evaluated (see Figure 2-12). The average percent of phosphorus in the algae at this location was 1.279 ± 0.135 % by weight, which is four times more than in algae from the other sites. The P input in the water at this location averaged 2.29 ± 0.28 mg L\(^{-1}\) (Table 2-9) during the sampling period, which was much higher than TDP levels at any of the other locations.

The P accumulation in biomasses harvested from different floway positions were not significantly different, except for the July 12 harvest. For this harvest, screen twenty biomass accumulated significantly more P than screen two (p-value = 0.043).
Table 2-8. Muskegon Wastewater Treatment Plant water nutrient levels. Total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP).

<table>
<thead>
<tr>
<th>Sample Date</th>
<th>TDN (mg L⁻¹)</th>
<th>TDP (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>07/11/11</td>
<td>6</td>
<td>2.69</td>
</tr>
<tr>
<td>07/19/11</td>
<td>8</td>
<td>2.23</td>
</tr>
<tr>
<td>07/25/11</td>
<td>8.2</td>
<td>2.13</td>
</tr>
<tr>
<td>08/02/11</td>
<td>9</td>
<td>2.42</td>
</tr>
<tr>
<td>08/08/11</td>
<td>8.2</td>
<td>2.58</td>
</tr>
<tr>
<td>08/15/11</td>
<td>5</td>
<td>2.05</td>
</tr>
<tr>
<td>08/22/11</td>
<td>8</td>
<td>1.94</td>
</tr>
</tbody>
</table>

2.4.6.3 Lake Erie

The percent of P in biomass grown using water from Lake Erie site ranged widely over the entire harvest period. Initially, the quantity of P in the algae was 0.415%, but as the harvest season progressed the quantity dropped and continued to be lower during the 2011 harvest period as compared to 2010 (Figure 2-12). Without water chemistry data for this area, it is difficult to speculate regarding reasons for the observed reduction. The percentage P in the Lake Erie algae is similar to the percentages in the Great Wicomico and York River algae (Figure 2-14).
The average percent P in algal biomass grown on Great Wicomico River floway one was 0.149 ± 0.045 wt % and on floway two the average was 0.156 ± 0.033 wt %. Although the P content in algal biomass harvested from floway one had a larger range (0.077 to 0.274 wt %), P levels were not significantly different between the floways. Algal biomass on floway two also decreased the total dissolved phosphorus concentrations (TDP) in water by 0.0026 mg L\(^{-1}\) more than algal biomass on floway one (see Table 2-2).

After CO\(_2\) addition was implemented on floway two, the lower section (receiving CO\(_2\) injection and nutrient addition) appeared to have higher P concentrations than the upper section (Figure 2-13). The average percent P in the lower section was 0.228± 0.057 wt %, while algae harvested from the upper section averaged 0.174± 0.031 wt %.
However, a student t-test was used to evaluate the data and the p-value was 0.08 (two tailed), which was not significant. Thus, the addition of CO$_2$ and nutrient injection did not significantly impact the P content of the algae.

Figure 2-13. Comparison of the phosphorus content in algae harvested from the (■) upper and (■) lower segments of floway two. Downstream portions of floway two were supplemented with CO$_2$ and the upper section of the floway served as the control.
The average percent of P in algae samples harvested from the York River location was 0.129 ± 0.037 wt%. The average percent P found in samples harvested from the Boat Basin location was 0.162 ± 0.045%, which was not significantly different from the amount found in the York River samples (Appendix A).

The percentage of P in the samples from the York River was similar to levels found in algae harvested from the Great Wicomico River site. These results are interesting because the total dissolved phosphorus levels in these locations are drastically different; total dissolved phosphorus (TDP) in the York River ranged from 0.02 to 0.07 mg L^{-1} (Table 2-3) and in the Great Wicomico River averaged 0.004 mg L^{-1}. These results indicate that P can be accumulated in the algae to a much higher level than is present in the water. TDP data from the York River site showed that the algal turf growing at this location...
site reduced TDP concentrations in the water more than algal turf at the Great Wicomico River site.

2.5 Summary

Algal biomasses produced from a variety of locations and water sources were evaluated for general nutrient elemental compositions, specifically carbon, hydrogen, nitrogen and phosphorus. The carbon fraction of the biomass was further partitioned into organic carbon and inorganic carbon. The elemental content of the algal biomass varied between each location. Biomass with the highest content of carbon, nitrogen and phosphorus was harvested from the Muskegon Wastewater Treatment Plant. Biomass harvested from the York River location had the lowest total nutrient content, except nitrogen levels were the lowest in algal biomass harvested from the Lake Erie site. At each individual site location, except for the York River sites, inorganic carbon levels were inversely correlated with organic carbon levels. At all locations Nitrogen tended to be positively correlated with organic carbon. Additionally, hydrogen levels were usually correlated with organic carbon levels. However in Goldsworth Pond algal biomass hydrogen was instead correlated with inorganic carbon levels, which may have been coincidental or related to HCO$_3^-$ stores in the algal biomass.

At a few of the locations experiments were carried out to evaluate the relationship between floway position and nutrient accumulation. Nutrient levels were generally greater in algal biomass grown further downstream on each floway. An experiment using CO$_2$ and nutrient additions to growing algal biomass at the Great Wicomico River site resulted in enhanced carbon, hydrogen and nitrogen levels in the harvested biomass.
To account for the organic carbon fraction of the algal biomass, carbohydrates were analyzed in samples collected from the freshwater locations. The carbohydrate profiles in the algal biomass consisted of glucose, galactose, ribose, mannose and xylose. Typically, glucose was the most abundant monosaccharide followed by galactose. Among the site locations the order of the remaining saccharides varied. The total quantity of saccharides also varied in each sample harvested.
2.6 Bibliography

(1) Fresenius, C. R.; Cohn, A. I. *Quantitative chemical analysis*; J. Wiley & sons, 1911; Vol. 2.

(2) Yin, C.-Y. *Fuel* 2011, 90, 1128–1132.


CHAPTER 3

SILICA, ASH, AND METAL CONTENT IN ALGAL BIOMASS

3.1 Introduction

3.1.1 Importance of Ash

When evaluating the possible uses of algal biomass for purposes such as alternative fuel production, feed, and fertilizer it is important to assess the chemical and physical properties of the biomass. Such properties can significantly affect the value and potential applications of the algal biomass. Ash is the proportion of biomass that is non-combustible and contains inorganic material. Generally, lower ash content is more favorable for biofuel applications. This is because increased ash content typically results in a reduction in the heating value of biomass fuels although the relationship is not directly proportional.\(^1\) Additionally, ash can cause slagging, fouling, bed agglomeration, and corrosion in combustion devices, which can reduce performance and damage equipment.\(^2\) Zevenhoven-Onderwater et al. (2006) found that non-combustible silicates, with their higher melting points, can form a sticky layer on other particles, gluing them together to form agglomerates.\(^3\)

The biomass elements forming ash are diverse and depend on biomass type, soil type and type of harvesting process. High alkali metal content in biomass causes ash components to be reactive, i.e. easily converted into new compounds during combustion, increasing the likelihood of interactions with sulfur and chlorine flue gases.\(^4,5\) Because
ash is a solid byproduct left after combustion, it builds up and must be removed and disposed of properly. Disposal can become even more complicated and expensive when toxic material such as heavy metals are present in the ash. All of these issues serve to reduce the economic efficiency of using biomass as a fuel source. In the following sections the two main components of ash, silica and metals, will be discussed in detail followed by their experimental quantitation in the collected biomass from different sites.

3.1.2 Silica

Although silicon is the second most abundant element in the earth’s crust, after oxygen, most of it is bound in solid forms that are not bioavailable, i.e. quartz or silicate minerals, and hence silica can be a limiting nutrient in aquatic systems. Naturally occurring silica can be classified into three groups: lithogenic, amorphous, and dissolved. Lithogenic silica is a terrestrial form of silica that is crystalline and comes from the lithosphere. Amorphous silica is a non-crystalline form of silica that comes from inorganic as well as biologically generated sources. The biological sources of silica are categorized as being biogenic, and the inorganic forms are terragenic. Dissolved silica (dSi), the aqueous form of silica, is the only readily bioavailable form.

Algae, especially diatoms, play a crucial role in the cycling of silicon (Si) in the environment. Diatoms are generally assumed to represent the most important Si sink in lakes and rivers. Diatoms take up the soluble (dSi) forms of silica: silicic acid as $\text{H}_3\text{SiO}_4$ and $\text{H}_4\text{SiO}_4$. A specific set of proteins then synthesize the dSi into nanospheres of biogenic silica that become the building blocks of frustules, the cell walls of diatoms. Once the algae die they fall to the bottom of the water column where they are either
buried in the sediment or undergo decomposition by bacteria.\textsuperscript{6,11} Long term geological cycling is responsible for returning silica to a crystalline form that may then reemerge at the earth’s surface through tectonic processes. The chemical weathering of rocks on land is responsible for the return of dSi to rivers and the ocean, thus completing the cycle.\textsuperscript{12}

Silicon is a fundamental nutrient required for the growth of algae as well as other plants. Silica has not traditionally been seen as an essential macronutrient, but Epstein \textit{et al.} (1999) found that plants deprived of silicon are structurally weaker and prone to abnormalities of growth, development, and reproduction. Silica is also the only nutrient that is not detrimental when collected in excess.\textsuperscript{13} The presence of Si in plants has been found to alleviate many abiotic and biotic stresses, leading to the incorporation of silicates into many fertilizers.\textsuperscript{14}

Algal biomass communities grown using the ATS™ systems are exposed to any contaminants in the water in which they are grown. Flowing water contains suspended material, both organic and inorganic, that could have collected along on the ATS™ floway. These particles may contain inorganic silicates that deposit on the growing biomass, introducing terragenic silica into the biomass samples. Because biogenic silica is an important nutrient for plant growth and terragenic silica is unavailable for plant growth, it is essential to determine the relative proportions of biogenic and inorganic silica in the biomass. Additionally, it is important to determine the total quantity of silica, both biogenic and terragenic (inorganic), because silica is non-combustible (the boiling point of silicon dioxide is 2230°C)\textsuperscript{15} and remains as ash upon combustion.

To assess the proportion of biogenic silica, DeMasters \textit{et al.} (1981) developed an alkaline extraction method. DeMasters \textit{et al.} based this technique on the observation that
aluminosilicates release silica slowly in a linear fashion as a function of time. Biogenic silica, which is amorphous, dissolves completely in an hour or two under alkaline extraction conditions, while inorganic silicates take much longer to dissolve.\(^{16}\) The difference in the dissolution rates of the two silicates allows them to be differentiated. During the alkaline digestion process the biogenic silica is rapidly hydrated and depolymerized to form soluble monosilicic acid or dSi.\(^{17}\)

Krausse \textit{et al} (1983) analyzed biogenic silica in algae using three different alkaline extraction procedures. The authors compared 0.2 M NaOH, 0.5 wt \% Na\(_2\)CO\(_3\), and 5 wt \% Na\(_2\)CO\(_3\) for accuracy and precision of silica extraction and found all methods to be suitable.\(^{17}\) Conley \textit{et al} (1992) used a wet alkaline digestion procedure to determine the quantity of diatoms, sponge spicules, and mineral (inorganic) silicates in the sediments of different Florida lakes.\(^{18}\) The wet alkaline digestion method was also investigated by Schlüter and Rickert (1998), who concluded that sequential extraction and continuous leaching were superior methods to single step methods, which do not provide a check on the progress of extraction.\(^{19}\)

3.1.3 Metals

Algae have been shown to have an affinity for metal bioaccumulation.\(^{20, 21, 22, 23}\) Bioaccumulation is an active process whereby removal of metals requires the metabolic activity of living organisms, whereas biosorption is a passive process and is not metabolically mediated.\(^{24}\) Doshi et al. (2008) showed that Cladophora \textit{sp.} (genus of green algae) can take up 347, 168, 819, and 504 mg g\(^{-1}\) (dry mass) of Cr\(^{3+}\), Cr\(\text{O}_7^{2-}\), Cu\(^{2+}\), and Ni\(^{2+}\), respectively.\(^{20}\) Cadmium bioaccumulation has been demonstrated using three
different species of algae: *Chlorella pyrenoidosa, Chlamydomonas reinhardii*, and *Chlorella vulgaris*. In addition, algae have been successfully used to monitor the heavy-metal variations in Zn and Cd concentrations as well as Co, Cr, Cu, Fe, Mn, Ni, and Pb levels in sea water.

The application of algal biomass communities for wastewater remediation has already been established and has potential advantages over conventional methods such as low operating costs and high efficiency of heavy-metal removal from dilute solutions. However, metal bioaccumulation has implications for downstream use of algae as a fertilizer or animal feed. Although the application of the algal biomass may increase the growth of crops, due to the addition of nitrogen and phosphorus to the soil media, heavy metals accumulated in the algal biomass could be taken up by the plants. The metal accumulation in edible parts of crop plants represents the principal route of toxic metal entry into the human food chain. Therefore, the US EPA regulates the concentration of heavy-metals in biomass applied for use as a fertilizer. Additionally, although the US FDA does not have a standard for heavy metal concentrations used in animal feed, other governments, such as the European Union, do have set standards. High heavy metal concentrations in algal biomass also have implications for the proper disposal of algal wastes after biofuel processing. Thus, to evaluate the potential applications for algal biomass it is necessary to determine the biomass-metal concentrations. In this study, lead, chromium, copper, molybdenum, cobalt, cadmium, and arsenic were analyzed in harvested biomass; however, mercury and nickel were not determined. Nickel was not analyzed because collected algal biomass was digested in nickel crucibles, which
certainly leached nickel into the biomass. Mercury was not analyzed due to potential instrument contamination.

The goal of this study was to provide general knowledge regarding the composition of ash in the collected biomass from different sites.

3.2 Experimental Details

Silica standard solution (1000ppm) EPA/ APHA analytical grade and yttrium ICP-MS Standard, (1,000 ppm Y) Y₂O₃ in 3% HNO₃ were both purchased from Ricca Chemical Company. Anhydrous sodium carbonate (99-101%) and potassium nitrate (99.0-100.5%) were purchased from Mallinckrodt Baker, Inc. Potassium tetraborate tetrahydrate (99+%) was purchased from Strem Chemicals. Nitric acid, ACS grade, sulfuric acid, ACS grade, 57 mm round aluminum weighing dishes, 50 mL polypropylene, and 14 mL polyethylene terephthalate centrifuge tubes were purchased from VWR international. All solutions were made up using 18.2 MΩ water from an in house system purchased from EMD Millipore Corporation.

3.3 Methods

3.3.1 Ash Analyses

To determine the quantity of ash in each algal biomass sample the ASTM E1755 standard test method for ash in biomass was employed. First, 57 mm round aluminum weighing dishes were put into a muffle furnace at 550°C for one hour. The weighing dishes were then cooled in a desiccator, marked for identification, and the weight
recorded as \( m_{\text{cont}} \). Each biomass sample was pulverized and ground into fine particles using a mortar and pestle. Then, 0.5-1.0 g of each ground algae sample was weighed into the previously heated aluminum weight boats and the mass recorded to the nearest 0.1 mg. Next, the samples were put into a muffle furnace at 105°C until the mass of the sample no longer changed with time. The mass of the sample and aluminum-weighing dish was then recorded as \( m_{\text{ar}} \). The dried algal biomass samples were then put in a muffle furnace at 550°C for 24 hours and then cooled in a desiccator. The samples were weighed and the mass was recorded as \( m_{\text{ash}} \). The ash content was calculated using the following equation:

\[
\% \text{ Ash} = \left[ \frac{m_{\text{ash}} - m_{\text{cont}}}{m_{\text{ar}} - m_{\text{cont}}} \right] \times 100
\]

Ash analysis was performed on each algal biomass sample in triplicate and the average of each ash analysis reported. The standard deviation of the three replicates for all ashed biomass samples was less than 0.1%.

### 3.3.2 Total Silica Analyses

The total quantity of silica in the algal biomass was analyzed using a method based on Reay and Bennett et al. (1987). A 10.0 mL solution containing 3.0% (w/v) potassium nitrate and 3.6% (w/v) potassium tetraborate was made up with 18.2 MΩ water and stored in a scintillation vial. Next, 10.0 mL nickel crucibles were submerged overnight in a 5% (v/v) solution of nitric acid, then soaked in 18.2 MΩ water for 30 minutes, and finally dried at 300°C for 10 minutes. Then, 0.4 mL of the potassium nitrate/potassium tetraborate solution was added to each crucible and heated at 100°C until all the solution had evaporated. Next, previously dried biomass samples were finely ground using a mortar and pestle and about 20-30 mg of each algae sample was
massed into the nickel crucibles. The crucibles were heated at 300°C for two hours to ash the biomass.

The crucibles were then removed from the heating element and 0.4 g (about 4 pellets) of potassium hydroxide was added to each crucible. Crucibles were again heated for 30 minutes at 400°C. Crucibles were allowed to cool to room temperature, and 1.0 mL of 18.2 MΩ water was added to each crucible and heated at 70°C for 15 minutes and allowed to cool. Next, the biomass and solution were washed into 50 mL PET centrifuge tubes using four 5.0 mL aliquots of 18.2 MΩ water. The total volume was 21 mL. The tubes were then capped and centrifuged at 11,000 rpm for 5 minutes. Then, 0.89 mL of each sample was removed and filtered through 0.45 µm PTFE membrane filters. The filtered aliquots were then put into a 14 mL PET centrifuge tube along with 0.11 mL of a 1000 ppm yttrium internal standard and a 5% (v/v) nitric acid solution was used to bring the sample volume to 11 mL. The samples were then labeled, capped, and stored in the freezer at 4°C until analysis by ICP-OES. Blank water samples were also analyzed for background silica levels.

3.3.3 Biogenic Silica Analyses

The determination of biogenic silica is assessed using a timed extraction of silica from the algal biomass. The quantity of biogenic silica was carried out using the methods developed by Conley and Schelske et al. (1993) and Demaster et al. (1981). The method requires that 20-30 mg of dried, pulverized, algal biomass be massed into 50 mL PET centrifuge tubes with the addition of 40 mL of 1.0 (wt/v) % Na₂CO₃ solution made up in 18.2 MΩ water. A small magnetic stir bar was added to each centrifuge tube, and the
tubes were heated and stirred in a water bath held at 85°C. Then, 1.00 mL subsamples were removed from the centrifuge tubes at 0, 1, 2, 3, 5 and 24 hours, filtered through a 0.45 µm PTFE membrane filter, and put into a 14 mL PET centrifuge tubes. Next, 0.11 mL of a 100 ppm yttrium internal standard was added to each centrifuge tube, and a 5% (v/v) nitric acid solution was added to bring the total sample volume up to 11.0 mL. The samples were then labeled, capped, and stored in the refrigerator at 4°C until analysis by ICP-OES. All harvested algal biomass samples analyzed were run in triplicate.

3.3.4 Silica ICP Analyses

Formerly prepared silica samples were analyzed using a Perkin Elmer Optima 4300 DV ICP-OES. The optical emission wavelength used to collect the Si data was 251.611 nm. A stock standard containing 1,000 ppm of SiO$_2$ was used to make working standards containing 0, 1, 5, 20, 60, and 100 ppm of SiO$_2$ in a solution containing 5% (v/v) nitric acid and 1 ppm of the internal standard, yttrium.

3.3.5 Biogenic Silica Results

Because there was a chance that harvested algal biomass samples could have contained other silica components, the experiment was run over a 24-hour period. According to the Conley et al. (1998), diatom biogenic silica generally dissolves within the first two hours, and silicate minerals take a longer time to dissolve. To distinguish the different silica-bearing constituents of the algal biomass community, the quantity of silica extracted during each sampling period (0, 1, 2, 5 and 24 hours) was plotted, and the diatom biogenic silica was estimated from the y-intercept of a least-squares regression
line of the 3, 5, and 24-hour regression line. The backwards extrapolation to time zero was used because mineral based silicates dissolve more slowly than BSiO$_2$.$^{29}$ Extrapolation of the linear ‘mineral dissolution line’ to time zero thus accounts for the contribution of non-biogenic silica and gives the BSiO$_2$ content of the sample.$^{29}$ An example is displayed in Figure 3-1. Silica in the algae is primarily stored in the form of hydrated silicon dioxide (SiO$_2$•H$_2$O)$_n$, so for analyses purposes Si is reported as SiO$_2$.

Figure 3-1. Hypothetical graph displaying the extraction of silica during digestion as a function of time. Algal biogenic silica is estimated from the y-intercept of the least-squares regression line (represented by the black dashed line).

3.3.6 Metal Analyses

To determine the heavy metal content in the algal community biomass, samples were prepared exactly as described in the Total Silica Analysis section, using the sodium
hydroxide fusion method. The samples were stored in a refrigerator at 4°C until analysis by ICP-OES. Blank water samples were also analyzed for background metal concentrations.

The metal samples were analyzed using a Perkin Elmer Optima 4300 DV ICP-OES. The optical emission wavelengths used to collect the data were 220.353 nm for lead, 267.716 nm for chromium, 327.393 nm for copper, 202.031 nm for molybdenum, 228.616 nm for cobalt, 228.802 nm for cadmium, and 193.696 nm for arsenic.

3.3.7 Mass Balance

The total biomass make-up, referred to as mass balance, was achieved for algal biomass sample AGHO61110 using lipid data from Puvandendran (2012) and monosaccharide data from Thompson (2011). The percentage of protein in the biomass was determined using Equation 3-1. All proteins were assumed to be albumin. The portion of carbon, and oxygen that were component of monosaccharides, lipids or proteins were determined using Equation 3-2. All other data was determined using the methods described here and in Chapter 2.

Equation 3-1. Protein calculation

\[
\text{Protein \text{ wt \%}} = \frac{N \text{ wt \%}}{N_{\text{protein}} \text{ wt \%}}
\]

where N is the percent nitrogen.

Equation 3-2. Calculation for the percentage of carbon, hydrogen and oxygen in each component

\[
(A) C_x = X \text{ wt \%} \times C_{\text{element \%}} \text{ wt \% in x}
\]
\[ (B) \ H_x = X \text{ wt \% of } H \text{ in } x \]

\[ (C) \ O_x = X \text{ wt \% of } O \text{ in } x \]

Where C is carbon wt \% H is hydrogen wt \% and O is oxygen wt \%. X stands for either the protein, lipid or monosaccharide component.

### 3.4 Results and Discussion

#### 3.4.1 Ash Analyses

Algal biomass community harvested from the Muskegon Wastewater Treatment Plant contain an average of 31 wt \% ash, which is by far the lowest percentage found in any of the algae growth locations (Figure 3-2). Algal biomasses collected from all locations at the York River have similar quantities of ash. This is noteworthy because the inorganic carbon levels, although not the largest constituent in the York River samples were higher than in the mini-flume, and inorganic carbon is a constituent of the ash.

The portion of ash in algal biomass harvested from Goldsworth Pond was around 67 wt \%, which is close to the percent found at the York River site. Algal biomass collected at both locations had similar proportions of total silica, inorganic carbon, and nitrogen, which could be why the ash levels are comparable.

The quantities of ash from the different floway locations at the Great Wicomico River site are not statistically significant. However, algal communities at this site do have a smaller portion of ash in comparison to the York River locations.
Figure 3-2. Average ash in algal biomass communities. Bars represent the cumulative average percentage of ash in samples collected from each location. The error bars represent the sample standard deviation of all analyses.

Ash analysis plots, such as the one presented in Figure 3-3, can be found for each sample location site in the Appendix. None of the ATS™ sample locations show any specific trends, i.e. seasonal variation, regarding the percent of ash present. The variation in ash throughout the sampling period is small for harvests from Goldsworth Pond and the York River floways. More variation is seen in harvests from the Great Wicomico, Boat Basin, and Muskegon Wastewater Treatment Plant locations. The ash variation at the Boat Basin location may be an artifact of having sediment incorporated with the biomass. This can occur if the samples are not harvested carefully. Additionally, the ash fluctuations at any location could be caused by changes in the algal-community species population, as species are known to fluctuate over time. Algal biomass ash composition has also been shown to fluctuate with changing temperatures, with higher
temperature causing an increase in ash among the same species.\textsuperscript{36} However, because there were no apparent seasonal trends regarding ash, temperature was likely not the dominant factor.

![Figure 3-3](image)

Figure 3-3. Percent ash in biomass samples harvested from Goldsworth Pond. Error bars represent the sample standard deviation of triplicate analyses.

### 3.4.2 Total Silicon Dioxide Analyses

Determining total silica in algal biomass samples is time consuming and expensive. Therefore it was not possible to determine the silica (Si) content of all the harvested biomass samples. To assess the general silica content in the biomass, selected samples from different times throughout the harvesting period were analyzed for total silica.
3.4.2.1 Goldsworth Pond

The level of SiO$_2$ in Goldsworth Pond algal biomass remained relatively stable throughout the entire sampling period, only ranging by 10% from June 11 2010 to July 8 2011 (Figure 3-4). The average percent SiO$_2$ was $61.877 \pm 3.791\%$ by total dry weight. These results were expected because the biomass also has a high percentage of ash which shows little variation over the harvesting period.

![Graph showing percent of silicon dioxide in algal biomass from Goldsworth Pond](image)

Figure 3-4. Percent of silicon dioxide in algal biomass harvested at the Goldsworth Pond location. Samples were run in duplicate, except in the case of AGH061811 and AGH070811, which were run in triplicate. Error bars represent the standard deviation.

3.4.2.2 Muskegon Wastewater Treatment Plant

Silicon dioxide levels were measured in biomass collected on three different harvest dates at the Muskegon Wastewater Treatment Plant (Figure 3-5). Samples from all harvest dates, except August 22, were collected from floway one, screen position one. The August 22 harvest was collected from floway three and screen position one. The
percent of SiO$_2$ in the collected samples ranged from 9.38 to 39.57% by dry weight, at this location. Statistical analysis of the data indicated that the average percent of silicon dioxide in these samples was significantly different at the $\alpha = 0.05$ level (two tailed t-test). The deviations could be caused by a variation in the silicic acid levels in the incoming water. However, the silica level in the effluent tested on June 6, August 2, and August 15, was stable with corresponding concentrations of 2.35, 2.48 and 2.32 mg L$^{-1}$.

The variation in silicon dioxide levels in the algal biomass could also indicate a change in the algal-turf community populations; unfortunately community populations were not recorded.

The ash content in samples harvested from this location is low, as expected from the low percent SiO$_2$ found in biomass at this location. Interestingly, the percentage SiO$_2$ in harvest AMS071211F1S2 (39.567 ± 1.367%) is higher than the percentage of ash in the samples (28.672± 2.56%). This could be a result of the biomass being improperly ground and homogenized prior to analyses being performed, which could lead to larger sediment particles being improperly excluded or included.
Figure 3-5. Percent of silicon dioxide in algal community biomass harvested from the Muskegon Wastewater Treatment Plant location. Samples were run in triplicate and error bars represent the standard deviation.

### 3.4.2.3 Lake Erie

The samples collected from the Lake Erie location were analyzed for total silicon dioxide content (Figure 3-6). Silicon dioxide levels ranged from $3.611 \pm 0.988$ to $12.513 \pm 1.377$ wt % by total dry mass for the 2010 harvests. The silicon dioxide level was elevated in samples harvested during the late fall months. The percentages of silicon dioxide in harvests ALE082219, ALE090710 and ALE093010 were not significantly different from one another ($\alpha=0.05$ level). However, harvests ALE101910 and ALE111910 have a significantly higher SiO$_2$ content.
Figure 3-6. Percent of silicon dioxide in algal biomass harvested at the Lake Erie location. Samples were run in triplicate and error bars represent the standard deviation. (■) Bars indicate biomass was harvested from floway one. (■) Bars indicate biomass was harvested from floway two.

In 2011 two floways were set-up to produce algal biomass. A student t-test was performed in order to determine if there was a difference between harvests from the different floways (see Table 3-1). The quantity of silicon dioxide was significantly higher in floway two for the June harvest, but floway one had a higher silicon dioxide content in the September harvests. No significant difference was found between floway one and two for the July harvest.
Table 3-1. Results of Lake Erie floway one and floway two comparison by student t-test. Starred values are statistically significant at the $\alpha \leq .05$ level.

<table>
<thead>
<tr>
<th>Harvest Code</th>
<th>Pair</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALE061411</td>
<td>F1&amp;F2</td>
<td>0.018*</td>
</tr>
<tr>
<td>ALE071311</td>
<td>F1&amp;F2</td>
<td>0.845</td>
</tr>
<tr>
<td>ALE090711</td>
<td>F1&amp;F2</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

3.4.2.4 Great Wicomico River

The average content of silicon dioxide in the lower section of floway two of the Great Wicomico River was $30.289 \pm 6.469$ wt %, and the average for the upper section was $47.357 \pm 2.162$ wt % (Figure 3-7). The lower section received carbon dioxide supplementation, while the upper section served as the control. Although it appears that the quantity of silicon dioxide in biomass harvested from the upper section is elevated in comparison to the lower section, this cannot be confirmed by statistical analysis due to the low number of samples analyzed. The average $\text{SiO}_2$ content in biomass harvested from floway one was $27.545 \pm 4.027$ wt %, which is more similar to the quantity found in the lower section of floway two than the upper section.

Total silicon dioxide fluctuations in the biomass harvested from floway two follow similar trends in algal biomass population fluctuations of Ochrophyta (diatoms) on the floway.\textsuperscript{34} Diatom populations were high in July, decreased through November, and then increased again through January and began to decline through the spring.\textsuperscript{34}
Figure 3-7. Percent of silicon dioxide in algal biomass harvested at the Great Wicomico River location. Samples were run in duplicate. (■) Bars indicate biomass was harvested from floway two. (▲) Bars indicate biomass was harvested from floway one.

### 3.4.2.5 York River

Silicon dioxide content in samples harvested from the Boat Basin and York River sites show differing levels of accumulation in the biomass (Figure 3-8). Although the percent of ash remains stable in the York River samples (~81 wt %), the proportion of SiO$_2$ in the biomass decreases by 34 wt % from July 14 to September 28. The average percent of SiO$_2$ in the Boat Basin biomass was 40.27 ± 3.08 wt %, and in York River biomass, it was 55.68 ± 17.51 wt %. The average percentages of silicon dioxide in biomasses collected from the York River locations were higher than what were found in Great Wicomico River location biomasses. Similarly, the percentages of ash were higher in York River samples than Great Wicomico River samples.
3.4.3 Biogenic Silica

3.4.3.1 Goldsworth Pond

The extractions of biogenic silica (BSiO$_2$) from biomass collected at the Goldsworth Pond location are displayed in Figures 3-9, 3-10 and 3-11. The percentages of biogenic and terragenic (inorganic) silica in biomass harvests can be found in Table 3-2. For the Goldsworth Pond site, only four of the collected harvests were analyzed for biogenic silica. The time and expense associated with the procedure reduced the number of harvests that could be analyzed. However, the goal of the experiment was to gather information regarding the general biogenic silica levels in the algal biomass communities harvested, which could be achieved without evaluating every harvest.
Table 3-2. Percent biogenic and terragenic silica in samples harvested from Goldsworth Pond, Muskegon Wastewater Treatment Plant, Lake Erie, the York River and the Great Wicomico River locations.

<table>
<thead>
<tr>
<th>Location</th>
<th>Harvest Code</th>
<th>BSiO₂ wt % (g g⁻¹)</th>
<th>Terragenic SiO₂ wt % (g g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goldsworth Pond</td>
<td>AGH0611110</td>
<td>2.23</td>
<td>64.73</td>
</tr>
<tr>
<td></td>
<td>AGH061811F2S4</td>
<td>1.01± 0.47</td>
<td>61.59</td>
</tr>
<tr>
<td>Muskegon Wastewater Treatment Plant</td>
<td>AMS071211F1S2</td>
<td>3.28 ± 0.26</td>
<td>36.28</td>
</tr>
<tr>
<td>Lake Erie</td>
<td>ALE082210</td>
<td>0.0 ± 0.30</td>
<td>3.62</td>
</tr>
<tr>
<td></td>
<td>ALE110910</td>
<td>0.0 ± 1.09</td>
<td>11.42</td>
</tr>
<tr>
<td></td>
<td>ALE061411F1</td>
<td>1.16 ± 0.68</td>
<td>23.61</td>
</tr>
<tr>
<td></td>
<td>ALE090711F1</td>
<td>0.75 ±0.93</td>
<td>10.73</td>
</tr>
<tr>
<td></td>
<td>ALE061411F2</td>
<td>2.20 ± 0.57</td>
<td>32.55</td>
</tr>
<tr>
<td></td>
<td>ALE090711F2</td>
<td>1.37 ± 0.18</td>
<td>3.26</td>
</tr>
<tr>
<td>York River</td>
<td>AVI071410YR</td>
<td>10.92</td>
<td>63.15</td>
</tr>
<tr>
<td></td>
<td>AVI100510YR</td>
<td>16.47 ± 0.66</td>
<td>35.71</td>
</tr>
<tr>
<td>Great Wicomico</td>
<td>AES022311F2D</td>
<td>16.22</td>
<td>13.61</td>
</tr>
<tr>
<td></td>
<td>AES052311F1D</td>
<td>27.59 ± 3.51</td>
<td>40.97</td>
</tr>
</tbody>
</table>
A decline in the quantity of silica extracted at the five-hour sampling time can be seen in the collected data (as seen in Figures 3-9, 3-10 and 3-11). Similar results can be seen in the time course extractions of samples from the other locations. However, the five-hour time sampling was not always only low, sometimes the three-hour sampling shows a drop in the Si quantity extracted. This indicates that the error is most likely systematic. The source of the error may come from the sampling process or it is possible that some of the silica may have precipitated out of solution. However, because replicates were used, the low extraction points were not removed.

![Figure 3-9 Percent biogenic silica extracted from Goldsworth Pond algal biomass harvest AGH061110.](image)
Figure 3-10. Percent biogenic silica extracted from Goldsworth Pond algal biomass harvest AGH061811F2S4. Each line represents the time course digestion of a single sample.

3.4.3.2 Muskegon Wastewater Treatment Plant

The biogenic silica component in the biomass collected from the Muskegon Wastewater Treatment Plant is similar to the percent in the Goldsworth Pond location biomass (see Table 3-2). All replicates show a similar quantity of silica extracted during the first three hours. One replicate shows a large deviation in silica content at the five- and 24-hour samplings (Figure 3-11). However, since the y-intercept is used to determine the quantity of biogenic silica, the differences in individual points do not greatly influence the calculated quantity of BSiO$_2$. Nevertheless, the digestion method has a lot of variation and must be interpreted carefully.
Figure 3-11. Percent biogenic silica in algal biomass harvest AMS071211F1S2. Each line represents the time course digestion of a single sample.

3.4.3.3 Lake Erie

The quantity of biogenic silica in algal biomass harvested from the Lake Erie site can be seen in Table 3-2. Biomass harvests ALE082210 and ALE110910 contained no detectable biogenic silica, possibly indicating a lack of diatoms in this biomass community. The only silica in these samples comes from inorganic sources. Figure 3-13 displays the time-course extraction of silica from harvest ALE082210 (extraction data of the other harvests can be found in Appendix A).
Figure 3-12. Percent of biogenic silica extracted from Lake Erie algal biomass harvest ALE082210. Each line represents the time-course digestion of a single sample.

3.4.3.4 York River

The quantity of BSiO$_2$ in the York River harvest was as much as tenfold higher than the measured quantity in the freshwater harvest locations (see Table 3-2). The percent of biogenic silica in harvest AVI100510YR was around five percent higher than that of harvest AVI071410YR. Figure 3-13 is representative of biogenic extraction plots from the York River and Great Wicomico River site locations (see Appendix A). Time extraction plots from these locations appear to level off after the three-hour extraction time. This is dissimilar to the plots of the freshwater locations, which show a linear release of silicon dioxide. The difference in the extraction plots might be due to the small amount of biogenic silica and the larger portion of terragenic silica in biomass collected from the freshwater locations.
3.4.3.5 Great Wicomico River

Table 3-2 gives the percent of biogenic and terragenic silica in the biomass samples evaluated from the Great Wicomico River site. The percentages of biogenic and terragenic silica are higher in the spring sample than in the winter. The difference in biogenic silica was probably due to different algal community populations. In spring the algal-community population showed a marked increase in Chlorophyta (green algae) and a decrease in Ochrophyta (diatoms) composition. The inverse is true in winter months. However, the biogenic silica in biomass collected from this location was measured on two different floways, known to have different algal communities, so they cannot be used to evaluate differences in the make-up of the species populations.
3.4.4 Heavy Metals

*Table 3-3 gives the US EPA concentration limits for heavy metals in biosolids to be applied to land for fertilizer. The concentrations of selenium, mercury and nickel were not analyzed in any of the algal biomass samples.*

Table 3-3. EPA pollution limits adapted from Table 2-1 A Plain English Guide to the EPA Part 503 Biosolids Rule. 37

<table>
<thead>
<tr>
<th>Pollutant</th>
<th>Ceiling Concentration Limits for All Biosolids Applied to Land (g g(^{-1})) (^{a})</th>
<th>% (g g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsenic</td>
<td>0.000075</td>
<td>0.0075%</td>
</tr>
<tr>
<td>Cadmium</td>
<td>0.000085</td>
<td>0.0085%</td>
</tr>
<tr>
<td>Chromium</td>
<td>0.003</td>
<td>0.3%</td>
</tr>
<tr>
<td>Copper</td>
<td>0.0043</td>
<td>0.43%</td>
</tr>
<tr>
<td>Lead</td>
<td>0.00084</td>
<td>0.084%</td>
</tr>
<tr>
<td>Mercury</td>
<td>0.000057</td>
<td>0.0057%</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>0.000075</td>
<td>0.0075%</td>
</tr>
<tr>
<td>Nickel</td>
<td>0.00042</td>
<td>0.042%</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.0001</td>
<td>0.01%</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.0075</td>
<td>0.75%</td>
</tr>
</tbody>
</table>

\(^{a}\) Pollutant allowed per unit amount of biomass on dry weight basis

3.4.4.1 Goldsworth Pond

Heavy-metal levels measured in the biomasses collected from the Goldsworth Pond site vary less than 0.01 wt % (see *Figure 3-14*) between harvests dates. An ANOVA statistical analysis of the data showed that the percent of chromium in harvest AGH061811F1S2 was significantly higher than the percent in harvest AGH070811F2S1.
(p-value < 0.001). Additionally, the percent of molybdenum was significantly higher in harvest AGH051311 than in harvest AGH070811F2S1 (p-value < 0.022). No other samples show significant differences in the percent composition of these heavy metals.

None of the samples analyzed contained any detectable lead. The quantity of arsenic in harvest sAGH051311, AGH051511, AGH061811F2S1, and AGH070811F2S1 were 0.0212, 0.0187, 0.0195 and 0.0171 wt %, respectively. The quantities of arsenic in the biomass were over the 0.0075 g g\(^{-1}\) (per unit amount of biomass on a dry weight basis) limit, set by EPA, for the allowed application of biosolids to land. The concentrations of the other tested heavy metals (cadmium, chromium, copper, molybdenum, and lead) were below the EPA limits. The concentration of metals in the biomasses harvested followed the general trend of \(\text{As} > \text{Cr} \approx \text{Cu} \approx \text{Co} \approx \text{Mo} > \text{Cd}\).
Figure 3-14. Percentages of metals in algal biomass harvested from the Goldsworth Pond site. (■) % Cr, (■) %Cu, (■) % Mo, (■) % Co, (■) % Cd, and (■) % As. All samples were run in triplicate and error bars represent the triplicate sample standard deviations.
Many factors may influence the availability of metals to algal biomass communities. The main physicochemical parameters are pH, salinity, temperature, light, particulate matter, and organic matter.\textsuperscript{38} Besides the variations in the available metal concentrations in the water, other factors such as water nutrient conditions, the stage of development and variation in growth and chemical composition of the algal communities may influence the pattern of accumulation.\textsuperscript{39} However, because these factors are unknown at this location, reasons for the variations in chromium and molybdenum levels in biomass are unclear.

\textbf{3.4.4.2 Muskegon Wastewater Treatment Plant}

Three algal biomasses harvested from the Muskegon Wastewater Treatment Plant were analyzed for heavy metal levels. The heavy metal composition breakdown can be found in Figure 3-18. None of the harvests analyzed contained any detectable lead. All the harvests had arsenic levels over the EPA allowed, 0.0075 g g\textsuperscript{-1} (per unit amount of biomass on a dry weight basis) limit, for the application of a biosolid to land (see Table 3-3). The total quantities of metals tested were the same in harvests from June 12 and August 8, at 0.0507 wt % on a total dry biomass basis. The August 22 harvest had a total percent metal concentration of 0.0384 wt % on a total dry biomass basis.
Figure 3-15. Percentages of metals in algal biomass harvested from the Muskegon Wastewater Treatment Plant location. () % Cr, () % Cu, () % Mo, () % Co, () % Cd, and ( ) % As. All samples were run in triplicate and error bars represent the triplicate sample standard deviations.
The concentration of metals in biomasses harvested follow the general trend As > Cr ≈ Cu > Co ≈ Mo > Cd. An ANOVA analysis revealed that the percent of each metal were not significantly different from one another in any of the harvests.

Table 3-4. Concentration of heavy metals measured in mg L\(^{-1}\) in the storage lagoon water at the Muskegon Wastewater Treatment Plant.  

<table>
<thead>
<tr>
<th>Sample Date</th>
<th>As (mg L(^{-1}))</th>
<th>Cd (mg L(^{-1}))</th>
<th>Cr (mg L(^{-1}))</th>
<th>Co (mg L(^{-1}))</th>
<th>Cu (mg L(^{-1}))</th>
<th>Pb (mg L(^{-1}))</th>
<th>Mo (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>07/06/11</td>
<td>&lt;0.005</td>
<td>&lt;0.0017</td>
<td>0.004</td>
<td>0.0015</td>
<td>0.0034</td>
<td></td>
<td>0.0119</td>
</tr>
<tr>
<td>08/02/11</td>
<td>&lt;0.005</td>
<td>&lt;0.0018</td>
<td>0.0031</td>
<td>0.0012</td>
<td>0.0031</td>
<td>&lt;0.003</td>
<td>0.0109</td>
</tr>
<tr>
<td>08/15/11</td>
<td>&lt;0.005</td>
<td>&lt;0.0019</td>
<td>0.0029</td>
<td>0.0011</td>
<td>0.0026</td>
<td>&lt;0.003</td>
<td>0.0109</td>
</tr>
</tbody>
</table>

The concentration of metals measured in the water at the Muskegon Wastewater Treatment Plant in mg L\(^{-1}\) during the time the algal biomasses were grown and harvested, can be found in Table 3-4. All the metal levels are much lower in the water than in the algal biomasses harvested, which indicates that these algal communities have bioaccumulated the heavy metals. The order of metal concentration in the input water (Mo > Cr ≈ Cu > Cd ≈ Co > As > Pb) was not comparable to the accumulation order in the algal biomass, indicating that the algal biomass do not accumulate metals according to the total aqueous concentrations in the water.
3.4.4.3 Great Wicomico River and York River

The Great Wicomico and York Rivers algal biomass, heavy metal levels are displayed in Figure 3-19. All the analyzed heavy metal levels for both locations are below the EPA allowed limits for application of a biosolid to land except for arsenic, which is 0.0168 and 0.0152 %, on a total dry mass basis, in the Great Wicomico and York River algae, correspondingly. Again, there was no measurable lead in samples from either location. The metal concentration levels in the York River algal biomass harvests are in the order of As > Cr ≈ Cu > Co ≈ Mo > Cd. The Great Wicomico river biomass sample had the identical order.
Figure 3-16. Percentages of metals in algal biomasses harvested from the Great Wicomico River and York River locations. (■) % Cr, (■) %Cu, (■) % Mo, (■) % Co, (■) % Cd, and (■) % As. All samples were run in triplicate and error bars represent the standard deviations.
The total metals concentration in the Great Wicomico River was 0.0507 wt % and the total in the York River was 0.0446 wt % on a total dry mass basis. An independent sample t-test was performed on the data, which found no difference in the percentage of any of the metals at the two locations. Interestingly, the total metal accumulation percent in the sample from the Great Wicomico River was the same as the total found in the July 8 and August 12 biomass harvests from the Muskegon Wastewater Treatment Plant. An ANOVA analysis was performed comparing the average percentages of each metal for the Muskegon Wastewater Treatment Plant and the Great Wicomico harvest, and no significant differences were found. This is interesting because the algal communities and water conditions at each of these sites were most likely very different.

Although these results are surprising, Conti and Cecchetti et al. (2003) measured some heavy metals in two algae species grown at different locations in Tyrrhenian Sea, central Italy. Two of the locations were considered “clean” (or unpolluted) and the other four were in contaminated waters. They found that the metal concentrations detected in P. pavonica and U. lactuca (two algae species) were homogeneous and did not indicate any differences among the sites examined. A study performed by Fytianos et al. (1999) evaluated the heavy metal concentrations in two species of algae, every month for over a year, and found the two species behaved similarly with respect to heavy metal accumulation, indicating that either species could be used to monitor the heavy metal profile of the water. From this it can be hypothesized that the composition of heavy metals in algal biomass has little to do with location and specific algal species and more to do with the biomass being generally classified as an algal community. This would explain why algal biomasses from the Great Wicomico River and Muskegon Wastewater
Treatment Plant have similar metal levels percentages. It would also explain why the metal up-take order, As > Cr ≈ Cu > Co ≈ Mo > Cd, is the same in the algal communities at each location.

3.4.5 Mass Balance

Data regarding possible fractions of algal biomass sample AGH061110 was gathered to evaluate mass balance. This samples was chosen as a representative because it had the most complete data set regarding the biomass constituents. The biomass was composed of 65 wt % ash and 35 wt % volatile organic material, or ash free dry mass. The constituents of ash can be seen in Figure 3-17 and all but 2 wt % of the ash portion was accounted for. However, not all metals were determined in the biomass.

The unknown portion of the ash free dry mass was much higher than in the ash fraction. Total organic carbon, hydrogen and nitrogen fractions made up only about half of the mass balance as indicated by Figure 3-18. Additionally about 4 wt % of the organic carbon fraction was unaccounted for by carbon in the lipid, protein and monosaccharide fractions of the biomass. Similarly, about half the hydrogen is unaccounted for in these fractions (monosaccharides, lipids, proteins). Other organic molecules, such as pigments, that were not analyzed may account for the missing carbon and hydrogen.
Figure 3-17. Mass balance of sample AGH061110. Center: Total ash and organic material in biomass. Left: Composition of ash. Right: Composition of organic material (or ash free dry mass). Standard error for all analyses are less than 1.0 wt %.
Figure 3-18. Organic fraction (or ash free dry mass) of biomass AGH061110. Left: total (■) organic carbon, (■) hydrogen, (■) nitrogen and (■) unknown fraction. Right: (■) monosaccharide carbon, (■) lipid carbon and (■) protein carbon fractions. (■) Monosaccharide hydrogen, (■) lipid hydrogen and (■) protein hydrogen fractions. (■) Protein nitrogen fraction. (■) Monosaccharide oxygen, (■) lipid oxygen and (■) protein oxygen fractions.
3.5 Summary

The ash proportions of the algal biomasses harvested from different sites were evaluated as well as some of the constituents of the biomass ash portion. The constituents of the ash evaluated were silica, arsenic, copper, cadmium, cobalt, molybdenum, lead, and chromium. The silica proportion of the biomass was fractioned into biogenic and terragenic portions.

Silica is the greatest contributor to the sample. The total silica content in collected biomass samples vary in range depending on location and time of collection. The total silica content was the most stable as a function of location and time in samples harvested from the Goldsworth Pond site. A time extraction method for determining the quantity of biogenic silica in algal biomass shows variation in the extracted quantity of silica over time. The method gave a general estimate of the portion of the total silica that was of biogenic origin.

Algal biomass samples were evaluated for arsenic, copper, cadmium, cobalt, molybdenum, lead, and chromium. None of the samples showed any detectable lead. Arsenic was found in the highest concentrations. The general metal concentration order among all samples tested was As > Cr ≈ Cu > Co ≈ Mo > Cd. Metals comprised between 0.045 wt% and 0.075 wt %, by total dry weight, of the biomass.
3.6 Bibliography


(11) Van Cappellen, P. Reviews in mineralogy and geochemistry 2003, 54, 357–381.


(37) A Plain English Guide to the EPA part 503 Biosolids Rule.


(40) *Unpublished data. 2010 and 2011 for the storage lagoon water at the Muskegon Wastewater Treatment Plant; Muskegon Wastewater Treatment Plant: Muskegon Wastewater Treatment Plant, 2011.*


CHAPTER 4
BIOCHAR APPLICATION OF ALGAL BIOMASS

4.1 Introduction

Biochar is a form of black carbon that is recalcitrant and resistant to decay. Biochar is comprised mainly of stable, aromatic forms of organic carbon, and cannot readily be returned to the atmosphere as CO$_2$, even under favorable environmental and biological conditions. Biochar is typically produced from the pyrolysis of biomass under limited oxygen conditions. Biochar has its origins in the ancient practice developed in Amazonia of creating “terra preta de indio,” or black earth. This seven thousand year-old practice took place by smoldering organic matter in an oxygen-deficient environment. The chars produced by this practice were used to increase the fertility of the soil.

Recently biochar has been investigated for reducing CO$_2$ emissions through carbon sequestration. Furthermore, biochar has been evaluated as a soil amendment and sorbent for metals and organic compounds. Biochars have been produced from an assortment of materials including wood, pine needles, orange peels, dairy-manure, and polyethylene terephthalate residues.

Algal biomass may represent an additional option for biochar production. Algal biochars tends to be rich in inorganic nutrients such as phosphorus, potassium, and calcium; this nutrient profile has encouraged its use as a soil amendment. Previous research on biochar formation from algae has mostly focused on the products of pyrolysis such as bio-oil and bio-gas, for energy production. Algal biomass that has previously been processed for biofuel or nutrient removal purposes could be utilized as a
biochar feedstock instead of being considered as a waste product. The biochar could be used for sorption of contaminants or enrichment of soil.

An already established method for the removal of contaminants from soil and water is to adsorb them onto activated carbon. Activated carbon has a large porous surface area (between 600 m$^2$ g$^{-1}$ and 1183 m$^2$ g$^{-1}$)\textsuperscript{16,17} and is capable of binding contaminants through a variety of physical and chemical processes.\textsuperscript{18} The only difference between charcoal and biochar lies in its use; charcoal is a fuel, and biochar has a nonfuel use.\textsuperscript{19} The shortcoming of using activated carbon is that it can be expensive,\textsuperscript{20} and once it becomes saturated with the adsorbate, it loses its sorption capacity and must be regenerated, which is an energy intensive process.\textsuperscript{21} Alternatively, waste-algal biomass represents a potential plentiful, low cost, sorption source, even if it, too, loses sorption capacity after saturation. The application of algae based biochar, whether whole or acid soluble carbohydrate-extracted, for the sorption of contaminants from water has not been tested.

The main objectives of this study were to evaluate properties of algae produced biochar and investigate the use of biochars produced at different temperatures for the sorption of organic contaminants from water. Previous studies found that pH plays an important role in the sorption of organic contaminants on biochar.\textsuperscript{22,23} Therefore, sulfuric acid-treated biochar was used to evaluate the sorption capacity of the algal biochar using organic compounds with different pK$_a$s. The compounds explored were 2-(4-(2-methylpropyl)phenyl)propanoic acid (pK$_a$= 4.91), 2,4-dinitroaniline (pK$_a$=18.46), 2-phenethylamine (pK$_a$=9.90) and 2-phenylethanol (pK$_a$=15.17). 2-(4-(2-methylpropyl)phenyl)propanoic acid, and 2,4-dinitroaniline were chosen because they are
known pollutants that persist in natural aquatic environments.\textsuperscript{24,25,26} The selection of 2-phenylethanol was made because of its high water-solubility and persistence in personal care products, which exist in domestic water supplies.\textsuperscript{27} 2-phenethylamine was chosen because it has a pharmacological profile similar to that of amphetamine, and it is the parent compound for abused drugs in the amphetamine class.\textsuperscript{28} Additionally, these compounds are similar in structure, as they all contain substituted aromatic rings (see Figure 4-1).

![Chemical structures of the compounds investigated for sorption onto biochar](image)

**Figure 4-1.** Chemical structures of the compounds investigated for sorption onto biochar

### 4.2 Experimental Details

*Materials.* 2-(4-(2-methylpropyl)phenyl)propanoic acid (≥ 99%) was purchased from Enzo Life Sciences. Meclofenamic acid sodium salt (≥ 99%) was purchased from
MP Biomedicals. 2,4-dintiroaniline analytical standard, activated charcoal (untreated, granular 4-8mesh), acetonitrile and methanol CHOMASOLV® (≤ 99.9%), acetic anhydride (≥99%), glacial acetic acid (≥99.7 %), dichloromethane (≥ 99.5%), and formic acid (98%) were purchased from Sigma-Aldrich. 2-phenylethanol (99%) was purchased from Acros Organics. 2-phenethylamine (≤99%) was purchased from Eastman Organics. 15.0 mL corning PET centrifuge tubes and sulfuric acid ACS grade were purchased from VWR international. Potassium hydroxide (pellets 87.8%) was purchased from J.T Baker. All solutions were made up using 18MΩ water from an in-house system purchased from EMD Millipore Corporation Billerica, MA. Ultra high purity grade 5.0 nitrogen gas was purchased from Airgas®. 2 mL GC autosampler vials with 9 mm PTFE septa caps were also purchased from VWR International.

4.3 Methods

4.3.1 Pre-treatment and Production of Biochar

Biomass harvested from the Great Wicomico River and Goldsworth Pond sites were used. Biomasses from these two locations were chosen because they have similar nutrient profiles (see Chapter 2) and ash content, although the water sources from which they were produced were different. Prior to use, the dried biomass was ground with a mortar and pestle to uniform size. About 3.0 g of each biomass were treated separately with 10 mL of 1.5% (v/v) sulfuric acid for two hours under sonication and then vacuum filtered through a Büchner funnel and rinsed with room temperature deionized water until the pH no longer changed (typically around pH 4). The treated biomasses were dried in a
104°C oven for 24 hours. The dried biomasses were reground and stored in a capped scintillation vial until pyrolysis treatment.

The dried sulfuric acid-treated and untreated biomasses, were placed in a 71 cm by 2.5 cm diameter glass tube terminated with Cajon fittings and placed in a Mini-Mite 1100C Linberg/Blue M tube furnace. High purity nitrogen gas flowed at a rate of 15 cm³ minute⁻¹ for five minutes to evacuate the oxygen in the tube. The same nitrogen flow rate was used during the pyrolysis treatment. The temperature of the furnace was increased at a rate of 25°C minute⁻¹ until the desired temperature was reached. The final temperature was held for 60 minutes, and then allowed to cool under flowing nitrogen gas. Biochar samples were produced at the four pyrolysis temperatures listed in Table 4-1.

Sorption of compounds on activated carbon was compared to biochar sorption experiments. The activated carbon was heated at 500°C under nitrogen gas, similar to how the biochars were produced prior to use.
Table 4-1. Pyrolysis temperatures and biomass sources.

<table>
<thead>
<tr>
<th>Pyrolysis Temperature (°C)</th>
<th>Treated biomass</th>
<th>Code</th>
<th>Untreated biomass</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>AGH</td>
<td>AGH200T</td>
<td>AGH</td>
<td>AGH200</td>
</tr>
<tr>
<td>200</td>
<td>AES</td>
<td>AES200T</td>
<td>AES</td>
<td>AES200</td>
</tr>
<tr>
<td>300</td>
<td>AGH</td>
<td>AGH300T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>AGH</td>
<td>AGH400T</td>
<td>AGH</td>
<td>AGH400</td>
</tr>
<tr>
<td>400</td>
<td>AES</td>
<td>AES400T</td>
<td>AES</td>
<td>AES400</td>
</tr>
<tr>
<td>500</td>
<td>AGH</td>
<td>AGH500T</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3.2 Biochar Characterization

4.3.2.1 Surface Area Analyses

The surface area, pore volume, pore size, and pore surface area of the Goldsworth Pond biochar samples were measured using a Nova® 2200 e Surface Analyzer by Quantachrome instruments. Brunauer–Emmett–Teller (BET) and Barrett-Joyner-Halenda (BJH) calculations were performed using Quantachrome NovaWin 2.0 software. All samples were degassed under vacuum at 393 K for 12 hours. Adsorption measurements were made under N2 gas at 77K from 0.025-0.99 P/P0. BET measurements were performed in triplicate, and BJH measurements were performed in duplicate.
4.3.2.2 pH Measurements

The pH of each biochar sample was measured using a pH meter (SevenGO pH meter SG2, Mettler Toledo). Biochar samples were stirred with deionized water for about one minute in a 1:11 ratio and allowed to settle for 30 minutes prior to pH measurements of the supernatant.

4.3.2.3 FE-SEM

Field emission scanning electron micrographs of the biomass samples were collected using a model S-4500 FE-SEM (Hitachi, Japan). All samples underwent a drying procedure as well as gold coating before being analyzed by FESEM.

4.3.2.4 Drying Procedure

A few milligrams of each biochar sample were placed into 20 mL scintillation vial followed by 1.0 mL of a solution made up of 1% osmium tetroxide in a 0.1 M phosphate buffer. This solution was used to fix, or preserve, the biomass sample. The vials were then capped and allowed to rest for 60 minutes. Next, most of the osmium solution was vacuumed off and the samples were incubated with 1-2 mL of a 0.1 M phosphate buffer for ten minutes. Again the excess solution was vacuumed off and an additional aliquot of the buffer was added for 10 minutes. This procedure was repeated once more, for a total of three times. Following this, a solution containing 50 % (v/v) ethanol was added to the vials for ten minutes and then vacuumed off. Additional 75 % (v/v), 90% (v/v) and 100% (v/v) solutions of ethanol were added for ten minutes each
and vacuumed off. Finally, the samples were dried in a vacuum oven for 24 hours and put into a desiccator until being plated with gold.

4.3.2.5 Gold Coating

The mounted dried biochar sample was placed in a gold splutter coater. The instrument was pumped down to 0.1 mbar, and then the samples were coated with gold using Argon gas for 2 minutes.

4.3.3 Ash and Elemental Analyses

To determine the quantity of ash in each biochar sample the ASTM E1755 standard test method for ash in biomass was employed. First, 1.0 g-0.5 g of each biomass sample was weighed into previously-heated aluminum weigh-boats, massed to 0.1 mg using an Ohaus Adventurer balance, and heated in a muffle furnace at 105°C until dried to a constant weight. The samples were then heated in air in a muffle furnace at 550°C for 24 hours and allowed to cool in a desiccator. Ash analysis was performed in duplicate on all samples.

The quantity of carbon, hydrogen, and nitrogen in each of the biochar samples was measured using a Truspec ® CHN analyzer from LECO Corporation. The oxygen content was determined by difference, assuming the biochar non-ash component to be composed only of C, H, N, and O. All were measured on an ash free dry mass basis. Biochar samples were each placed in a 20 mL glass scintillation vial and heated at 104 °C for 1-2 hours, capped, and allowed to cool in a desiccator prior to being analyzed. 50 mg-100 mg of each sample were weighed to the nearest tenth of a microgram on a Sartorius
balance, into a tin foil cup. Biochar samples were analyzed in triplicate, and the CHN contents were averaged.

4.3.4 Sorption Experiments

2-(4-(2-methylpropyl)phenyl)propanoic Acid. A standard solution of 21.6 mg L\(^{-1}\) 2-(4-(2-methylpropyl)phenyl)propanoic acid was prepared by adding a measured amount of 2-(4-(2-methylpropyl)phenyl)propanoic acid into a volumetric flask and adding a 10% (v/v) methanol and 18 MΩ water solution. The methanol was used to reduce the risk of microbial growth.

30 mg samples of biochar were massed into capped 14 mL centrifuge tubes, which were then filled with 10 mL of the 21.6 mg L\(^{-1}\) 2-(4-(2-methylpropyl)phenyl)propanoic acid stock solution. A PTFE-coated magnetic stir bar was also added to each centrifuge tube. The samples were run in triplicate. To test whether 2-(4-(2-methylpropyl)phenyl)propanoic acid adsorbs to the side of the centrifuge tube or the stir bar, a centrifuge tube containing only the 2-(4-(2-methylpropyl)phenyl)propanoic acid standard and a stir bar was used as a control. Additionally, a centrifuge tube containing 30 mg of activated charcoal and the 2-(4-(2-methylpropyl)phenyl)propanoic acid stock solution were used for comparison. The samples were continuously agitated on a stir-plate at 1100 rpm for 24 hours. The pH of the solution was not adjusted or monitored during the experiment. Next 1.0 mL samples were extracted from each centrifuge tube at different times to evaluate the rate of sorption. The quantity of 2-(4-(2-methylpropyl)phenyl)propanoic acid was analyzed using HPLC equipped with a UV detector set at 230nm.
**2,4-dinitroaniline.** A 21.6 mg L\(^{-1}\) solution of 2,4-dinitroaniline was prepared by dissolving 5.4 mg of 2,4-dinitroaniline in 250 mL of 10 % (v/v) methanol solution. The biochar samples were then prepared in the same way as the 2-(4-(2-methylpropyl)phenyl)propanoic acid samples but with 10 mL of 2,4-dinitroaniline instead of 2-(4-(2-methylpropyl)phenyl)propanoic acid. A blank control was run, containing only the 2,4-dinitroaniline and a stir bar. Activated charcoal was also incubated with 2,4-dinitroaniline solution for comparison. Next, 1 ml samples were taken from all samples at times of 0 minute, 1 minute, 2 hours, and 24 hours. The samples were run in triplicate and analyzed by HPLC at 230nm.

**2-phenylethanol.** A standard solution of 21.73 mg L\(^{-1}\) of 2-phenylethanol was prepared in a 10 % (v/v) methanol solution. The biochar samples were prepared as previously stated with the substitution of the 10 mL of the 2-phenylethanol standard. A blank control was run, containing only the 2-phenethanol and a stir bar. For comparison to the biochar, activated charcoal was incubated with the 2-phenylethanol solution. Samples were run in triplicate and analyzed by HPLC at 200 nm.

**2-phenethylamine.** A standard solution of 21.6 mg L\(^{-1}\) of 2-phenethylamine was prepared in a 10 % (v/v) methanol solution. The biochar samples were prepared as previously stated with the substitution of the 10 mL of the 2-phenethylamine standard. A blank control was run, containing only the 2-phenethylamin and a stir bar. For comparison to the biochar, activated charcoal was incubated with the 2-phenethylamin solution. Samples were run in triplicate and analyzed using GC/MS.

2-(4-(2-methylpropyl)phenyl)propanoic acid, 2,4-dinitroaniline, and 2-phenethanol sorption experiments were analyzed using a Shimadzu CBM20A/Lite HPLC.
System equipped with a SPD-20A spectrometer. The 1.0 mL samples were filtered through 0.22μm nylon filters, and 2.99 mg L⁻¹ Meclofenamic acid was used as an internal standard for HPLC analysis. The samples were made up in 50:50 acetonitrile:water. Separation was achieved using an Agilent Zorbex SB-C18 (2.1x150mm, 5μm) column. 2-(4-(2-methylpropyl)phenyl)propanoic acid analysis was carried out using a 60:40 acetonitrile: 0.1% formic acid mobile phase with a flow rate of 0.800 mL min⁻¹ for 5 minutes. 2,4-dintiroaniline was analyzed using a 55:45 acetonitrile: 0.1% formic acid mobile phase with a flow rate of 0.800 mL min⁻¹ for 3.8 minutes. Analysis of 2-phenylethanol was carried out using a 49:51 acetonitrile: 0.1% formic acid mobile phase with a flow rate of 0.900 mL minutes⁻¹ for 5 minutes.

2-phenethylamine sorption experiments required derivatization by acetylation prior to analysis by CG/MS. The derivatization procedures were performed by transferring 1 mL aliquots of each of the samples into test tubes and adding 37.5 μL of a 2-phenethanol internal standard. The internal standard was made by adding 11.2 μL of 2-phenylethanol into a 500 mL volumetric flask and bringing it up to volume using 18 MΩ water. Next, 100 μL of glacial acetic acid was added to each test tube and vortex mixed at 1700 rpm for 10 seconds. Then, 2.0 mL of acetic anhydride was added, and the tubes were mixed and allowed to react for 45 minutes at room temperature. 5.0 mL of water was added to each tube to decompose excess acetic anhydride. The test tubes were put in an ice bath to cool. Next, 2.0 mL of dichloromethane was added and vortex mixed for 10 seconds. The phases were allowed to separate, and then mixed again for 10 seconds. The solution phases were allowed to separate for an additional 15 minutes and the top, aqueous layer, was removed by pipette. Then, 5.0 mL of a 3.5 M KOH solution was
added to each test tube to increase the pH. The test tubes were vortex mixed again, and the phases were allowed to separate. The aqueous top phase was then removed, and the dichloromethane layer was pipetted into GC auto sampler vials for analysis.

GC/MS analysis was performed using a HP 6890 Model equipped with a 5973 N MSD mass selective detector and an HP 7683 Series auto sampler. MSD Chemstation software was used to evaluate the chromatography. Separation was completed using a 30m x 0.32mm x 0.25μm Stabilwax (PEG) column from Restek. High purity helium was used as the carrier gas. The GC inlet temperature was kept at 240 °C with inlet purge of 10.4 mL minute$^{-1}$ starting at 0.5 minutes. The column flow was 15.5 mL minute$^{-1}$ of helium. The pressure was set to 8.47 psi with a split ratio of 5:1. The initial column temperature was 120 °C, held for 0.5 minutes and increased 15 °C minutes$^{-1}$ until reaching 175 °C, then the temperature was increased 10 °C minute$^{-1}$ until reaching the final temperature of 225°C and held for 2.5 minutes, for total run time of 11.67 minutes. The transfer line from the GC to the MSD detector was held at 240°C. The mass selective detector was run in scan mode from m/z 50-300 with a solvent delay of 3.5 minutes. The detector was calibrated according to the autotune parameters.

4.3.5 Quantitation

All quantitation was performed by first creating calibration curves using a set of four standards to check linearity of signal. The peak area of each analysis was integrated using the available system software and double-checked by hand. The quantity of compound in each sample was then calculated using the internal standard method.
4.3.6 Statistical Analyses

All the data presented are averages and standard deviations of triplicate analysis, unless stated otherwise. Means, stand deviations, and correlation coefficients were all calculated using Microsoft Excel 2008 software. ANOVA analysis was done using SPSS 16.0 (SPSS, 2007) with a significance level of \( p < 0.05 \).

4.4 Discussion and Results

4.4.1 Biochar Characterization

4.4.1.1 BET and BJH

The surface area of the AGH500T was 18.39 \( \text{m}^2 \text{g}^{-1} \), which was significantly higher than the surface of area of the biochars produced at lower temperatures. The surface areas of the lower temperature biochars were not significantly different from those of the unheated sulfuric acid treated biomass. These results suggest that the degree of carbonization does not increase until reaching pyrolysis temperatures greater than 400°C. Previous research by Grierson et al. (2011) on biochar production of unicellular marine diatom *Tetraselmis chui* showed similar surface area measurements (19 \( \text{m}^2 \text{g}^{-1} \)) at 500°C.\(^{29}\) However, biochar analysis done by Bird et al. (2011) found lower surface areas (1.15 \( \text{m}^2 \text{g}^{-1} \) to 4.26 \( \text{m}^2 \text{g}^{-1} \)) for algal biochar produced over the temperature range of 307°C to 512°C.\(^{12}\) Additionally, Wang et al. (2012) found that the surface area of biochar produced from *Chlorella vulgaris* at 500°C was 2.4 \( \text{m}^2 \text{g}^{-1} \).\(^{13}\) The high surface area of the AGH500T biochar sample and the relatively small percentage of carbon could possibly be the result of aromatic compounds creating pore spaces as they volatilize.\(^{5}\)
The surface areas of the untreated biochar samples (AGH200 and AGH500) are lower than the corresponding surface areas of the sulfuric acid treated biochars (AGH200T and AGH500T), indicating that the sulfuric acid treatment increases the surface area of the biochar. In contrast, the pore volume as calculated by BJH method, remains the same for the sulfuric acid treated biomass, the lyophilized biomass, and both treated biochars and untreated biochars produced at 200°C and 500°C. Suggesting that the heat treatment, not the chemical treatment, increases the pore volume.
Table 4-2. BET and BJH analyses of samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface Area $\text{m}^2 \text{ g}^{-1}$</th>
<th>Pore Volume $\text{mL} \text{ g}^{-1}$</th>
<th>Pore Radius Å</th>
<th>Pore Surface Area $\text{m}^2 \text{ g}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated Charcoal</td>
<td>600-800 $^{30}$</td>
<td>0.44 $^{31}$</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Lyophilized biomass</td>
<td>3.62 ±0.39</td>
<td>0.01</td>
<td>6.32</td>
<td>8.08</td>
</tr>
<tr>
<td>$\text{H}_2\text{SO}_3$ Treated Biomass</td>
<td>3.71 ±0.06</td>
<td>0.01</td>
<td>10.72</td>
<td>1.97</td>
</tr>
<tr>
<td>AGH200</td>
<td>3.51 ±1.17</td>
<td>0.02</td>
<td>27.04</td>
<td>2.97</td>
</tr>
<tr>
<td>AGH500</td>
<td>5.30 ±2.19</td>
<td>0.04</td>
<td>31.17</td>
<td>9.77</td>
</tr>
<tr>
<td>AGH200T</td>
<td>3.69 ±0.46</td>
<td>0.02</td>
<td>6.24</td>
<td>7.37</td>
</tr>
<tr>
<td>AGH300T</td>
<td>3.58 ±0.40</td>
<td>0.02</td>
<td>6.25</td>
<td>6.72</td>
</tr>
<tr>
<td>AGH400T</td>
<td>4.94 ±0.37</td>
<td>0.02</td>
<td>31.37</td>
<td>6.38</td>
</tr>
<tr>
<td>AGH500T</td>
<td>18.39 ±1.39</td>
<td>0.04</td>
<td>16.32</td>
<td>8.72</td>
</tr>
</tbody>
</table>

**4.4.1.2 FE-SEM**

FE-SEM images of AGH biochars as well as images of the untreated lyophilized biomass and sulfuric acid treated biomass prior to pyrolysis treatment are displayed in Figure 4-2. The FE-SEM images were unclear and difficult to obtain due to excess charging. The images do not provide enough information about the structure of the biochar and biomass to draw any conclusion regarding the chemical or thermal treatments.
Figure 4-2. SEM images of (A) lyophilized algae biomass (B) $\text{H}_2\text{SO}_4$ treated biomass (C) AGH200T (D) AGH300T (E) AGH400T (F) AGH500T.
4.4.1.3 Ash and Elemental Analyses

Results of compositional and characteristic analyses of biochar are shown in Table 4-3. The ash content of the algal biomass sample exhibited very little change with the 1.5% H$_2$SO$_4$ treatment. However, the carbon and hydrogen percentages were reduced. The reduction is likely due to the hydrolysis of acid and water soluble saccharides from the biomass.\textsuperscript{32, 33} Acid treatment catalyzes the cleavage of the glycosidic bonds between the monomers that compose the polysaccharides. Once the monosaccharides are liberated, they are extracted from the biomass during the washing step. The fractions of carbon, hydrogen, and oxygen that constitute the monosaccharides are then lost from the biomass, which would account for the diminished carbon and hydrogen content in the treated biomass.

The increase in the percentage of ash in the biochar samples appears to be positively associated with increasing pyrolysis treatment temperatures. As pyrolysis treatment temperatures increase so does the percentage of weight lost during the treatment. The loss can be attributed to the enhanced volatilization of organic material with greater energy input.

The carbon content in the algal biomasses on an ash free dry mass basis was 65.63 wt %. This is high in comparison to other biomass substrates such as corn stover and spruce wood, which have a corresponding carbon content of 49.3 wt % and 51.9 wt % on an ash free dry mass basis.\textsuperscript{34} The carbon content of the biochar decreases with pyrolysis treatment. Bird \textit{et al.} (2011) saw similar reductions in carbon content in biochar production from macroalgae.\textsuperscript{12} These results are contrary to the results of Kim \textit{et al.} (2012), who found that the carbon content of pitch pine biochar increased from 63 to 90
wt % ash free dry mass, from 300 to 500°C. The reduction in carbon content in the biochars produced in this study with increasing temperature were probably due to the loss of volatile compounds.\textsuperscript{35}
Table 4-3. Percent mass loss with pyrolysis treatment, ash, and CHN content in Goldsworth Pond algal biochar.

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>Pyrolysis (wt.%)</th>
<th>% Ash</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>O*</th>
<th>H/C</th>
<th>O/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>6.64</td>
<td>n/a</td>
<td>71.512</td>
<td>18.700±0.071</td>
<td>2.69±0.02</td>
<td>1.6243±0.014</td>
<td>4.48</td>
<td>0.14</td>
<td>0.24</td>
</tr>
<tr>
<td>1.5% treatment</td>
<td>3.29</td>
<td>n/a</td>
<td>71.08</td>
<td>15.125±0.0957</td>
<td>1.95±0.18</td>
<td>1.628±0.336</td>
<td>10.23</td>
<td>0.13</td>
<td>0.68</td>
</tr>
<tr>
<td>AGH200T</td>
<td>3.29</td>
<td>4.90</td>
<td>68.745</td>
<td>15.725±0.050</td>
<td>2.16±0.30</td>
<td>1.520±0.035</td>
<td>11.90</td>
<td>0.14</td>
<td>0.76</td>
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<td>AGH300T</td>
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<td>10.00</td>
<td>76.175</td>
<td>14.000±0.173</td>
<td>1.59±0.03</td>
<td>1.432±0.118</td>
<td>6.80</td>
<td>0.11</td>
<td>0.49</td>
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<tr>
<td>AGH400T</td>
<td>4.42</td>
<td>11.77</td>
<td>74.685</td>
<td>12.367±0.603</td>
<td>2.59±0.18</td>
<td>1.632±0.079</td>
<td>8.72</td>
<td>0.21</td>
<td>0.70</td>
</tr>
<tr>
<td>AGH500T</td>
<td>4.52</td>
<td>19.95</td>
<td>87.97</td>
<td>6.997±0.136</td>
<td>1.70±0.05</td>
<td>1.2013±0.138</td>
<td>2.10</td>
<td>0.24</td>
<td>0.30</td>
</tr>
</tbody>
</table>

O* - Oxygen content was determined by difference.
According to Peng et al. (2011), changes in the H/C and O/C ratios can be used to measure the degree of aromaticity of the biochar. A decrease in H/C and O/C ratios suggests a greater degree of aromaticity. The H/C and O/C ratios in AGH400T (H/C=0.21, O/C=0.71) and AGH500T (H/C=0.24, O/C=0.30) are higher than the ratio in the untreated biomass (H/C=0.14, O/C=0.24), indicating, by this measure, that the degree of aromaticity has decreased. The ratio of H/C in the treated biomass is only slightly lower (at 0.13) than the ratio in the untreated biomass. This result is not unexpected, because the sulfuric acid treatment should not affect the degree of aromaticity. The O/C ratios show no trends with respect to pyrolysis treatment temperature. However, the percentage of oxygen in the biochar decreases with increasing pyrolysis treatment temperature.

The nitrogen content in all the biochar samples remains stable with increasing heat treatment. This is because the nitrogen is likely fixed, primarily as protein in the raw biomass and within heterocyclic aromatic ring structures in the pyrolized biomass. These sp² bonds require more energy to break than sp bonds, causing nitrogen to be retained. The nitrogen, carbon, and hydrogen levels in the all the biochar samples produced are significantly different at the α <0.05 level (one-way ANOVA analysis).

4.4.2 Sorption Experiments

In all sorption tests conducted the blank control showed no significant sorption to the sides of the centrifuge tube or stir bars. The quantity of each compound in the
controls varied by less then 0.1% over the 24-hour testing period. For the sorption test performed using the charcoal standard, the concentration of each compound in solution (2,4-dintrotoluene, 2-(4-(2-methylpropyl)phenyl)propanoic acid, 2-phenethylamine and 2-phenylethanol) was below the level of detection (<1 mg L\(^{-1}\)) after the 30 minute sampling. This indicates that the charcoal removed 100% of each compound at the experimental concentration of 7.2 mg g\(^{-1}\) (for statistical analyses refer to Table 4-4).

Sorption experiments carried out on biochar samples AGH and AES which did not receive the sulfuric acid treatment showed no sorption of the 2-(4-(2-methylpropyl)phenyl)propanoic acid over the 24-hour test period. Sorption experiments were also conducted using each biochar feedstock after they were subjected to different pyrolysis temperatures were tested for each biochar feedstock, 200 and 400°C, and neither showed any significant difference from the quantity of 2-(4-(2-methylpropyl)phenyl)propanoic acid found in the blank control sample.

4.4.2.1 Sulfuric Acid Treated Great Wicomico River Site Biochars

Sorption of 2-(4-(2-methylpropyl)phenyl)propanoic acid was carried out on AES biochar samples produced at 200 and 400°C that underwent the sulfuric acid treatment. Figure 4-3 shows the sorption profiles of these samples over 24 hours. Although some sorption of 2-(4-(2-methylpropyl)phenyl)propanoic acid is observed for the 200°C biochar at the 5- and 180-minute samplings, little sorption is seen at the 24-hour sampling. The reduction in sorption is not immediately clear but it may be because the compound partitioned back in the aqueous phase. Additional samples were taken at 48-hours in order to verify that the biochar did not resorb the 2-(4-(2-
methylpropyl)phenyl)propanoic acid. The concentration of 2-(4-(2-methylpropyl)phenyl)propanoic acid sorbed on the biochar at 48-hours was $0.51 \pm 0.50$ mg g$^{-1}$, which was not significantly different than the 24-hour samplings. Because there was very little sorption on the biochar, no further experiments were conducted using the Great Wicomico location algal biomass.

![Figure 4-3. Sorption of 2-(4-(2-methylpropyl)phenyl)propanoic acid on Great Wicomico 1.5% sulfuric acid treated biochar produced at 200 and 400 °C.](image)

AES200T, and AES400T.
Table 4-4. Student t-test paired differences between compound sorption on the biochar and charcoal.

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>2,4-dinitroaniline</td>
<td>2-(4-(2-methylpropyl)phenyl)propanoic acid</td>
<td>2-phenethylamine</td>
<td>2-phenylethanol</td>
<td>Charcoal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Charcoal P-value</td>
<td>Blank P-value</td>
<td>Charcoal P-value</td>
<td>Blank P-value</td>
<td>Charcoal P-value</td>
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<td>AGH200T</td>
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<td>0.021</td>
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<td>0.007</td>
<td>&lt;0.000</td>
<td>0.169</td>
</tr>
<tr>
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<td>0.009</td>
<td>0.009</td>
<td>0.007</td>
<td>&lt;0.000</td>
<td>0.192</td>
</tr>
<tr>
<td>AGH400T</td>
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<td>0.009</td>
<td>0.0001</td>
<td>0.006</td>
<td>&lt;0.000</td>
<td>0.066</td>
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<tr>
<td>AGH500T</td>
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<td>0.002</td>
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<td>0.043</td>
<td>&lt;0.000</td>
<td>0.029</td>
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<td>AES400</td>
<td></td>
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<td>0.004</td>
</tr>
</tbody>
</table>
4.4.2.2 Sulfuric Acid Treated Goldsworth Pond Site Biochars

The sorption of 2,4-dinitroaniline, 2-phenethylamine, 2-(4-(2-methylpropyl)phenyl)propanoic acid, and 2-phenylethanol in mg L\(^{-1}\) are plotted for each biochar temperature over a 24-hour period in Figures 4-4 through 4-6. The basic 2,4-dinitroaniline compound (pKa of 18.46) sorbs measurably on all treated biochars. The concentration of sorbed compound after 24-hour equilibration was 2.8, 3.1, and 3.1 mg g\(^{-1}\) on AGH200T, AGH300T, and AGH400T, respectively. ANOVA analysis shows that these concentrations are not statistically different. 4.2 mg g\(^{-1}\) of the 2,4-dinitroaniline sorbed on AGH500T biochar after 24 hours, which translates to removal of 58% of the compound from solution. The enhanced sorption capacity for the basic compound is significantly enhanced for this 500 °C treated biochar compared to biochars subjected to lower temperatures.
Figure 4-4. Sorption of 2,4-dinitraniline on sulfuric acid treated AGH biochars. AGH200T, AGH300T, AGH400T, and AGH500T. Error bars represent the standard deviations of triplicate samples.

The concentration of 2-phenethylamine sorbed on biochars AGH200T and AGH300T was 0.72 mg g\(^{-1}\). Similarly, the concentration of 2-phenethyamine on biochar AGH400T was 1.08 ± 0.30 mg g\(^{-1}\), which was not significantly different than the concentration found in the biochars produced at lower temperatures. The concentration sorbed on biochar AGH500T was 1.60 ± 0.01 mg g\(^{-1}\). ANOVA statistical analyses indicate that the concentration of 2-phenethylamine sorbed was significantly higher on biochar AGH500T than on biochars AGH200T and AGH300T (p-value = 0.001, and 0.048, respectively). However, the concentration sorbed on biochar AGH400T was not significantly different.
Figure 4-5. Sorption of 2-Phenethylamine on sulfuric acid treated AGH biochars. AGH200T, AGH300T, AGH400T and AGH500T. Error bars represent the standard deviations of triplicate samples.

The sorption of 2-phenylethanol was minimal on all the biochar samples produced (see Figure 4-6). The maximum sorption concentration of 0.63 ± 0.07 mg g⁻¹ was observed for biochar sample AGH300T. ANOVA analysis indicated that there was no significant difference in the 2-phenylethanol concentrations sorbed on AHG200T, AGH400T, and AGH300T.
The lack of sorption of both the 2-phenylethanol and 2-phenethylamine is perhaps due to the higher water solubility of both compounds, at 20 g L\(^{-1}\) and 4.3 g L\(^{-1}\) respectively. In contrast, the solubilities of 2-(4-(2-methylpropyl)phenyl)propanoic acid and 2,4-dinitroaniline are lower at, 0.049 g L\(^{-1}\) and 0.06 g L\(^{-1}\), each. Compounds with higher water solubilities are more likely to partition into the aqueous phase, resulting in less sorption.\(^{39}\) Other researchers have suggested that this “hydrophobic effect” is responsible for the sorption of hydrophobic compounds on biochars.\(^{40,41,42}\)

Another explanation for the lack of sorption is that neither 2-phenylethanol nor 2-phenethylamine are strong π- electron donors or π- electron acceptors systems. The π-π electron-donor-acceptor interactions are responsible for the strongly sorbing aromatic pi-systems found on black carbon (i.e., soot, char, and charcoal).\(^{41,43}\) Traditionally, -OH and -NH\(_2\) substitutions on aromatic rings deactivate the ring causing it to become a π-
electron acceptor, but in this case the alkyl group further removes the functional groups from the ring. Alkyl groups act as ring activators, causing the ring to become a π electron donor. However, the combination of effects on rings causes the aromatic systems to be neither strongly electron deficient nor electron rich.

The sorption of 2-(4-(2-methylpropyl)phenyl)propanoic acid on biochar, AGH200T is significantly higher than sorption on biochar, AGH400T (see Figure 4-6 and Table 4-4). Sorption of 2-(4-(2-methylpropyl)phenyl)propanoic acid on biochar, AGH300T, is significantly higher than on biochars AGH400T and AGH500T. Biochar AGH300T and AGH200T sorbed 2-(4-(2-methylpropyl)phenyl)propanoic acid concentrations of 3.73 ± 0.57 and 3.64 ± 0.33 mg g⁻¹, respectively. The 300°C treated biochar had the lowest H/C ratio of the biochars, which indicates that it had the greatest degree of aromaticity. Further, 2-(4-(2-methylpropyl)phenyl)propanoic acid is a π-electron donating system, due the alkyl functional groups donating electrons to the ring system and the increased aromaticity on the 300°C biochar could lead to π-π electron-donor-acceptor bonding interactions between the 2-(4-(2-methylpropyl)phenyl)propanoic acid and the biochar surface. According to Keiluweit et al. (2009) π-π electron-donor-acceptor interactions have been proposed as relevant for the adsorption of π-donating contaminants to π-accepting sites in organic soil components and on chars produced from maple wood and charcoal.⁷,⁴¹,⁴²
Figure 4-7. Sorption of 2-(4-(2-methylpropyl)phenyl)propanoic acid on sulfuric acid treated AGH biochars. AGH200T, AGH300T, AGH400T, and AGH500T. Error bars represent the standard deviations of the samples.
4.5 Summary

The production of biochar produced from acid treated algae was investigated by evaluating the physical and chemical properties of chars produced at different temperatures under pyrolysis treatment. Surface area analysis of the biochars revealed that the surface area and pore size does not increase significantly until treatment at temperatures of 500°C. The treated biochar samples all had a lower immersion pH than the compounds chosen for the sorption experiments. The basic (2,4-dintiroaniline) and acidic (2-(4-(2-methylpropyl)phenyl)propanoic acid) compounds sorbed on the treated biochar to a much greater extent than the more neutral, hydrophilic compounds. The mechanism of sorption of these compounds needs further investigation to determine the forces responsible (i.e. π-π donor/acceptor, electrostatic forces, or hydrophobic effect). The results from this study indicate that algal residue represents a possible additional sources for activated carbon for sorption of contaminates from an aqueous environment and another possible use for algae biomass produced for removal of nutrients from water.
4.6 Bibliography


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(30) Product Information: Activated Charcoal **2006**.


CHAPTER 5

ANALYTICAL METHOD COMPLICATIONS: COLORIMETRIC SILICA ANALYSIS AND BOEHM TITRATION

5.1 Introduction

This chapter discusses problems associated with two analytical methods; the Boehm Titration and the molybdenum blue colorimetric method for determining silica in water. The Boehm Titration is commonly used to analyze oxygenated surface groups on biochar. Upon using the Boehm Titration to analyze biochar, we found that the method may be inappropriate for the identification of oxygenated functional groups on biochar. In this chapter the Boehm Titration is evaluated using a set of standard oxygenated functional groups to assess the method.

The molybdenum blue colorimetric method for analyzing silica in water was originally utilized to analyze the silica content in algal biomass after the silica was extracted from the biomass and solubilized into water. During the analysis the concentration of silica in the biomass was determined to be over 100 wt %. The high results were suspected to be the consequence of interferences present in the matrix. The following section discusses possible sources of the interference.
5.2 Colorimetric Silica Analysis

A common analytical method for the determination of silica in soil, sediments, and mineral samples is to prepare these samples by alkaline fusion at high temperatures followed by dissolution of the silica in water for analysis by colorimetry, atomic absorption or gravimetry.\textsuperscript{1} Gravimetric procedures have been deemed not sensitive enough for the determination of less than 5 mg of silica.\textsuperscript{2} Colorimetric methods are more conventional and convenient to use.\textsuperscript{1,3} The customary colorimetric procedure for silica relies on the formation of the yellow (color) silicomolybdate complex produced when ammonium molybdate reacts, in an acidic media, with the soluble silica (H\textsubscript{2}SiO\textsubscript{4}).\textsuperscript{4,5} In a refinement, the silicomolybdate complex can then be reduced to form the molybdenum blue color. The molybdenum blue method has been reported to be sixteen times more sensitive than the molybdenum yellow procedure.\textsuperscript{6}

Many authors have evaluated the molybdenum blue procedure for the determination of silica in aqueous samples.\textsuperscript{1} A major criticism of the method is that other substances, such as phosphorus and iron, can interfere or react with the molybdenum complex.\textsuperscript{2} Additionally, silicomolybdate formation is complicated by multiple factors such as pH, amount of molybdate used, and quantity of other electrolytes present.\textsuperscript{7} Nevertheless, as it currently stands the colorimetric molybdenum blue method is the ASTM Standard Test Method for Silica in Water and is still used by the US EPA to test silica levels in lake waters.\textsuperscript{8,9} In previously referenced publications, the only interferences mentioned are phosphorus and high levels of iron. However, the quantity of iron and phosphorus that lead to the interferences is not specified.
Further complicating matters, Thayer et al. (1930), analyzed interference of ferrous and ferric iron with the molybdenum reagents alone and with the addition of silica and found that 250 mg L\(^{-1}\) Fe\(^{2+}\) caused the solution to turn brown, and 25 mg L\(^{-1}\) of Fe\(^{3+}\) combined with silica caused the solution to turn yellow\(^{10}\). King et al. (1933) concluded that the presence of iron in solution with silicomolybdic acid produced a greenish tint that could not be matched against the silicate standard and was not proportional to the amount of silica present.\(^{11}\) Fogg et al. (1958) suggested that iron levels not exceed 1.0 ppm in solutions being analyzed with colorimetric molybdenum blue method.\(^{12}\) King et al. (1933) also found that the presence of arsenate caused the silicomolydenum blue solution to become darker after 20 minutes. Fortunately, the arsenic (and phosphorus) interference can be removed by the addition of oxalic, citric, or tartaric acid.\(^{6}\) Germanium has also been reported to interfere with this colorimetric method.\(^{13}\)

Moreover, the standard methods (ASTM and EPA) for determining dissolved silica mention that the presence of salt (NaCl) affects the color formation of the molybdenum complex and leads to negative errors.\(^{8,9}\) The absorbance of silica decreased linearly with increased quantities of NaCl salt.\(^{14}\)

In the following work the molybdenum blue colorimetric method, prescribed by Reay and Bennett et al. (1987) and Conley and Schelske et al. (1993), for analyzing silica in plant material and sediment was used to measure silica in the algal biomass.\(^{15,16}\) Spectrometric results obtained using this method showed the presence of an interference in the analyses. Because the source of the interference was unknown, we conducted the following experiments to examine possible sources:

- Fe\(^{2+}\) was evaluated with molybdenum blue reagents alone and also in the presence of
The addition of iron to the matrix of the silica molybdenum blue method was monitored over time using UV/Vis spectroscopy.

Many references suggest a “salt error” with the molybdenum blue method. However, none of the studies that evaluated the “salt error” assessed the addition of NaCl with the molybdenum blue reagents alone so, NaCl was added to silica matrix prior to analysis.

5.3 Experimental

Materials. Ammonium molybdate tetrahydrate (81-83%) ACS grade, sodium bisulfite dried, and oxalic acid dihydrate (99.9%) ACS grade were all purchased from Millinckrodt Baker, Inc. Sodium sulfate (98%+) ACS grade, hydrochloric acid (37%) ACS grade, NaCl (<99%) and iron (II) chloride (98%) were all purchased from Sigma-Aldrich. Silica standard (1000ppm) EPA/ APHA analytical grade was purchased from Ricca Chemical Company. 1-amino-2-naphthol-4-sulfonic acid (98%+) was purchased from Eastman Organic Chemicals. All solutions were made up using 18.2MΩ water from an in-house system purchased from EMD Millipore Corporation.

5.4 Methods

All colorimetric experiments were performed according to the procedure developed by ASTM D859-10 Standard Test Method for Silica in Water.

Amino-Naphthol-Sulfonic Acid-Solution: 0.5 g of 1-amino-2-naphthol-4-sulfonic acid was dissolved in 50 mL of a solution containing 1g of sodium sulfite (Na₂SO₃).
After dissolving, the solution was added to 100 mL of a solution containing 30 g of sodium hydrogen sulfite (NaHSO₃). The final solution was then made up to 200 mL using 18.2MΩ water and stored in plastic bottles. The solution was fresh each day.

*Ammonium Molybdate Solution:* 7.5 g of ammonium molybdate was dissolved in 100 mL of 18.2MΩ water.

*Oxalic Acid Solution:* 10 g of oxalic acid were dissolved in 100 ml of 18 MΩ water.

*HCl solution:* Mixing one volume of 18.2MΩ water with one volume of hydrochloric acid made a 50:50 (v/v) solution of hydrochloric acid and water.

### 5.4.1 Colorimetric Analyses Procedure

Silica standards were prepared by diluting the 1000 ppm silica stock (Ricca Chemical Company) to the desired concentration (0, 0.5, 1, 5, and 6 mg L⁻¹) with 18.2MΩ water. Blank controls were prepared with 18.2MΩ water.

25.0 mL of each of the silica standards were transferred quantitatively, by pipette, into 50.0 mL polypropylene centrifuge tubes. In quick succession, 1 mL of 50 (v/v)% HCl and 2 mL of the ammonium molybdate solution were added to the solution and mixed by hand. After exactly 5 min, 0.75 mL of the oxalic acid solution was added and again the solution was mixed. After one minute, 1.0 mL of the amino-naphthol-sulfonic acid solution was added and mixed. The solution was allowed to stand for 10 minutes prior to measurement. Measurements were made using a Perkin Elmer Lambda Spec 20. The absorbance was collected from 400 to 950nm, with $\lambda_{\text{max}} \approx 820$nm.
5.4.2 Iron (II) Interference

A stock solution of 250 mg L$^{-1}$ iron (II) was prepared by dissolving 0.056 g of FeCl$_2$ in 1.0 L of water. The iron stock solution was then diluted with 18.2 MΩ water to produce iron standards containing 1, 10, 20 mg L$^{-1}$ Fe$^{2+}$. Each iron standard was then added to a silica solution containing 0, 1, 2, or 6 mg L$^{-1}$ of SiO$_2$.

5.4.3 NaCl Interference

Solutions containing 1.0 % (w/v), 5.0 % (w/v) and 10.0 % (w/v) NaCl were evaluated using the molybdenum blue colorimetric method without the addition of silica. Additionally, a solution containing 1 mg L$^{-1}$ SiO$_2$ and 5.0% (w/v) NaCl was evaluated using this colorimetric method.

5.5 Results and Discussion

5.5.1 Iron (II) Interference

The absorbance spectra of the 1.0, 2.0 or 6.0 mg L$^{-1}$ silica molybdenum blue solution (displayed in Figure 5-2 through Figure 5-6) shows the addition of either 1, 10 or 20 mg L$^{-1}$ of Fe$^{2+}$ to a standard quantity of silica (1.0, 2.0, or 6.0 mg L$^{-1}$ SiO$_2$). For this experiment all solutions were allowed to react for at least 10 minutes prior to analysis.

The addition of iron (II) to the silica matrix causes an overall reduction in the absorption spectrum (see Figure 5-2). This result is similar to the findings of another study that showed the addition of iron (III) reduced the optical-density of molybdenum blue solutions over the wavelength range in which maximum optical density occurs.
Interestingly, when the same quantity of iron was added to different quantities of silica the reduction in the absorbance maximum was not linear. When 1 mg L\(^{-1}\) Fe\(^{2+}\) was added to 1 mg L\(^{-1}\) SiO\(_2\), the lambda max was reduced by 0.178 absorbance units, and when the same amount of Fe\(^{2+}\) was added to the 2 mg L\(^{-1}\) SiO\(_2\) solution, the lambda max was reduced by 0.233 absorbance units. Larger quantities of Fe\(^{2+}\) reduced the absorbance spectrum even more. In the case of the 2 and 5 mg L\(^{-1}\) SiO\(_2\) samples, the addition of 10 mg L\(^{-1}\) Fe\(^{2+}\) reduced the absorbance spectra more than the addition of 20 mg L\(^{-1}\) Fe\(^{2+}\).

These are curious results, although upon one repeat analysis similar results were observed. However, there was a small difference in the observed lambda max by an average of 0.065 ± 0.036 absorbance units.

It was observed that the color formation was slow to develop, even after ten minutes. The experiment was repeated with an adjustment to the amount of time allowed to lapse between the introduction of the molybdenum blue reagents and the time the solutions were analyzed by the spectrometer. The solutions were allowed to sit for exactly 30 minutes before determination, instead of just ten minutes as recommended in the ASTM method.

*Figure 5-3* displays the visible spectrum of different concentrations of silica and iron after 30 minutes (*Figures 5-4 and 5-5*). From these spectra it can be seen that absorbance maxima with or without the addition of Fe\(^{2+}\) are similar. The addition of the Fe\(^{2+}\) ion to the solution did not result in severe changes in the observed spectra, and the additional reaction time clearly reduced the effect of iron (II) on the absorbance spectra.
Figure 5-1. Absorbance of different concentrations of SiO$_2$ from 400 to 950nm. (■) Is the absorption spectra of the 1 mg L$^{-1}$ SiO$_2$ solution, (■) is the absorption spectra of the 2 mg L$^{-1}$ solution, and (●) is the absorption spectra of the 6 mg L$^{-1}$ solution.
Figure 5-2. Ten-minute absorbance spectra of silica with the addition of Fe\(^{2+}\) at different concentrations. Top figure 1 mg L\(^{-1}\) silica, middle figure 2 mg L\(^{-1}\) silica, and bottom figure 6 mg L\(^{-1}\), with (■) no Fe\(^{2+}\) addition, (▲) 1 mg L\(^{-1}\) Fe\(^{2+}\), (■) 10 mg L\(^{-1}\) Fe\(^{2+}\), and (■) 20 mg L\(^{-1}\) Fe\(^{2+}\).
Figure 5-3. Thirty-minute absorbance spectra of silica with the addition of Fe$^{2+}$ at different concentrations. Top figure: 1 mg L$^{-1}$ silica, Middle figure: 2 mg L$^{-1}$ silica and Bottom figure: 5 mg L$^{-1}$ silica with (■) no Fe$^{2+}$ addition, (▲) 1 mg L$^{-1}$ Fe$^{2+}$, (△) 10 mg L$^{-1}$ Fe$^{2+}$, and (■) 20 mg L$^{-1}$ Fe$^{2+}$.
Figure 5-4. $\lambda_{\text{max}}$ absorbance of 1 mg L$^{-1}$ silica with Fe$^{2+}$ after (●) 10 minutes and (■) 30 minutes.

Figure 5-5. $\lambda_{\text{max}}$ absorbance of 2 mg L$^{-1}$ silica with Fe$^{2+}$ after (●) 10 minutes and (■) 30 minutes.
An additional experiment was performed to evaluate changes in the absorbance spectra of the silicomolybdenum blue complex with the addition of the Fe$^{2+}$ ion. The goal was to see how the absorbance lambda max changed as time progressed. For this experiment the silicomolybdenum complex with the addition of iron, was monitored at the $\lambda$ max absorbance of 820 nm for a period of 60 minutes. Additionally, the absorbance of the silicomolybdenum blue complex without the addition of Fe$^{2+}$ was monitored for comparison.

*Figure 5-6* shows a 60 minute period monitoring the absorbance of the silicomolybdenum blue complex alone and with the addition of 10 mg L$^{-1}$ Fe$^{2+}$. It is obvious from the figure that the iron (II) affects the rate of color formation. The $\lambda$ max absorbance value for the silica solution alone plateaus after ~1000 seconds, whereas the addition of iron to the solution not only lowers the total absorbance max, but retards the rate of color formation. From this data it can be concluded that iron (II) changes the development rate of the silicomolybdenum blue complex. Allowing the reaction to proceed for only ten minutes, as recommended by the EPA and ASTM methods, in the presence of iron (II) would cause incorrect values of SiO$_2$ to be reported.
Figure 5-6. Time dependence monitoring of silicomolybdenum blue complex with (●) and without (□) the addition of Fe$^{2+}$.
5.5.2 Interference of NaCl

Figure 5-7 shows the absorbance spectra, after 10 minutes, of 1.0, 5.0 and 10.0 % (w/v) solutions of NaCl in the presence of the molybdenum blue reagents. None of the solutions displayed any color to the naked eye. A 0.5 % (w/v) solution of NaCl was also evaluated but showed no absorption. From the figure it can be seen that the salt shows very little absorption, even at the 10 % (w/v) concentration. The spectra of the 1.0 and 5.0 % (w/v) solution are very similar, achieving the lambda max absorbance of 0.023 absorbance units at 828 nm and 829 nm, respectively. The 5.0 % (w/v) NaCl solution has a lambda max absorbance of 0.072 absorbance units at 832 nm. It is interesting that the lambda max migrates to longer wavelengths as the concentration of NaCl increases.

An additional experiment was performed to determine the effect of NaCl on the silicomolybdenum blue complex. The absorbance spectrum shows a lower lambda max when NaCl is added (see Figure 5-8). However, if the shoulder feature (620-650nm) were used to determine the absorbance then the salt interference would cause a slight over estimation of SiO₂. The lambda max of the NaCl+SiO₂ solution is 0.144 at 822 nm, and the lambda max of the SiO₂ alone is 0.156, at 814 nm. Again the presence of the NaCl shifts the lambda max to longer wavelengths.
Figure 5-7. Absorbance spectra of different concentrations of NaCl with the molybdenum blue reagents from 400 to 900 nm. (■) 1.0 % (w/v) NaCl, (▲) 5.0 % (w/v) NaCl and (▲) 10 % (w/v) NaCl.
Figure 5-8. Absorbance spectra of (■) 1mg L$^{-1}$ SiO$_2$ and (▲) 1mg L$^{-1}$ SiO$_2$ and 5.0 % (w/v) NaCl.
There has been speculation that the “salt effect” is likely due to kinetic factors. Experiments conducted in this study did not evaluate any kinetic interactions, but future studies should do so. Other authors have suggested that it is the ionic strength that affects the method. Zhang et al. (2004) found that the linear dynamic range of silica in a 1.0 M NaCl solution was about one half of that in water. From this work it is difficult to conclude what exact interaction NaCl has with the molybdenum. Nevertheless, changes in the absorbance features, specifically the shoulder feature and $\lambda$ max position with the addition of NaCl, support the ionic strength hypothesis.

5.6 Summary

The molybdenum blue colorimetric method for the determination of silica using Amino-Naphthol-Sulfonic Acid reagents was assessed for possible interference with NaCl and iron (II). Results of the iron (II) experiments indicate that rate of the molybdenum blue color formation is slowed by the presence of iron. Results from the NaCl experiment indicated that high levels of NaCl in the solution matrix might cause over and underestimations of total silica depending on the wavelength used for analysis. In conclusion, use of this method for determining silica in a complex matrix may not be appropriate and results should be viewed with caution.
5.7 **Boehm Titration**

Knowledge of the quantity and quality of functional groups on the surface of the carbonized materials helps to determine adsorptive behavior, properties such as hydrophilicity, and prediction of catalytic performance. B.H. Boehm first developed an analytical method for characterizing and approximating oxygenated surface functional groups on carbonized material (*Figure 5-9*). The foundation for concluding only surface groups react in the titration method came from the theory that all the carbon atoms within the three-dimensional diamond lattice or the graphite layer structure are joined by covalent bonds and only free valences at the surface can be saturated by oxygen. The method is based on the titration of functional groups on carbon prepared from sugar charcoal (charcoal produced from sugar) using different strength bases. According to Boehm (1964),

NaOH ($pK_a=15.74$) neutralizes all acidic functional groups, Na$_2$CO$_3$ ($pK_a=10.25$) is capable of neutralizing carboxylic acids and lactonic groups, and NaHCO$_3$ ($pK_a=6.37$) being the weakest base, only reacts with carboxylic acid groups. Using the information gained from doing titrations with each base, the quantity of each functional group can be deduced.

(p.670)

Originally Boehm also included a titration with sodium ethoxide, which was supposed to titrate acids with a $pK_a < 20.58$, but it is no longer included by investigators because it reacts with water and requires oxygen-free conditions. Thus, the existence of weak acid groups on the surface of carbonized materials leads to inherent problems with the
quantification and qualification of oxygenated surface groups.

Figure 5-9. Functional groups that are titrated using the Boehm Titration Method.
*Lactones can be of multiple ring sizes

The Boehm Titration Method has been used for the characterization of activated carbons and biochars (also known as black carbons).\textsuperscript{26,27} It is popular because it is inexpensive, quick, and believed to give reproducible results.\textsuperscript{28} One major criticism of the Boehm Titrations is that it fails to take into account other oxygenated surface functional groups that may form during pyrolysis treatment.\textsuperscript{29,30} For instance, the method does not account for the presence of quinone, ether, esters, or anhydride groups.\textsuperscript{24} Furthermore, some weakly acidic groups will not be accounted for because their pK\textsubscript{a}s are higher than that of the strongest base (NaOH) used for titration. For example, alcohols that typically have pK\textsubscript{a}s of around 16 are non-titratable by NaOH.\textsuperscript{31} Other surface groups that are decidedly not carboxylic, phenolic or lactonic in character, such as those previously mentioned, but have a pK\textsubscript{a} below that of the titration bases, will also be titrated using the Boehm Titration Method. While all of these issues seem apparent, the Boehm titration remains the most common method for assessing oxygenated surface groups.\textsuperscript{26,25-32}
This study attempts to evaluate the selectivity of the Boehm titration using standard compounds with known oxygenated functional groups. The oxygenated functional group in the heptadecanoic acid standard is classified as a carboxylic acid (Table 5-1). The oxygenated functional group on δ-valerolactone is classified as a lactone. Finally, phenol is used as a standard to test the titration of phenolic groups. Hypothetically, the titration of each of these standards using the series of titration bases should lead to identification of only the specific functional group present on that compound.

Table 5-1. Standard Compounds for Boehm Titration.

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Titrant</th>
<th>Standard</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylic Acid</td>
<td>HCO$_3^-$</td>
<td>Heptadecanoic Acid</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Lactone</td>
<td>CO$_3^{2-}$</td>
<td>δ-Valerolactone</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Phenol</td>
<td>OH</td>
<td>Phenol</td>
<td><img src="image" alt="Structure" /></td>
</tr>
</tbody>
</table>
5.8 Experimental Method

Boehm Titration Materials. Sodium hydroxide ACS grade (≥97%) and potassium hydrogen phthalate ACS grade (≥99.95%) were purchased from VWR International. Sodium carbonate ACS grade (≥99.95%) was purchased from Mallinckrodt. Sodium bicarbonate ACS grade (≥99.7%) was purchased from Fisher Chemical. Hydrochloric acid ACS grade (37%) was purchased from EMD Millipore. Heptadecanoic acid (≥98%) and δ-valerolactone (technical grade) were purchased from Sigma-Aldrich. Phenol, ACS grade, was purchased from Spectrum Chemicals. Sodium carbonate and sodium bicarbonate were dried in an oven at 105°C and kept in a desiccator. All other reagents were used as received.

5.9 Boehm Titration Method

5.9.1 Preparation of Solutions

The procedure for the titration of surface oxide groups was based on the method developed by Boehm. Each day, fresh 0.05 M solutions of NaOH, Na₂CO₃, NaHCO₃, and HCl were made up. All solutions were prepared in 18.2 ΩM water, degassed for two hours by sonication. Na₂CO₃ and NaHCO₃ were dried overnight at 105°C and cooled in a desiccator prior to use. The NaOH solutions were standardized by titration using potassium hydrogen phthalate as the indicator. For the NaOH standardization, 0.1 g of potassium hydrogen phthalate was weighed to the nearest 0.1 mg into 40 mL of distilled water and two drops of phenolphthalein indicator were added. HCl was standardized by measuring out 25 mL of HCl into an Erlenmeyer flask with the addition of 2 drops of
phenolphthalein indicator. The HCl solution was then titrated with standardized NaOH base. All standardizations were carried out in triplicate, and the results were averaged.

**Statistics**

Mean and standard deviations were calculated from the triplicate runs using Microsoft Excel 2008. Error propagations were also calculated but were found to be smaller than the standard deviation errors.

**5.9.2 Boehm Titration of Standards**

The Boehm Titration Method was based off of the method developed by Boehm et al. (1964). For each titration the individual standard was weighed into a 50.0 mL capped polypropylene centrifuge tube together with 45 mL of 0.05 M of one of the base solutions (NaOH, NaHCO₃, or Na₂CO₃). The centrifuge tubes were then agitated for 24 hours on an orbital shaker. The samples were gravity filtered through Watman No. 42 filter paper, and 10.0mL of the supernatant was pipetted into a 125 mL Erlenmeyer flask. Next 20.0 mL of the 0.05 M HCl solution was added to the flasks containing the 0.05 M NaHCO₃ supernatant. Then, 30.0 mL of 0.05 M HCl was added to the flasks containing the Na₂CO₃ supernatant. HCl was not added to the flask containing the NaOH supernatant.

The acidified supernatant solutions were allowed to equilibrate for one hour prior to beginning the titration. The solutions were then back-titrated using 0.05 M NaOH. Back titrations were performed to validate endpoint determination. Titrations were performed under constant stirring to ensure uniform mixing. A pH meter, calibrated each
day using a series of standard buffers, was used to determine the endpoint of the titration, considered to be pH 7.0 held for at least 60 seconds. Each titration was performed in triplicate.

5.9.3 Surface Group Calculations

In order to determine the quantity of surface groups on the titrated samples the following equations were used from Oickle et al. (2010):

Equation 5-1. Titrated molar functional groups

\[
\begin{align*}
\text{(A)} & \quad n_{fg} = \frac{n_{HCl}}{n_{B}} [B] V_{B} - \left( [HCl] V_{HCl} + [NaOH] V_{NaOH} \right) \frac{V_{B}}{V_{a}} \\
\text{(B)} & \quad n_{fg} = [B] V_{B} - [HCl] V_{HCl} \frac{V_{B}}{V_{a}}
\end{align*}
\]

Where \([HCl]\) and \([NaOH]\) and \(V_{HCl}\) and \(V_{NaOH}\) are the concentrations and volumes of HCl and NaOH solutions added used to acidify the supernatant and to back-titrater, respectively. \(V_{B}\) and \([B]\) denote the volume and concentration of the base mixed with the standard prior to titration. The value of \(V_{a}\) is the volume of sample (10.0 mL) taken from \(V_{B}\). The symbol \(n_{fg}\) represents the moles of the functional group standard that reacted with the base during the incubation time. \(n_{HCl} \over n_{B}\) is the molar ratio of HCl to the reaction base. This is used to account of the diprotic Na\(_2\)CO\(_3\) reaction.
base. Equation (B) was only used for the titrations of the NaOH supernatant. (p.1254-1255)\textsuperscript{33}

Once \( n_{fg} \) for each reaction bases are determined, simple subtraction allows for the determination of carboxylic acid, lactones, and phenol groups. Therefore, Equation 5-2. Functional groups

\[
\text{(C)} \quad n_{fg} (\text{Na}_2\text{CO}_3 \text{ supernatant}) - n_{fg} (\text{NaHCO}_3 \text{ supernatant}) = n_{fg} \text{ of lactones mol}
\]

\[
\text{(D)} \quad n_{fg} (\text{NaOH supernatant}) - n_{fg} (\text{Na}_2\text{CO}_3 \text{ supernatant}) = n_{fg} \text{ of phenols mol}
\]

\[
\text{(E)} \quad n_{fg} (\text{NaHCO}_3 \text{ supernatant}) = n_{fg} \text{ of carboxyl mol}
\]

This information can then be used to calculate the quantity of each type of functional group per gram of standard by dividing the number of moles by the mass of the standard incubated in solution.\textsuperscript{33}

\textbf{5.10 Results and Discussion}

The results from the titrations of each representative standard compounds (phenol, heptadecanoic acid, and \( \delta \)-valerolactone) are displayed in Table 5-2 (and Figure 5-10). For the titration of the phenol standard material, the quantity of phenolic functional groups was calculated to be 10.62 mmol g\textsuperscript{-1} but the titration only indicated1.27± 0.06 mmol g\textsuperscript{-1} of phenolic functional groups. The sodium hydroxide base did not titrate any functional groups on the phenol standard, although it should have. The results may have been due to the phenol analyte reacting too slowly with titrant in the direct titration causing the solution to appear to not contain acidic functional groups.
Results from the titration of heptadecanoic acid (standard carboxylic acid) were in the range of the predicted quantity of carboxylic acid groups, but the standard deviation between sample titrations was high (4.32±0.61 mmol g⁻¹). Because heptadecanoic acid can be titrated by all bases, using Equation 5-2 would yield functional groups that were clearly not present in solution, so the equation was not used. Curiously, even though the pKₐ of heptadecanoic acid is 4.78, ³⁴ and it can be titrated by all three bases, the back-titration of the Na₂CO₃ did not expose the existence of any functional groups, and the back-titration of NaHCO₃ led to the identification of about half (2.27 ± 0.03 mmol g⁻¹) of the carboxylic groups. However, titration with the strongest base, NaOH, did yield the correct quantity.

Table 5-2. Results of the Boehm titration of the standard compounds.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Phenol (mmol g⁻¹)</th>
<th>Lactone (mmol g⁻¹)</th>
<th>Carboxylic acid (mmol g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>Calcd. 10.63</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Act. 1.27 ±0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heptadecanoic Acid</td>
<td>Calcd. 0</td>
<td>0</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>Act. 0</td>
<td>0</td>
<td>4.32 ±0.61</td>
</tr>
<tr>
<td>δ-Valerolactone</td>
<td>Calcd. 0</td>
<td>9.98</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Act. 0</td>
<td>2.86 ±0.03</td>
<td>9.40 ±0.02</td>
</tr>
</tbody>
</table>
Figure 5-10. Titrated (■) vs. calculated (■) functional groups for the standard compounds. (A) phenol (B) δ-valerolactone and (C) heptadecanoic acid. Error bars represent the standard deviation of triplicate analyses.
To represent a standard lactonic group compound, δ-valerolactone was used for the Boehm Titration analysis. Lactones are cyclic esters, most favored in strain-free five- or six member rings (δ-valerolactone is a six-member ring). Lactonization is an equilibrium process, but δ-lactones are so readily formed that the carboxylic acid can provide the acid catalyst. When the δ-valerolactone is incubated with the 0.5 M HCl it does not undergo acid hydrolysis and remains in the closed ring form. Alternatively, the hydrolysis of a lactone to the corresponding hydroxy acid can proceed under alkaline conditions. However, because the acid incubated δ-valerolactone solution is back titrated during the Boehm titration process to the neutral pH of 7, there is not enough excess base in the solution to hydrolyze the lactone, and additionally δ-valerolactone is known to exist in the ring form even at pH 7.4.

The calculated quantity of titratable lactonic groups for the δ-valerolactone standard was 9.99 mmol g⁻¹, but the actual titrated quantity was only 2.86 ±0.03 mmol g⁻¹. Carboxylic acid groups were also titrated using this method (9.40 ±0.02 mmol g⁻¹) even though none were predicted. The sodium hydroxide base did not titrate any functional groups, probably due to the slow reaction between the analyte and titrant.

The results obtained using the Boehm Titration Method here are erroneous and erratic. For experiments performed using the phenol and lactone standard compounds (phenol and δ-valerolactone) the calculated quantity of functional groups did not correspond to the quantity of titrated functional groups. In the case of δ-valerolactone, carboxylic acid functional groups were indicated that should not exist in the standard solution. Salame et al. (2001) found that when the Boehm Titration was compared to a potentiometric titration of the same material and the results did not correlate well.
The authors attributed the difference to the possibility that there were species on the carbonized surface that contain heteroatoms, different from oxygen, and whose pKₐ's corresponds to the pKₐ of carboxylic acid. The original Boehm manuscript that explains the logic behind the use of this particular method concluded the functional groups detected by the method explain, at most, 50% of the analytically determined oxygen content. Additionally, the Boehm titration was developed to examine surface oxides on black microcrystalline carbon only. Although the Boehm titration is widely used, it has not been standardized using a material with known surface properties, which makes it difficult to interpret or compare Boehm titration results reported in the literature. Consequently, using the Boehm Titration Method to deduce specific information about biochar surfaces may be misleading. Additional research is needed to determine how to best use the method.

5.11 Summary

The Boehm Titration Method was evaluated using three different standards: heptadecanoic acid, phenol, and δ-valerolactone. For the phenol and lactonic model compounds, the Boehm Titration Method failed to titrate the phenolic and lactonic functional groups. Review of the literature shows that there is no published standard for evaluating the method. The results from these sets of analyses leads to the conclusion that this particular method needs significant study before it can be used to characterize biochar or black carbon surface functional groups.
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CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

In an effort to improve water quality and enhance energy sustainability, algal biomass communities have been implemented to harness the power of the sun, capture carbon dioxide, nitrogen, and phosphorus, and produce a valuable product. This thesis focuses on characterizing algal turf biomass grown in different environmental conditions and evaluating variations in the algal biomass composition. The information is crucial to develop optimal uses for the algal biomass. For instance, biomass grown on wastewater effluent tends to be high in carbon and low in ash, making it a suitable biofuel. Instead of primarily dealing with biofuel-feedstock components, this research also tackles the problem of managing the residual material. Here the biomass has been evaluated for use in nutrient recapture, as a fertilizer feedstock, and as a biochar for the sorption of chemical contaminants from aqueous solutions.

6.1 Major Finding and Recommendations

Algal biomass communities can be produced at most locations. The water conditions under which algae are produced can vary while still producing biomass with high nutrient content (nitrogen and phosphorus). This is promising information for the biofuel industry, because it means that the cultivation of algal biomass for biofuel purposes can be linked with nutrient removal, and the residual biomass may have value in other markets.
Algae produced from the Muskegon Wastewater Treatment plant presents the best overall profile with respect to nutrient content and recycling value of the algal biomass communities assessed in this study. This biomass has the lowest overall ash content and total silica content, which is not surprising because the silica quantity is generally directly correlated with the amount of ash. Lower ash content gives greater value to the biomass because it means there will be less material to dispose after biofuel processing. The Muskegon wastewater grown algal biomass had the highest phosphorus content (>1.0 wt. %) and also had high nitrogen and organic carbon level, exceeding those found in a previous study that evaluated the nutrient content in algae produced from wastewater, which reported phosphorus, nitrogen, and (total) carbon levels of 0.5, 4 and 50 wt %, respectively.\(^1\) The overall value of the wastewater-produced algal biomass as feed or fertilizer feedstock would thus be higher in comparison to the other biomass grown at other sites in this study. However, the high nitrogen content in wastewater-produced algae is associated with greater release of a potent greenhouse gas, \(\text{N}_2\text{O}\), from the direct combustion of biofuels.\(^2\)

The relationship between nutrient levels in the algal growth medium and nutrient levels in the associated algal biomass has previously been investigated.\(^3,4\) However, to the best of my knowledge, none of these studies have evaluated the relationship between organic and inorganic carbon content in the algal biomass. This study demonstrates that an inverse relationship exists between the organic carbon and inorganic carbon content in the algal biomasses. Although the reason for this relationship was not determined, prior work suggests that algae develop a DIC concentrating mechanism and store more inorganic carbon when grown under low dissolved inorganic carbon (DIC) conditions.\(^5\)
Therefore, the inverse correlation is hypothesized to be due to low DIC levels in the water, which would lead to more storage of inorganic carbon in the algal cells. When DIC levels are high, inorganic carbon is readily fixed from the water and excess inorganic carbon is not stored in the algal biomass. Unfortunately, DIC was not measured at the study sites, so this hypothesis could not be tested in this study. Future studies should focus on determining the relationship between organic and inorganic carbon by manipulating exposure to high and low levels of DIC and evaluating the resulting inorganic and organic carbon content in the algal biomass.

6.1.1 Flowway Position Analyses

Subsets of data were collected in an attempt to evaluate the accumulation of nutrients at different positions along the floway. This information could be of interest to an ATSTM system user, because it could help determine optimal flowway length. The flowway position experiments performed at Goldsworth Pond and the Muskegon Wastewater Treatment Plant showed no significant difference in the phosphorus content with flowway position. Conversely, nitrogen, and carbon levels were significantly lower at the start of the floway compared to the end of the floway. These results were contrary to the original hypothesis stating that higher nutrients concentrations would be found in biomass grown near the beginning of the floway. This hypothesis was based on the notion that nutrient concentrations would be higher closer to the water inlet and lower as the water flowed down the floway, because the algal biomass would remove nutrients from the water. However, the flowways used in this study were 7.4m shorter than those used at large ATSTM installations with long (loom) floways, where nutrient content was
reported to show some dependence on floway position. This hypothesis could not be fully tested because the nutrient content in the water at each position was not routinely tested during the growth experiments. However, the limited data collected at Goldsworth Pond showed insignificant nutrient removal as water flowed over the length of the short, 15.6 m long, floway.

6.1.2 Carbon Dioxide Addition Experiment

The addition of CO$_2$ to algal growth media of algae produced in a lake has been previously found to enhance productivity and carbon content and decrease the nitrogen and phosphorus content in filamentous green algal biomass. Therefore, algal biomass composition was determined in this study for algal communities produced with CO$_2$ supplementation to assess how CO$_2$ changed the biomass composition. Carbon dioxide additions to floways at the Great Wicomico River site were found to increase organic carbon and hydrogen levels in the harvested biomass. Notably, CO$_2$ addition did not appear to have any significant effect on phosphorus or inorganic carbon accumulation in the biomass. Inorganic carbon may have been decreased by CO$_2$ addition, but insufficient data was collected to determine this with certainty. There is also some evidence that CO$_2$ supplements may have reduced the total silica content in the algal biomass, but more data is needed to confirm this.

6.1.3 Limitations and Recommendations

The work presented here only begins to touch upon the numerous studies needed to evaluate the utilization of algal biomass for pollution reduction and biofuel production.
Although this work shows that multiple locations have the potential to produce biomass with high nutrient content, it lacks measurements of nutrient removal from the water and productivity rates were not determined at the various sites. These questions need to be answered to determine the feasibility of growing algae to be used for nutrient recapture at a specific location. Another drawback of this study was the limited number of samples available for some of the experiments, which makes it difficult to draw broad conclusions.

The value of the biomass, in broad terms, has been considered in this work but information about the quantity and types of bioactive compounds present in the algal biomass would allow fuller assessment of potential biomass uses. For example, it would be helpful to more fully characterize the carbohydrate content of the algal biomass, specifically, the nature and distribution of polysaccharides. Variations in the monosaccharide composition were observed for algal biomass from different locations, but the causes of the variability were not determined.

### 6.1.4 Heavy Metals

Heavy metals accumulation in the biomass was generally the same in all locations, with As > Cu ≈ Cr > Co ≈ Mo > Cd. This suggests that there is a preferred order of metal uptake by algae communities. None of the algae harvests evaluated had any detectable amount of lead. The quantity of arsenic detected in the algal harvests was high, 0.012-0.021 wt %, and could pose a concern for direct application of the biomass as a fertilizer. The source of the arsenic was not investigated; it might be present in sediments
or particulates that collect on the turf from the water column or could be bioaccumulated by the algae.

It does appear that heavy metals bioaccumulated in algal samples harvested from the Muskegon Wastewater treatment plant location. At this location the metal levels in the water were measured and were found to be lower than those in the corresponding algal biomass. The bioaccumulation order did not correspond to the order of metal concentrations in the water.\textsuperscript{10}

A previous study evaluated the metal concentrations in river water, river sediment, and algae \textsuperscript{11}. That study found the metal accumulation order and concentrations in the benthic algae were similar to those found in the river sediment, but not the river water.\textsuperscript{11} Those results are in agreement with the conclusions presented here. We can thus confidently conclude that metal accumulation order in algae does not follow the concentration order in the water.

\subsection*{6.1.5 Biogenic Silica}

The time-course digestion method was used to estimate the quantity of biogenic silica in the algal samples collected from different locations. Algal biomass samples from the freshwater locations have significantly lower biogenic silica content than the samples harvested from the freshwater/saltwater mixed locations. Because biogenic silica is linked directly to diatoms,\textsuperscript{13} these results suggest that the freshwater locations had lower diatom populations.

The data gathered using the time-course extraction method show a great deal of variation in the quantity of silica liberated. However, according to other studies the
digestion method is the best method available for analyzing biomass that contains significant amounts of mineral interferences.\textsuperscript{14, 15} Because algal-turf biomass is a complex matrix, some variation in the extracted quantity of biogenic silica is expected. Future analyses should include more replicates to better understand variation in results. Additionally, it would be helpful to correlate the algal taxonomic classifications to the biogenic silica content.

6.1.6 Limitations and Recommendations

Major limitations of the results of the biogenic-silica and heavy-metal analyses were due to (1) limited number of harvests analyzed, (2) lack of data on the composition of the growth medium and (3) incomplete species characterization of the algal biomass. The accumulation of heavy-metals study would also have benefited from metal uptake studies, which could help determine the uptake capacity of the biomass. The high content of heavy metals and silica has poor implications for the direct conversion of biomass to fuel and for recycling of the biomass as animal feed or fertilizer. To better understand how environmental factors affect the accumulation of metals, future studies should investigate how the influence of nutrient levels, temperature, light, and rate of harvesting influence the accumulation of silica and heavy metals in the algal biomass.

6.1.7 Recycling Algal Biomass as Biochar

The production of biochar produced from the residual algal biomass after acid hydrolysis was investigated by evaluating the physical and chemical properties of chars produced at different temperatures under anaerobic pyrolysis treatment. Surface area
analyses of the biochars revealed that the surface area and pore size do not increase significantly until treatment at elevated temperatures (500°C). Treated biochar samples had lower immersion pH values compared to compounds chosen for the sorption experiments. Both basic (2,4-dintiroaniline) and acidic (2-(4-(2-methylpropyl)phenyl)propanoic acid) compounds sorbed on the treated biochar to a much greater extent than the more neutral hydrophilic compounds. The mechanism of sorption of these compounds (i.e. π-π donor/acceptor, electrostatic forces, or hydrophobic effect) requires further investigation. 16, 17, 18 The results from this study indicate that algal residue represents a possible additional source of activated carbon for sorption of contaminants from water. This suggests another possible use for algae turf biomass produced primarily for nutrient removal from water.

6.1.8 Limitations and Recommendations

Biochar represents a promising avenue whereby algal biomass residue can be recycled and repurposed. Future work should evaluate algal biochars produced after the extraction of all carbohydrates, not just the acid and water labile portions, as well as after extraction of lipid or protein portions of the biomass. Other methods of biochar production, such a hydrothermal liquefaction, should also be considered.19

Because algal biomass is high in nutrients that are useful for plant growth, algal biochar could be applied as a soil amendment. As the biomass undergoes heat treatment, the non-volatile nutrients become more concentrated, increasing the value of the biochar as a fertilizer. Additionally, the pores produced in the biochar during pyrolysis could increase the water holding capacity of the soil.
Another aspect of biochar production that needs to be evaluated is the production and identification of volatile organic compounds produced during pyrolysis. For example, pyrans and furans, which are typically produced from the depolymerization of cellulose and lignin, have been identified. \(^20\) Other compounds such as benzene have also been produced during biochar production. \(^21\) Assessing the production of these compounds could provide insights into the reactions occurring in the biomass during processing.

### 6.2 Interferences

#### 6.2.1 Colorimetric Interferences

Iron (II) and NaCl were investigated as potential interfaces in the determination of silica by the molybdenum blue colorimetric method. The presence of iron (II) in the silica solution matrix causes the amount of silica to be underestimated if the absorption measurement is made after the addition of the molybdenum blue reagents. Monitoring of the blue complex with and without the addition of iron (II) indicates that the rate of color formation is impeded by the Fe\(^{2+}\) cation, such that even after 60 minutes the color may not be fully developed. Investigation into the “salt error” demonstrates that the presence of NaCl causes an underestimation of the silica concentration at lower wavelength and an overestimate at higher wavelengths. The change in the absorbance spectra might be caused by ionic strength, but additional studies are needed to confirm this observation.

When the algal biomass was analyzed for silica using the molybdenum blue colorimetric method, the quantity of silica measured was overestimated. Further investigation suggests that interferences were responsible for the overestimation. Because
the silica content was overestimated by the colorimetric method, it is not likely that iron (II) or NaCl were the actual causes of the interference although they were present in the solution matrices.

6.2.2 Conclusion and Recommendations

Methods that are commonly used in the literature are often perpetuated without further evaluation. Therefore, it becomes necessary to validate these “standard analytical methods” from time to time to ensure their validity. From the studies presented in this work, it can be concluded that silica analysis with complex matrixes requires prior determination of possible interferences, which can be time consuming and difficult. Therefore, it is advisable to use more specific analytical methods such as ICP, where fewer interferences are likely to exist, rather than unspecified techniques. Future research in this area should focus on determining the kinetics behind the iron (II) interference and the mechanism behind the spectrum shift in the presence of NaCl.

6.2.3 Boehm Titration

An attempt to validate the accuracy of the Boehm titration for measuring the number and type of oxygenated groups present in a set of standard materials was investigated. The quantities of functional groups calculated using the titration did not agree with the theoretically calculated values. Rather, the quantity of functional groups calculated using the titration was lower and sometimes functional groups were indicated that were not present.

The original Boehm titration method was developed to evaluate carbon produced
from pure sugar charcoal and was never standardized. The work presented in the original manuscript explained that the method might have limitations. However, many authors have continued to use the method to determine the types and quantities of surface functional groups in various organic materials. Until the Boehm titration is validated and standardized, it should only be used to evaluate the total quantity of acid and basic functional groups on a given material. It should not be used to identify the functional characteristics of specific surface sites.
6.3 Bibliography


(10) *Unpublished data. 2010 and 2011 for the storage lagoon water at the Muskegon Wastewater Treatment Plant; Muskegon Wastewater Treatment Plant: Muskegon Wastewater Treatment Plant.*


Table 7-1. The ultimate analysis data of algae biomass grown at Goldsworth Pond, Kalamazoo, MI.

<table>
<thead>
<tr>
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<th>Hydrogen</th>
<th>Nitrogen</th>
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<td>2.20 ±0.02</td>
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<tr>
<td>AGH051710</td>
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<td>2.17 ±0.08</td>
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<td>AGH061110</td>
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<td>14.02</td>
<td>2.54 ±0.07</td>
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<td>AGH073010</td>
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<td>1.88 ±0.05</td>
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<tr>
<td>AGH090110</td>
<td>4.2 ±</td>
<td>19.92</td>
<td>3.91 ±0.13</td>
<td>2.14 ±0.03</td>
</tr>
<tr>
<td>AGH091010</td>
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<td>3.10 ±0.12</td>
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<td>Magnesium</td>
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<td>2.87 ± 0.00</td>
<td>2.06 ± 0.05</td>
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<tr>
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<tr>
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<tr>
<td>AGH070811F4S4</td>
<td>5.7 ± 0.6</td>
<td>17.41 ± 3.28</td>
<td>2.49 ± 0.04</td>
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</table>

*Asterisks next to sample names indicate that the inorganic carbon content was not obtained directly, but was estimated by averaging the inorganic carbon content of samples harvested from a similar time period and location on the floway.*
Figure 7-1. Goldsworth Pond ultimate analysis floway comparison. (■) inorganic carbon, (■) organic carbon, (■) hydrogen, and (■) nitrogen make up of algae grown at Goldsworth Pond, Kalamazoo, MI. Samples were harvested from different screen locations along different floways. Error bars represent sample standard deviations for triplicate analyses.

Table 7-2. The ultimate analysis of algal biomass harvested from floways located at the Muskegon wastewater treatment plant, Muskegon, MI. All samples were run in triplicate, except where noted by a lack of standard deviation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inorganic Carbon</th>
<th>Organic Carbon</th>
<th>Hydrogen</th>
<th>Nitrogen</th>
</tr>
</thead>
<tbody>
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<td>AMS071211F1S2</td>
<td>12.30 ±1.71</td>
<td>27.43</td>
<td>5.76 ±0.28</td>
<td>7.00 ±0.08</td>
</tr>
<tr>
<td>AMS071211F1S8</td>
<td>11.95 ±0.82</td>
<td>25.68</td>
<td>5.51 ±0.15</td>
<td>6.38 ±0.11</td>
</tr>
<tr>
<td>AMS071211F1S10*</td>
<td>11.77 ±</td>
<td>30.96</td>
<td>6.35 ±0.22</td>
<td>7.37 ±0.23</td>
</tr>
<tr>
<td>AMS071211F1S15</td>
<td>11.07 ±0.81</td>
<td>28.07</td>
<td>5.82 ±0.04</td>
<td>6.84 ±0.12</td>
</tr>
<tr>
<td>AMS071211F3S2</td>
<td>9.67 ±1.14</td>
<td>28.83</td>
<td>5.77 ±0.19</td>
<td>6.05 ±0.17</td>
</tr>
<tr>
<td>Sample Name</td>
<td>Value ± Error</td>
<td>Value ± Error</td>
<td>Value ± Error</td>
<td>Value ± Error</td>
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<tr>
<td>AMS071211F3S8</td>
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<td>24.78</td>
<td>5.87 ± 0.03</td>
<td>6.31 ± 0.02</td>
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<td>AMS071211F3S10</td>
<td>12.41 ± 2.37</td>
<td>24.99</td>
<td>5.78 ± 0.04</td>
<td>7.11 ± 0.06</td>
</tr>
<tr>
<td>AMS071211F3S20</td>
<td>13.90 ± 0.99</td>
<td>28.17</td>
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<td>34.30</td>
<td>4.04 ±</td>
<td>8.91 ±</td>
</tr>
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<td>AMS072911F3S10</td>
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<td>33.94</td>
<td>4.36 ± 0.15</td>
<td>7.94 ± 0.43</td>
</tr>
<tr>
<td>AMS081211F1S2</td>
<td>2.16 ± 0.18</td>
<td>35.20</td>
<td>4.82 ± 0.38</td>
<td>7.18 ± 0.33</td>
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<tr>
<td>AMS081211F1S20</td>
<td>1.92 ± 0.11</td>
<td>28.64</td>
<td>5.58 ± 0.35</td>
<td>7.77 ± 0.20</td>
</tr>
<tr>
<td>AMS082211F1S2</td>
<td>3.16 ± 0.19</td>
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<td>4.40 ± 0.37</td>
<td>5.76 ± 0.20</td>
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<tr>
<td>AMS082211F1S10</td>
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<td>31.48</td>
<td>4.88 ± 0.23</td>
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<td>AMS082211F1S20</td>
<td>4.69 ± 0.54</td>
<td>32.81</td>
<td>4.88 ± 0.64</td>
<td>7.66 ± 0.85</td>
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</table>

*Asterisks next to sample names indicate that the inorganic carbon content was not obtained directly, but was estimated by averaging the inorganic carbon content of samples harvested from a similar time period and location on the floway.
Figure 7-2. Ultimate analysis of algal biomass grown in wastewater. (■) inorganic carbon, (■) organic carbon, (■) hydrogen, and (■) nitrogen make up of algae grown at the Muskegon wastewater treatment plant, Muskegon, MI. Samples were harvested from different screen locations along different flowways. Error bars represent sample standard deviations for triplicate analyses.

Table 7-3. The ultimate analysis data of algal biomass grown at the Great Wicomico River, VA. All samples were run in triplicate, except where noted by a lack of standard deviation.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Inorganic Carbon</th>
<th>Organic Carbon</th>
<th>Hydrogen</th>
<th>Nitrogen</th>
</tr>
</thead>
<tbody>
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<td>3.11 ±0.04</td>
<td>1.82 ±0.11</td>
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<tr>
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<td>3.58 ±0.01</td>
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<td>2.55 ±0.38</td>
<td>2.24 ±0.01</td>
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<tr>
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<td>2.71 ±0.01</td>
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<td>10.66</td>
<td>2.43 ±0.20</td>
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</tr>
<tr>
<td>AES080210F1</td>
<td>4.70 ±0.33</td>
<td>15.41</td>
<td>2.98 ±0.01</td>
<td>1.66 ±0.02</td>
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<td>AES121110F2D</td>
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<td>3.76 ±0.04</td>
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<td>3.31 ±0.22</td>
<td>3.12  ±0.05</td>
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<tr>
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</tr>
<tr>
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<td>16.30</td>
<td>4.47 ±0.13</td>
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</tr>
<tr>
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<td>24.91</td>
<td>4.81 ±0.22</td>
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<td>7.69  ±0.21</td>
<td>19.28</td>
<td>4.03 ±0.09</td>
<td>2.77  ±0.04</td>
</tr>
</tbody>
</table>

*Asterisks next to sample names indicate that the inorganic carbon content was not obtained directly, but was estimated by averaging the inorganic carbon content of samples harvested from a similar time period.
Figure 7-3 Great Wicomico floway one ultimate analysis. Proportion of biomass collected from the Great Wicomico River, VA that is comprised of . (■) inorganic carbon, (□) organic carbon, (■) hydrogen, and (▲) nitrogen. Error bars represent sample standard deviations for triplicate analyses.
Figure 7-4. Great Wicomico floway 1 plot of organic carbon vs. inorganic carbon.

Figure 7-5. Great Wicomico floway 2 plot of organic carbon vs. organic carbon for samples that did not receive CO$_2$ injection.
Figure 7-6. Great Wicomico floway two ultimate analyses. Portion of biomass collected from the Great Wicomico River, VA that is comprised of (■) inorganic carbon, (□) organic carbon, (●) hydrogen, and (■) nitrogen. Error bars represent sample standard deviations for triplicate analyses.

CHN analysis of the data from the lower section of floway two shows that the organic carbon content in the algal biomass harvested on May 10, 2011 was the highest at 24.9 wt. %. The corresponding inorganic carbon content in this harvest was 5.94 wt. %, which was low in comparison to other harvests. The sample with the smallest proportion of organic carbon (9.55 wt %) was harvested on March 12, 2011 and this sample also...
displays an inverse relationship between organic carbon and inorganic carbon (12.02 wt. \%) content. Statistical evaluation confirms that the variables are highly correlated with an $r$-value of -0.976 and a $p$-value $< 0.000$. Further statistical evaluations of nitrogen and hydrogen nutrient levels show no correlation between any other nutrients.

Figure 7-7. Great Wicomico River lower floway two inorganic carbon vs. organic carbon.
Table 7-4. The ultimate analysis of algal biomass harvested from floways located at the York River, VA. All samples were run in triplicate, except where noted by a lack of standard deviation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inorganic Carbon</th>
<th>Organic Carbon</th>
<th>Hydrogen</th>
<th>Nitrogen</th>
</tr>
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<tbody>
<tr>
<td>AVI060109BB</td>
<td>3.3 ±0.0</td>
<td>5.68</td>
<td>1.54 ±0.14</td>
<td>1.67 ±0.02</td>
</tr>
<tr>
<td>AVI082109BB</td>
<td>8.6 ±0.7</td>
<td>4.30</td>
<td>2.35 ±0.02</td>
<td>1.57 ±0.02</td>
</tr>
<tr>
<td>AVI082809BB</td>
<td>3.4 ±0.0</td>
<td>8.12</td>
<td>2.08 ±0.04</td>
<td>1.57 ±0.02</td>
</tr>
<tr>
<td>AVI062110BB</td>
<td>3.6 ±0.0</td>
<td>9.75</td>
<td>2.31 ±0.02</td>
<td>1.19 ±0.04</td>
</tr>
<tr>
<td>AVI062910BB</td>
<td>3.1 ±0.0</td>
<td>12.91</td>
<td>2.56 ±0.01</td>
<td>1.63 ±0.01</td>
</tr>
<tr>
<td>AVI071910BB</td>
<td>3.2 ±0.1</td>
<td>14.50</td>
<td>2.72 ±0.02</td>
<td>1.45 ±0.05</td>
</tr>
<tr>
<td>AVI081710BB*</td>
<td>3.3 ±</td>
<td>11.37</td>
<td>2.55 ±0.00</td>
<td>1.75 ±0.03</td>
</tr>
<tr>
<td>AVI083110BB</td>
<td>3.7 ±0.01</td>
<td>12.47</td>
<td>2.65 ±0.06</td>
<td>1.86 ±0.01</td>
</tr>
<tr>
<td>AVI091810BB*</td>
<td>3.3 ±</td>
<td>8.84</td>
<td>1.79 ±0.02</td>
<td>1.48 ±0.01</td>
</tr>
<tr>
<td>AVI092910BB*</td>
<td>3.3 ±</td>
<td>13.89</td>
<td>2.30 ±0.01</td>
<td>2.21 ±0.03</td>
</tr>
<tr>
<td>AVI060810YR</td>
<td>3.6 ±0.0</td>
<td>5.53</td>
<td>1.57 ±0.01</td>
<td>1.63 ±0.02</td>
</tr>
<tr>
<td>AVI062110YR</td>
<td>3.5 ±0.0</td>
<td>6.44</td>
<td>1.65 ±0.01</td>
<td>0.97 ±0.01</td>
</tr>
<tr>
<td>AVI071410YR</td>
<td>3.3 ±0.3</td>
<td>4.81</td>
<td>1.39 ±0.10</td>
<td>0.85 ±0.00</td>
</tr>
<tr>
<td>AVI071910YR</td>
<td>4.3 ±0.1</td>
<td>8.10</td>
<td>1.33 ±0.67</td>
<td>0.98 ±0.00</td>
</tr>
<tr>
<td>AVI080910YR</td>
<td>3.5 ±0.15</td>
<td>4.81</td>
<td>1.64 ±0.04</td>
<td>0.17 ±0.00</td>
</tr>
<tr>
<td>AVI081710YR</td>
<td>3.4 ±0.06</td>
<td>5.19</td>
<td>1.47 ±0.05</td>
<td>0.91 ±0.04</td>
</tr>
</tbody>
</table>
AVI083110YR  4.1 ±0.24  4.60  1.52 ±0.00  0.97 ±0.9
AVI090710YR*  3.7 ±  6.48  1.42 ±0.07  1.17 ±0.04
AVI091410YR*  3.7 ±  9.66  1.83 ±  1.56 ±0.02
AVI092810YR  5.7 ±  7.15  1.78 ±0.07  1.45 ±0.12
AVI100510YR  4.4 ±0.7  5.37  1.44 ±0.01  1.14 ±0.03

* Asterisks next to sample names indicate that the inorganic carbon content was not obtained directly, but was estimated by averaging the inorganic carbon content of samples harvested from a similar time period.

Figure 7-8. Ultimate analysis of Lake Erie algal biomass. (■) Carbon, (■) hydrogen, and (■) nitrogen nutrient analysis of algal biomass harvested from Lake Erie, Buffalo, NY. Error bars represent sample standard deviations for triplicate analyses.
Figure 7-9. Goldsworth Ponds percent phosphorus analysis at different distances on the floway. Floway and screen location are plotted along the x-axis. The harvest code is labeled on the z-axis.

Figure 7-10. Percentage of phosphorus in algae grown at the Muskegon Wastewater Treatment Plant. The floway number and screen number are plotted along the x-axis and the Harvest code is plotted along the z-axis.
Figure 7-11. Percent phosphorus in algae harvest from floway one at the Great Wicomico River site. Each data point is an average of two analyses. Error bars represent the sample standard deviation of duplicate analysis.
Figure 7-12. Percent Phosphorus in algae harvest from floway two at the Great Wicomico River Location. Each data point represent the average of two analysis, data points with error bars represent the standard deviation of samples with triplicate analyses.
Figure 7-13. Percent Phosphorus in algae harvested from the York River location. Data points represent the average of three analysis. Error bars represent triplicate sample standard deviation.

Figure 7-14. Percent Phosphorus in algae harvest from the York River Boat Basin location. Data points represent the average of three analysis. Error bars represent triplicate sample standard deviation.
Figure 7-15. Percent ash in algae samples Muskegon Wastewater Treatment Plant. Ash percentages are displayed for samples collected from this site. Percent ash is the average of two samples except in the case of AMS071211F1S2, AMS071211F3S2, and AMS081211F3S21, which were run in triplicate. Error bars represent the standard deviation.

Figure 7-16. Percent ash in algae samples from the Great Wicomico River flow-way one. Ash percentages are displayed for samples collected from this site. Error bars represent standard deviations.
Figure 7-17. Percent ash in algae samples from the Great Wicomico River flow-way two site. Ash percentages are displayed for samples collected from this site. Error bars represent standard deviations.

Figure 7-18. Percent ash in algae samples from the lower section of flow-way two at Great Wicomico River site. Ash percentages are displayed for samples collected from this site. Error bars represent standard deviations.
Figure 7-19. Percent ash in algae samples from the York River site. Ash percentages are displayed for samples collected from this site. Error bars represent standard deviations.
Figure 7-20. Percent ash in algae samples from the Boat Basin site. Ash percentages are displayed for samples collected from this site.

Figure 7-21. Percent ash in algae samples from the Lake Erie site. Ash percentages are displayed for samples collected from this site. Error bars represent standard deviations.
Figure 7-22. Percent biogenic silica extracted from Lake Erie algae biomass during the time course digestion of harvest ALE0110910. Each line represents the time course digestion of a single sample.

Figure 7-23. Percent biogenic silica extracted from Lake Erie algae biomass during the time course digestion of harvest ALE061411F1. Each line represents the time course digestion of a single sample.
Figure 7-24. Percent biogenic silica extracted from Lake Erie algae biomass during the time course digestion of harvest ALE090711F1. Each line represents the time course digestion of a single sample.

Figure 7-25. Percent biogenic silica extracted from Lake Erie algae biomass during the time course digestion of harvest ALE061411F2. Each line represents the time course digestion of a single sample.
Figure 7-26. Percent biogenic silica extracted from Lake Erie algae biomass during the time course digestion of harvest ALE090711F2. Each line represents the time course digestion of a single sample.

Figure A-27. Arabinose multipoint calibration.
Figure A-28. Galactose multipoint calibration.

Figure A-29. Glucose multipoint calibration.
Figure A-30. Mannose multipoint calibration.

Figure A-31. Xylose multipoint calibration
Figure A-32. Phosphate multipoint calibration.

Figure A-33. Silicon dioxide multipoint calibration curve.