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Carbon Limitation in Periphytic Algal Wastewater Treatment Systems

Brandon J. Furnish

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BRANDON J FURNISH

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ABSTRACT

As global eutrophication poses an ever increasing threat to water quality new techniques must be implemented to improve the sustainability of natural resource consumption. Wastewater treatment facilities (WWTF) are designed to destroy pathogens, remove particulates, lower oxygen demanding substances, and reduce nutrients from influent waste water to avoid the degradation of receiving waters. WWTF are generally effective, however they are mostly inadequate at removing nutrients. When wastewater derived nutrients such as nitrate and phosphate are discharged to receiving waters, they stimulate algal blooms. Algal blooms can reduce dissolved oxygen, block sunlight for submerged aquatic plants, and render state waters unusable for recreational activities; these issues can negatively impact ecological systems and local economies. One experimental approach to reducing nitrogen and phosphorus in wastewater effluent uses algae as a tertiary treatment system. Algae are photosynthetic organisms that not only remove nutrients from wastewater, but they produce biomass for biofuels. Research is needed to increase the productivity of algae in tertiary treatment systems to make WWTF more ecologically sustainable and economically viable. Because algae in tertiary wastewater treatment systems are typically not limited by nutrients, the algae may become limited by dissolved inorganic carbon. To test this hypothesis, this study characterized the effects of adding carbon dioxide to lab scale, recirculating wastewater algal treatment floways. Eight 61 cm x 121 cm x 5.1 cm (height x length x diameter) recirculating floways, lined with unglazed clay tiles were administered gaseous carbon dioxide for 18 days while eight more were left untreated as controls. The floways, which were administered carbon dioxide, had a 41% greater algal dry

mass, and 43% greater ash free dry mass compared to controls. By day 18 phosphate and total phosphorus concentrations in treated floways averaged 67% and 39% lower than controls respectively. Nitrate and total nitrogen concentrations in treated floways averaged 37% and 10% lower than controls respectively. The results demonstrated that carbon dioxide stimulates algae in tertiary wastewater treatment floways. Because floways administered carbon dioxide had a magnitude lower pH than control floways, a secondary experiment was designed to determine if lower pH stimulated algal production. Replicating the previous experiment, recirculating (8L, n=8) floways were treated with 2N hydrochloric acid daily to lower their target pH to 6.48. A pH 7 solution of HCl and sodium hydroxide (n=8) was administered to floways (n=8) and others were left unmanipulated (n=8). After 18 days the acidified floways had 51% less dry algal mass compared to controls. The only nutrient concentration to differ significantly was nitrate which was 17% higher in acidified floways compared to controls. A final experiment was conducted to determine if another carbon source could stimulate algal production. For the third experiment, floways (n=8) were spiked with 8 g/L of sodium bicarbonate ($NaHCO_3$, a carbon source) or left untreated for controls (n=8). After 18 days the floways administered $NaHCO_3$ showed no difference in nutrient removal compared to control floways. However, these floways did have a 50% increase in volatile solids, a measure of algal biomass, and 39% greater dry algal mass. The first experiment supported the hypothesis that algae may become carbon limited but confounding variables left assigning causality impossible. The second experiment directly assessed the pH effect on algae while the third experiment confirmed the carbon limitation hypothesis by manipulating dissolved inorganic carbon. By assessing each variable a stronger

case can be made for the benefits of algal wastewater treatment floways such as reducing global eutrophication and improving water quality with a sustainable system.

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INTRODUCTION

Eutrophication, the nutrient over enrichment of natural waters, has degraded many aquatic ecosystems globally (Smith 2003). Phosphorus, a limiting nutrient often involved in eutrophication, has increased by 75% globally compared to preindustrial levels in terrestrial and freshwater ecosystems (Bennet et al. 2001). This phosphorus loading translates into approximately 13 Tg/yr of phosphorus accumulating in freshwaters and surface soils, ultimately effecting marine environments (Bennet et al. 2001). Nutrient over-enrichment can cause algal blooms, reduce water quality, and decrease aquatic biodiversity (Smith 2009). In the US, nutrient loading, the primary cause of eutrophication, accounts for nearly 60% of water quality impairments. In order to solve the problems associated with eutrophication, countermeasures that prevent the introduction of nutrients into aquatic ecosystems must be implemented.

Surface waters can become enriched in nutrients when they receive discharges of domestic sewage, effluent from industrial production, or runoff from agricultural fields and impervious surfaces (Correll 1998). This pollution accelerates the natural process of aquatic ecosystem enrichment and is referred to as cultural eutrophication (Smith et al. 2009). When nutrient concentrations become elevated in bodies of water, planktonic algal blooms can blanket the epilimnion, blocking light from reaching submerged aquatic plants (Havens 2008). If left untreated, the aquatic plants, which need this source of energy for photosynthesis, will be displaced. In the absence of aquatic submerged plants, sediments can be disturbed more easily, causing increased turbidity, ultimately affecting other aquatic species (Henley et al. 2000). Thus, cultural eutrophication can alter the biodiversity and shift the structure of entire ecosystems (Scheffer et al. 2001).

The detrimental effects of cultural eutrophication are not limited to aquatic plants and wildlife. In lakes and reservoirs, algal blooms, key indicators of cultural eutrophication, can inhibit anthropogenic uses by emitting odors, releasing toxins, and blocking navigable areas (Paerl et al. 2001). To reduce the nutrients that enter state surface or ground waters, the US Environmental Protection Agency (EPA) amended the Clean Water Act to regulate pollutants being discharged by point sources (Pickrel 2004). EPA mandated that total maximum daily loads be set to limit nutrients, sediments, and effluent temperatures, for all impaired state waters (Birkeland 2001). Wastewater treatment facilities (i.e., point sources) are required to implement technologies to reduce nutrients, chemicals, and biological material from their effluent water prior to discharge to achieve the mandates of the Clean Water Act (Copeland 1999).

Municipal wastewater treatment facilities were designed to treat water from sewer lines connected to households, businesses, and factories. In some instances, storm water is also treated if the area has a combined storm water sewer system. Most wastewater treatment facilities operate a two-stage process referred to as primary and secondary treatment (Pescond 1992). The primary stage removes solids using grit chambers/filters and settling tanks. Aeration basins, and clarifiers are typical of the second stage of treatment and are designed to remove dissolved organic carbon (Washington 2004). This treatment stage uses chemicals, such as aluminum sulfate and polymerics, to coagulate organic particles to facilitate their removal in sedimentation basins (Brostow et al. 2009). These secondary processes remove only a portion of the nitrogen or phosphorus (Dodds et al. 2010). Primary and secondary treatment processes typically ensure nutrient levels in effluent meet federal and state regulations, but these

technologies show only 78% removal efficiency for a key cause of cultural eutrophication, total phosphorus (Jetten et al. 1997).

To further reduce nutrient concentrations in discharges, some water treatment facilities have expanded their treatment process to include a tertiary stage (Wang et al. 2009). During this treatment stage, the use of biological processes, like denitrification, are often implemented to achieve lower nutrient concentrations (Henze, 1991). Where applicable, wetlands have been constructed as a sustainable solution to further treat wastewater effluent before its discharge to natural ecosystems (Solano et al. 2004).

Algae can be used in tertiary treatment systems to remove nitrogen and phosphorus due to their high affinity for these nutrients (De-Bashan et al. 2002). One of the key advantages of algae is that they can remove nitrogen and phosphorus down to much lower concentrations in water than chemicals (Su et al. 2011). Chemical removal of phosphorus from wastewater has been reported to have an average removal efficiency of 47% (Rockne et al. 2006). Jetten et al. (1997) reported phosphorus and nitrogen removal efficiencies of 78% and 44% respectively using chemical treatments. When algae are used as a tertiary treatment of wastewater the removal efficiency of phosphorus has been shown to be as high as 97% (Craggs et al. 1996). The economic benefits of algae treatment systems are numerous as they do not require chemicals and there are low disposal costs since algae can be used for bioenergy production (Zamolloa et al. 2011).

Algae produced in the treatment process can be harvested as a renewable biomass resource that can be used in various applications (Adey et al. 2011). For example, oils extracted from algae can be converted to biodiesel (Hossain et al. 2008), dried algae can be incinerated to

generate heat for power production (Jacob et al. 2015), or wet algal biomass can be anaerobically digested to make biogas (Bohutskyi and Bouwer 2013). The widespread use of algal biomass to generate energy could reduce our dependence on non-renewable fossil fuel-based energy sources (Hossain et al. 2008) and make the wastewater treatment process more sustainable (Zamalloa et al. 2011).

Another attractive characteristic of algal treatment systems is that they reduce carbon dioxide, an atmospheric greenhouse gas. Like other autotrophic organisms, algae need carbon to maintain photosynthesis. Atmospheric carbon dioxide diffuses into water and serves as the source of dissolved inorganic carbon (DIC) for aquatic autotrophs such as algae (Liue et al. 2010). As algae absorb the DIC, more carbon dioxide from the atmosphere can diffuse into the water, to reestablish the air-water carbon equilibrium. In this way, algal photosynthesis reduces atmospheric carbon dioxide (Moreira and Pires 2016).

Although atmospheric carbon dioxide readily diffuses into water (Bolin 1960), it is possible that during high rates of photosynthesis, such as those found in algal treatment systems, algae may become carbon limited (Liang et al. 2013). Infusing carbon dioxide into wastewater provides more carbon substrate for planktonic algae and yields additional lipids for biofuels (Woertz et al. 2009). Mulbry et al. (2008) used algal floways, to grow algae on a two-dimensional surface and observed the effects of infusing carbon dioxide into dairy manure effluent. In their floway treatment systems, wastewater traveled the length of a raceway to facilitate attached algae nutrient uptake. While carbon dioxide stimulated additional algal biomass, the cause of this effect could not be determined in this study (Mulbry et al. 2008). The assessment of causality in most carbon dioxide infusion studies is impossible because carbon dioxide alter pH and DIC

simultaneously. However, Cole et al. (2017) addressed the importance of DIC augmentation by testing the effects of different carbon supplies on the filamentous algae *Oedogonium intermedium* in high rate algal ponds. The study's results were consistent with the DIC limitation hypothesis because experiments showed an 87% and 89% increase in algal biomass when molasses and carbon dioxide (respectively) were used as carbon sources.

Carbon dioxide plays a key role in the bicarbonate buffering system in water. Dissolved carbon dioxide transforms immediately into carbonic acid which lowers the pH of water. Carbonic acid is then transformed into bicarbonate and carbonate (Fig. 1). While excessively low pH could cause problems for algae, in algal wastewater treatment systems, high pH is the more common stressor (Chen et al 1994). During periods of rapid photosynthesis, algae remove carbon dioxide and bicarbonate from the water, causing an imbalance in the carbonate-bicarbonate buffer. This process liberates hydroxide and raises pH. High pH values can negatively affect algal growth and productivity (Moheimani 2013). Azov et al. (1982) regulated the pH of wastewater using carbon dioxide and found that planktonic algae have a pH optimum for maximum productivity. For example, *Scenedesmus obliquus* (a colonial green alga) had its highest productivity when carbon dioxide maintained pH at 7.5.

Thus, there exists clear evidence showing the benefits of adding carbon dioxide to highly nutrient enriched algal treatment systems (Azov et al. 1982, Woertz et al. 2009, Moheimani 2013). Infusing carbon dioxide into wastewater provides more carbon substrate for photosynthesis (Cole et al. 2017) and simultaneously moderates pH (Moheimani 2013). Because of the dual effects of carbon dioxide, questions remain about the mechanism by which it improves the productivity of algal treatment systems (i.e., reducing carbon limitation or

suppressing elevated pH). The goal of this study was to determine if carbon dioxide stimulated algal production in algal treatment systems and if so, by what mechanism (i.e., pH regulation versus DIC augmentation). A strong inference-type hypothesis framework (Platt 1964) was evaluated using a series of three experiments. The first experiment infused carbon dioxide into wastewater to determine if it increases algal productivity and enhances nutrient removal. This initial experiment was to test and verify carbon dioxide effects in previous studies. A second experiment maintained pH at near neutral levels using carbon-free acid to evaluate how pH control affects algal productivity and nutrient removal in highly enriched wastewater treatment systems. The third experiment added DIC in the form of sodium bicarbonate to determine DIC effects on algal growth and nutrient removal capacity. If the pH hypothesis is correct, manipulating the pH (i.e., experiment 2) will increase algae productivity without providing an additional carbon source. If the carbon limitation hypothesis is correct, adding sodium bicarbonate (i.e., DIC) will increase algal productivity without affecting pH (i.e., experiment 3). In order to control external variables such as light levels, grazers, and temperature, experiments were conducted indoors using a series of bench-scale, recirculating algal floway systems.

Methods

General Experimental Conditions

To conduct the carbon infusion experiments, replicated experimental wastewater treatment floways were constructed of cylindrical polyvinyl chloride pipes (PVC: 61 cm x 121 cm x 5.1 cm, height x length x inside pipe diameter, Fig. 2). A 7.62 cm wide opening was cut into the top of each floway so that the wastewater received light from grow lights placed tangential to

the direction of flow. Each flume was filled with approximately 7 L of wastewater collected from a clarifier at Columbus Water Works Wastewater Treatment Facility in Columbus, Georgia (USA). The wastewater was collected on the start date of each experiment. Thirty-two (2cm x 2cm) unglazed ceramic tiles were placed inside the floways as substrates for periphytic algae. Each flume was fashioned with a flow-controlled air-line and aeration stone to circulate the wastewater (Fig. 2). To establish uniform flow conditions, air injection rates were adjusted until a 3 cm x 0.5 cm x 0.01 cm (height x length x thickness) piece of polyethylene sheeting submerged 2 cm in the circulating wastewater was deflected approximately 45 degrees. Flume water levels were monitored daily, and Milli-Q® Ultrapure water was added to maintain a constant volume of water without introducing additional nutrient or ions. Light for photosynthesis was provided using 6 1.2 m x 38.1 mm (length x diameter) GE™ F40 T12 grow lights which produced a color appearance of 3100K at 1900 Lumens. The lights were set on a 12-hour time interval to simulate night and day and were placed on the floways tangential to the direction of flow. Instead of using algal seed stock, algae in the wastewater was allowed to colonize and grow naturally.

Physicochemical Measurements

To characterize nutrient conditions, 80 mL of water were collected from each floway, placed in Whirl-Paks™, and preserved by adding one drop of concentrated sulfuric acid (36N). Milli-Q® water and Hach® effluent wastewater standards were preserved in the same manner and used as method blanks and standard controls. Samples were stored at $4^{o}C$ until analysis, which was no longer than 30 days after collection.

Directly before analysis, preserved wastewater samples were raised to 25°C and to pH 7 using 0.1 N sodium hydroxide. For nitrate analysis, samples were analyzed using a cadmium reduction method (Hach® Method 8039). Phosphate samples were analyzed using a molybdate ascorbic acid colorimetric reaction (Hach® Method 8048). Total phosphorus was analyzed after acid persulfate digestion using a molybdate ascorbic acid colorimetric analysis of resulting phosphate with Hach® Total Phosphorus Test 'N Tube™ Vials (Method 8190). Total nitrogen was analyzed using an alkaline persulfate digestion followed by nitrate analysis using chromotropic acid reaction (Hach® Method 10071). A Hach® DRB200 block was used to heat test tubes for the total phosphorus (150°C, 30min) and total nitrogen digestion period (105°C, 30min). The concentration of all nutrient species was quantified using a Hach® DR 2700 spectrophotometer. For quality assurance and control, a HACH wastewater standard was used to determine the accuracy of the procedures.

The temperature and pH of each flume's wastewater was recorded daily using a Hach® EC10 which was recalibrated when readings differed from pH 7 standard by more than 0.02. The probe was calibrated using Hach® pH standard solutions 4.01, 7.00, and 10.01. Light intensity was measured at the beginning, middle, and end of each floway's opening using a Li-Cor® Quantum LI-190R sensor and LI-1400 Data Logger.

On the last day of each experiment, three random rows of tiles were selected from each floway, one was analyzed for dry mass, ash-free dry mass, and chlorophyll. Samples were stored frozen (-4°C) in Whirl-Paks™ prior to analysis, which was no longer than 30 days after collection.

Quantifying Algal Biomass & Species Composition

To prepare filters for dry mass and ash-free dry mass measurements, pre-rinsed filters (GF/F, 0.7 μm, Wyvern Scientific) were placed in numbered, pre-weighed aluminum weigh pans, ignited at 550^oC for 15 minutes in a muffle furnace, and cooled in Drierite™ filled desiccators before being weighed to the nearest 0.1mg. Drierite™ was placed inside the scale chamber to minimize moisture wicking during weighing. Algae were rinsed and scraped off tiles onto filters using Milli-Q® water and a Kartell™ spatula before being dried in an oven for 24h at 105^oC. Dry mass was determined after filters were cooled in Drierite™ filled desiccators and weighed (± 0.1 mg). To estimate ash-mass, dry mass filters with algae were then ignited at 550^oC for 30 min. in a muffle furnace, cooled in desiccators, and weighed to the nearest 0.0001 mg using a Mettler Toledo AT400 Precision Digital Balance. To calculate the ash-free dry mass the weight of the ashed algae was subtracted from the dry weight of the algae (EPA Method 150.1).

To examine species composition, algae samples were collected from random tile locations on days 9 and 18 from each flume and observed using a Leica DM500 microscope at 400x magnification. Images of the algae were taken through the ocular of the microscope using an iPhone 7 Plus digital camera.

Experiment 1: Carbon Dioxide Infusion

To characterize the effects of carbon dioxide infusion on nutrient removal and algal growth, wastewater in half of the floways were infused with 100% food grade gaseous carbon dioxide. Eight floways, selected using a random number generator, were administered carbon dioxide using an aeration stone submerged downstream of the tiles (Fig. 2 & 3). Eight other

floways were left untreated as controls. The experiment was conducted indoors for 18 days from Sept. 10, 2016 through Sept. 28, 2016.

Experiment 2: pH Manipulation

Because carbon dioxide infusions reduce pH as well as increase dissolved organic carbon, a second experiment was designed to determine if pH influences nutrient uptake and algal biomass production. Eight floways, selected at random, were administered 0.5 N HCl daily to maintain an average pH of 6.4 (n=8). This pH was calculated to be the average pH of the eight floways infused with carbon dioxide in experiment 1. To control for the addition of chloride when adding HCl to the pH treatment floways, another eight floways, selected at random, were administered a neutralized solution (pH = 7) of sodium hydroxide (NaOH) and HCl daily (n=8). The volume of the pH neutral solution added daily was calculated as the average volume of HCl added to the eight floways in order to maintain a pH of 6.4. An additional eight floways were left untreated as experimental controls (n=8). The experiment was conducted from March 12, 2017 to March 29, 2017. Ash-free dry mass were excluded from this experiment analyzed because results indicated problems with moisture wicking that made the measurements unreliable.

Experiment 3: Sodium Bicarbonate Addition

The sodium bicarbonate addition experiment was designed to investigate the efficacy of adding an alternative carbon source to wastewater other than gaseous carbon dioxide. Eight floways, selected at random, were administered 8 g of sodium bicarbonate. Eight floways were

used as unmanipulated controls. The experiment was conducted from July 22, 2017 to August 8, 2017.

Alkalinity, a measure of water's ability to resist changes in pH, is often controlled by the bicarbonate buffer system. Since most of the DIC in water is part of the buffer system, alkalinity can provide an indicator of carbon content, particularly when strong bases are absent (Dickson 1981). This measurement was used to determine how much, if any, sodium bicarbonate needed to be added to treated floways to maintain the target dissolved inorganic carbon concentrations. Alkalinity of all floways was measured on days 1, 9, and 18 using a HACH® EC-10 pH probe by titrating 1.6N sulfuric acid into the sample and measuring the volume required to achieve a pH = 4.5. Based on the results of White's 2012 experiment the alkalinity of sodium bicarbonate treated flumes was also measured on day 2 to check that the target alkalinity (800 mg/L as calcium carbonate) was achieved.

Statistical Analysis

To assess the outcomes of the experiments, average nutrient concentrations (i.e., nitrate, phosphate, total nitrogen, and total phosphorus) were compared among treatments using repeated measures analysis of variance models (RM ANOVA). This approach was necessary since multiple measurements were made from each floway throughout each experiment. Nitrate and phosphate concentrations were analyzed for days one, nine, and eighteen. Total nitrogen, and total phosphorus concentrations were analyzed for days 1 and 18 for experiment 1 (carbon dioxide infusion) and days 1, 9 and 18 for experiment 2 and 3 (pH manipulation and sodium bicarbonate addition). Because dry mass and ash-free dry mass were measured only on the last

day of the experiments, a one-way analysis of variance (ANOVA) was used to assess treatment effects. Initial pH and temperatures were analyzed using a RM ANOVA. Light intensity was analyzed using an ANOVA. To determine where differences occurred between groups, pairwise post-hoc comparisons were made using the Bonferroni method which corrects for the number of pairwise corrections. All statistical analysis was performed using JASP Version 0.8.

Results

General Experimental Conditions

To compare baseline testing conditions across experiments, initial samples were collected and analyzed after wastewater was distributed to the floways. Initial temperatures of the wastewater showed significant differences between experiments (Bonferroni, $p < 0.001$, Fig. 4-A). The carbon dioxide infusion and sodium bicarbonate addition experiments had similar average temperatures 25.1 ± 0.1 °C (mean ± 1SD) and 25.2 ± 0.2 °C respectively (Bonferroni, $p = 0.587$). However, temperature of water used in the pH variable experiment averaged 20.1 ± 0.5 °C, significantly lower compared to the other experiments (Fig. 4-B, Bonferroni, $p < 0.001$ for all).

Initial wastewater pH also differed among experiments. The pH experiment's wastewater had a significantly higher initial average pH 7.6 ± 0.2 (Bonferroni, $p < 0.001$ for all) than wastewater from the carbon dioxide infusion experiment and sodium bicarbonate addition 7.1 ± 0.4 and 6.7 ± 0.4 respectively (Fig. 4-B).

Likewise, nutrient concentrations varied among experiments. Initial nitrate, total nitrogen, and total phosphorus concentrations differed across all experiments (Bonferroni, $p < 0.001$ for all, Fig. 5, Table 1). Initial nitrate concentrations were highest in the pH variable

experiment at 4.4 ± 0.4 mg/L (Bonferroni, $p < 0.001$ for all); nitrate composed 61% of the total nitrogen in the wastewater (Fig. 5). Wastewater from the carbon dioxide infusion and sodium bicarbonate addition experiments had initial nitrate concentrations that accounted for 31% and 28% of total nitrogen respectively (Fig. 5). The sodium bicarbonate experiment had the highest initial phosphate concentrations at 4.39 ± 0.37 mg/L (51% of total phosphorus). In the carbon dioxide infusion and pH variable experiments, phosphate concentrations composed 88% and 61% of total phosphorus respectively. No statistically significant difference was found when comparing initial phosphate concentrations between the carbon dioxide infusion and pH experiment (Bonferroni, $p = 0.092$, Fig. 5) and no difference between the pH and sodium bicarbonate experiments (Bonferroni, $p = 0.089$, Fig. 5). However, initial phosphate concentrations in the carbon dioxide experiment were 43% less than those of the sodium bicarbonate experiment (Bonferroni, $p < 0.001$, Fig. 5).

The experimental floways were dominated by filamentous green algae in each experiment. The two most abundant taxa were always *Ulothrix zonata*, and *Oedogonium* sp. Diatoms were also present but were far less abundant.

Experiment 1: Carbon Dioxide Infusion

The effectiveness of infusing wastewater with gaseous carbon dioxide was determined by comparing algal biomass between treatments (with carbon dioxide infusions) and controls (no infusion). Floways infused with carbon dioxide averaged 41% greater algal dry mass compared to controls (ANOVA, $p = 0.002$, Fig. 6-A, Table 2). Similarly, ash-free dry mass for treated floways averaged 43% greater than controls (ANOVA, $p = 0.003$, Fig. 6-B, Table 3).

To further evaluate the effects of carbon limitation in algal floways, nutrient concentrations were compared across treatments. The carbon dioxide treated floways had a significantly lower nitrate concentration averaging 37% less than controls (RMANOVA, $p = 0.023$, Fig. 7-A, Table 5). Treated floways averaged 67% lower phosphate concentrations versus controls (RMANOVA, $p = 0.013$, Fig. 7-B, Table 5). There existed no statistically significant difference between treated and control floways for total nitrogen (RMANOVA, $p = 0.864$, Fig. 7-C, Table 5). Total nitrogen concentrations were only 10% lower in treated floways versus controls. In contrast, total phosphorus concentrations were 39% lower in treated floways relative to controls (RMANOVA, $p = 0.003$, Fig. 7-D, Table 5).

Along with analyzing treatment effects on nutrient concentrations, temporal changes were also compared among all dates for all nutrient forms (RMANOVA, $p < 0.001$ for all, Table 5). Floways averaged 69% nitrate removal from days 1 to 9 and 87% removal from days 9 to 18 (Bonferroni, $p < 0.001$ for both). Phosphate removal averaged 58% from days 1 to 9 and a further 93% from days 9 to 18 (Bonferroni, $p < 0.001$ for both). Total nitrogen declined by 90% over the course of the experiment (RMANOVA, $p < 0.001$, Table 5). Similarly, floway systems averaged 89% total phosphorus removal by day 18 (RMANOVA, $p < 0.001$, Table 5).

This study also characterized how the interaction between time and treatment influenced nutrient concentrations. Significant interactions between treatment and date were observed for nitrate (RMANOVA, $p < 0.001$) and total phosphorus (RMANOVA, $p = 0.038$, Table 5). Treated floways averaged 66% greater nitrate removal from days 1 to 9 compared to controls. From days 9 to 18 treated floways nitrate removal rate became 81% less than controls. There was no significant interaction between treatment and time for phosphate (RMANOVA, $p = 0.279$) or total

nitrogen (p = 0.108, Table 5). Carbon dioxide infused floways averaged 37% greater phosphate removal than controls from days 1 to 9 but decreased to 33% less than controls from day 9 to 18.

Experiment 2: pH Manipulation

To assess the effects of pH on algal productivity, treated and control floways were analyzed for algal biomass growth and reductions in nutrient concentration. Dry algal mass differed significantly among pH treatments (RMANOVA, p = 0.002, Table 2). Floways that were administered HCl had 51% less dry algal mass compared to controls (Bonferroni, p = 0.045, Fig. 8-A, Table 2). Floways treated with the neutralized solution had similar dry mass compared to controls (Bonferroni, p = 0.549, Fig. 8-A, Table 2).

While algal biomass was the primary variable for characterizing pH effects, nutrient concentrations in this experiment were also measured. The only nutrient concentrations to differ significantly between treatments were nitrate (RMANOVA, p = 0.028, Table 6) and total nitrogen (RMANOVA, p = 0.042, Table 6). Pairwise comparisons showed no significant differences among any pairs of treatments for nitrate concentrations ($p_{\text{Bonferroni}} > 0.1$ for all). Total nitrogen concentrations for HCL and neutralized solution treated floways averaged 7% (Bonferroni, p = 0.059) and 9% (Bonferroni, p = 0.183) lower concentrations respectively when compared to controls (Fig. 9-C, Table 6). HCl treated floways averaged 3% greater total nitrogen concentrations when compared to floways treated with neutralized solution (Bonferroni, p = 0.999, Fig. 9-C, Table 6). Neither phosphate nor total phosphorus concentrations differed significantly among treatments (RMANOVA, p = 0.087 and p = 0.348 respectively, Fig. 9-B, Table 6).

Time was a key factor affecting nutrient removal was recorded for all analyzed species (RMANOVA, $p < 0.001$ for all, Table 6). Nitrate concentrations declined by an average of 36% from days 1 to 9 and a further 92% from days 9 to 18 (Bonferroni, $p < 0.001$ for both). During this experiment, floways averaged 52% phosphate removal from days 1 to 9 and 64% from days 9 to 18 (Bonferroni, $p < 0.001$ for both). Total nitrogen concentrations were reduced by 32% from days 1 to 9 and 84% from days 9 to 18 (Bonferroni, $p < 0.001$ for both). Floways averaged 24% total phosphorus removal from days 1 to 9 and a further 66% from days 9 to 18 (Bonferroni, $p < 0.001$ for both).

Total phosphorus showed the only statistically significant interaction between treatments and time (RMANOVA, $p < 0.001$, Table 6). Floways treated with HCl, NaOH and HCl, and controls averaged total phosphorus removal rates at 0.10 mg/L/d, 0.25 mg/L/d, and 0.17 mg/L/d respectively from days 1 to 9 and increased to 0.29 mg/L/d, 0.38 mg/L/d, and 0.40 mg/L/d respectively from days 9 to 18. No significant interaction was found for nitrate (RMANOVA, $p = 0.369$), phosphate (RMANOVA, $p = 0.928$), or total nitrogen (RMANOVA, $p = 0.377$, Table 6).

Experiment 3: Sodium Bicarbonate Addition

Adding sodium bicarbonate as an alternative carbon source caused algal dry mass and ash-free dry mass to increase significantly compared to controls. Bicarbonate treated floways averaged 39% greater algal dry mass compared to controls (ANOVA, $p < 0.001$, Fig. 12-A, Table 2). Furthermore, ash-free dry mass was on average 50% greater in bicarbonate treated floways versus controls (ANOVA, $p < 0.001$, Fig. 12-B. Table 4).

While algal productivity increased significantly in treated floways, the addition of sodium bicarbonate had no significant effect on nutrient concentrations. Concentrations averaged 6% and 3% lower in treated floways compared to controls for nitrate (RMANOVA, $p = 0.515$) and phosphate respectively (RMANOVA, $p = 0.519$, Fig. 11-A, Fig. 11-B, Table 7). Concentrations of total nitrogen averaged 1% higher in treated floways versus controls (RMANOVA, $p = 0.478$) while total phosphorus concentrations averaged 7% lower in treated floways comparatively (RMANOVA, $p = 0.124$, Fig. 10-C, Fig. 11-D, Table 7).

The effectiveness of the recirculating floways system for nutrient removal was analyzed by comparing nutrient concentrations among days 1, 9, and 18. Nitrate, Phosphate, total nitrogen and total phosphorus showed significant declines for all dates tested (RMANOVA, $p < 0.001$ for all, Table 7). Essentially no nitrate removal occurred between days 1 and 9 (Bonferroni, $p = 0.999$) but there was an 87% removal from days 9 to 18 (Bonferroni, $p < 0.001$). Similarly, there was no phosphate removal from days 1 to 9, however a 59% removal was documented from days 9 to 18 (Bonferroni, $p < 0.001$). Total nitrogen concentrations declined 48% by day 9 while an additional 74% removal occurred between days 9 and 18 (Bonferroni, $p < 0.001$ for both). From days 1 to 9 total phosphorus concentrations were reduced by 21% then from days 9 to 18 concentrations dropped another 58% (Bonferroni, $p < 0.001$ for both).

For this experiment there were no significant interactions between treatments and time for any nutrient species measured (Table 7).

Discussion

Algal photosynthetic activity depends on the availability of carbon, nitrogen, and phosphorus (Barsanti 2014). Whereas phosphorus and nitrogen are derived primarily in watershed processes such as runoff, carbon is often supplied by the carbonate bicarbonate buffer system through diffusion of atmospheric carbon dioxide into water (King 1970). While the lack of phosphorus and nitrogen can impede algal productivity in oligotrophic freshwater lakes (Miller et al. 1974), less is known about conditions that cause carbon availability to limit photosynthesis (King 1970). Theoretically, during periods of rapid algal growth (i.e., high light and nutrient availability), algal uptake of dissolved inorganic carbon during photosynthesis could deplete local sources of carbon faster than diffusion can replenish it (King 1970). Thus, carbon could become the limiting factor in algal productivity in nutrient rich waters such as those found in tertiary algal wastewater treatment systems.

This study examined carbon limitation in a series of wastewater floway experiments. Results showed that infusing gaseous carbon dioxide in wastewater increased dry algal biomass by 41%. This finding is consistent with the hypothesis that algal productivity can become carbon limited during heightened states of photosynthesis. Cole et al. (2014) reported increased algal biomass (~40%) when they infused gaseous carbon dioxide into circulating phytoplankton dominated wastewater ponds. Similarly, Park et al. (2011) infused wastewater-filled high rate algal ponds with gaseous carbon dioxide which resulted in a 30% increase in micro-algal productivity. Studying the effects of wastewater supplemented with carbon dioxide on algal lipid production in stirred 1L bottles, Woertz et al. (2009) recorded a 38% increase in algal productivity

when carbon dioxide was added to the wastewater. Increases in biomass are not the only effect that might be expected when algae productivity is enhanced with carbon dioxide.

To characterize other important effects of carbon dioxide infusion on algae wastewater treatment systems, this study analyzed nutrient concentration changes and calculated nutrient removal rates. The addition of carbon dioxide resulted in greater removal rates of nitrate, total nitrogen, phosphate and total phosphorus concentrations (37%, 10%, 67% and 39% respectively) relative to controls. Sutherland et al. (2015) observed a 25% decrease in nutrient removal when adding carbon dioxide to wastewater in high rate algal ponds. Mulbry et al. (2008) found no significant difference in nutrient removal when carbon dioxide was supplemented in dairy effluent. Wastewater infused with carbon dioxide in the high rate algal ponds of the Park and Craggs (2011) experienced a 17% decrease in nitrogen removal which they concluded was the result of reduced ammonia volatilization. Park et al. (2011) found ammonia volatilization was enhanced as the pH of wastewater increases. These studies indicate that carbon dioxide infusions, which acidify wastewater, cause a reduction in ammonia volatilization and thus reduce overall nitrogen removal.

The magnitude of ammonia volatilization is also dependent on the type of wastewater being treated. Secondarily treated municipal wastewater has nitrogen primarily in the form of nitrate (2.0 – 4.4 NO3 – N mg/L) rather than ammonia (0.4 ± 0.1 mg/L, mean ± SD from Columbus Water Works, Inc. December 2016 – December 2018). In contrast primary treated municipal wastewater, used in the Southerland et al. (2015) and Park et al. (2011), has nitrogen primarily as ammonia and ammonium (~55 $NH_3 - N$ mg/L, ~60 $NH_4 - N$ mg/L). Because this experiment studied secondarily treated wastewater, there were lower ammonia concentrations and a

reduced likelihood of ammonia volatilization. Thus, nitrogen removal from these algal floways systems was from mechanisms other than ammonia volatilization (e.g., algal uptake or denitrification).

Woertz et al. (2009) observed >99% phosphate removal when gaseous carbon dioxide was infused into wastewater with multi-species algae cultures. Similarly, Adey et al. (1993) reduced phosphate and total phosphorus concentrations by 93% and 83% respectively using algal turf scrubbers™ to remove phosphorus from natural waters. Comparatively, this study showed a 99% phosphate removal rate in floways infused with gaseous carbon dioxide and 86% removal rate for controls. Total phosphorus concentrations were reduced by 94% and 83% in treated and controlled floways respectively in the carbon dioxide experiment.

Because the addition of carbon dioxide to wastewater effects both pH and carbon content of the water, most experiments, including experiment one reported here, are confounded by a second uncontrolled variable. To better isolate the effects of carbon limitation versus pH on algal productivity in wastewater floways, multiple experiments need to be conducted to test each variable separately.

During photosynthesis, algae uptake local carbon from the water in the form of carbon dioxide (at low pH values only) and bicarbonate (Ghirardi et al 200). Active photosynthesis causes the pH of the surrounding water to shift the dominate form of carbon to carbonate (Goldman et al. 1972). Because it is difficult for most species of freshwater algae to uptake carbon in the form of carbonate (King 1970), algal productivity could be limited during elevated pH conditions typical of algal treatment systems. During the carbon dioxide infusion experiment (i.e., exp. 1), carbon

dioxide maintained the pH of the wastewater at pH = 6.45 whereas controls averaged pH = 9.25 by the end of the experiment. This difference in pH and carbon availability during carbon dioxide infusions confounds the interpretation of the experiment's results. To evaluate the importance of this confounding pH effect, a second experiment was designed to control pH using carbon-free acids (hydrochloric acid) to see if pH regulation alone controls algal productivity in wastewater floways.

The second experiment maintained a near constant pH = 6.45 using hydrochloric acid additions in treatments. Counter to expectations, the acidification treatment resulted in a 51% decrease in algal biomass compared to controls. This finding did not support the pH hypothesis in the strong inference model that pH reductions improve algal productivity. Azov (1982) reported a 65% increase in algal biomass for *Scenedesmus obliquus* and a 95% increase in *Chlorella vulgaris* when carbon dioxide was used to maintain algal cultures at a pH of 7. Although, Azov (1982) attributed the increase in biomass to pH regulation, this finding is possibly due to an increase in the availability of dissolved inorganic carbon in the infusion treatments. *Oedogonium,* a green filamentous algae, grown in tumble cultures infused with flue gas (pH between 8.5 and 7.5) grew 40% more biomass compared to controls (Cole et al. 2014). In contrast, Moheimani (2012) observed no change in *Tetraselmis sueccica* biomass (i.e., microalga) compared to controls when carbon dioxide was used to regulate the pH of the wastewater inside six-liter glass vessels. The biomass of both *Thalassiosira pseudonana* and *Thalassiosira oceanica* diatoms in batch cultures decreased by 10% at the regulated pH 9.4 compared to controls and carbon dioxide infused cultures at pH 7.9 in the Chen et al. (1994) study. The decrease in biomass was

hypothesized to result from a decrease in available inorganic carbon (e.g., carbon dioxide and bicarbonate) as pH levels increased (Chen et al. 1994).

Similar to the biomass study, the pH experiment did not match expectations related to nutrient removal. The addition of hydrochloric acid in this pH experiment decreased nitrate removal by 17% compared to controls which was both biologically and statistically significant. Acid treatments had no detectable effect on total nitrogen removal nor phosphate/total phosphorus removal. Liang et al. (2013) added sodium hydroxide and hydrochloric acid to regulate wastewater pH (pH = 7) on the productivity of *Chlorella vulgaris* cultures and reported an 8% increase in nitrogen removal. This result was attributed to higher algal biomass as measured by Chl a.

To more directly examine the importance of dissolved inorganic carbon limitation for algae, a third experiment was conducted that tested algal responses to an alternative carbon source, sodium bicarbonate, which has a limited effect on wastewater pH. This study found that addition of sodium bicarbonate increased dry algal mass by 39%. Final mean alkalinities for sodium bicarbonate and control floways were 804 mg/L as $CaCO_3$ and 217 mg/L as $CaCO_3$ respectively indicating that sodium bicarbonate increased DIC by more than 3-fold. Alkalinity remained relatively constant for treated and control floways throughout the experiment. It is important to note that there was no significant difference in pH between treated and control floways. The results of this experiment are consistent with the hypothesis that during heightened states of photosynthesis, algae become carbon limited.

Other research has shown that sodium bicarbonate can be used as an alternative to carbon dioxide, yet not as effective, as a source of DIC for algae (Gardner et al. 2013). These

findings are possibly due to differences among algal species in their affinity for carbon dioxide versus bicarbonate, which can be more difficult to uptake (King 1970). However, White et al. (2012) reported that sodium bicarbonate additions at 1 g/L resulted in a 90% increase in algae biomass in cultures of the microalgae *Tetraselmis sueccica* and *Nonnochloropsis salina*.

The additional algal biomass in the sodium bicarbonate treatments was expected to remove more nutrients, however, it had no significant effect on nitrogen or phosphorus removal compared to controls. After day 9 of the experiment it was discovered that a few midge larvae had invaded the control floways and were consuming algae. It is unclear how these grazers would affect nutrient removal from the water column. The absence of a treatment effect on nutrient removal is particularly perplexing given that biomass production rates were very similar between experiments; experiment 1 showed strong nutrient removal rates (Fig. 7) while experiment 3 had lower rates of nutrient removal (Fig. 11).

Conclusion

While several studies have reported that carbon dioxide infusions in algal wastewater treatment systems stimulate algal productivity (Woertz et al. 2009, Park et al. 2011, Cole et al. 2014), few have identified the causal mechanism (see Cole et al. 2017 for an exception). This study confirmed earlier experiments showing that carbon dioxide stimulates algal productivity in periphytic algae wastewater treatment systems. Furthermore, the experiments show inconvertible evidence that the mechanism causing this effect is carbon-limitation rather than pH moderation. Experiments revealed that maintaining a constant, near-neutral pH using HCl had no effect on algal biomass or nutrient removal and thus was inconsistent with the hypothesis that pH moderation controls algal growth in wastewater treatment systems. Furthermore,

experiments showed that adding a dissolved inorganic carbon source, sodium bicarbonate, stimulated algal biomass comparable in magnitude to that of carbon dioxide.

The reduced availability of dissolved inorganic carbon may be a result of heightened states of photosynthesis in nutrient enriched wastewater treatment systems. In these systems algae rapidly remove usable carbon and cause a shift in the buffer system to relatively bio-unavailable carbonate as the pH increases. This study showed that the addition of carbon dioxide or bicarbonate replenishes the dissolved inorganic carbon needed to maintain the productivity of filamentous algae

These findings emphasize the value of injecting carbon into algal treatment floways. Carbon augmentation can improve treatment efficiency by increasing nutrient uptake, growing more biomass for biofuel, and shrinking the size of the system's footprint. Carbon enrichment could make algal wastewater treatment more economically viable and reduce the need for utilities to invest in costly and less sustainable alternative technologies for nutrient removal. Algae could also be harvested more frequently and produce greater amounts of biomass for biofuel production (Park et al. 2011). For wastewater treatment facilities that contain bioreactors, the source of carbon dioxide could come from gases scrubbed from the anaerobically produced biogas. Thus, algal treatment floways could be used to improve carbon capture. Thus, this study's results confirm that carbon augmentation has the potential to improve the sustainability of nutrient recovery during wastewater treatment. Technologies that implement natural processes, such as algal wastewater treatment floways, to reverse the effects of anthropogenic influence on the environment stand to lead the way in natural resource management and remediation. The widespread adoption of algal treatment technologies may

help solve the growing global challenge of nutrient runoff and its resulting eutrophication of our fresh and estuarine ecosystems (Callahan et al. 2018).

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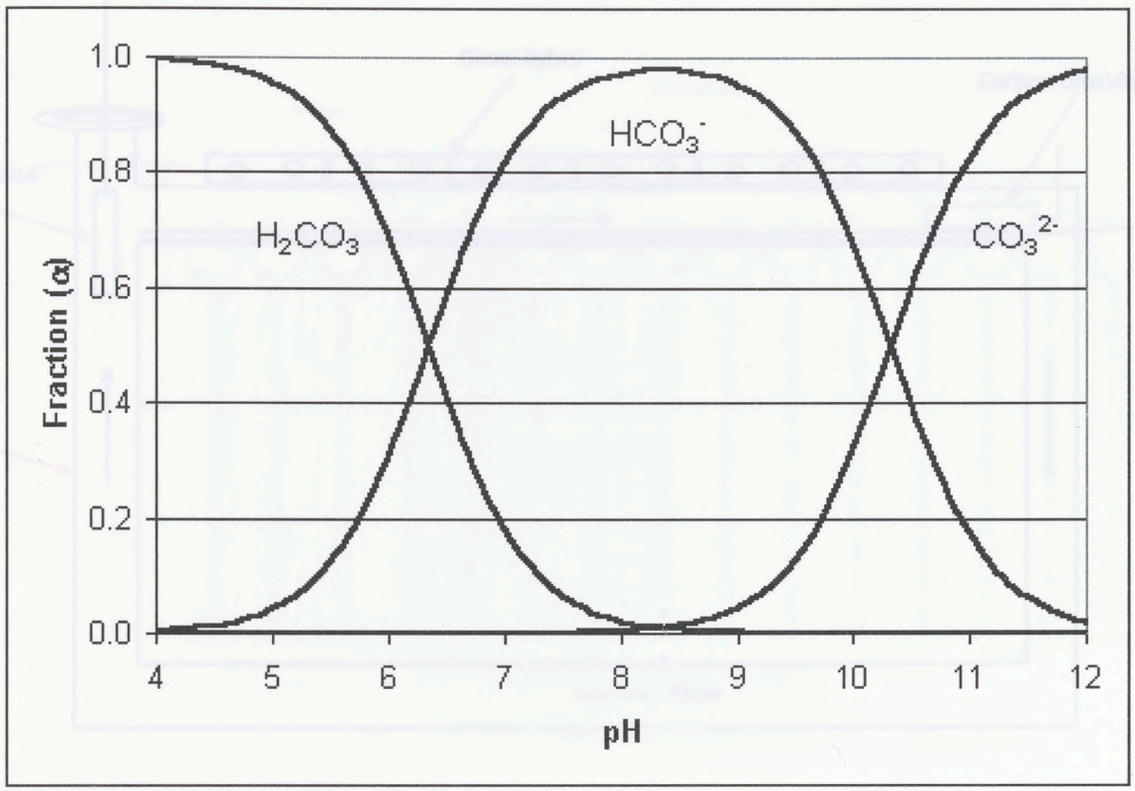

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Figure 1: Graph of fraction of DIC forms vs pH

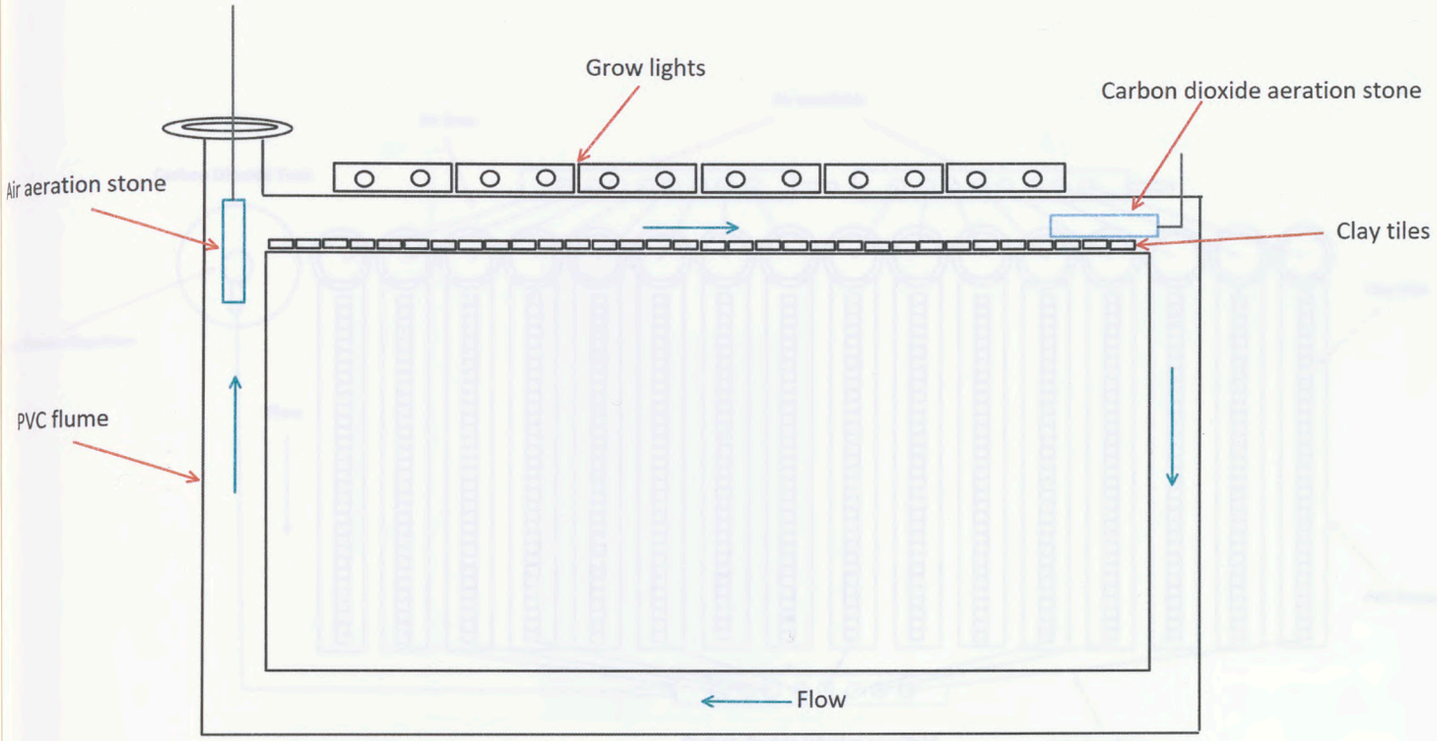

Figure 2: Sideview of a recirculating floway used in the carbon dioxide experiment

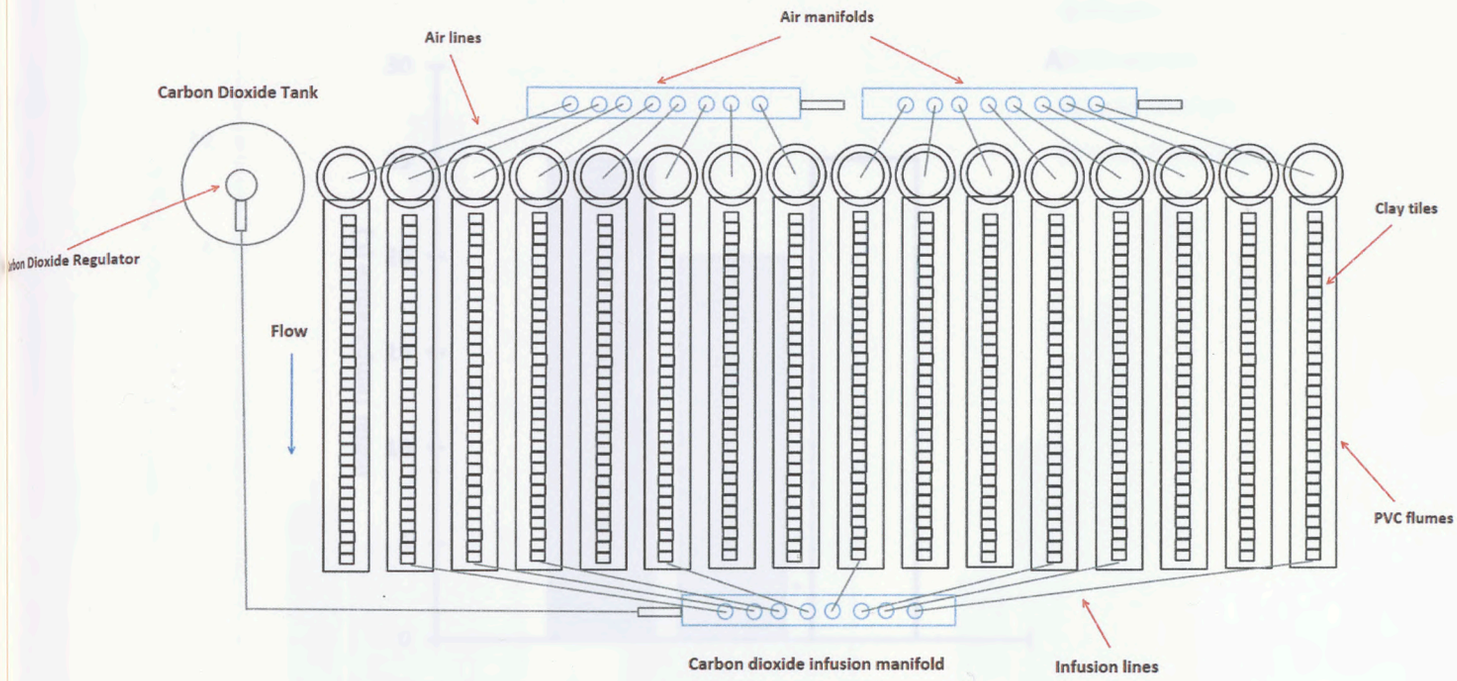

Figure 3: Top view without grow lights of recirculating floways shown used in the carbon dioxide infusion experiment.

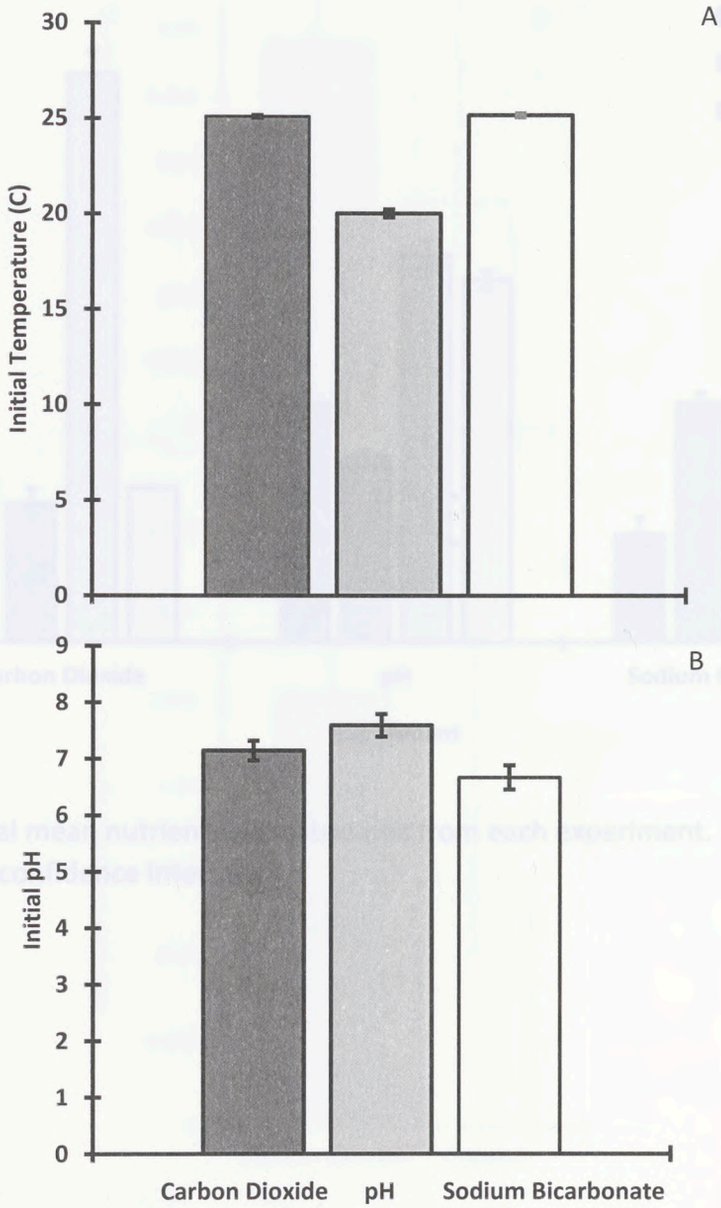

Figure 4: Initial mean temperature (A), and pH (B) of wastewater from each experiment. Error bars indicate 95% confidence intervals.

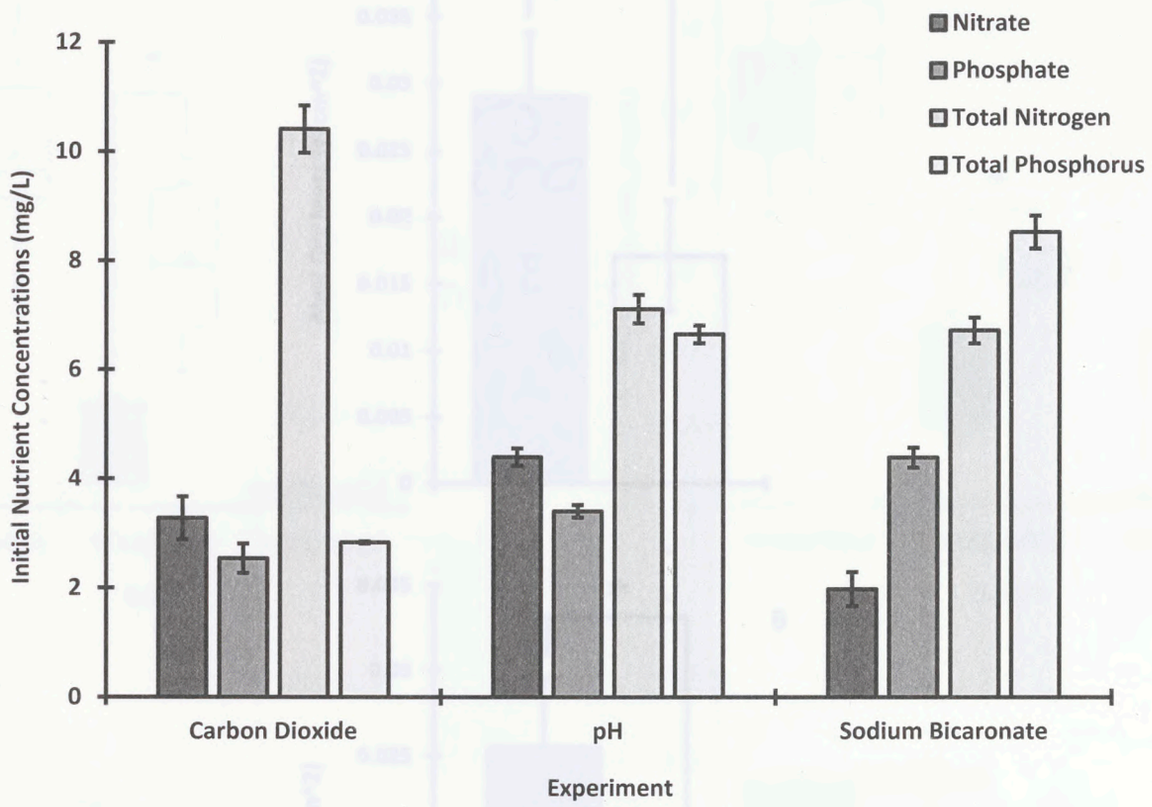

Figure 5: Initial mean nutrient concentrations from each experiment. Error bars indicate 95% confidence intervals.

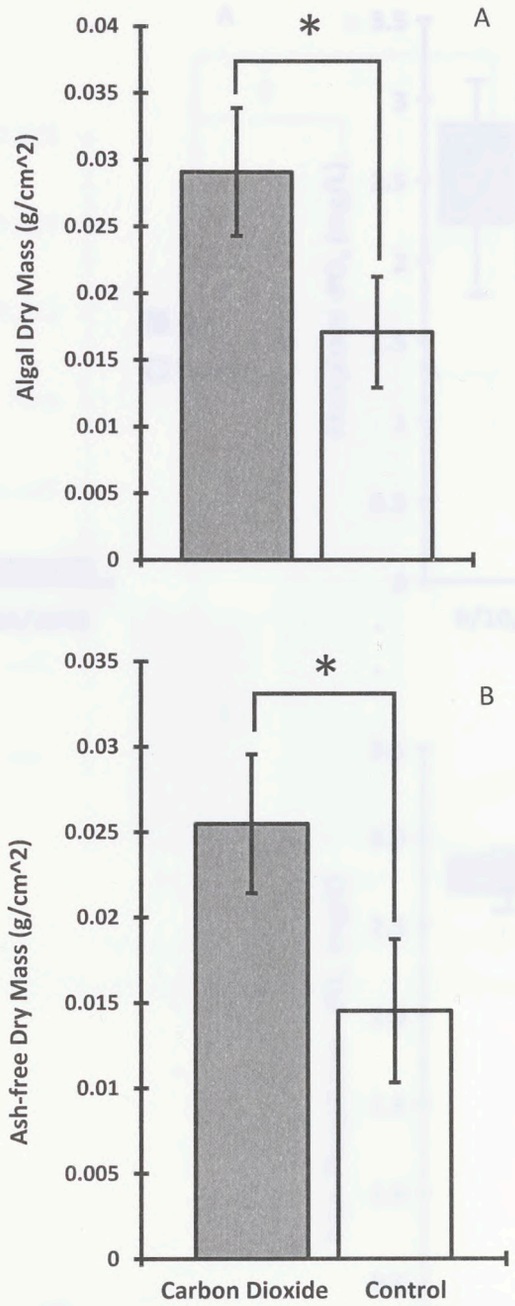

Figure 6. Mean algal dry mass (A) and ash-free dry mass (b) for the carbon dioxide infusion experiment. Error bars represent 95% confidence intervals. Star linked bars denote statistical significance between treatments.

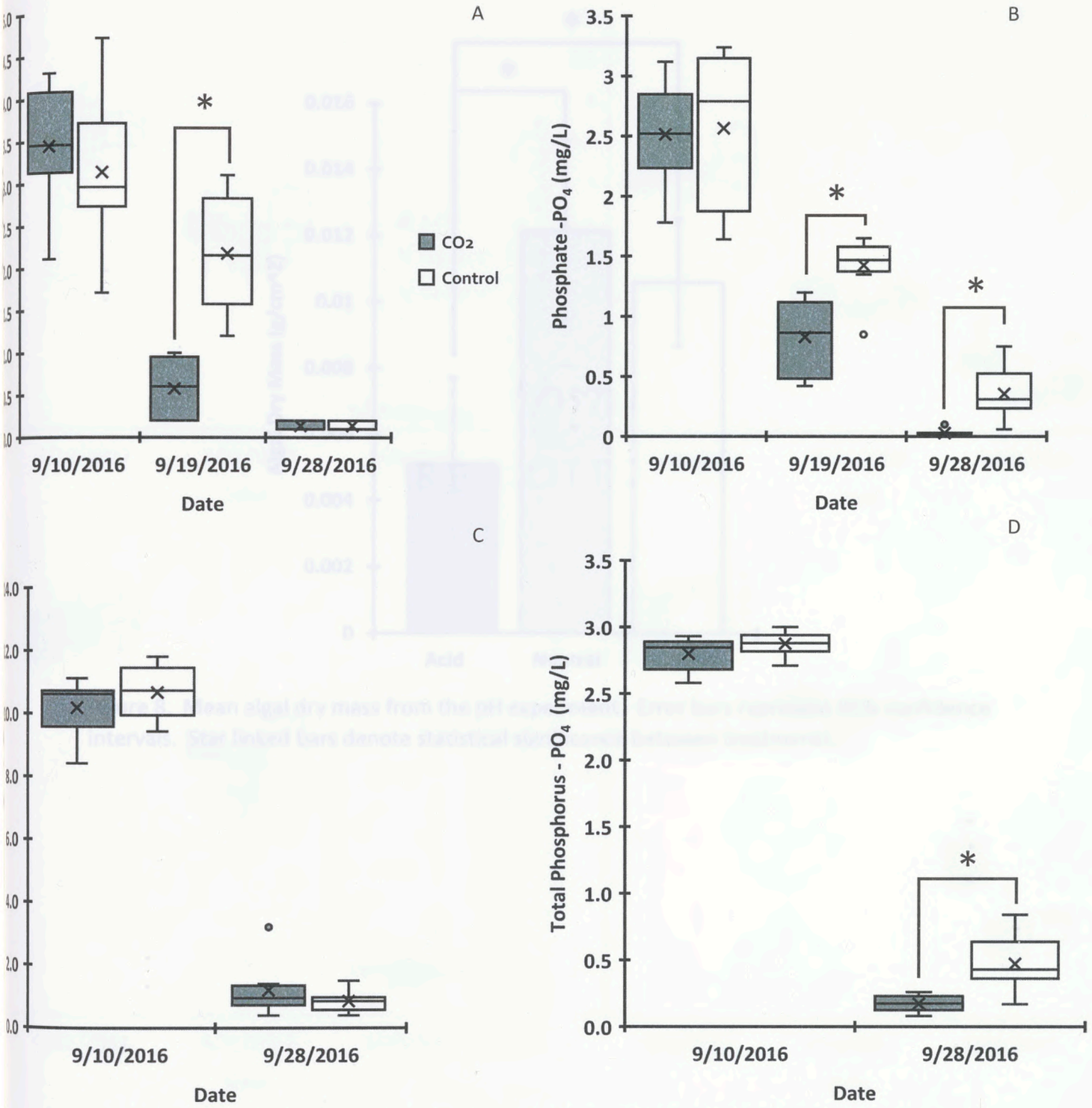

Figure 7. Box plot of nitrate (A), phosphate (B), total nitrogen (C), and total phosphorus (D) concentrations (mg/L) from carbon dioxide infusion experiment. Error bars represent 90% quartiles. Lower, middle, and upper portions of box are 25th, 50th, and 75th percentiles respectively. Points outside the boxes represent outliers that are greater than 1.5 times the interquartile range. Star linked bars denote statistical significance between treatments.

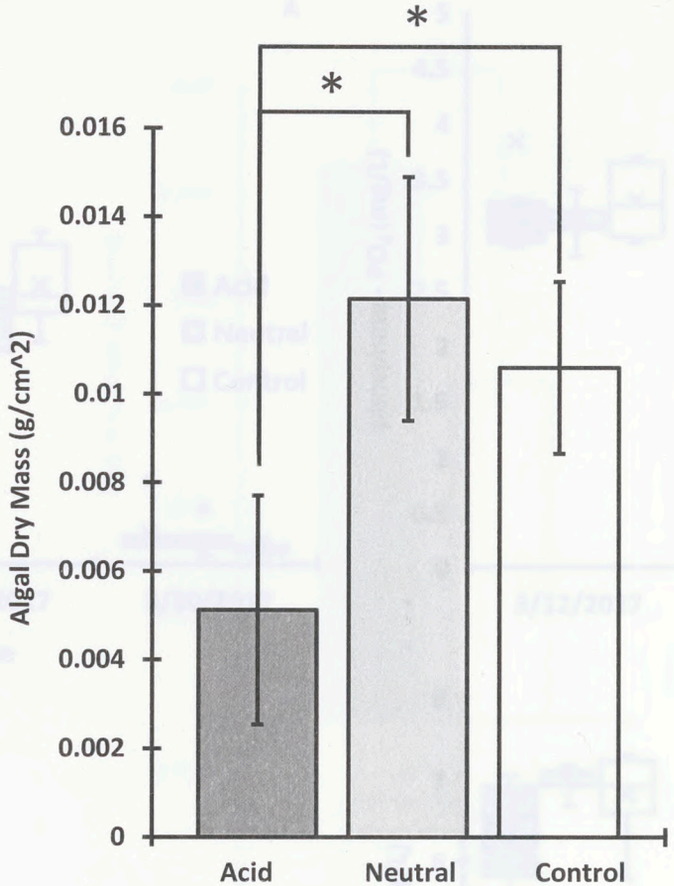

Figure 8. Mean algal dry mass from the pH experiment. Error bars represent 95% confidence intervals. Star linked bars denote statistical significance between treatments.

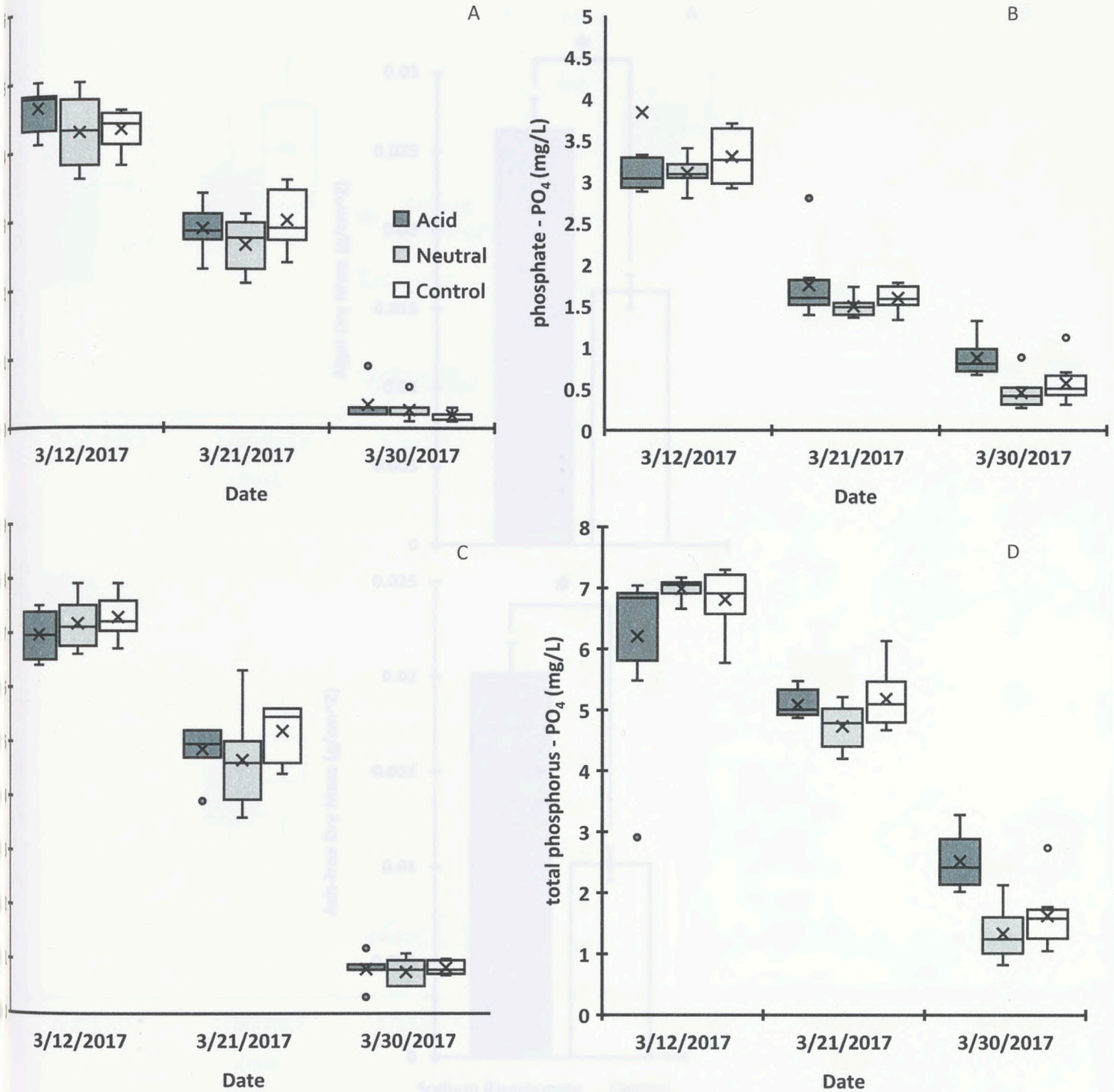

Figure 9. Box and whisker plots showing nitrate (A), phosphate (B), total nitrogen (C), total phosphorus (D) concentrations (mg/L) from the pH experiment. Error bars represent 90% quartiles. Lower, middle, and upper portions of box are 25th, 50th, and 75th percentiles respectively. Points outside the boxes represent outliers that are greater than 1.5 times the interquartile range.

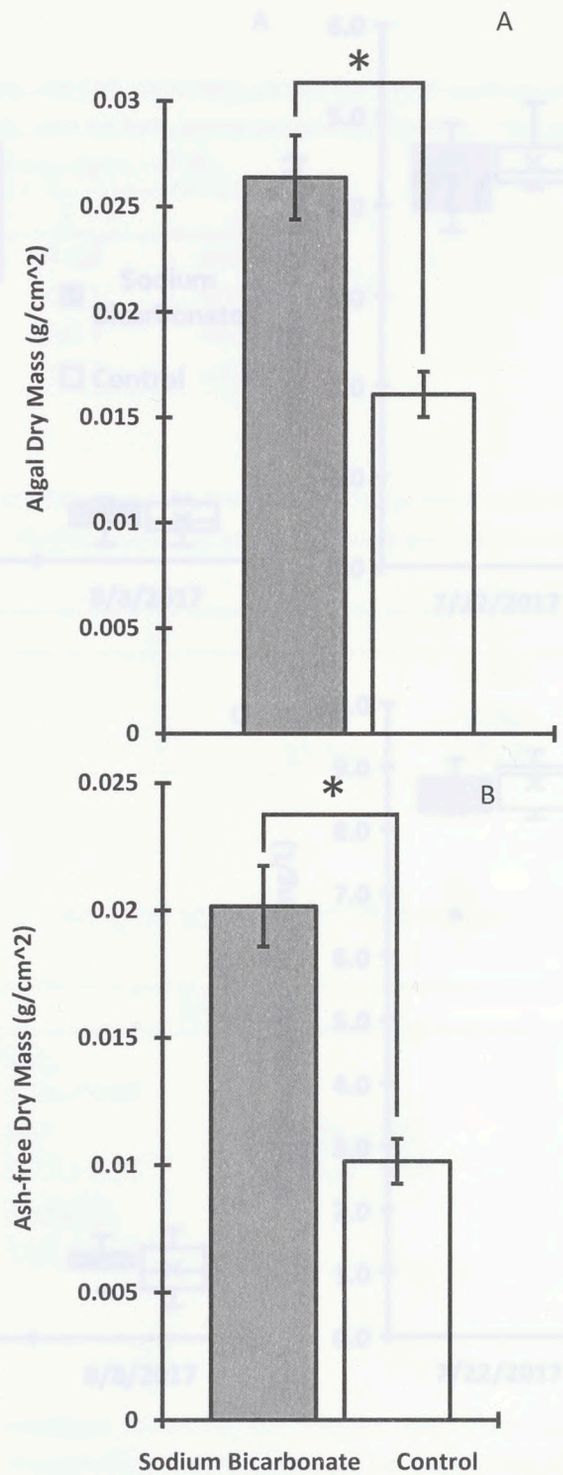

Figure 10. Mean algal dry mass (A) and ash-free dry mass (B) from the sodium bicarbonate addition experiment. Error bars represent 95% confidence intervals. Star linked bars denote statistical significance between treatments.

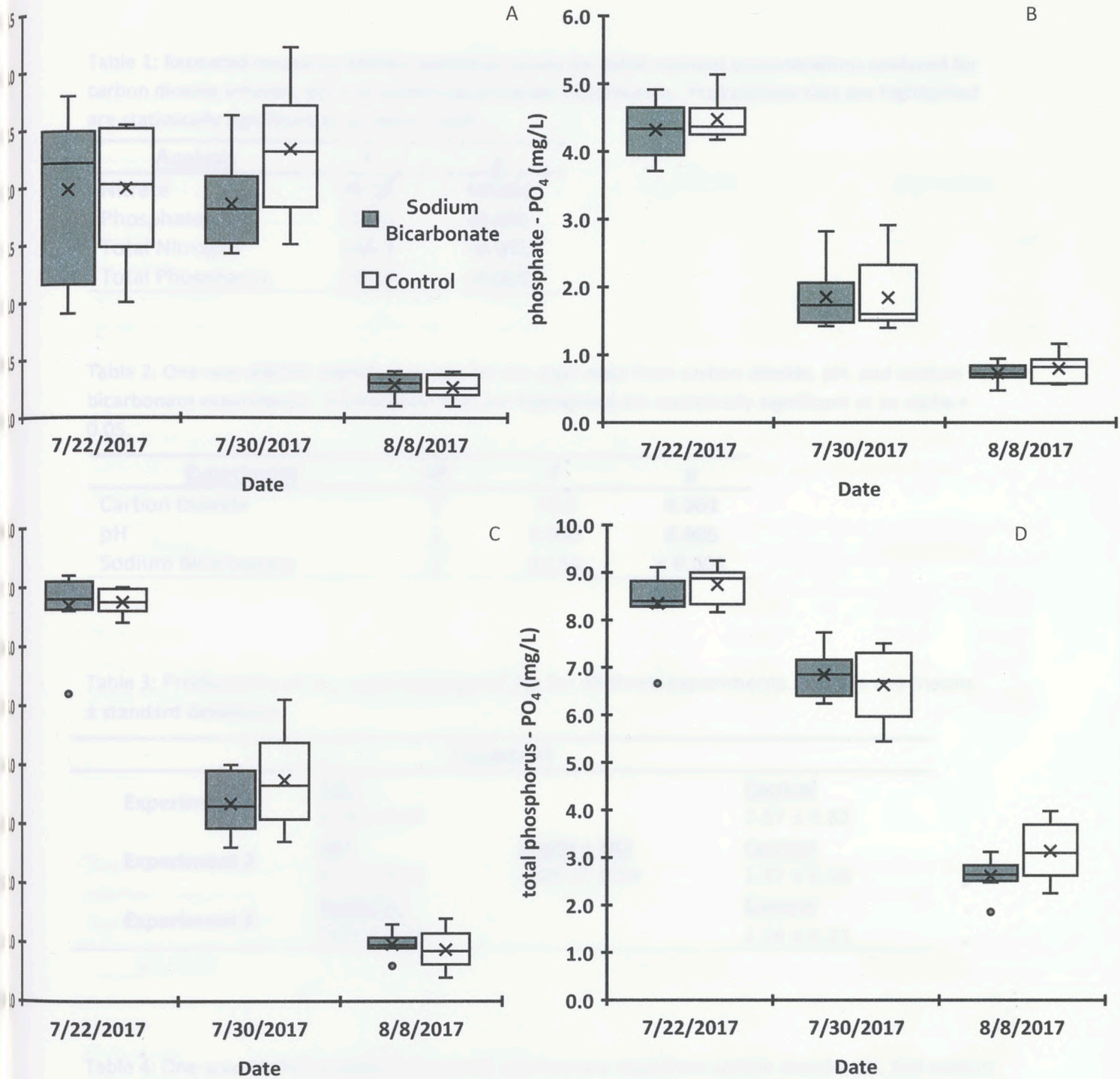

Figure 11. Box and whisker plots showing nitrate (A), phosphate (B), total nitrogen (C), total phosphorus (D) concentrations (mg/L) from the sodium bicarbonate addition experiment. Error bars represent 90% quartiles. Lower, middle, and upper portions of box are 25th, 50th, and 75th percentiles respectively. Points outside the boxes represent outliers that are greater than 1.5 times the interquartile range.

Table 1: Repeated measures ANOVA statistical results for initial nutrient concentrations analyzed for carbon dioxide infusion, pH, and sodium bicarbonate experiments. Probabilities that are highlighted are statistically significant at an alpha = 0.05.

Analysis	F	p
Nitrate	55.02	**<0.001**
Phosphate	16.22	**<0.001**
Total Nitrogen	164.3	**<0.001**
Total Phosphorus	272.5	**<0.001**

Table 2: One-way ANOVA statistical results for dry algal mass from carbon dioxide, pH, and sodium bicarbonate experiments. Probabilities that are highlighted are statistically significant at an alpha = 0.05.

Experiment	df	F	p
Carbon Dioxide	2	13.9	**0.002**
pH	2	6.953	**0.005**
Sodium Bicarbonate	2	83.54	**< 0.001**

Table 3: Productivity of dry algal mass ($g/m^2/d$) for all three experiments. Values are means ± standard deviations.

	Treatment		
Experiment 1	CO_2		Control
	4.04 ± 0.94		2.37 ± 0.82
Experiment 2	HCl	NaOH + HCl	Control
	0.71 ± 0.51	1.69 +/- 0.54	1.47 ± 0.38
Experiment 3	$NaHCO_3$		Control
	3.67 ± 0.39		2.24 ± 0.21

Table 4: One-way ANOVA statistical results for ash-free dry mass from carbon dioxide, pH, and sodium bicarbonate experiments. Probabilities that are highlighted are statistically significant at an alpha = 0.05.

Experiment	df	F	p
Carbon Dioxide	2	12.94	**0.003**
Sodium Bicarbonate	2	123.1	**<0.001**

Table 5: Repeated measures ANOVA statistical results for date, treatment, and their interaction for nutrients measured during the carbon dioxide infusion experiment. Probabilities that are highlighted are statistically significant at an alpha = 0.05.

Analysis	Date			Treatment			Interaction		
	df	*F*	*p*	df	F	*p*	*df*	*F*	*p*
Nitrate	2	93.446	**<0.001**	2	8.356	**0.023**	2	17.424	**<0.001**
Phosphate	2	258.643	**<0.001**	2	10.786	**0.013**	2	1.399	0.279
Total Nitrogen	2	1270.161	**<0.001**	2	0.032	0.864	2	3.405	0.108
Total Phosphorus	2	4189.027	**<0.001**	2	18.884	**0.003**	2	6.507	**0.038**

Table 6: Repeated measures ANOVA statistical results for date, treatment, and their interaction for nutrients measured during the pH manipulation experiment. Probabilities that are highlighted are statistically significant at an alpha = 0.05.

Analysis	Date			Treatment			Interaction		
	df	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
Nitrate	2	1246.62	**<0.001**	2	4.673	**0.028**	4	1.116	0.369
Phosphate	2	76.44	**<0.001**	2	2.916	0.087	4	0.215	0.928
Total Nitrogen	2	710.995	**<0.001**	2	3.995	**0.042**	4	1.097	0.377
Total Phosphorus	2	378.627	**<0.001**	2	1.14	0.348	4	6.51	**<0.001**

Table 7: Repeated measures ANOVA statistical results for date, treatment, and their interaction for nutrients measured during the sodium bicarbonate addition experiment. Probabilities that are highlighted are statistically significant at an alpha = 0.05.

Analysis	Date			Treatment			Interaction		
	df	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
Nitrate	2	91.149	**<0.001**	1	0.469	0.515	2	3.042	0.080
Phosphate	2	304.544	**<0.001**	1	0.462	0.519	2	0.492	0.622
Total Nitrogen	2	667.853	**<0.001**	1	0.563	0.478	2	1.363	0.288
Total Phosphorus	2	681.465	**<0.001**	1	3.056	0.124	2	1.648	0.228

Appendix A

Dissolved Nutrient Levels in Wastewater Floways

Table 1 : Dissolved nutrient levels in floways from Carbon Dioxide experiment

Date	Day	Flume	Treatment	Nitrate (NO_{3-} N mg/L)	Phosphate (PO_4^{3-} mg/L)	Total Nitrogen (N mg/L)	Total Phosphorus ($PO_4^{3-} mg/L$)
9/10/2016	1	1	Control	2.7	2.94	11.8	3.00
9/10/2016	1	2	Carbon Dioxide	2.1	2.86	9.5	2.79
9/10/2016	1	3	Carbon Dioxide	3.1	3.12	8.4	2.59
9/10/2016	1	4	Carbon Dioxide	3.4	2.76	10.6	2.86
9/10/2016	1	5	Control	2.9	1.64	10.9	2.95
9/10/2016	1	6	Carbon Dioxide	4.2	1.78	11.1	2.90
9/10/2016	1	7	Control	2.8	3.12	10.6	2.94
9/10/2016	1	8	Control	3.8	3.24	11.6	2.82
9/10/2016	1	9	Carbon Dioxide	3.7	2.82	10.7	2.66
9/10/2016	1	10	Control	1.7	2.64	9.9	2.85
9/10/2016	1	11	Control	4.7	1.92	9.4	2.72
9/10/2016	1	12	Carbon Dioxide	3.2	2.28	9.7	2.85
9/10/2016	1	13	Carbon Dioxide	3.5	2.28	10.7	2.94
9/10/2016	1	14	Control	3.0	1.86	10.2	2.85
9/10/2016	1	15	Control	3.4	3.16	10.8	2.93
9/10/2016	1	16	Carbon Dioxide	4.3	2.22	10.6	2.84
9/19/2016	9	1	Control	2.6	1.58	NA	NA
9/19/2016	9	2	Carbon Dioxide	0.5	0.42	NA	NA
9/19/2016	9	3	Carbon Dioxide	0.2	0.43	NA	NA
9/19/2016	9	4	Carbon Dioxide	0.2	0.80	NA	NA
9/19/2016	9	5	Control	1.8	1.58	NA	NA
9/19/2016	9	6	Carbon Dioxide	0.2	1.20	NA	NA
9/19/2016	9	7	Control	2.5	1.65	NA	NA
9/19/2016	9	8	Control	1.2	1.45	NA	NA
9/19/2016	9	9	Carbon Dioxide	0.8	0.93	NA	NA
9/19/2016	9	10	Control	3.1	1.46	NA	NA
9/19/2016	9	11	Control	2.9	1.48	NA	NA
9/19/2016	9	12	Carbon Dioxide	1.0	1.08	NA	NA
9/19/2016	9	13	Carbon Dioxide	1.0	0.64	NA	NA
9/19/2016	9	14	Control	1.8	1.35	NA	NA
9/19/2016	9	15	Control	1.5	0.85	NA	NA

Date	Day	Flume	Treatment	Nitrate ($NO_3 - N\ mg/L$)	Phosphate ($PO_4^{3-} mg/L$)	Total Nitrogen ($N\ mg/L$)	Total Phosphorus ($PO_4^{3-}\ mg/L$)
9/19/2016	9	16	Carbon Dioxide	0.7	1.13	NA	NA
9/28/2016	18	1	Control	0.1	0.28	1.0	0.40
9/28/2016	18	2	Carbon Dioxide	0.2	0.01	0.9	0.12
9/28/2016	18	3	Carbon Dioxide	0.2	0.01	0.8	0.16
9/28/2016	18	4	Carbon Dioxide	0.1	0.02	1.2	0.24
9/28/2016	18	5	Control	0.1	0.22	0.8	0.35
9/28/2016	18	6	Carbon Dioxide	0.1	0.03	1.0	0.20
9/28/2016	18	7	Control	0.1	0.75	0.4	0.84
9/28/2016	18	8	Control	0.2	0.59	0.9	0.70
9/28/2016	18	9	Carbon Dioxide	0.1	0.01	3.2	0.08
9/28/2016	18	10	Control	0.2	0.30	0.5	0.42
9/28/2016	18	11	Control	0.2	0.32	1.5	0.45
9/28/2016	18	12	Carbon Dioxide	0.1	0.10	0.7	0.26
9/28/2016	18	13	Carbon Dioxide	0.1	0.03	1.4	0.14
9/28/2016	18	14	Control	0.1	0.33	0.8	0.44
9/28/2016	18	15	Control	0.1	0.06	0.9	0.17
9/28/2016	18	16	Carbon Dioxide	0.2	0.01	0.4	0.19

Table 2: Dissolved nutrient levels in floways from pH variable experiment.

Date	Day	Flume	Treatment	Nitrate ($NO_3 - N\ mg/L$)	Phosphate ($PO_4^{3-}\ mg/L$)	Total Nitrogen ($N\ mg/L$)	Total Phosphorus ($PO_4^{3-}\ mg/L$)
3/12/2017	1	1	Control	4.4	3.70	7.6	7.03
3/12/2017	1	2	Acid	4.8	3.04	7.3	7.04
3/12/2017	1	3	Neutralized	3.8	3.24	7.5	7.10
3/12/2017	1	4	Acid	5.0	3.04	6.9	6.86
3/12/2017	1	5	Neutralized	4.6	3.04	7.9	7.05
3/12/2017	1	6	Acid	4.1	3.20	7.0	6.93
3/12/2017	1	7	Neutralized	5.0	3.06	7.5	6.91
3/12/2017	1	8	Neutralized	4.8	3.08	7.3	7.17
3/12/2017	1	9	Acid	4.7	9.30	7.5	2.91
3/12/2017	1	10	Control	4.4	2.92	7.0	7.23
3/12/2017	1	11	Neutralized	4.0	3.40	6.6	7.06
3/12/2017	1	12	Acid	4.6	3.00	6.8	6.81
3/12/2017	1	13	Neutralized	4.6	2.80	6.9	6.91
3/12/2017	1	14	Control	3.8	2.92	6.7	7.30
3/12/2017	1	15	Acid	4.2	2.88	6.4	6.87
3/12/2017	1	16	Neutralized	3.8	3.12	6.9	7.05
3/12/2017	1	17	Control	4.4	3.68	7.9	7.18
3/12/2017	1	18	Control	4.0	3.16	7.5	6.56
3/12/2017	1	19	Control	4.6	3.14	7.1	6.79
3/12/2017	1	20	Control	4.6	3.36	7.1	6.61
3/12/2017	1	21	Control	4.4	3.52	7.3	5.77

Date	Day	Flume	Treatment	Nitrate ($NO_3 - N\ mg/L$)	Phosphate ($PO_4^{3-}\ mg/L$)	Total Nitrogen ($N\ mg/L$)	Total Phosphorus ($PO_4^{3-} mg/L$)
3/12/2017	1	23	Acid	4.8	3.32	7.4	6.78
3/12/2017	1	24	Acid	4.8	2.90	6.4	5.48
3/20/2017	9	1	Control	2.8	1.75	5.4	5.56
3/20/2017	9	2	Acid	2.8	1.84	4.7	5.01
3/20/2017	9	3	Neutralized	2.3	1.53	4.3	4.55
3/20/2017	9	4	Acid	2.3	1.39	3.9	4.93
3/20/2017	9	5	Neutralized	2.3	1.36	3.6	4.20
3/20/2017	9	6	Acid	2.7	1.50	4.8	4.87
3/20/2017	9	7	Neutralized	2.6	1.54	5.0	5.05
3/20/2017	9	8	Neutralized	3.0	1.53	4.4	4.92
3/20/2017	9	9	Acid	3.4	1.73	5.1	5.43
3/20/2017	9	10	Control	2.8	1.69	4.4	4.73
3/20/2017	9	11	Neutralized	2.1	1.44	6.3	4.65
3/20/2017	9	12	Acid	3.1	2.80	4.7	5.47
3/20/2017	9	13	Neutralized	2.9	1.44	5.0	5.21
3/20/2017	9	14	Control	3.6	1.50	5.6	5.17
3/20/2017	9	15	Acid	3.1	1.56	5.2	5.03
3/20/2017	9	16	Neutralized	3.1	1.73	4.8	4.93
3/20/2017	9	17	Control	3.0	1.58	5.5	6.13
3/20/2017	9	18	Control	3.5	1.55	5.6	4.99
3/20/2017	9	19	Control	2.4	1.33	4.5	5.08
3/20/2017	9	20	Control	3.3	1.78	5.6	5.11
3/20/2017	9	21	Control	2.7	1.59	4.9	4.67
3/20/2017	9	22	Neutralized	2.9	1.38	3.8	4.35
3/20/2017	9	23	Acid	2.8	1.59	5.2	4.92
3/20/2017	9	24	Acid	2.9	1.60	5.2	5.00
3/30/2017	18	1	Control	0.1	0.51	0.8	2.74
3/30/2017	18	2	Acid	0.2	0.69	0.3	2.02
3/30/2017	18	3	Neutralized	0.3	0.31	0.5	1.21
3/30/2017	18	4	Acid	0.3	0.81	0.9	2.34
3/30/2017	18	5	Neutralized	0.3	0.52	0.5	1.65
3/30/2017	18	6	Acid	0.3	0.67	0.8	2.50
3/30/2017	18	7	Neutralized	0.1	0.32	1.1	1.14
3/30/2017	18	8	Neutralized	0.2	0.49	0.8	1.28
3/30/2017	18	9	Acid	0.3	0.80	0.8	2.72
3/30/2017	18	10	Control	0.1	0.42	1.0	1.18
3/30/2017	18	11	Neutralized	0.2	0.34	0.5	0.82
3/30/2017	18	12	Acid	0.2	0.92	0.9	2.09
3/30/2017	18	13	Neutralized	0.2	0.88	1.0	2.13
3/30/2017	18	14	Control	0.2	0.54	0.7	1.77
3/30/2017	18	15	Acid	0.3	1.32	0.8	2.94
3/30/2017	18	16	Neutralized	0.6	0.50	0.9	1.46
3/30/2017	18	17	Control	0.3	1.12	0.7	1.61
3/30/2017	18	18	Control	0.2	0.50	0.8	1.49
3/30/2017	18	19	Control	0.2	0.45	0.9	1.56
3/30/2017	18	20	Control	0.2	0.70	1.0	1.60
3/30/2017	18	21	Control	0.2	0.31	0.8	1.05
3/30/2017	18	22	Neutralized	0.2	0.27	0.8	0.97
3/30/2017	18	23	Acid	0.9	1.00	1.2	3.28
3/30/2017	18	24	Acid	0.2	0.78	0.8	2.27

Table 3: Dissolved nutrient levels in floways from Sodium Bicarbonate experiment

Date	Day	Flume	Treatment	Nitrate ($NO_3 - N\ mg/L$)	Phosphate ($PO_4^{3-}\ mg/L$)	Total Nitrogen ($N\ mg/L$)	Total Phosphorus ($PO_4^{3-}\ mg/L$)
7/22/2017	1	1	yes	0.9	4.65	7.1	8.37
7/22/2017	1	2	yes	2.5	4.54	7.1	8.33
7/22/2017	1	3	no	2.0	4.54	6.6	8.17
7/22/2017	1	4	yes	2.3	4.14	6.6	8.80
7/22/2017	1	5	yes	2.8	4.06	5.2	6.66
7/22/2017	1	6	no	2.0	4.18	6.9	8.60
7/22/2017	1	7	no	1.3	4.37	6.4	8.25
7/22/2017	1	8	no	2.5	4.35	6.6	9.01
7/22/2017	1	9	no	2.5	4.67	6.7	8.83
7/22/2017	1	10	yes	2.4	4.64	6.7	8.42
7/22/2017	1	11	no	2.0	4.22	6.8	9.25
7/22/2017	1	12	yes	0.9	4.92	6.9	8.27
7/22/2017	1	13	yes	1.9	3.91	7.2	9.11
7/22/2017	1	14	yes	2.1	3.71	6.7	8.83
7/22/2017	1	15	no	2.5	4.36	7.0	8.92
7/22/2017	1	16	no	1.0	5.14	7.0	8.95
7/30/2017	9	1	yes	2.0	1.65	4.0	7.74
7/30/2017	9	2	yes	2.1	1.42	3.4	6.35
7/30/2017	9	3	no	2.2	1.40	3.8	6.35
7/30/2017	9	4	yes	1.5	1.43	2.6	6.24
7/30/2017	9	5	yes	2.6	1.82	2.9	6.83
7/30/2017	9	6	no	2.2	1.55	3.3	5.44
7/30/2017	9	7	no	1.5	1.66	4.0	6.94
7/30/2017	9	8	no	2.7	1.54	3.5	6.48
7/30/2017	9	9	no	2.4	1.50	3.0	5.84
7/30/2017	9	10	yes	1.9	1.61	4.0	7.25
7/30/2017	9	11	no	2.7	1.65	4.5	7.16
7/30/2017	9	12	yes	1.4	1.98	3.2	6.55
7/30/2017	9	13	yes	1.7	2.09	3.0	6.92
7/30/2017	9	14	yes	1.5	2.82	3.6	6.88
7/30/2017	9	15	no	1.7	2.55	0.0	7.36
7/30/2017	9	16	no	3.2	2.91	0.0	7.51
8/8/2017	18	1	yes	0.4	0.75	1.0	2.68
8/8/2017	18	2	yes	0.4	0.72	0.9	2.63
8/8/2017	18	3	no	0.3	0.73	0.8	3.03
8/8/2017	18	4	yes	0.3	0.47	0.6	1.86
8/8/2017	18	5	yes	0.3	0.66	0.9	2.48

Date	Day	Flume	Treatment	Nitrate ($NO_3 - N\ mg/L$)	Phosphate ($PO_4^{3-}\ mg/L$)	Total Nitrogen ($N\ mg/L$)	Total Phosphorus ($PO_4^{3-}\ mg/L$)
8/8/2017	18	7	no	0.4	0.56	0.9	2.92
8/8/2017	18	8	no	0.2	0.60	0.7	2.52
8/8/2017	18	9	no	0.2	0.93	1.0	3.46
8/8/2017	18	10	yes	0.2	0.94	1.1	2.89
8/8/2017	18	11	no	0.4	0.57	0.6	2.25
8/8/2017	18	12	yes	0.3	0.70	0.9	2.59
8/8/2017	18	13	yes	0.1	0.72	1.3	2.66
8/8/2017	18	14	yes	0.3	0.87	1.0	3.12
8/8/2017	18	15	no	0.1	0.91	0.4	3.77
8/8/2017	18	16	no	0.3	1.17	1.4	3.98

Appendix B

Algae Mass

Table 1: Algae mass in grams collected from one randomly selected tile per flume from Carbon Dioxide experiment.

Date	Flume	Treatment	Dry Algal Mass (g)	Ashed Algal Mass (g)	AFDM (g)
9/28/2016	1	Control	0.0134	0.0027	0.0107
9/28/2016	2	Carbon Dioxide	0.0377	0.0034	0.0343
9/28/2016	3	Carbon Dioxide	0.0192	0.0032	0.016
9/28/2016	4	Carbon Dioxide	0.0392	0.0082	0.031
9/28/2016	5	Control	0.0246	0.0021	0.0225
9/28/2016	6	Carbon Dioxide	0.0292	0.0032	0.026
9/28/2016	7	Control	0.0092	0.0024	0.0068
9/28/2016	8	Control	0.0172	0.0027	0.0145
9/28/2016	9	Carbon Dioxide	0.0342	0.0039	0.0303
9/28/2016	10	Control	0.0176	0.0018	0.0158
9/28/2016	11	Control	0.0276	0.0025	0.0251
9/28/2016	12	Carbon Dioxide	0.0274	0.002	0.0254
9/28/2016	13	Carbon Dioxide	0.0257	0.0027	0.023
9/28/2016	14	Control	0.0153	0.0023	0.013
9/28/2016	15	Control	0.0188	0.0026	0.0162
9/28/2016	16	Carbon Dioxide	0.0258	0.003	0.0228

Table 2: Dry algal mass in grams collected from one randomly selected tile per flume from pH variable experiment.

Date	Flume	Treatment	Dry Algal Mass (g)
3/30/2017	1	Control	0.0088
3/30/2017	2	Acid	0.0127
3/30/2017	3	Neutralized Solution	0.0097
3/30/2017	4	Acid	0.0061
3/30/2017	5	Neutralized Solution	0.0160
3/30/2017	6	Acid	0.0089
3/30/2017	7	Neutralized Solution	0.0162

Date	Flume	Treatment	Dry Algal Mass (g)
3/30/2017	8	Neutralized Solution	0.0168
3/30/2017	11	Neutralized Solution	0.0145
3/30/2017	12	Acid	0.0067
3/30/2017	13	Neutralized Solution	0.0057
3/30/2017	14	Control	0.0058
3/30/2017	15	Acid	0.0034
3/30/2017	16	Neutralized Solution	0.0105
3/30/2017	17	Control	0.0109
3/30/2017	18	Control	0.0098
3/30/2017	19	Control	0.0128
3/30/2017	20	Control	0.0137
3/30/2017	21	Control	0.0138
3/30/2017	22	Neutralized Solution	0.0130
3/30/2017	23	Acid	0.0051
3/30/2017	24	Acid	0.0085

Table 3: Algae mass in grams collected from one randomly selected tile per flume from Sodium Bicarbonate Experiment.

Date	Flume	Treatment	Dry Algal Mass (g)	Ashed Algal Mass (g)	AFDM (g)
8/8/2017	1	Sodium Bicarbonate	0.0235	0.0067	0.0168
8/8/2017	2	Sodium Bicarbonate	0.0226	0.0024	0.0202
8/8/2017	3	Control	0.0185	0.0057	0.0128
8/8/2017	4	Sodium Bicarbonate	0.0273	0.0057	0.0216
8/8/2017	5	Sodium Bicarbonate	0.0286	0.0061	0.0225
8/8/2017	6	Control	0.0144	0.0053	0.0091
8/8/2017	7	Control	0.017	0.0059	0.0111
8/8/2017	8	Control	0.015	0.0048	0.0102
8/8/2017	9	Control	0.0172	0.0071	0.0101

Date	Flume	Treatment	Dry Algal Mass (g)	Ashed Algal Mass (g)	AFDM (g)
8/8/2017	10	Sodium Bicarbonate	0.0272	0.0075	0.0197
8/8/2017	11	Control	0.0173	0.0075	0.0098
8/8/2017	12	Sodium Bicarbonate	0.0286	0.0094	0.0192
8/8/2017	13	Sodium Bicarbonate	0.0304	0.0067	0.0237
8/8/2017	14	Sodium Bicarbonate	0.024	0.0055	0.0185
8/8/2017	15	Control	0.0155	0.0058	0.0097
8/8/2017	16	Control	0.0145	0.0056	0.0089

Appendix C

Alkalinity of Wastewater in Recirculating Floways

Table 1. Summary of alkalinity values from wastewater in recirculating floways during Sodium Bicarbonate test. Eight grams of $NaHCO_3$ was added to treated floways on 7/22/2017 and alkalinity was recorded for said floways 24 hours later, then at the end of the experiment.

Date	Flume	Treatment	pH	P - Alkalinity	T - Alkalinity
7/22/2017	1	Sodium Bicarbonate	8.14	0	100
7/22/2017	2	Sodium Bicarbonate	8.12	0	200
7/22/2017	3	Control	8.15	0	100
7/22/2017	4	Sodium Bicarbonate	8.12	0	150
7/22/2017	5	Sodium Bicarbonate	8.16	0	150
7/22/2017	6	Control	8.18	0	150
7/22/2017	7	Control	8.20	0	350
7/22/2017	8	Control	8.14	0	150
7/22/2017	9	Control	8.09	0	250
7/22/2017	10	Sodium Bicarbonate	8.16	0	200
7/22/2017	11	Control	8.17	0	150
7/22/2017	12	Sodium Bicarbonate	7.99	0	200
7/22/2017	13	Sodium Bicarbonate	8.17	0	250
7/22/2017	14	Sodium Bicarbonate	8.18	0	250
7/22/2017	15	Control	8.18	0	200
7/22/2017	16	Control	8.15	0	50
7/23/2017	1	Sodium Bicarbonate	9.17	0	1400
7/23/2017	2	Sodium Bicarbonate	9.16	0	700
7/23/2017	3	Control	8.11	0	NA
7/23/2017	4	Sodium Bicarbonate	9.17	0	800
7/23/2017	5	Sodium Bicarbonate	9.20	0	800
7/23/2017	6	Control	8.18	0	NA
7/23/2017	7	Control	8.17	0	NA
7/23/2017	8	Control	8.12	0	NA
7/23/2017	9	Control	8.08	0	NA
7/23/2017	10	Sodium Bicarbonate	9.24	0	800
7/23/2017	11	Control	8.08	0	NA
7/23/2017	12	Sodium Bicarbonate	9.13	0	800
7/23/2017	13	Sodium Bicarbonate	9.24	0	800
7/23/2017	14	Sodium Bicarbonate	9.25	0	700
7/23/2017	15	Control	8.07	0	NA
7/23/2017	16	Control	8.04	0	NA
8/8/2017	1	Sodium Bicarbonate	9.70	0	700
8/8/2017	2	Sodium Bicarbonate	9.52	0	850
8/8/2017	3	Control	9.62	0	200
8/8/2017	4	Sodium Bicarbonate	9.44	0	800
8/8/2017	5	Sodium Bicarbonate	9.77	0	800

Date	Flume	Treatment	pH	P - Alkalinity	T - Alkalinity
8/8/2017	6	Control	9.48	0	250
8/8/2017	7	Control	9.39	0	200
8/8/2017	8	Control	9.38	0	250
8/8/2017	9	Control	9.31	0	200
8/8/2017	10	Sodium Bicarbonate	9.59	0	750
8/8/2017	11	Control	9.54	0	250
8/8/2017	12	Sodium Bicarbonate	9.63	0	850
8/8/2017	13	Sodium Bicarbonate	9.76	0	850
8/8/2017	14	Sodium Bicarbonate	9.58	0	850
8/8/2017	15	Control	9.92	0	200
8/8/2017	16	Control	9.23	0	200

CARBON LIMITATION IN PERIPHYTIC ALGAL WASTEWATER TREATMENT SYSTEMS

By

Brandon J Furnish

2018

Dr. Troy Keller, Chair — 12/7/2018 Date

Dr. Clifton Ruehl, Member — 12/7/2018 Date

Dr. Stacey Blersch, Member — 12/7/18 Date

Signature Page Approved:

Committee Chair:

DATE DUE

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