Lactic Acid Production from Filamentous Algae grown using Aquaponics Wastewater

by

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Abstract

Algal Turf Scrubber (ATS) systems are a known method of treating nitrogen and phosphorous rich wastewater by cultivating green filamentous algae biomass. One novel source of this wastewater is from an aquaponics system, where fish and vegetable crops are grown in an aquatic environment connected by the movement of the water from the fish to the plants for their nutrient sources. The wastewater from the fish is potent in nitrates, phosphates, and ammonium that the plants thrive on by pulling these compounds from the water. However, often there are still lingering nutrients in the water afterwards, so ATS lanes can be attached at the end of the system for further nutrient recovery, while also potentially producing additional value products from algal biomass. The filamentous algal species typical in ATS systems are often rich in carbohydrates. As such, this algal biomass has the potential to be used as feedstock for lactic acid fermentation from lactic acid producing bacteria (LAB). The fermentation process typically requires both a nutrient source, usually composed of forms of nitrogen, and a carbohydrate source, in the form of monosaccharides or simple sugars. The nitrogen serves as a building block for the growth of the LAB while the sugars provide the energy for the LAB to conduct the fermentation. In this study, algal biomass cultivated on aquaponics wastewater on laboratory-scale indoor ATS units was studied for use as the carbohydrate source for lactic acid fermentation production. A dilution experiment was first performed to investigate the growth yield of higher carbohydrate concentration algae over a range of concentrations of the aquaponics wastewater. Results showed that the half wastewater, half tap water dilution yielded the highest yield of green filamentous algae with a mean ash free dry weight productivity of 7 $g/m^2/day$, based upon weekly harvests. Production of the algae biomass at the half dilution

yielded an additional 1 kg of the dried biomass. The fermentation experiment tested the suitability of the different algal biomasses harvested as a carbohydrate source by measuring both the concentration of lactic acid present within the system and the yield of lactic acid from the available sugars from samples taken over the course of the fermentation. Results of the fermentation experiments indicated that algae grown while under half dilution ATS conditions and without a customary heat and pressure pretreatment produced lactic acid concentrations of 20 g/L and a yield of 80% lactic acid from the available sugars. These results outstripped the other fermentations trials ran, with the lower quality green algae biomass from the dilution experiment at 15 g/L lactic acid concentration and algae biomass from the half dilution production that were pretreated with heat and pressure at 17g/L concentration. Further comparisons to predominantly cyanobacteria algae biomass initially grown from the ATS system at 8 g/L and cucumber residues at 18 g/L lactic acid concentrations were favorable, while waste paper mill sludge had near double the concentration with up to 45 g/L lactic acid produced. These results suggest that there is potential for filamentous algae for use as a carbohydrate feedstock for lactic acid fermentation due to high carbohydrate availability that merits continued research.

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LA	Lactic acid
LAB	Lactic acid producing bacteria
ATS	Algal Turf Scrubber
YE	Yeast extract
Ca	Calcium carbonate
AFDW	Ash free dry weight

Chapter 1: Introduction

From plant residues in agriculture to accumulated solids from wastewater, waste is a constant factor in most production processes and cultivation methods, and typically needs further processing to minimize the impact on the local environment when discharged. This includes wastewater produced during the cultivation of fish, such as Nile tilapia in an aquaponics system. Aquaponics is the coupled production of fish aquaculture and the production of plants, usually vegetable crops (Pinho et al., 2017) and is an approach for controlled high density food production that has gained considerable interest in recent years (Estrada-Perez et al., 2018). The vegetables require nitrogen and phosphorus to grow, which are provided by the fish wastewater (Kim et al., 2020), compensating for the lack of soil while growing the vegetable crop, , as the soil is the usual source of nutrients for plants grown terrestrially. These nutrients are found and utilized in compounds like nitrates, nitrite, ammonia and phosphates (Effendi et al., 2020) making vegetable cultivation a logical next step to both maximize economic returns and reduce waste treatment (Lam et al., 2015)

However, this wastewater is usually still rich in nutrients comprising nitrogen and phosphorus (Kim et al., 2020) which can cause algal blooms in local waterways if released into the environment without treatment. These blooms deprive the environment of essential oxygen in the water once the algae decays and cause die offs of local species unable to adapt to the new conditions, with some blooms potentially producing potent toxins (Blakey et al., 2015).

Fish crops and vegetable production are not the only way to recover and utilize nutrients in the aquaponics system. A slightly more unorthodox option is to cultivate algae in a pond or

Algal Turf Scrubber® [1] (ATS) System using the nutrients present within the wastewater. The algae need many of the same nutrients as the vegetables and therefore make a logical secondary step for unused nutrients in wastewater streams. Studies involving algae wastewater treatment cover numerous sources, including oyster production (Ray et al., 2015), dairy manure (Mulbry et al., 2008), and citrus production (D'Aiuto et al., 2015). The resulting algal biomass has the potential to be utilized to produce a variety of products, from nutrient supplements in animal feed and biofuels to pharmaceuticals and chemical compounds (Adey et al., 2013).

One of the chemical compounds of interest is lactic acid, which is produced by the fermentation of a nitrogen source and a carbohydrate source by lactic acid producing bacteria (LAB). Lactic acid is a common chemical used in a variety of common processes, such as the cosmetic, pharmaceutical, food, and chemical industries (Pleissner et al., 2017), in addition as a feedstock to produce polylactic acid, a biopolymer that is greener and biodegradable compared to traditional petrochemical derived plastics (Talukder et al., 2012, Abdel-Rahman et al., 2011). Various sources of waste products have been investigated for their use in the lactic acid fermentation process, from restaurant food waste (Pleissner et al., 2017) and vegetables such as carrots and beet root (Gardner et al., 2001) to fish waste (Shi et al., 2018) and even microalgae biomass (Kwan et al., 2015). However, ATS grown filamentous algae itself has not featured as prominently in the literature, let alone algae also cultured from aquaponics wastewater.

Thus, if algae cultivated from surplus aquaponics wastewater can function as a suitable source of carbohydrates for the fermentation of lactic acid, this additional stage can potentially be incorporated into the aquaponics wastewater treatment system as an additional added value

[1] Algal Turf Scrubber® and ATS™ are registered trademark names by HydroMentia, Ocala, FL.

product and nutrient recycle option. Development of this pathway needs further study and research.

1.2 Purpose of Research

The purpose of this research is to investigate the suitability of filamentous algae grown from aquaponics wastewater as a feedstock for lactic acid fermentation performed by lactic acid producing bacteria (LAB). This task was performed by cultivating algae within an indoor Algal Turf Scrubber (ATS) system and covers a dilution experiment undertaken to promote the emergence and predominance of green filamentous algae species from the algal turf community. The biomass harvested from the ATS was collected and prepared for lactic acid fermentation by LAB and tested for overall lactic acid yields and the efficiency of conversion from available sugars to lactic acid. It is expected that algae with rich carbohydrate content will produce higher yields of lactic acid compared to poorer quality algae.

1.3 Goals and Objectives

The goal of this study is to investigate and optimize the productivity of the ATS algal turf community and to determine whether algae grown from aquaponics wastewater is viable as a feedstock for lactic acid fermentation, while also testing for what additional treatments during fermentation would further boost lactic acid yields. The objectives to accomplish this task are as follows:

1. Quantify algae productivity from cultivation in an indoor ATS system fed with wastewater from an aquaponics system;

2. Analyze the relationships nutrient concentration of the wastewater, time, and between dilutions and lanes have on algal productivity and ash content and whether they are significant;
3. Quantify lactic acid yield from lactic acid fermentation using algae biomass from algae growth experiments and accomplished with LAB and the whether the differences between treatment options are statistically significant.

Chapter 2: Literature Review

2.1 Aquaponics

The aquaponics approach, which co-cultivates fish and vegetables using waste nutrients from fish production to fertilize plant crop production, is a well-established method of maximizing food production profits while also reducing waste treatment and disposal costs (Fang et al., 2017). The specific fish species and vegetable crop can be varied to suit local climate and management practices, with the species cultivated ranging from common carp (Fang et al., 2017) and oysters (Ray et al., 2015) for fish, to Pak choi (Fang et al., 2017) Swiss chard and kale (Addy et al., 2017) for vegetables.

The fish require regular feeding, and the feed is either digested and excreted as water or resides within the fish tank before decomposing. In most aquaculture systems, the suspended solids are filtered out in an adjacent settling tank or some other technology and treated separately, leaving dissolved nutrients still present in the effluent stream (Addy et al., 2017). This produces a high nutrient load wastewater that is then fed into plant cultivation, eliminating the need for additional fertilizer and supplements for crops. If it is not completely consumed within the plant cultivation, this water with a much lower nutrient load can be recirculated to the fish production following further treatment and sterilization or collected for further downstream use or disposal (Aquilino et al., 2020**)**. Further downstream use might entail additional cultivation, crop scenarios, even constructed wetlands for further recovery of nutrients and biomass production that potentially generates additional economic value (de Farias Lima et al., 2019**).**

2.2 Algae Cultivation

The continued presence of these nutrients in the water stream if not fed back into the system is still a potential problem, and this can be remedied with algae cultivation for additional biomass growth and nutrient treatment. Algae are known to be a reliable option for wastewater treatment (Liu et al., 2020), and algae cultivation is frequently studied for nitrogen and phosphorus removal (Stevčić et al., 2019; Ross et al., 2018**)**. These nutrients are most frequently available in the forms of nitrate, nitrite, ammonium, and phosphate, which are ideal for algal uptake, and treatment with algae also has impact on other contaminants and pollutants in wastewaters, such as metals (Craggs et al., 1996). These nutrients are utilized by the algae for growth, and therefore contribute to remediating wastewaters through removal and fixation into the algal biomass (Valeta & Verdegem, 2015). This suggests that the addition of an algae cultivation step after the vegetable production in an aquaponics system is appealing, due to the continued presence of nitrogen and phosphorous in the waste stream that have the potential continued value as fertilization nutrients.

Algae cultivation for wastewater remediation has been investigated using a variety of approaches, reactor designs, and algal taxa and species. The species of algae that have been studied range over the breadth of morphologies and cell types, from single cell microalgae species such as *Chlorella sp.* (Addy et al., 2017) and *Scenedesmus sp.* (Guerrero-Cabrera et al., 2014) to more colonial filamentous macroalgae species *Zygnema sp.* (Zelibor et al., 1988) and *Oedogonium sp.* (Lawton et al., 2017). Algae is hardy and capable of handling a variety of different sources of water, and for growth require the water, accompanying nutrients, and light, with waste and nutrient sources studied including municipal wastewater (Sandefur et al., 2011),

dairy wastes (Mulbry et al., 2008), horticultural wastewater (Liu et al., 2016), and natural waters (Mulbry et al., 2010), among others.

The microalgae species, particularly *Chlorella sp.* were common studies for biofuel applications due to the high lipid content within the biomass (Newby et al., 2016) but have also been studied as a protein source for animal feedstocks, and as a plasticizer, or promoting flexibility, as a revenue stream (Kwan et al., 2015). This is in addition to lactic acid production, which will be examined in more depth later.

Most algae species are usually cultivated in a monoculture, or a single species exclusively raised alone, due to that algae species exhibiting the properties that are most desired by the experiment that can be diluted or obscured if combined with other algae species. This can apply to microalgae species or filamentous species to reflect the goal of the project, such as *Chlorella sp.* due to their lipid content for biodiesel production. This monoculture method is feasible due to the cultivation system usually being closed to external factors such as predation and contamination (Newby et al., 2016). This is usually accomplished in a photobioreactor and a light source, with the algae sealed from the environment with the wastewater providing the nutrients over the course of the growth phase (Guerrero-Cabrera et al., 2014).

The photobioreactor is not the only method of culturing microalgae, as Addy et al. (2017) uses an open raft system to cultivate *Chlorella sp.* along with an aquaponics system centered on tilapia and swiss chard. The microalgae species was chosen to reduce the residual nitrogen and phosphorous levels in the system, and outperformed the vegetables grown in the system in terms of nitrogen removal rate due to their higher nitrogen content in the algae (Addy et al., 2017). Fang et al. (2017) also concluded that an algal based aquaponics system growing *Chlorella vulgaris* in a photobioreactor had a 13% higher nitrogen utilization rate, or a higher rate of

nitrogen removal, than a model media filter aquaponics system growing pak choi, a type of Chinese cabbage. These results signify the potency of the microalgae in nutrient removal, making it an attractive alternate to normal decontamination measures for the wastewater stream, while cultivating biomass for further experiments.

2.3 Algal Turf Scrubber Algae Cultivation

Macroalgae is also capable of recovering nutrients from wastewater but requires a different method of cultivation from microalgae. A common variety for study is the green filamentous algae species, many of which are benthic and require a surface to attach to, where they remain in place and filter the nutrients from the surrounding water delivered to them (Ekong et al., 2019**)**. This can be accomplished via a pond or raceway structure for continuous exposure to the wastewater.

A common method for cultivating filamentous green algae is called the Algal Turf Scrubber (ATS), which are flow way channels with a material substrate covering the bottom of the channel to provide an anchoring surface for the benthic filamentous algae (Adey et al., 2013). Wastewater is continuously recirculated over the lane in a thin layer with periodic surges provided by a mechanism to simulate waves on a shore to better stimulate growth (Ray et al., 2015). The wastewater in the system is sometimes collected in a reservoir at the foot of the lane and is pumped back to the top of the lane, ensuring the continuous recirculation (Liu et al., 2016). These lanes can provide algal growth continuously provided the wastewater is replenished in the reservoirs. ATS systems are typically colonized by residential indigenous community of benthic filamentous algae, which grow over time to form a complex multiple layer turf periphyton community (Adey et al., 2013; Ekong et al., 2019**)**. The algal biomass is

harvested regularly via mechanical means, usually by scraping or with a vacuum (Sandefur et al., 2011) to yield biomass with higher solids content than similar microalgae biomass (Higgins & Kendall, 2012). Following the harvest, the algal turf community repopulates quickly from residual anchor cells on the substrate, resulting in algal biomass growth that can be maintained near indefinitely, provided sufficient nutrient availability. As it grows, the algal community utilizes dissolved nutrients from the overlying water, and the periodic harvests of the biomass removes them from the water system, resulting in cleaner water downstream and an algal biomass with relatively high solids content (Adey et al., 2011).

In studies on applications of ATS, the source of the wastewater has been variable, similar to the microalgae cultivation, with studies covering such sources as horticulture (Liu et al., 2016), agricultural runoff (Bohutskyi et al., 2016), and even oyster aquaculture facility wastewater (Ray et al., 2015). In these studies, a range of macroalgae taxa have been observed as dominant, including *Stigeoclonium sp.* (Liu et al., 2016), *Oedogonium sp.* (Lawton et al., 2017), and *Cladophora sp.* (Sandefur et al., 2011), depending on the water source and local environment conditions. For all these systems, the community was self-generated from a local indigenous inoculum (Aston et al., 2018), and the community that formed was akin to a polyculture scenario of multiple species. These macroalgae species are adept at removing the excess nutrients from the wastewater (Liu et al., 2016) due to their sessile nature and large surface area to volume ratio (Adey et al., 2013). The algal biomass is easily dewatered for further processing, such as a feedstock for biomethane production (Bohutskyi et al., 2016) or for other bioenergy and biomaterial reactions (Lawton et al., 2017). This is due to the algae producing long filaments that extend downstream, allowing the algae to absorb nutrients from the water all along the length of the algae as it floats in the stream.

However, unlike in microalgae studies, studies on ATS macroalgae have found a polyculture community, or many species of algae coexisting, as opposed to a monoculture since most large scale lanes are outdoors which makes maintaining a strict monoculture highly difficult due to potential contamination from external species and predation from grazers (Newby et al., 2016). The same study also suggests that a polyculture makes the whole assemblage more resistant to environmental changes due to the abundance of species dominating the system should another species diminish. This makes the composition of the filamentous algae culture cultivated from an ATS lane a veritable cornucopia of elements and components (Bohutskyi et al., 2016), leading to the emergence of different species depending on environmental conditions and water chemistry.

2.4 Algae Species Present

Since the algae community in many ATS lanes is a polyculture, there can be a plethora of different algae species present at any given time. This diversity enables the culture to adapt to changes with the system, such as seasonal change, water chemistry differences, or nutrient availability (D'Aiuto et al., 2015**)**. As such, if the community is not actively controlled for a specific algae species, the community needs constant monitoring to identify the dominant species shift within the system. The turf community cultured often develops to contain such green algae species like *Cladophora sp., Rhizoclonium sp., Microspora sp.,* and *Tribonema sp,* not to mention the numerable other families and species of algae like diatoms or cyanobacteria that compose a minority of the overall biomass (Adey et al., 2013).

Often, the dominant species shifts over the course of the operation and seasonally, depending on the environmental temperatures, nutrient presence, and time from startup. The turf

community would often shift from cyanobacteria and diatoms to green filamentous algae species depending on environmental conditions and the presence of other competing microorganisms (Craggs et al., 1996). These species directly impact the quality of the biomass harvested, from varying ash content levels (Aston et al., 2018**)**, to varying levels and forms of carbohydrates or lipids (Grayburn et al., 2013**)**. Often, the green algae prevalent in many ATS systems has been shown to be *Cladophora sp.* and *Rhizoclonium sp.* in the fall to the *Microspora sp.* and *Tribonema sp.* in the winter (D'Aiuto et al., 2015**)**, showing the variability of biomass production over the seasons (D'Aiuto et al., 2015**)**.

2.5 Lactic Acid Fermentation

Lactic acid can be produced from two different methods: chemical synthesis or microbial fermentation, with microbial fermentation the more popular process (Abdel-Rahman et al., 2011). The production of lactic acid via microbial fermentation requires a lactic acid producing bacteria (LAB) species, along with a carbon and nitrogen source (Pleissner et al., 2017). The species of lactic acid producing bacteria studied are predominantly *Lactobacillus sp.* (Gordeeva et al., 2017), but other species have been examined, such as *E. coli* strains (Abdel-Rahman et al., 2013) and *Pediococcus sp.* (Gardner et al., 2001). LAB are studied more often compared to other species and processes (Abdel-Rahman et al., 2011), since it is a known commodity for safety in industrial production and product generation (Abdel-Rahman et al., 2013).

The carbon source LAB require most often takes the form of sugars, which are broken down by the bacteria for the energy necessary to power the fermentation process, while the nitrogen source provides the nutrients the bacteria need to propagate and therefore continue the fermentation. The sugars and nutrients available in the feedstock for the fermentation have a

significant impact on the quantity and efficiency of the lactic acid production, thus feedstocks that have high amounts of these sugars and nitrogen compounds make for more desirable sources. Additionally, the structural complexity of the feedstock has a direct impact on the magnitude of the lactic acid produced in the fermentation, with more complex biomass requiring more energy to breakdown, like woody biomass and material with thicker cell walls, and access the nutrients within, if even possible by the LAB, while simpler biomass removes that need for additional energy expenditure that would otherwise be spent on the fermentation of the readily available nutrients.

Since feedstocks for lactic acid fermentation need to provide both a source of easily accessible carbon and nitrogen, a variety of sources have been studied for either additional recyclability or higher yields, frequently food waste and other biomass options, such as algae (Kwan et al., 2015) and catfish and tilapia manure (Shi, Li, & Blersch, 2018; Shi, Li, Guan, et al., 2018).

Gardner et al. (2001) evaluated the results of varying LAB strain compositions for lactic acid and other product production when cultured in a vegetable juice mixture of carrot, cabbage, beet, and onion. The study found that a combination of the LAB *Lactobacillus plantarum, Pediococcus acidilactici,* and *Leuconostoc mesenteroides* had the fastest acidification rate and maintained the resultant lactic acid and sugars intact for up to 90 days (Gardner et al., 2001). The different species had different niches and roles, so the combination of the three accomplished more than a singular species could alone.

Similarly, Pleissner et al. (2017) tested mixed food waste, containing noodles, potatoes, meat, and others, blended together into homogeneity, as a feedstock for *Lactobacillus sp.* and *Streptococcus sp.* The study used simultaneous saccharification and fermentation (SSF) which

combines the breakdown of complex organic compounds and the fermentation of the resulting sugars simultaneously, with minimal enzyme addition. They found that the *Streptococcus sp.* performed best directly converting the food waste into lactic acid, especially when food solid ratios were higher and had the added benefit of producing predominantly lactic acid (Pleissner et al., 2017).

Shi, Li, & Blersch, (2018) and Shi, Li, Guan, et al. (2018) explored lactic acid yields from using catfish and tilapia manure wastes as both carbohydrate and nutrient feedstock, respectively, for lactic acid fermentation. Both waste products are readily available in their respective cultivations and so offer a low-cost input for a feedstock in fermentation experiments. The catfish waste was successful as a feedstock for both nutrients and carbohydrates and holds promise for combination with richer carbohydrate sources to reduce costs while maintaining yields. The tilapia waste was used in conjunction with varying carbohydrate sources and compared with catfish waste and yeast extract for efficiency in providing nutrients to the fermentation. While producing lower yields than the standard yeast extract when used with glucose, the tilapia waste was more promising in conjunction with the more complex cellulosic matter. These studies indicate that biomass collected from aquaculture waste or low priority products can be repurposed to produce a high value product to help offset overall operation costs while reducing waste production.

Abdel-Rahmen et al. (2011) explored lignocellulose, or complex organic biomass in the forms of cellulose, hemicellulose, and lignin often found in plant matter, including hardwoods, grasses, and produce, as a potential feedstock. The study found that although the lignocellulose could provide the required sugars for lactic acid fermentation, the requirement of expensive

enzymes to breakdown the bonds in the lignocellulose into simpler sugars via hydrolysis prevents its use as an industrial mainstay.

Conversely, Shi et al. (2015) examined the potential for Southern Pine wood chips and paper mill sludge, otherwise common paper mill waste products, to provide carbohydrates for lactic acid fermentation. A mix of the hemicellulose sugars obtained from the wood chips and the paper sludge produced lactic acid yields upwards of 60 g/L. This is promising as this process continues the themes of taking byproducts from established systems and producing value products from otherwise lost costs.

These studies are primarily focused on reducing biomass waste produced from common processes and everyday actions, while also identifying potential uses for the waste in ways beyond disposal and neglect. This indicates that the feedstock for microbial lactic acid fermentation can be varied and derive from unorthodox sources, provided the source material can facilitate the nutrient requirements of the bacteria.

2.6 Lactic Acid Fermentation of Algae

Algae, especially photosynthetic green algae species, can be potentially used as a biomass source for the microbial lactic acid fermentation process, like the vegetable biomass studies. This is due to the potential for the algae to contain high levels of glucose, xylose, and other carbohydrates that the LAB can breakdown for easy energy (Shi et al., 2015**)**. This can be an attractive approach since algae is generally much less complex than the vegetable biomass, while still containing the glucose and other desirable sugars, making it easier for the bacteria to get energy while limiting energy expense it could otherwise be using in fermentation. While not as

popular a source as traditional plant matter, some studies have begun examining algae's potential as a carbon source for the fermentation of lactic acid.

Talukder et al. (2012) looked at *Nannochloropsis salina*, a microalgae species, as both a microbial lactic acid fermentation feedstock and as a lipid production source. In order to induce glucose and xylose production, the biomass was hydrolyzed with sulfuric acid, then treated with hexane to remove lipids from the biomass. The defatted algal biomass was then used as a feedstock for the lactic acid fermentation process. The resulting analysis found that the yield of lactic acid compared to the sugar present in the defatted biomass was around 92% at its peak efficiency but would require further modifications to increase the volumetric yield of 0.45 g/l/h for a more cost-effective method of producing lactic acid.

Abdel-Rahman et al. (2013) reviews and analyzes microbial lactic acid fermentation advancements and potential applications in all facets of the process, from the lactic acid producers to potential feedstocks and advanced techniques to better harvest lactic acid. Microalgae is mentioned to be both a lactic acid producer, as well as a feedstock for other lactic acid producers. The species mentioned as a lactic acid producer include *Scenedesmus obliquus* and *Nannochlorum sp.* with the *Nannochlorum sp.* seeming promising. The feedstock biomass sources are mentioned as having the capacity for being highly abundant with the species *Hydrodictyon reticulum* said to consist of around 47.5% reducing sugars, including 35% glucose (Abdel-Rahman et al., 2013).

Aston et al. (2018) analyzed ash content produced from marine filamentous algae cultivated by ATS lanes. While primarily focused on the effects of the application of aqueous alkaline extraction on the algal biomass to remove ash content, the study found that the ash had a significant capacity to influence buffering capacity (Aston et al., 2018). This suggests that ash

content resident in algae biomass will help maintain a stable pH while undergoing lactic acid fermentation, with the magnitude of the effect being reliant on the amount present and can be supplemented by the additional basic buffering agents, such as calcium carbonate.

That is not to say that ATS algal biomass is without difficulties. The algal species present in the lanes can emerge and recede based on a number of factors, such as light level being too low or too high for too long, or the temperature of the water, as lanes left out in the summer sun will frequently be less productive or shift in species composition (Mulbry et al., 2008). This is in addition to generally higher levels of ash content within the algae biomass due to the open nature of the lanes, especially in outdoor systems (Adey et al., 2013). The ash takes the place of useful compounds that lower the effectiveness of the algae as a source for chemical processes. There is also the danger for a local species to enter the system and dominate the algae community away from more desirable species.

If macroalgae can be a viable source of nutrients for the lactic acid fermentation process, similar to their microalgae cousins, the overarching aquaponics system can be made that much more financially lucrative and environmentally friendly with the addition of Algal Turf Scrubber lanes and the filamentous algae they produce. Otherwise, the macroalgae will have less appeal as a lactic acid source material, except as a teaching resource or for further study while still providing a secondary filter step for the wastewater. However, further cost analyses would need to be performed to determine overall impact and effectiveness.

Chapter 3: Methods

The study is focused on the growth of algal biomass in laboratory-scale ATS lanes for use as the feedstock for lactic acid fermentation. The approach was first to investigate the optimum conditions for algae growth in aquaponics wastewater by following a dilution experiment. Next, the dilution that showed the highest productivity was employed to produce algal biomass to supply subsequent fermentation experiments. Finally, the biomass was used in lactic acid fermentation under various treatments and amendment conditions to investigate the conditions resulting in the greatest yield of lactic acid.

3.1 Aquaponics Wastewater Source

The water for the experiment is sourced from an aquaponics system on the campus of Auburn University that produces Nile Tilapia and vegetables for on-campus dining and experimentation. Approximately 10,000 tilapia are raised in two indoor15 m^3 tanks within a 10m x 33m greenhouse, with regular daily feedings using commercial feed consisting of 36% crude protein and additions of a hydrated lime slurry to control the optimal pH of 6.5. The system uses a modified biofloc biofiltration system where around 5% of the tank volume is replaced daily with fresh water and microbes within the system help control ammonia levels by nitrification into nitrate. Water from the tank is also moved continuously via airlift pump to a series of two gravity clarifiers outside the greenhouse that remove large particles present from the wastewater, which include uneaten feed, fecal matter produced by the fish and microbial biofloc generated in the tank.

Clarified water is then pumped one way by demand into an adjacent 10m x 33m greenhouse to irrigate various vegetable crops, such as cherry tomatoes and cucumbers through a system of drip feeds and planters. Water intake from the tilapia tank is controlled by an irrigation controller which can be adjusted as called for by the experiment. Water quality, such as pH and electrical conductivity, was monitored within the greenhouse and corrected as needed.

Excess unused wastewater from the plant irrigation in the plant greenhouse is collected by a gravity drain system into a series of four 100 gal underground sumps. These sumps collect the irrigation drainage from the various zones in the greenhouse to allow for experimentation of different system variables for the plant crops in the plant greenhouse. Water from these sumps in turn is pumped on demand by float switches to an open above ground 250 gallon collecting and mixing tank located near the plant greenhouse. The collecting tank provides water on demand to an outdoor experimental ATS system located at the facility, in addition to the indoor ATS system described in the experiment.

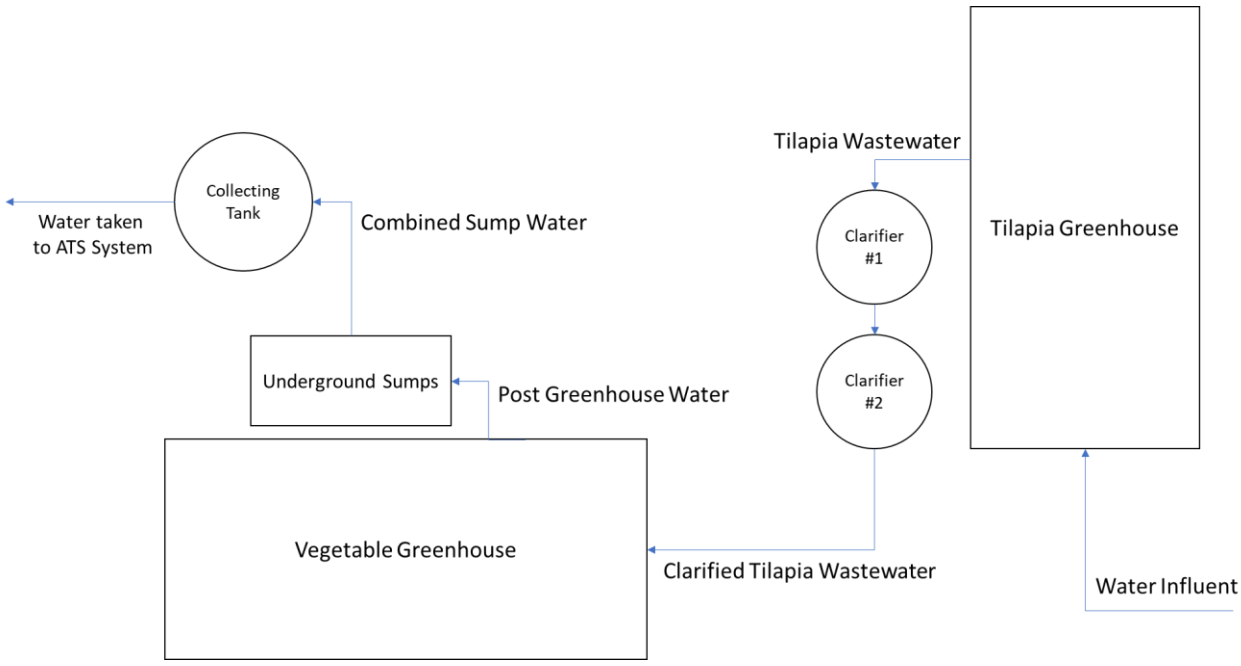

Figure 3.1. Overhead scheme of the aquaponics system and water flow from influent into the tilapia tanks to transportation for use in the ATS.

Figure 3.2. Pictures of the individual components of the aquaponics system, clockwise from top left: the tilapia greenhouse, the outdoor clarifiers, underground sump cover and above ground collecting tank and the vegetable greenhouse.

3.2 Algal Turf Scrubber Experimental System

The algal turf scrubber (ATS) system used for these experiments was maintained indoors in the laboratory and consisted of four plastic PVC residential rain gutters acting as lanes, labelled lanes 1, 2, 3, and 4, respectively, parallel with each other. Each lane had 2 Lithonia

Lighting shop lights with Philips F32T8/TL950 fluorescent bulbs hanging on average 7.6 cm over the lanes to provide light for the algae, with timers used for a sixteen-hour light, eight-hour dark cycle to represent a normal day night cycle. The lanes are 274 cm long and with a 5 cm wide polyethylene netting mesh (Industrial Netting XV1672) laid within and along the lane to act as a colonizing substrate for the algae to attach and grow. The total growth area provided by the mesh screen and under the overhead lamps was 1368 cm^2 for each lane. The water for the systems is provided and stored in five-gallon bucket reservoirs at the foot of each lane, with magnetic drive centrifugal pumps (Danner Supreme Classic MD3, Danner Manufacturing Inc.) and PVC pipe and vinyl hose plumbing moving the water back to the head of the lane as a recirculating system. The system runs continuously until powered off for harvest, which was performed every 7 to 10 days and thus affording an average growth time of seven to ten days. The water is disposed after each harvest in its entirety and replaced using freshly acquired water from the collecting tank connected to the aquaponics system described previously and treated as the phase of algae production required.

The algae community within the ATS was predominantly cultivated by the various algae species transported into the system from external water sources, like the aquaponics system sourcing the experiment, making the algae representative of indigenous algae species including the ever present cyanobacteria and diatoms, as well as various filamentous algae species. Intermittent algae samples taken from the community and examined under the microscope revealed that species such as *Cladophora sp.*, *Rhizoclonium sp.*, *Microspora sp.*, and *Oscillatoria sp.*, were variably present and predominant over the course of the productivity experiments.

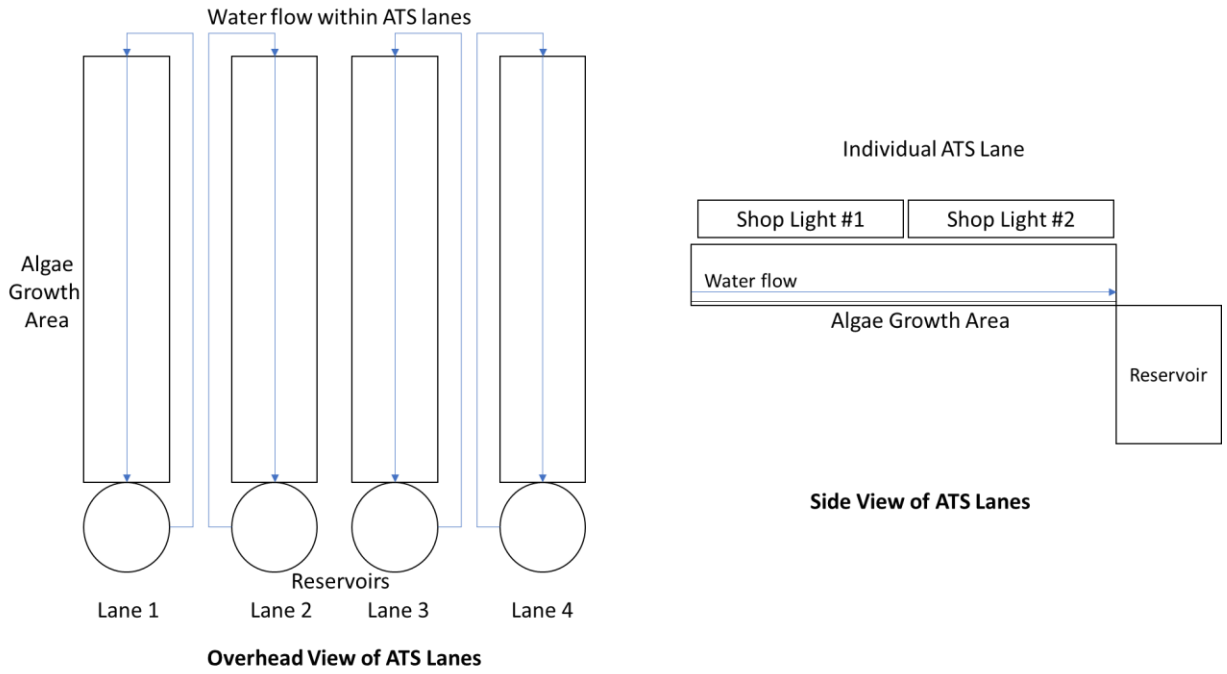

Figure 3.3. Left to right: Overhead and side plan views of the indoor ATS system.

Figure 3.4. Pictures of the indoor ATS system used in the experiment, left to right: the four lanes used in the experiment and a side view of an individual lane.

The harvest procedure begins with the power being cut to the system to allow as much water as possible to drain back into the reservoir tank, since the algae is difficult to move and work with if not allowed to initially drain. A plastic particleboard scraper is used to mechanically scrape the algae that grows on the side of the lane into the center and to slowly

move and corral the accumulating algae biomass down the lane and towards the reservoir. This biomass is collected and placed on a drying rack consisting of two different meshes, one a fine mesh bag and an underlying wider plastic mesh that acts as a support to hold the mesh and algae over a plastic trash bin to collect the water dripping from the algae. Most of the remaining algae in the ATS lane is collected using a vacuum pump with a small hose and vacuum flask and added to the prior biomass to collectively drain most of the remaining moisture. Once the algae is dry enough to be rolled into a loose ball for transport, the collected algae biomass is taken and placed on a final drying rack and mesh and allowed to air dry over the next couple days to an average moisture content of 10%. The water in the reservoirs was disposed of at the end of each harvest and replaced with new water from the aquaponics system and treated as necessary for each lane. This process is repeated every seven days for each lane. The total harvest's worth of the algae from all four lanes is taken a few days later to be weighed, labelled, and stored individually in sample bags for future lactic acid experiments.

3.3 Dilutions

The raw water collected from the aquaponics system tended to produce algae that was most similar to cyanobacteria and other non-green filamentous algae when utilized in the ATS system, as determined through observations and lactic acid fermentation tests on preliminary algae growth trials not reported here.

A dilution test was performed to investigate the effect on growth rates of filamentous green algae species that would potentially provide the qualities desired in the lactic acid fermentations. The test consisted of a growth experiment using various dilutions of the same source wastewater from the aquaponics systems. The water collected from the aquaponics

system was diluted with dechlorinated tap water created by thoroughly mixing upwards of 30 gallons of municipal tap water in a plastic waste bin with approximately 1 mL of a concentrated sodium thiosulfate solution, a standard dechlorinating solution to minimize adverse chlorine effects on the microbiota. The solution consisted of 25 g sodium thiosulfate crystals (Proline Water Conditioner) and 500 mL deionized (DI) water and mixed until the crystals were fully dissolved. The dilutions for each lane were produced by mixing the amounts of the pretreated tap water and the raw wastewater together in a 5-gallon bucket as called for by the dilution using a smaller 2 L measuring bucket. The DI water dilutions tested were undiluted, 2x, 4x, and 8x for a full strength, 1/2 strength, 1/4 strength, and 1/8 strength wastewater concentrations, again respectively, and will be referred to interchangeably in regards to dilution. These dilutions were used for lanes 1, 2, 3, and 4, respectively, and were in effect for 11 harvests and growth periods from April 17, 2019 to August 22, 2019.

3.4 Algae Production Phase

The half dilution of wastewater and half dechlorinated tap water produced the highest algae biomass yields while the filamentous green algae species were dominant (see Results and Discussion section). Thus, the remaining lanes were transitioned to this dilution for biomass production for the subsequent lactic acid fermentation tests, which covered 13 harvests and growth periods from September 3, 2019 to January 15, 2020.

3.5 Nutrient Analysis of Aquaponics Wastewater

Nutrient levels were tracked throughout the aquaponics system to gauge the system's effectiveness in removing common eutrophication compounds like nitrate and phosphate. Water

samples from the aquaponics system at the system's water influent, the fish tank, the outdoor clarifier, and the underground sump outside plant greenhouse were collected every 7 to 10 days over the course of a year (Kalvakaalva 2020). The water samples were then filtered at 0.2 mm and stored at -80℃ until the end of sampling period and were then analyzed for soluble ion concentrations via high pressure liquid chromatography (HPLC; Shimadzu Prominence System,Kyoto, Japan) using an anion exchange column (Dionex AS22, ThermoFisher Scientific, USA) and ion suppressor (Dionex AERS 500, ThermoFisher Scientific, USA) (Kalvakaalva 2020). The final concentrations are recorded in Table 3.1.

Table 3.1. Raw aquaponics wastewater nutrient levels over the course of both dilution and production phases of algal biomass cultivation. (Kalvakaalva 2020).

Date	Nitrate mg/L	Phosphate mg/L	Chloride mg/L
1/9/2020	178.11	21.83	45.65
12/5/2019	129.06	20.56	40.71
11/14/2019	84.33	18.45	24.62
11/8/2019	103.01	19.99	31.12
10/30/2019	117.48	21.95	39.20
10/16/2019	385.70	32.86	171.74
10/9/2019	210.39	24.41	110.71
10/2/2019	145.37	20.21	71.78
9/25/2019	129.95	25.52	83.11
9/18/2019	104.13	16.04	62.62
9/11/2019	415.37	37.78	142.43
9/4/2019	411.87	42.98	71.27
8/28/2019	395.73	52.31	91.06
7/24/2019	529.87	44.10	118.96
7/10/2019	563.43	34.62	259.00
6/19/2019	480.25	32.10	173.30
6/12/2019	346.63	28.41	95.30
5/22/2019	380.42	33.24	77.34
5/15/2019	498.19	37.71	97.36
5/10/2019	277.17	28.51	63.19
5/3/2019	431.93	17.35	137.00
4/26/2019	195.75	17.55	101.19

However, since the water sampling dates do not align perfectly with the dates of water collection for the ATS system, a method to approximate the water nutrients levels was used in order to better understand the effects of the nutrient levels on the algal productivity and ash content of the system. This was accomplished by taking the dates that fell close to the date the water was gathered for each harvest, usually the day of or day before harvest, and recreating the list of dates with the corresponding harvest dates. This led to some harvests from both the dilution and production phases not having a corresponding set of nutrient levels as shown in Figure 3.5. This was approximated by interpolating the two closest surrounding dates and nutrient levels for the missing date and using the resultant nutrient levels for that date as shown in Table 3.2.

Dilution Phase	
Water	Algae
Test Date	Harvest
"8/15/2019"	8/22/2019
7/24/2019	8/15/2019
7/10/2019	7/27/2019
"6/27/2019"	7/11/2019
6/19/2019	6/27/2019
6/12/2019	6/20/2019
"6/6/2019"	6/13/2019
"5/30/2019"	6/6/2019
5/15/2019	5/30/2019
5/3/2019	5/16/2019
4/26/2019	5/3/2019

Production Phase	
Water	Algae
Test Date	Harvest
1/9/2020	1/15/2020
"12/21/2019"	1/5/2020
"12/11/2019"	12/21/2019
12/5/2019	12/11/2019
"11/23/2019"	12/4/2019
11/14/2019	11/23/2019
11/8/2019	11/13/2019
10/30/2019	11/6/2019
"10/23/2019"	10/30/2019
10/16/2019	10/23/2019
9/25/2019	10/16/2019
9/11/2019	9/27/2019
9/4/2019	9/10/2019

Figure 3.5. Dates where the water sampling data does not align with ATS harvest dates. The highlighted and quotation mark dates are the missing data dates. The harvest date is the day the biomass is harvested, and the next harvest's water would be used for the harvest, gathered either that day or the day before.

Table 3.2. Dates and nutrient data used for the interpolation of the missing water quality measurements. The dates in quotations are the missing data dates and the red nutrient level data are the interpolated results of the surrounding dates with recorded data.

Date	Nitrate mg/L	Phosphate mg/L	Chloride mg/L
1/9/2020	178.11	21.83	45.65
"12/21/2019"	151.48	21.14	42.96
"12/11/2019"	137.47	20.78	41.55
12/5/2019	129.06	20.56	40.71
"11/23/2019"	106.70	19.51	32.66
11/14/2019	84.33	18.45	24.62
10/30/2019	117.48	21.95	39.20
"10/23/2019"	251.59	27.41	105.47
10/16/2019	385.70	32.86	171.74
9/4/2019	411.87	42.98	71.27
"8/28/2019"	445.55	49.26	101.42
7/24/2019	529.87	44.10	118.96
7/10/2019	563.43	34.62	259.00
"6/27/2019"	511.94	33.06	205.95
6/19/2019	480.25	32.10	173.30
6/12/2019	346.63	28.41	95.30
"6/6/2019"	356.29	29.79	90.17
"5/30/2019"	367.55	31.40	84.18
5/15/1019	498.19	37.71	97.36

3.6 Productivity measurements

The yield of the algal biomass produced from the growth experiments does not clearly convey the quality of the algae for fermentation, as the fermentation focuses on the available sugars in the biomass and the algae biomass is composed of other components that are less desirable and do not actively contribute to the reaction. One method to account for this is to discount the moisture and inorganic components of the biomass for calculating the available algae matter for future utilization.

The algae biomass harvested from the two growth experiments were tested for moisture content and ash percentage. The algae was air dried for 48 hours after harvest, and analysis of the residual moisture content of the algae was performed by weighing and running

approximately 0.5g of algae biomass in an Ohaus MB45 Moisture Analyzer. The analyzer returned a percentage that constituted the moisture presence in the sample, on average 10% of the total air-dried algae biomass across the different experiments. The ash content represents the portion of algae that is composed of inorganic material and was tested by following standard ash content procedure. This entails taking the moisture free algae after being run through the moisture analysis and weighing the combined algae and weighing pan. The algae and pan were placed in a muffle furnace, which heats the contents to 500°C before slowly lowering the temperature back down to the starting 100°C temperature. The algae and pan were removed and weighed after allowing to cool to room temperature within a desiccator. The ash content is then calculated by dividing the combined algae and pan weight by the algae and pan weight pre vaporization for an ash percentage.

The production of usable algae biomass in the growth experiments was measured by determining the algal productivity, P, in g/m^2/day,

$$P = \frac{Y}{A * t}$$

where Y is the dry weight of the algae available for fermentation in grams, A is the growth area of the ATS lane containing the algae in square meters, and t is the number of days grown in the system, as determined by the harvest interval. Y is determined by subtracting the moisture and ash contents from the air-dry weight of the harvested algae samples. As stated previously, A is 0.1368 m^2 for each lane and t was usually 7 days.

3.7 Lactic Acid Fermentation

The algae harvested from the ATS lanes needed additional preparation before utilization in the fermentation experiments. The air-dried algae biomass was ground using a coffee grinder

to break down larger algae clumps to promote accessibility for the microbes later in the fermentation. The fermentation tests required algae predominantly ground to a size, that when sifted, was contained between 1 mm opening mesh and 250 μm opening mesh. Algae particle size too large is harder for accessibility in fermentation procedures and particle sizes too small are little more than dust and consolidate when settling, causing a limitation on mass transport during the fermentation process.

Algae from the early dilution experiment and from the production phase were chosen for lactic acid fermentation experiments and had approximately 70-80% of the biomass from each harvest mixed together by shaking a large container to form a composite sample, since individual harvests did not produce enough algae to support a fermentation experiment. This composite biomass also had its moisture content and ash percent measured through the methods described earlier. This algae biomass was combined with deionized water to create a slurry at 20% and 10% solid loading, dilution phase feedstock and production phase feedstock, respectively, since higher solid loadings were observed to become too viscous for later samplings.

Pretreatment via heat and pressure was performed due to fermentation sources usually needing additional preparation to yield higher fermentation yields and was subsequently tested to gauge whether it was even necessary for the algae biomass to perform optimally for the fermentation procedure. This was tested by having two batches of the production phase algae slurry described previously with one batch receiving pretreatment and the other without. The pretreatment was performed with a Parr Instrument Series 4560 Mini Reactor that continuously stirred the algae slurry while heating to 160°C and pressure cooking from the resulting pressure build-up for 30 minutes.

Fermentation reactions were performed in 100mL glass serum vials. A total volume of 50mL for each serum vial was composed of predominantly algae slurry, in addition to 3mL of inoculum containing *Lactobacillus pentosus* (ATCC-8041) and cellulase enzyme (Novozyme C-tec2) at around 15 FPU/g-solid enzyme loading, in addition to the presence or absence of 0.75g yeast extract (YE) and a varying amount of calcium carbonate ($CaCO_3$) calculated from the available sugars within the algae biomass of the sample at a rate of 0.55g $CaCO_3$/ g sugar. The cellulase is used to hydrolyze the polysaccharides in the algae biomass into their monosaccharide components for easier bacteria use. The yeast extract provides additional nutrients to the lactic acid producing bacteria (LAB) and the $CaCO_3$ to sugar ratio is designed to compensate for the change in pH from the production of lactic acid which is connected to the utilization of the sugar within the system. This is to ensure the lactic acid fermentation process goes to completion by buffering the solution to prevent the lactic acid produced from lowering the pH too low and inhibiting the fermentation.

Each algae phase feedstock fermentation vial treatment was done in duplicate, with vials that had no added YE or $CaCO_3$, vials with YE but no $CaCO_3$, vials with $CaCO_3$ but no YE, and vials with both YE and $CaCO_3$. In addition, vials with algae with no pretreatment in addition to the presence of YE and $CaCO_3$, were also run in the pretreated algae fermentation to gauge if the pretreatment was required for optimal lactic acid yields. Due to results from the pretreated fermentation, the fermentation test was later repeated with algae that was not pretreated. All vials were then subjected to the addition of nitrogen gas to drive out as much oxygen in the vial as possible to ensure anaerobic conditions before sealing with caps and sterilized by autoclave at 121℃ for 15 minutes.

After the serum vials were prepped, sealed, and sterilized, they were placed in an incubator-shaker set to 37°C and shaken at 200 rpm. Approximately 0.6 to 1 mL samples of the vials were taken at set hour increments with a syringe and needle and placed into microcentrifuge vials. The sampling times range from samples taken every six hours during the first 24 hours of the fermentation to 12-hour increments for the next two days, to eventually every 24 hours afterwards, stopping when the vials cease to produce byproduct carbon dioxide gas when the needle head of the syringe was inserted into the cap, an indication the fermentation is still operating. The fermentation process usually takes four to five days to reach the end of the lactic acid production within the vials due to the depletion of available sugars within the feedstock.

Samples were collected for every vial and frozen in a freezer at -20°C until all samples were collected. The samples required dilution for the High Performance Liquid Chromatography (HPLC) to read, so the thawed samples were microcentrifuged at 18000 rpm and approximately 150 microliters of supernatant collected and diluted with 450 microliters DI water using pipettes, with the resulting dilution factor recorded for each vial. This was done to remove all solids from the sample, since even small particles can interfere with the HPLC process by clogging the columns used in analyzing the samples. The samples were then transferred to glass vials designed for the HPLC and capped.

The HPLC is able to test for various components of a mixed liquid sample by pumping the liquid at a constant flow through a column that contains numerous tiny beads that are specialized to interact with specific compounds. The HPLC detects the interaction between the beads and the liquids and records the time it takes for the liquid components to travel through the column, resulting in the retention time. The HPLC then produces a retention time versus

intensity graph, with the retention time identifying the component and the area under the peaks indicating the amount of the component.

The HPLC system used for both the lactic acid and sugar concentrations analysis in the study was comprised of an autosampler, LC-20 AD pump, and a RID-10A detector (Li et al., 2018). In order to identify both the sugars within the algae samples and the yields of lactic acid from the fermentation, the HPLC requires two differently designed columns, the Aminex HPX-87P and the Aminex HPX-87H, to test for sugars and organic acids like lactic acid, respectively. The lactic acid fermentation analysis was performed with a mobile phase consisting of 5nM sulfuric acid at 0.6mL/min and the Aminex HPX-87H column was kept at 45℃ (Li et al., 2018). The sugar concentration analysis was performed with a mobile phase of nano-pure water also running at 0.6mL/min, while the Aminex HPX-87P column was at 85℃ (Li et al., 2018).

At the start of the HPLC, vials are run that contain known quantities of the sugars or lactic acid to establish a baseline reference for comparison with the unknown levels within the samples. The sugars analysis tested which sugars were potentially available within the algae biomass, which were glucan, xylan, galactan, arabinan, and mannan as discussed previously and were performed first to help gauge whether the available sugars within the biomass would be enough for fermentation before proceeding to the fermentation tests and recorded. The lactic acid samples would then be run afterwards, this time with solutions with known lactic acid levels to provide a comparison to the unknown fermentation samples. These known quantity vials of both sugars and lactic acid are then followed by vials of the fermentation samples taken over the course of the fermentation. Between each vial containing a sample for the HPLC to run, a blank containing DI water is run to flush the system between each sample. As the HPLC runs through the samples, a series of the retention time versus intensity charts are generated for each sample

and require analysis to determine the presence and quantity of the relevant component within the sample. These resultant values were then exported to Excel and made into charts and tables for further analysis.

The fermentation results were reported based on the lactic acid concentration of the fermentation vial, as determined by the HPLC and converted to g/L, and lactic acid yield, or the percent of lactic acid over the amount of available sugars within the feedstock. Since sugars are one of the primary drivers of the fermentation in that they provide the energy for the fermentation, yield is a check to see how effectively the biomass is being utilized as a feedstock. The sugar level is represented by the glucan percent of the biomass, as determined by the HPLC sugars analysis, since glucan is the primary provider of energy out of the five different carbohydrates tested.

3.8 Statistical Analyses

The ash free dry weight productivity and ash content data for the dilution and production phase algal growth were analyzed for the effect and significance that cultivation factors such as nitrate, phosphate, chloride, and the N:P ratio in the aquaponics wastewater had on productivity, in addition to the effect of time and the impact of dilution for the dilution phase and lane for the production phase. The nutrient levels and time were analyzed using linear regression, while the dilutions and lanes were compared by One-Way ANOVA and post-hoc Tukey Pairwise Comparisons to determine the variance between dilution or lane. These analyses were performed using Minitab 19.

The lactic acid yields of the dilution phase algae and the pretreated and unpretreated production phase algae were analyzed by One-Way ANOVA to establish the impact and

significance of each treatment containing or not YE and Ca had on lactic acid yields. Tukey Pairwise Comparisons were also performed to gauge whether any of the specific treatments had more of an impact than the others. These analyses were also performed using Minitab 19.

Chapter 4: Results and Discussion

4.1 Algal Productivity Experiments

Results were obtained from two sets of growth trials. Dilution experiments were performed to investigate the role of nutrient concentrations on growth rates and biomass productivity. A production trial was performed using one wastewater dilution ratio to expand biomass production to support subsequent fermentation trials. Results from each of these growth experiments yield perspective into algal biomass cultivation from aquaponics wastewater.

4.2 Dilution Experiment

Results from the dilution experiment tracked the ash free dry weight (AFDW) productivity of the algae biomass over time from the four different dilutions of aquaponics wastewater across the four lanes in the ATS system. Results showed a range of productivities correlating with dilution ratio (Figure 4.1). The AFDW productivity was generally higher at the 1/2 strength concentration, producing higher biomass productivities of green filamentous algae species than the other three concentrations. Similarly, the undiluted condition generally showed high productivity as well, whereas greater dilution ratios (1/4 and 1/8 concentrations) exhibited lower productivity. A general increasing trend in productivity was observed throughout the time of the experiment for all trials, with productivity values starting low in the ranges of 0 to 4 g $m^{-2}d^{-1}$ across all dilutions in April and May and eventually reaching in the range of 6 to 12 g $m^{-2}d^{-1}$ in August.

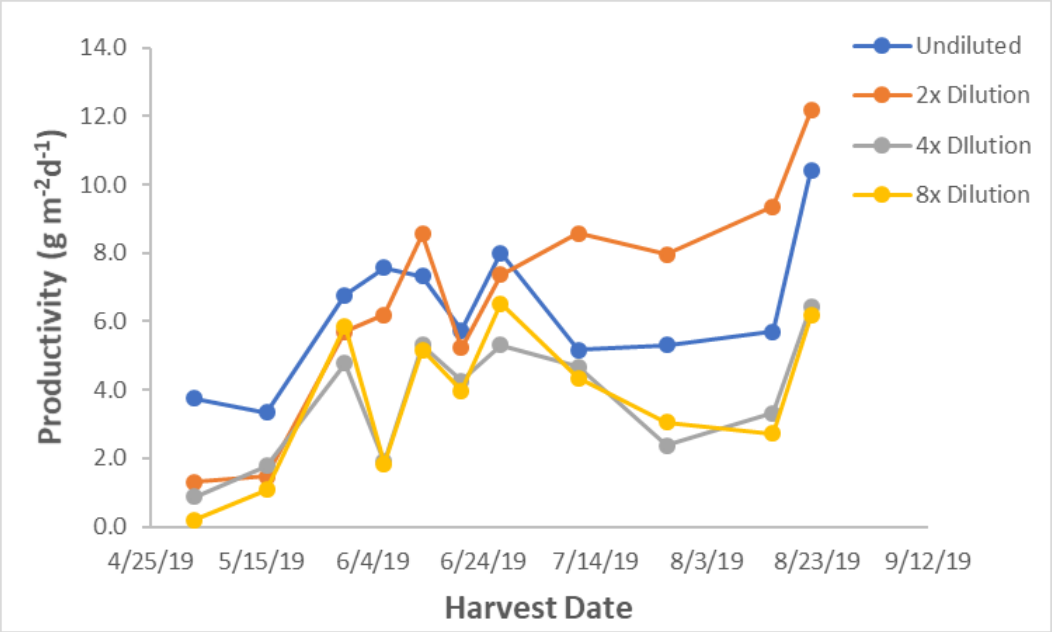

Figure 4.1. Ash free dry weight productivity of the algae grown at the various dilution strengths over the course of the dilution experiment.

The total dry weight productivity, or the moisture free dry weight of the algae harvested without excluding the inorganic portion of the algae biomass, results and trajectories are nearly identical to the total ash free dry weight productivity, as seen in Figure 4.2, with higher overall productivities of around 2 to 3 g $m^{-2}d^{-1}$ each lane and similar overall placements for all four lanes. These results are to be expected when the inorganic material within the algae biomass are not deliberately excluded from the overall algal biomass harvested.

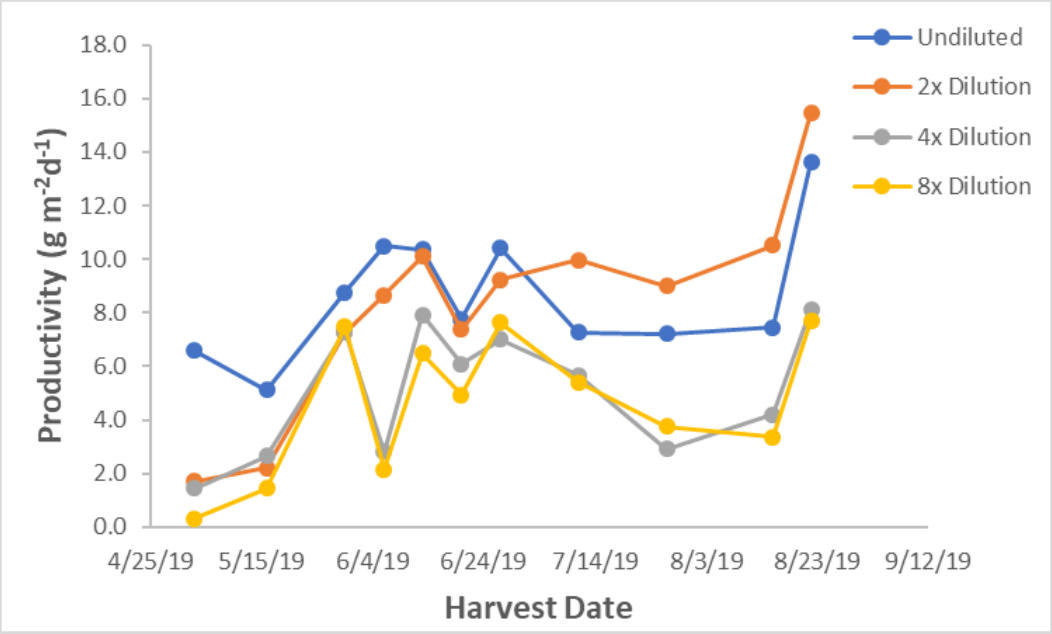

Figure 4.2. Total dry weight productivity of the various dilutions over time in the dilution experiment. Note that the ash is included in this measurement, resulting in higher overall yields.

A linear regression analysis on the trend in the time series for each trial condition shows that all four conditions are not significant (Table 4.1), suggesting that there is not a significant trend of increasing productivity over time (Appendices A.1, A.2, A.3, A.4).

Table 4.1. Linear regression comparisons between dilutions' ash free dry weight productivity over time.

Dilution	Degrees of Freedom	F statistic	P value
Undiluted	10	3.18	0.108
2x	10	2.20	0.172
4x	10	3.16	0.109
8x	10	2.20	0.172

Mean AFDW productivities across the entire time showed that greater productivity in the undiluted and ½ concentration dilutions, 6.28 g $m^{-2}d^{-1}$ (s=1.93) and 6.71 g $m^{-2}d^{-1}$ (s=3.10), respectively, and lower productivities in the 1/4 and 1/8 concentration dilution conditions, 3.73 g $m^{-2}d^{-1}$ (s=1.70) and 3.72 g $m^{-2}d^{-1}$ (s=2.03), respectively (Figure 4.3). One-Way ANOVA analysis of the effect of dilution on productivity indicates that dilution does have a statistically

significant effect (Degrees of Freedom: 43, F statistic: 5.10, P value: 0.004) (Appendix A.5). Post-hoc analysis by Tukey's comparisons test found that the 2x dilution and undiluted treatments were grouped together, and treatments undiluted, 4x and 8x dilutions were grouped together, showing that the 2x dilution was the most significantly different of the four treatments (Appendix A.5).

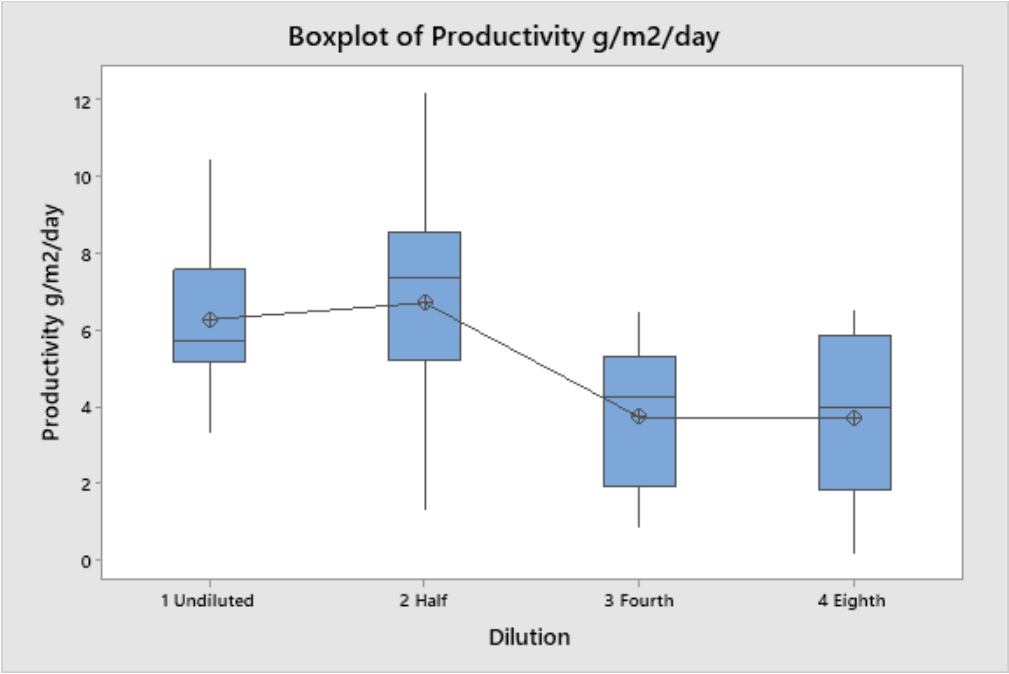

Figure 4.3. Boxplot of ash free dry weight productivity compared across the four dilution concentrations tested. The points connected by lines are the means for each set of data.

Analysis by linear regression showed that the dilution experiment AFDW algal productivity across all four dilutions was significantly affected by all four nutrients tested: nitrate, phosphate, chloride, and N:P ratio (Table 4.2) (Appendices A.6, A.7, A.8, A.9).

Table 4.2. Linear regression analysis comparisons of all dilution phase ash free dry weight algal productivity values over select nutrient levels present in the aquaponics wastewater.

Nutrient	Degrees of Freedom	F statistic	P value
Nitrate	43	11.78	0.001
Phosphate	43	23.57	0.000
Chloride	43	4.49	0.040
N:P Ratio	43	6.58	0.014

The half dilution algal ash free dry weight productivity was the highest of the four dilutions tested, with the undiluted dilution second. These results back up the visual observations during the harvest periods where the 2x dilution seemed to produce noticeably higher quality and quantity filamentous green algae, while the biomass from the undiluted dilution, while high quantity, was lacking in the green filamentous algal community displayed by the half dilution, with the species present seemingly more like cyanobacteria and similar less complex structure species. The higher dilutions, 4x and 8x, were together in producing the lowest algal productivity, with both lanes having near identical ranges and means. This would suggest that these dilutions are too dilute and thus low in nutrient availability to produce the higher productivity of the algae community seen in the undiluted and 2x dilutions.

These results are interesting in that they indicate that the dilutions have a significant impact on overall productivity, and this was confirmed via One-Way ANOVA that put significant impact of dilution on productivity (Appendix A.5). However, that the 2x dilution was the most productive indicates that more nutrient availability is not the sole determining factor, otherwise the undiluted would be the highest. This suggests that some reduction of the wastewater strength promotes the advantageous algae biomass seen in the 2x dilution and not the undiluted. Further analysis of the effect of time on productivity via ANOVA showed little significance (Appendices A.1, A.2, A.3, A.4), indicating little seasonal impact on productivity, which makes sense due to the indoor ATS system. The significance of all four nutrients tested: nitrate, phosphate, chloride, and N:P ratio on productivity (Appendices A.6, A.7, A.8, A.9) gives additional credence to the wastewater having impact on the algae community, with fine tuning required for determining the exact optimal zone.

The dilution experiment succeeded in producing additional filamentous green algae for further experimentation, although additional testing to more firmly establish parameters is needed to produce the filamentous green algae consistently, such as confirming optimal nutrient levels preferred by the algae and further diligence in collecting and harvesting the system to minimize human error.

4.3 Production Phase

During the production phase trials, algae was grown on aquaponics solution at the 2x dilution as determined from the dilution experiment across all four ATS lanes. Generally, productivity was similar across all four lanes, with slight variation across the lanes. The ash free productivity was overall highest in Lane 3 over the course of the timeframe. The three remaining lanes tended to trade places in order of productivity with Lane 1 tending the lowest towards the end of the experiment. The overall values fluctuated between lows of 4 g $m^{-2}d^{-1}$ and highs of 10 g $m^{-2}d^{-1}$ with the lanes trending together downward at the end.

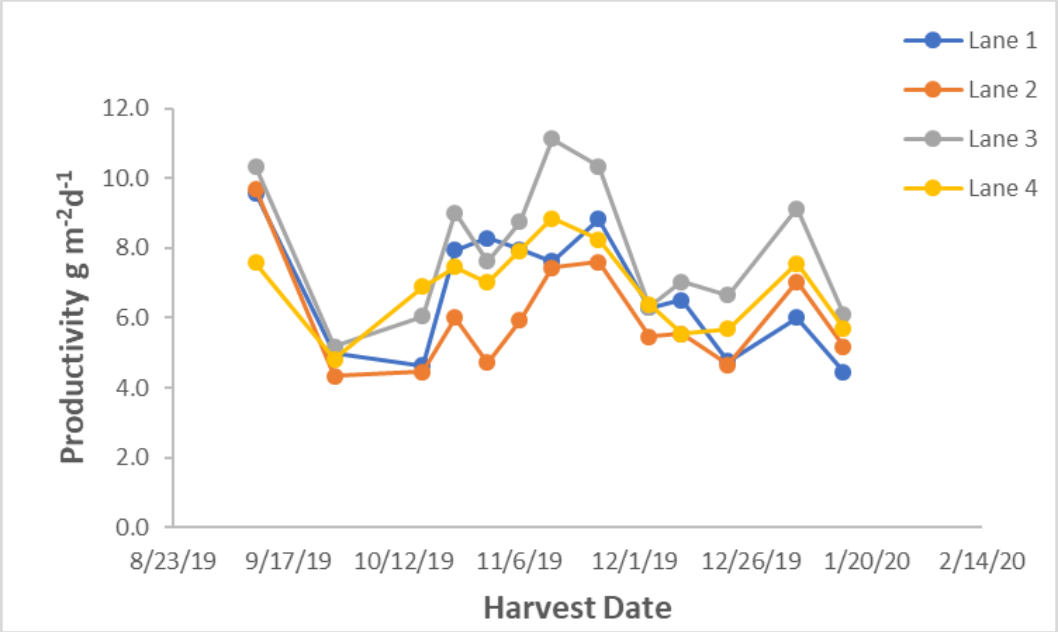

Figure 4.4. Production phase ash free dry weight productivity over time. Each lane ran at 1/2 dilution as determined from the dilution experiment.

The total dry weight productivity of the algae reflected similar results to the ash free productivity, with Lane 3 again being the highest overall productivity and the other three lanes trading places over the course of the phase. Again, the overall values were a few grams higher and results trended the same way over time.

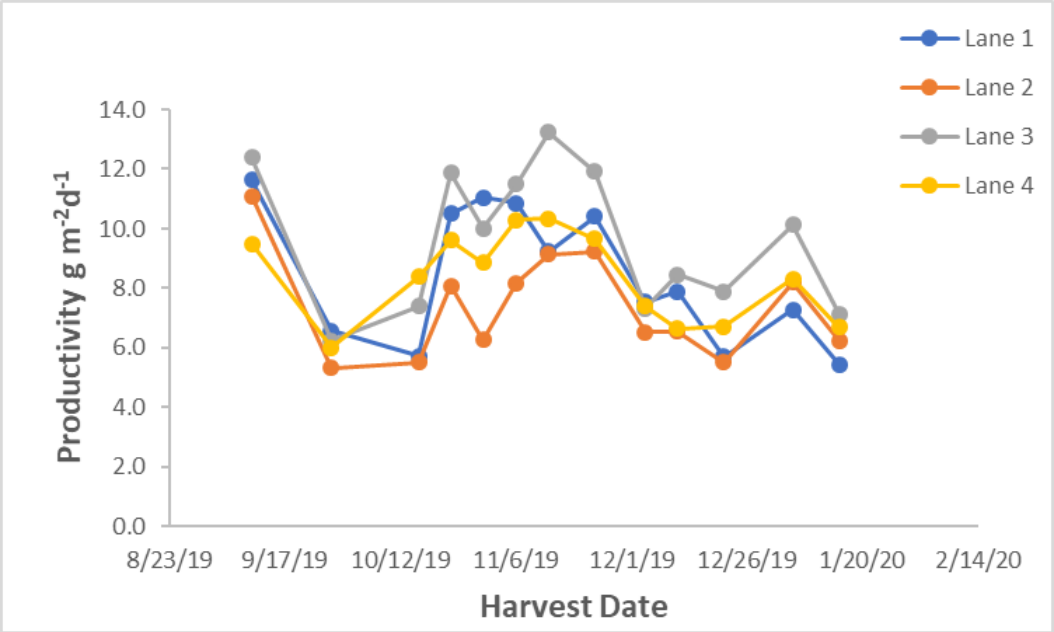

Figure 4.5. Production phase total dry weight productivity over time. Ash content is not excluded like ash free dry weight productivity.

A linear regression analysis on the trend in the time series for each lane's productivity shows that the trends in all four lanes are not statistically significant, suggesting that there is not a trend of increasing productivity over time (Table 4.3) (Appendices A.10, A.11, A.12, A.13).

Table 4.3. Linear regression comparisons between lanes' ash free dry weight productivity over time.

Lane	Degrees of Freedom	F statistic	P value
1	12	2.69	0.129
2	12	0.53	0.482
3	12	0.26	0.618
4	12	0.28	0.607

Mean AFDW productivities over time showed greatest productivity in Lane 3, at 7.98 g $m^{-2}d^{-1}$ (s=1.86), with Lanes 1, 2, and 4 more alike at slightly lower productivities, with 6.76 g $m^{-2}d^{-1}$ (s=1.66), 6.01 g $m^{-2}d^{-1}$ (s=1.50), and 6.90 g $m^{-2}d^{-1}$ (s=1.15), respectively (Figure 4.6) (Appendix A.14). One-Way ANOVA analysis of the effect of lane on productivity indicates that lane does have a statistically significant effect (Degrees of Freedom: 51, F statistic: 3.24, P value: 0.030) (Appendix A.14). Post-hoc analysis by Tukey's comparisons test found that Lanes 3, 4, and 1 were not significantly different, and Lanes 4, 1, and 2 were not significantly different, indicating that Lane 3 was most significantly different to Lane 2 as the highest and lowest productivity mean lanes (Appendix A.14).

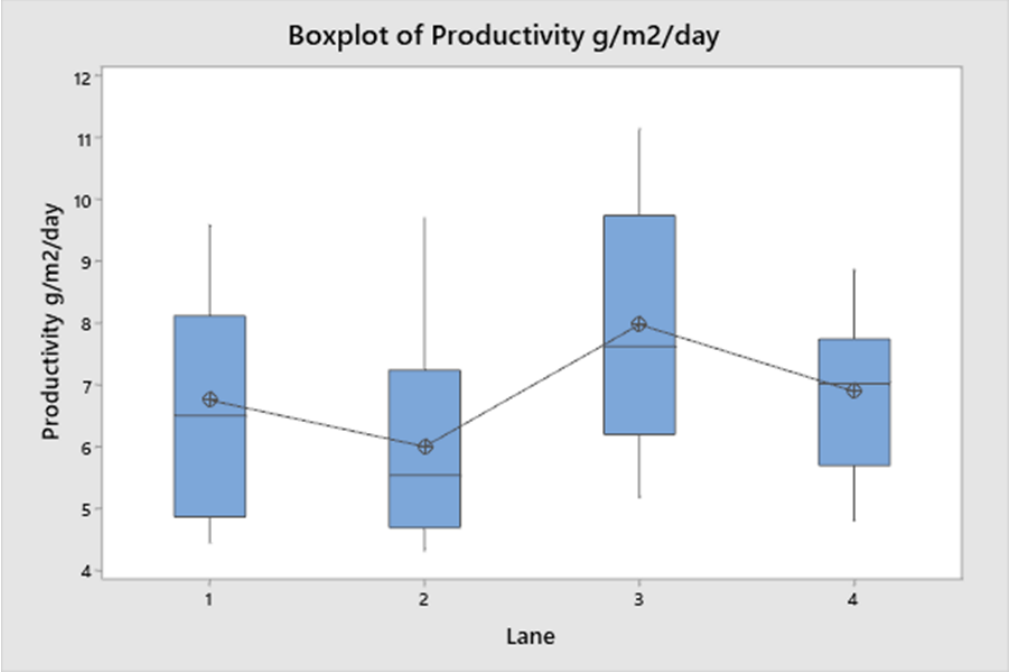

Figure 4.6. Boxplot of ash free dry weight productivity compared across the four lanes of the production phase. The points connected by lines are the means for each set of data.

Analysis by linear regression showed that the production phase AFDW productivity across all four lanes was not significantly affected any of the four nutrients tested: nitrate, phosphate, chloride, and N:P ratio (Table 4.4) (Appendices A.15, A.16, A.17, A.18).

Table 4.4. Linear regression analysis comparisons of all production phase ash free dry weight productivity over listed nutrient levels present in the aquaponics wastewater.

Nutrient	Degrees of Freedom	F statistic	P value
Nitrate	51	0.03	0.873
Phosphate	51	0.38	0.543
Chloride	51	1.27	0.265
N:P Ratio	51	0.42	0.521

The 2x dilution was implemented across the four ATS lanes for the production phase and succeeded in producing more than ample algae biomass for the final set of fermentation experiments. Interestingly, there is a degree of separation between the ATS lanes, indicating that the system is not as uniform as desired. Lane 3 had the highest productivity lane of the four and Lane 2, the original 2x dilution lane for the dilution experiment, had the lowest overall productivity. This was confirmed via ANOVA indicating that the individual lanes had a significant impact on productivity (Appendix A.14).

This is interesting in that even with this lower yield compared to the other lanes in the production phase, the high productivity from the dilution experiment could have been even more pronounced if the lane was more in line with the other lanes. This also suggests that the algae quality in the half dilution was such that it was able to compensate and excel even in a significantly lower quality environment lane, indicating that it was the algae desired.

The production phase was able to accomplish the goal of producing green filamentous algae biomass for fermentation experiments, even with unexpected deficiencies in the individual lane quality. This should be remedied for future work by effectively resetting the lanes after work conclusion and reexamination of the components and replacing or fixing any problems found. This would also be caught sooner in a control experiment to establish the baseline of the system. However, this was not performed as it was not expected to be necessary due to the continual use of the ATS system without problem.

4.4 Algal Composition: Ash Content

Ash percentages are the inorganic material within the algae and is a general indicator of what parts of the algae are unavailable for use in fermentation experiments. During the dilution experiment, growing algae produced high ash percentages early in the process and trended down over time. 2x dilution tended to have the lowest ash percent values and undiluted the highest, with 4x and 8x dilutions near interchangeable as the middle values by the final month.

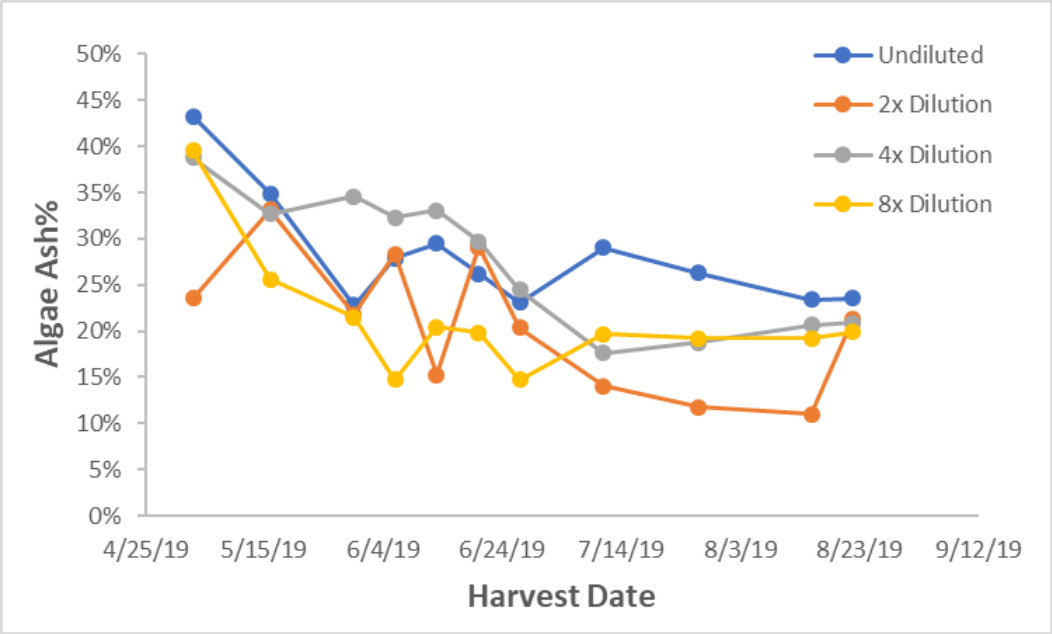

Figure 4.7. Algae biomass ash percent over time for each dilution from the dilution phase.

A linear regression analysis on the trend in the time series for each dilution shows that the effects time has on the undiluted, 2x dilution, and the 4x dilution are significant, but not for 8x dilution (Table 4.5) (Appendices B.1, B.2, B.3, B.4), suggesting that there is most likely a trend of decreasing ash content over time.

Table 4.5. Linear regression comparisons between dilutions' ash content over time.

Dilution	Degrees of Freedom	F statistic	P value
Undiluted	10	7.55	0.023
2x	10	5.77	0.040
4x	10	33.49	0.000
8x	10	3.65	0.089

Mean ash content across the dilution phase showed higher algae biomass ash percentages in the undiluted and 4x dilutions, at 28.2% (s=5.9%) and 27.6% (s=7.0%), respectively, and lower percentages in the 2x and 8x dilution conditions, at 20.9% (s=7.0%) and 21.3% (s=6.4%), respectively (Figure 4.8). One-Way ANOVA analysis of the effect of dilution on ash percent indicates that dilution does have a statistically significant effect on ash percent (Degrees of Freedom: 43, F statistic: 3.56, P value: 0.022) (Appendix B.5). Post-hoc analysis by Tukey's comparisons test found that all four dilutions were not significantly different and grouped together, indicating that the ash content across the dilutions are similar (Appendix B.5).

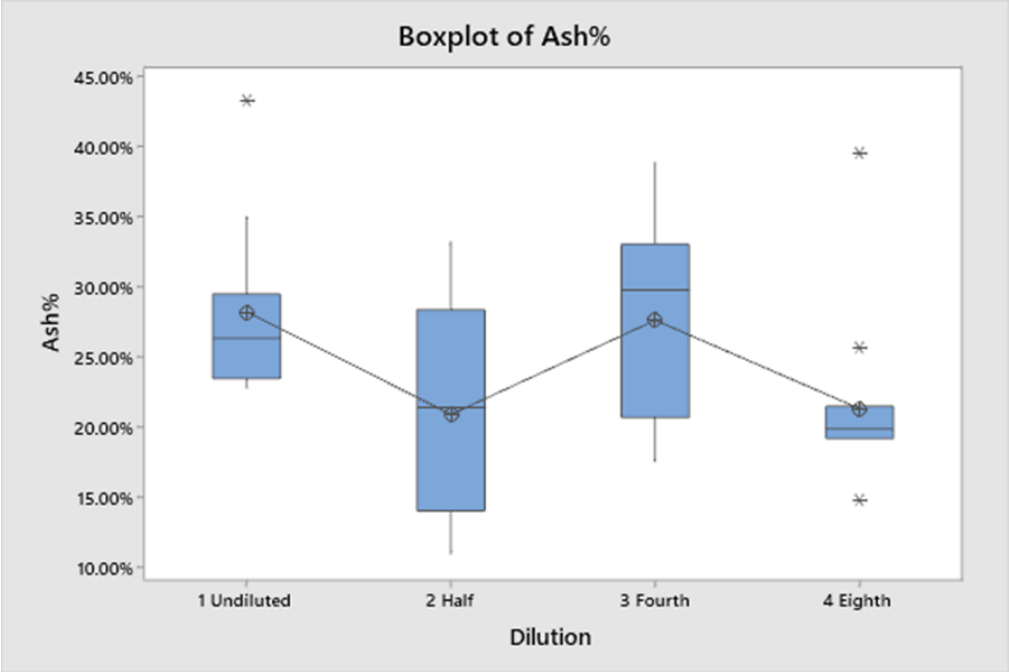

Figure 4.8. Boxplot of ash content compared across the four dilution concentrations of the dilution phase. The dots connected by lines are the means for each dilution. The asterisks were data points flagged as outliers.

Analysis by linear regression showed that the dilution phase algae ash content across all four dilutions was not significantly affected by any of the four nutrients tested: nitrate, phosphate, chloride, and N:P ratio (Table 4.6) (Appendices B.6, B.7, B.8, B.9).

Table 4.6. Linear regression analysis comparisons of all dilution phase dilutions' ash content over listed nutrient levels present in the aquaponics wastewater.

Nutrient	Degrees of Freedom	F statistic	P value
Nitrate	43	0.05	0.816
Phosphate	43	0.00	0.966
Chloride	43	0.16	0.688
N:P Ratio	43	0.66	0.419

Ash percentage content of algae grown during the production phase was generally consistent across all lanes and throughout the time of production. The production phase using the 1/2 concentration dilution produced algae that followed similar trajectories across the lanes, with Lanes 3 and 4 tending to be lower than Lanes 1 and 2 while the average ash percent stayed below 20% for all four lanes towards the end of the experiment.

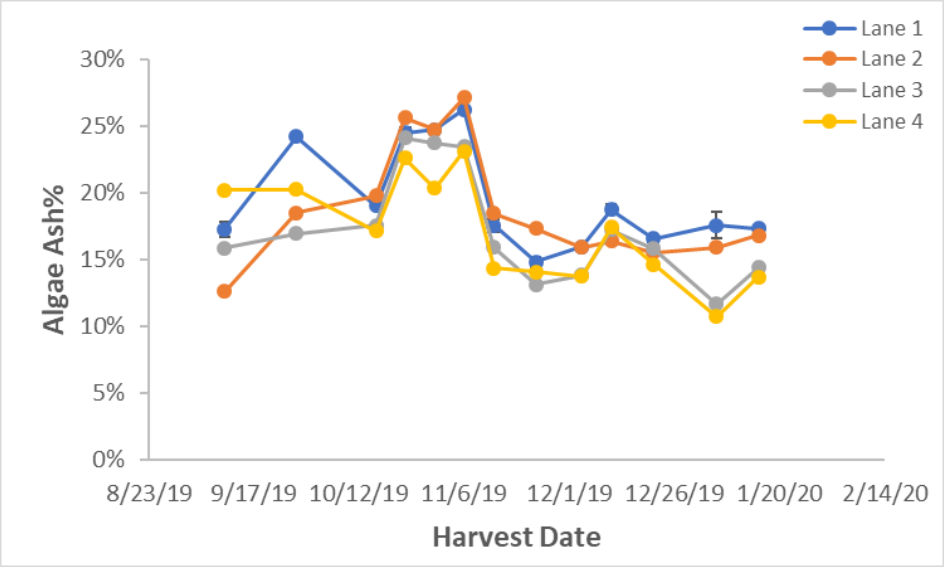

Figure 4.9. Ash percent over time in the algae biomass from the production phase for each lane.

A linear regression analysis on the trend in the time series for each lane in the production phase shows that Lanes 1, 2, and 3 are not significantly affected by time, but Lane 4 is (Table 4.7) (Appendices B.10, B.11, B.12, B.13).

Table 4.7. Linear regression comparisons between the production phase lanes' algae ash content over time.

Dilution	Degrees of Freedom	F statistic	P value
Lane 1	12	2.73	0.127
Lane 2	12	0.58	0.462
Lane 3	12	2.69	0.129
Lane 4	12	12.27	0.005

Mean ash content across the production phase showed higher algae biomass ash percentages in Lanes 1 and 2, at 19.6% (s=3.9%) and 18.9% (s=4.4%), respectively, and slightly lower percentages in Lanes 3 and 4, at 17.2% (s=4.1%) and 17.1% (s=3.9%), respectively (Figure 4.10). One-Way ANOVA analysis of the effect of lane on ash percent indicates that the individual lane does not have a statistically significant effect on ash percent (Degrees of Freedom: 51, F statistic: 1.17, P value: 0.331) (Appendix B.14). Post-hoc analysis by Tukey's comparisons test found that all four dilutions were not significantly different and grouped together, indicating that the ash content across the dilutions are similar (Appendix B.14).

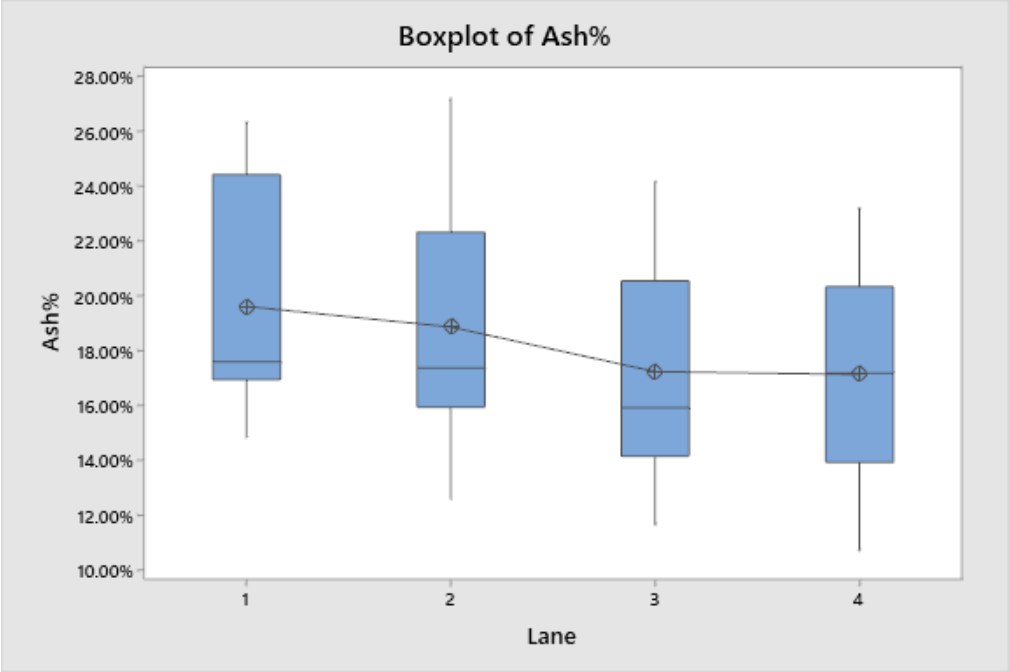

Figure 4.10. Boxplot of ash content compared across the four lanes of the production phase. The dots connected by lines are the means for each dilution.

Analysis by linear regression showed that the production phase algae ash content across all four lanes was significantly affected by all four nutrients tested: nitrate, phosphate, chloride, and N:P ratio (Table 4.8) (Appendices B.15, B.16, B.17, B.18).

Table 4.8. Linear regression analysis comparisons of all production phase lanes' ash content over listed nutrient levels present in the aquaponics wastewater.

Nutrient	Degrees of Freedom	F statistic	P value
Nitrate	51	6.81	0.012
Phosphate	51	5.03	0.029
Chloride	51	23.54	0.000
N:P Ratio	51	8.08	0.006

The dilution phase algae ash content is noteworthy in that percentages across the dilutions start very high at the start of the experiment, up to 40%, before dropping steadily until they even out around 20% towards the end of the experiment. Ash would compose the inorganic matter both within and on the algae, so the high yields across the dilutions can represent both the

matter within the water attaching to the algae biomass while being harvested, and the species of algae that make up the community having higher inorganic content, such as diatoms, even when examination under a microscope revealed no significant presence. If the particulates in the water are causing the ash content spikes, then the higher dilution lanes would have lower ash content due to the lower levels of wastewater and thus inorganic particles, and the undiluted the highest. However, since the half dilution tended to be the lowest ash percent, especially towards the end, we can assume the ash is better reflective of the algae communities of each lane and that the lane 2, half dilution algae would have better fermentation results than the algae from the other dilutions due to the lower ash content enabling more productive components to be present and potentially contribute to the fermentation.

As the production phase had all four ATS lanes at the half dilution, we would assume that the ash content would be similar throughout the experiment. For the most part, the lanes were similar though most of the timeframe, as mentioned previously when analyzed via Tukey Pairwise Comparison revealing no major differences between lanes, with deviations most noticeable when graphed at the beginning. This observed discrepancy could be explained at the beginning of the time frame of the experiment by the algae communities adjusting to the new dilution over the course of a few weeks, thus the community would be in the process of shifting dominant algae species. Other changes could be explained by variations within the lanes themselves, such as differences in light level over the lanes or the presence or lack thereof of certain bacteria or algae accumulated over time. Considering the relative uniformity of the results over time, we can assume that the algae species dominant in the lanes were relatively similar so the ash percentages would remain close together.

4.5 Algae Composition: Sugars Analysis

Table 4.9. Sugar analysis of the dilution phase and production phase algae biomass used in the fermentation experiments. Prelim green is the early dilution phase algae and Prod green is the production phase algae biomass.

Algae	Glucan	Xylan	Galactan	Arabinan	Mannan	Ash
Prelim Green	9.80%	1.90%	4.60%	0.00%	2.30%	25.60%
Prod Green	26.03%	1.20%	2.90%	1.71%	4.41%	14.77%

All algal biomass from the two growth experiments were analyzed for fermentable simple sugars. Results of the analysis are listed in Table 4.9. The five polysaccharides listed are common carbohydrates used as energy in lactic acid fermentation and their presence serves as a good indicator for whether a carbohydrate source is suitable or not. The values represent the mass percentage each sugar composes of the total biomass in the system with the ash accounting for the inorganics present. Prelim Green is a composite sample of dilution phase algae from harvests early in the experiment and used in the dilution phase green algae fermentation and Prod Green is the composite sample of algal biomass produced from the production phase and was used in both treated and untreated production phase algae fermentations.

Glucan is the most common carbohydrate across the three samples and accounts for the majority of the energy contribution to the fermentation process. The ash percent indicates how much of the algae is occupied by non-sugar inorganics and can be taken as unusable when measuring suitability. The production phase algae showed the highest glucan percentage and lowest ash content, while the preliminary green algae from the early dilution study has lower glucan and higher ash. The other four sugars are relatively low and varied between the algae samples and tend to not compensate for lower glucan levels in the poorer algae samples.

The sugars present within the algae represent their suitability as a carbohydrate source in lactic acid fermentation. The algae samples were tested before use in lactic acid fermentation via the HPLC to quantify what sugars were present and their amount, with the precise working of the

HPLC described later. Glucan is by far the most common and widely used sugar source, so the individual glucan level can be representative of the ability of the algae to work as a feedstock and is used when calculating the conversion of lactic acid from the available sugars

The production phase algae was rich in glucan and low in ash content, suggesting that the algae would perform better in lactic acid fermentation than the preliminary green algae. The other four sugars were noticeably lower, but as stated previously, glucan is treated as representative of the sugars present. Alongside the sizable decrease in ash percentages across the algae samples, this suggests that the production phase green filamentous algae is superior as a feedstock to the dilution phase algae and thus the dilution experiment and production phase algae growth succeeded in providing higher carbohydrate content algae biomass for the fermentation experiments.

4.6 Fermentation Experiment: Dilution Phase Algae

A composite algae sample was taken from across the various ATS lanes over the early stages of the dilution experiment and served as the algae biomass for each fermentation sample. Results from fermentation under a range of conditions, with and without yeast extract (YE) and with or without calcium carbonate ($CaCO_3$), are shown in Figures 4.11 and 4.12. Each fermentation had one, both, or neither nutrient to gauge the influence each of the nutrients had on the resulting fermentation yield and with each combination represented within the legend in Figure 4.11. The two samples that had YE were higher in max concentration, 15.0 g/L for the sample excluding $CaCO_3$ and 14.0 g/L for the one with $CaCO_3$, with yields approximately 70%. The other two trials without YE were near identical at lower max concentrations of 13.5 g/L and lower 63% yields.

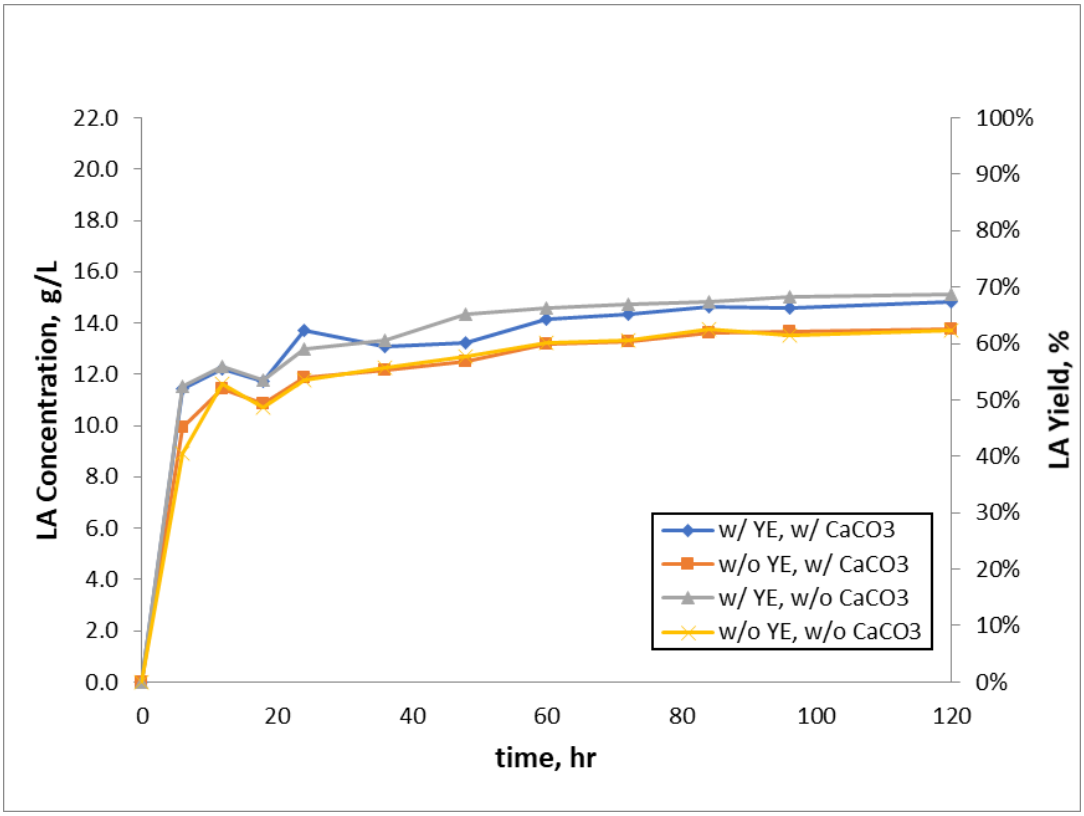

Figure 4.11. Lactic acid concentrations and yield percent of the different fermentation samples and the presence or absence of different additional treatments to sample for dilution phase algae biomass. YE stands for yeast extract and $CaCO_3$ for calcium carbonate, the usual additives to increase fermentation yields.

Final lactic acid concentrations at time 120 hours of the dilution phase algae showed higher lactic acid concentrations for the 'with YE and $CaCO_3$' treatment, at 14.8g/L, and the 'with YE, but no $CaCO_3$' treatment at 15.1g/L, and slightly lower yields for the 'with $CaCO_3$, but no YE' treatment at 13.8g/L and the 'neither YE nor $CaCO_3$' treatment at 13.7g/L (Figure 4.12). One-Way ANOVA analysis of the effect of treatment on lactic acid concentration indicates that the differences between treatments are significantly affecting concentrations (Degrees of Freedom: 43, F statistic: 3.09, P value: 0.038) (Appendix C.1). Post-hoc analysis by Tukey's comparisons test found that all four treatments were not significantly different and grouped together, indicating that the yields between the treatments are similar (Appendix C.1).

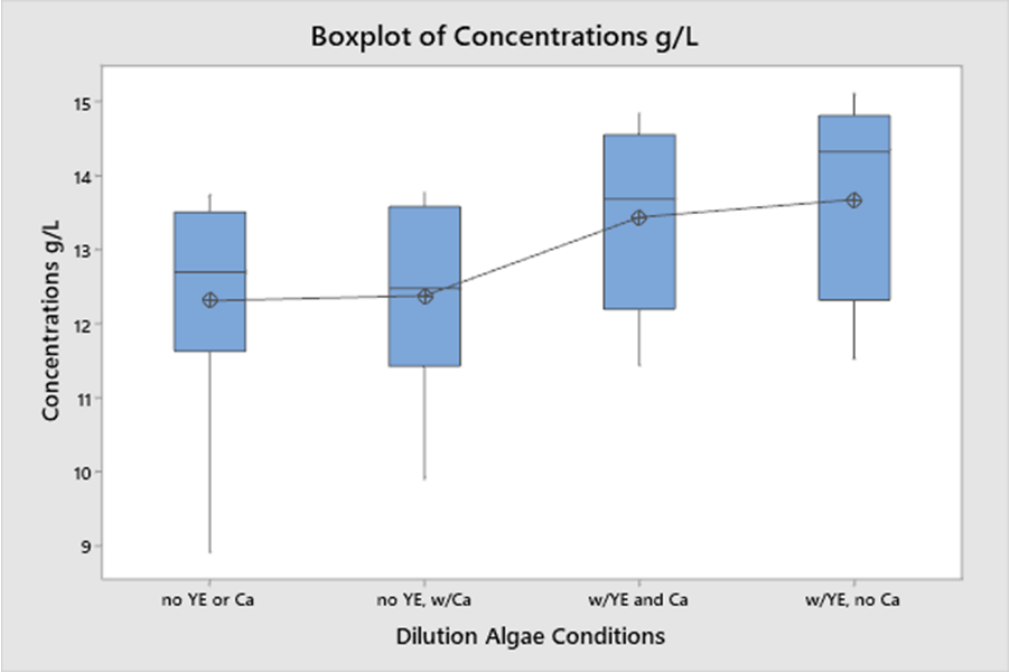

Figure 4.12. Box plot of lactic acid concentrations from dilution phase algae feedstock across treatments. YE stands for yeast extract and Ca stands for calcium carbonate, $CaCO_3$. The dots connected by lines are the means for each treatment.

The algae for this fermentation was gathered from the ATS lanes during the early phase of the Dilution Phase of the algae growth experiment and compiled into a composite sample. Since both fermentations with YE are higher in concentration and yield than the two without, we can assume that the YE has a beneficial effect on the fermentation. Calcium carbonate ($CaCO_3$) did not seem to have much effect on the fermentations, whether by its presence or absence. This can indicate that the levels of lactic acid within the system did not necessitate the addition of an additional basic buffer to prevent the acidic inhibition of the process from the elevated lactic acid presence. Given that the final concentrations between treatments was found to be very similar, we can also assume that this algae feedstock will give similar results regardless of treatment and thus maximizing concentration using YE or $CaCO_3$ is not entirely necessary for future fermentations of this biomass.

4.7 Fermentation Experiment: Production Phase Pretreated Algae

A composite sample of algae from across the production phase of 1/2 strength concentration dilution wastewater was gathered and pretreated with heat and pressure, with a fifth sample for the algae with yeast extract (YE) and calcium carbonate (Ca), but without the heat and pressure pretreatment. Results from the fermentation trials are shown in Figures 4.13 and 4.14. The highest max LA concentration was observed for untreated algae, with a final concentration of 18 g/L and a yield of 70%. The trial with YE present and no Ca showed the highest concentration, with final lactic acid concentrations around 16.0 g/L and yield of around 65%. Other trial conditions, including both YE and Ca present, with Ca and no YE, and neither YE nor Ca are close together as the collective lowest around 12-13 g/L concentration and 50-55% yield.

The no pretreatment with both YE and Ca fermentation interestingly resulted in higher results than any of the treated samples with concentration levels of 18.0 g/L and yield of 70%, indicating that higher lactic acid yields would be achieved with the biomass without the heat and pressure treatment. This would be explored with the next set of fermentation runs.

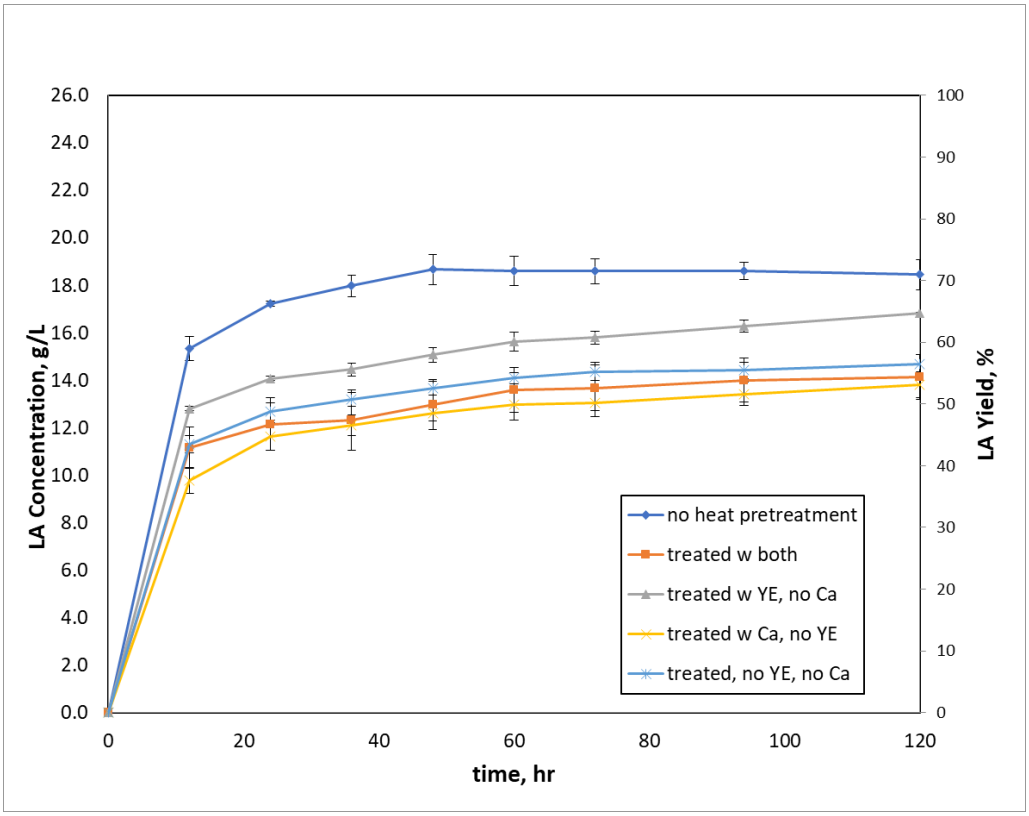

Figure 4.13. Lactic acid concentrations and yield percent of the different fermentation samples and the presence or absence of different additional treatments to sample for production phase algae biomass. YE means yeast extract and Ca calcium carbonate.

Final lactic acid concentrations across the production phase algae showed the highest final lactic acid concentration for the 'no heat pretreatment' treatment, at 18.5g/L, followed by the 'treated, w YE, no Ca' treatment at 16.8g/L, and the 'treated, no YE, no Ca' treatment at 14.7g/L, while the 'treated, w both (YE and Ca)' treatment and 'treated, w Ca, no YE' treatments were the lowest at 14.1g/L and 13.8g/L, respectively (Figure 4.13, Appendix C.2). One-Way ANOVA analysis of the effect of treatment on lactic acid concentration indicates that the differences between treatments are significantly affecting yields (Degrees of Freedom: 39, F statistic: 27.78, P value: 0.000) (Figure 4.14, Appendix C.2). Post-hoc analysis by Tukey's comparisons test found that the unpretreated treatment grouped alone, while the 'treated, w YE, no Ca' and 'treated, no YE, no Ca' treatments were grouped together, and that the 'treated, no

YE, no Ca', 'treated, w both (YE and Ca)', and 'treated, w Ca, no YE' were grouped together, indicating that the unpretreated treatment is the most significantly different in terms of concentration from the collected treatments(Appendix C.2).

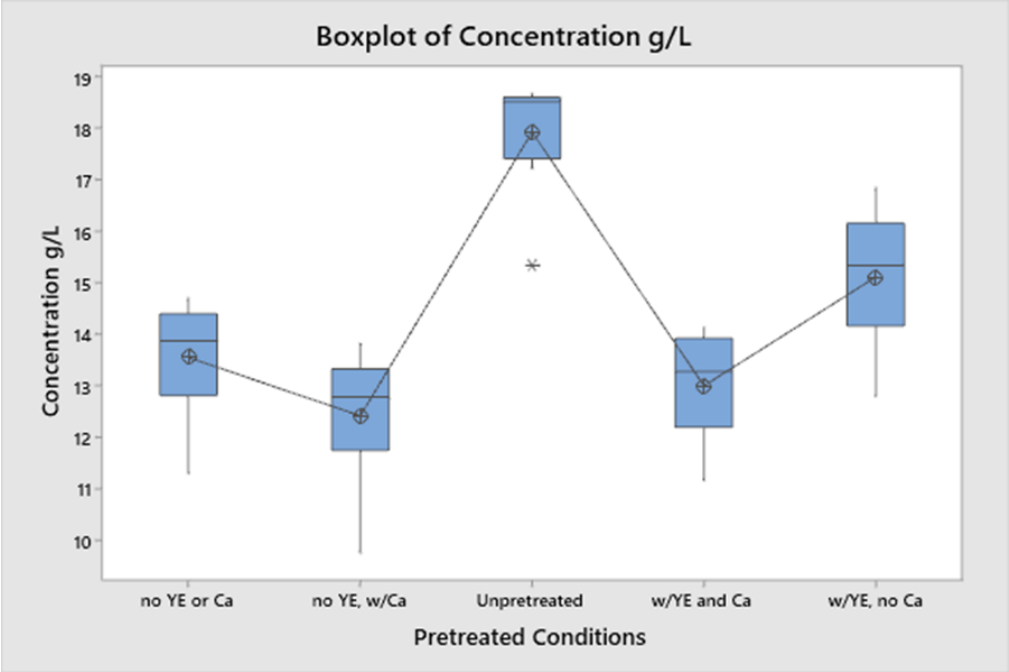

Figure 4.14. Box plot of lactic acid concentrations from the pretreated production phase algae feedstock fermentation across treatments. YE stands for yeast extract and Ca stands for calcium carbonate, $CaCO_3$. The dots connected by lines are the means for each treatment.

The treated fermentation samples were relatively similar across the different fermentations, with the most noticeable exception the sample with YE yet no Ca that had the highest treated results in concentration and yield. However, the untreated algae fermentation is the biggest surprise in both LA concentration and yield by greatly outperforming the treated samples. Prior tests also indicated that samples that receive both YE and Ca would perform better than those lacking in one or both additional nutrients, yet this fermentation had middling results. This would seem to indicate that there may be user error either in the preparation of the algae slurry used in the fermentation or while taking samples over the fermentation. The

performance of the other sample that had YE would suggest this, as Ca tends to have negligible impact unless paired with YE in less sugar dense samples.

The untreated sample was designed to demonstrate the effectiveness of the pretreatment in breaking down the structure of the algae and freeing the sugars within for easier access for the LAB. This sample should have been noticeably lower in concentration and yield than the treated samples, but its higher lactic acid concentration performance called that into question, even when the addition of both YE and Ca to the sample are accounted for. This may suggest that the sugars within the algae were degraded or transformed during the treatment thus lowering the quantity and quality of the readily available sugars for use in the fermentation, impacting the final concentration and yields. This would also signify that the algae are generally simpler in structure compared to woody biomass (Chao et al., 1999) enough to either not require pretreatment or a less harsh treatment, since prior tests indicate pretreatment is beneficial to both concentration and yield.

4.8 Fermentation Experiment: Production Phase Untreated Algae

Following the results of the nontreated algae sample from the pretreated fermentation, a second fermentation test centered on leaving the algae untreated was subsequently performed. Samples from the same composite algae biomass used in the treated algae fermentation were utilized as the carbohydrate source in fermentation trials. Results are shown in Figure 4.15, where YE and Ca stand for yeast extract and calcium carbonate, respectively, and their presence or absence from each fermentation test is listed in the legend. The fermentation sample with both YE and Ca showed the greatest maximum concentration at around 20.0 g/L and a yield efficiency of 80%. The second highest result was from the treatment that contained Ca but no

YE with a concnetration of 16.5 g/L and a yield of 65%. The sample without either nutrient additive and the sample with YE but no Ca together had the lowest performance, with concentrations of 13.0 g/L and 14.5 g/L and yields in the range of 50% and 55%, respectively.

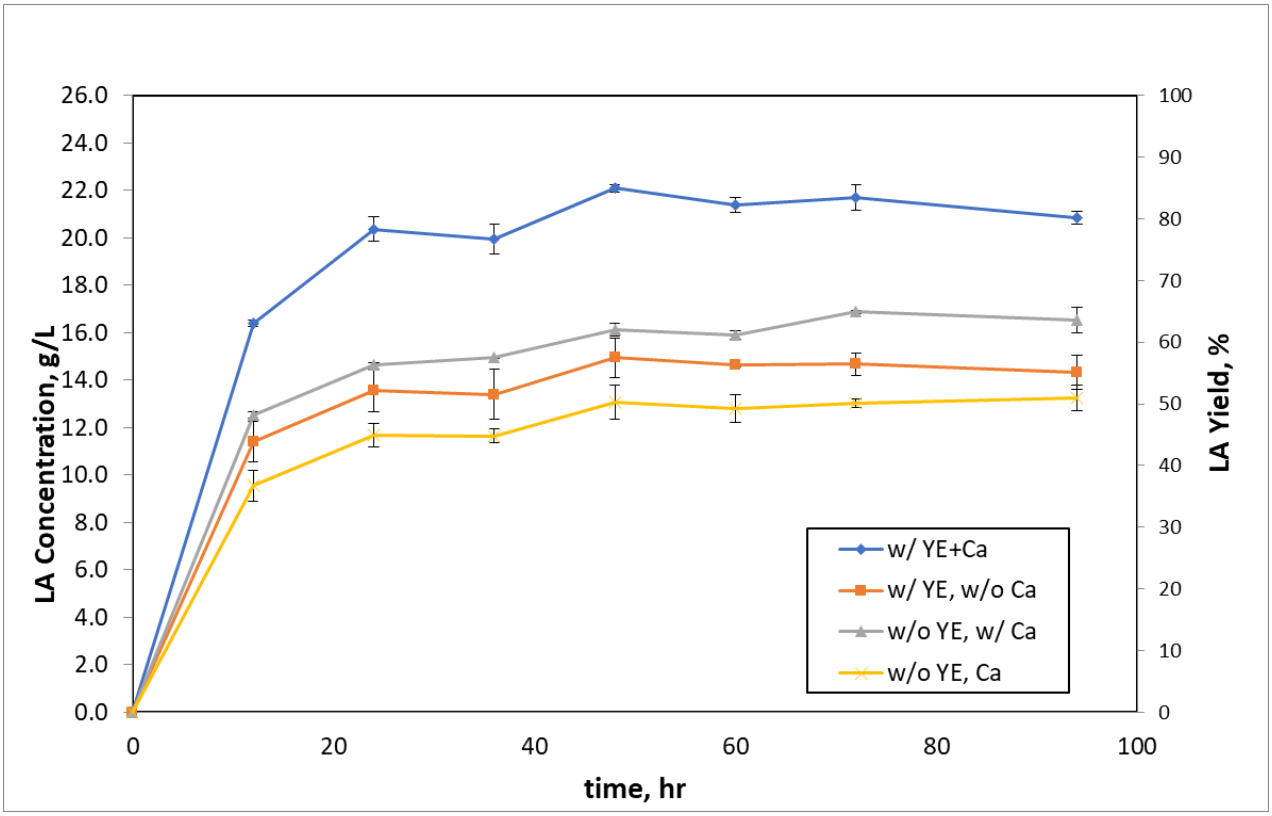

Figure 4.15. Lactic acid concentration and yield from production phase algae biomass over time. The individual fermentation samples represent the presence or absence of YE and Ca in each.

Final lactic acid concentrations across the production phase untreated algae showed the highest lactic acid concentrations for the 'w/ YE and Ca' treatment, at 20.9g/L, followed by the 'no YE, w/ Ca' treatment at 16.5g/L, while the 'w/ YE, no Ca' treatment at 14.3g/L, and the 'neither YE or Ca' treatment at 13.2g/L were the lowest (Figure 4.15, Appendix C.3). One-Way ANOVA analysis of the effect of treatment on lactic acid concentration indicates that the differences between treatments are significantly affecting concentrations (Degrees of Freedom: 27, F statistic: 38.85, P value: 0.000) (Figure 4.16, Appendix C.3). Post-hoc analysis by Tukey's

comparisons test found that the ‘w/ YE and Ca’ was grouped alone, while the ‘no YE, w/Ca’ and ‘w/YE, no Ca’ treatments were grouped together, and the ‘w/YE, no Ca’ and ‘no YE or Ca’ treatments were grouped, indicating that the ‘w/YE and Ca’ treatment is the most significantly different in terms of concentration from the treatments (Appendix C.3).

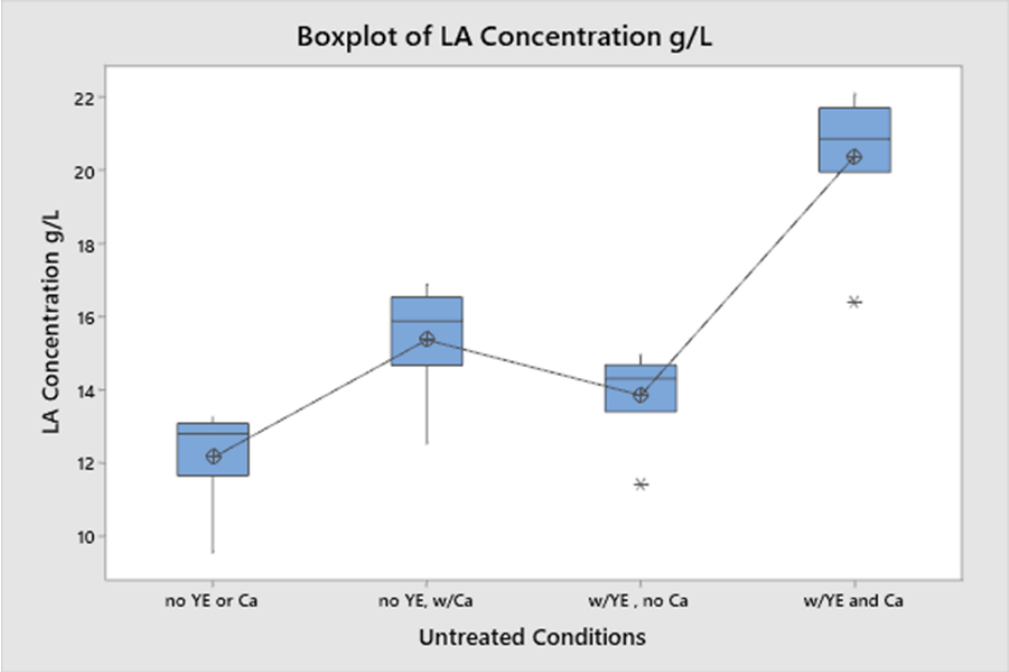

Figure 4.16. Box plot of lactic acid concentrations from the unpretreated production phase algae feedstock fermentation across treatments. YE stands for yeast extract and Ca stands for calcium carbonate, $CaCO_3$. The dots connected by lines are the means for each treatment. The asterisks are the data points considered outliers.

The untreated sample from the pretreated algae fermentation had both YE and Ca applied and performed the highest in lactic acid yield and led to running a second set of fermentation tests using the algae biomass without pretreatment. Through the One-Way ANOVA analysis, it was found that treatment had significant effect on lactic acid yields, which is readily apparent through comparison of yields that the YE and Ca combined treatment had the most impact on lactic acid concentration.

The concentrations seem to indicate that Ca is more important in buffering the solution due to the high concentrations of lactic acid causing the process to inhibit itself and thus continuing the fermentation process. As opposed to needing more YE to kick start the LAB into producing lactic acid, this suggests that the algae is providing the necessary nutrients to maintain the fermentation. The addition of YE seems to be needed once the solution is buffered appropriately from the Ca addition to begin further boosting the concentration of lactic acid and yield of the fermentation, as seen from the YE added sample only being around 1 g/L higher concentration with similar yields than the base algae. As mentioned previously, the lactic acid concentrations from untreated filamentous algae were unexpected but hold the most promise for further research.

Additional lactic acid concentration comparisons were made to compare the filamentous algae to other feedstocks for fermentation. Alongside the three lactic acid fermentations using the dilution and production phase algae biomass, a preliminary fermentation was performed using predominantly cyanobacteria algae biomass grown within the system before the experiment. Additionally, a lactic acid fermentation using cucumber residues collected from a cucumber crop grown in the aquaponics system and data from the fermentation of paper sludge in Shi et al. (2015) were collected. The preliminary algae fermentation was a test to determine whether algae biomass could function as a feedstock in lactic acid fermentation and the cucumber and paper sludge fermentations were to provide comparisons to biomass that had known high carbohydrate levels. Table 4.10 details the primary differences between the feedstocks, namely the lactic acid concentration, the yield percent of lactic acid to sugars, and the glucan and ash percentages of the feedstock, with the glucan percent representing the readily available sugars within the biomass.

Table 4.10. Comparisons between different lactic acid fermentation feedstocks. Prelim green and Prod green treated and untreated are the feedstocks covered previously. Prelim algae is the initial algae biomass produced from the ATS system. Note that the values of the Prelim and Prod Green sources are of the treatments that had the highest lactic acid yields.

Source	LA Concentration g/L	LA Yield%	Glucan %	Ash %
Prelim Green	15.1	69	9.8	25.6
Prod Green treated	16.8	65	26.03	14.8
Prod Green untreated	22.1	85	26.03	14.8
Prelim Algae	8.9	80	10.1	24.8
Cucumber Residue	17.8	89	17.9	24.1
Paper Sludge[1]	45.5	86	47.6	34.5

[1]*Data from Shi et al. (2015).*

The paper sludge had the highest lactic acid concentration of the combined grouping, with 45.5 g/L lactic acid produced at an 86% yield. This is not surprising since the glucan percentage of the biomass was over 47% of the total biomass, making for a readily available energy source for lactic acid fermentation, even with the higher ash percent of 35%. As noted previously and seen in Table 4.10, the untreated production phase algae was the highest producer of lactic acid from the experiments at a peak concentration of 22 g/L lactic acid with a 85% yield rate with a glucan percent of 26% and 15% ash content. This indicates that total glucan, and thus overall sugar availability, has a high impact on the yield of lactic acid in a fermentation.

The cyanobacteria that largely composed the earliest algae harvests had the lowest lactic acid concentration, in addition to one of the lowest glucan contents of the sources. The cucumber residues results were mainly to display the need for pretreatment for more complexly structured feedstocks and the high returns that the pretreatment would yield. The results shown are for the pretreated biomass, and the results placed it amongst the better algae yields, hinting that the filamentous algae can be comparable to cucumber residues and potentially other production wastes from cultivations.

Chapter 5: Conclusions

5.1 Summary

The data gathered from the research experiments showed that green filamentous algae could be successfully cultivated via an indoor ATS system in such quantities that could be used for the fermentation of lactic acid using lactic acid producing bacteria. The algae biomass had high lactic acid concentrations, that at their best, were superior when compared to other algae biomass and cucumber residues, and only being outstripped by highly processed waste paper mill sludge, as reported in the literature. This makes continued research into this algae biomass an appealing prospect in further improving lactic acid production from an otherwise unassuming algae crop.

The algal growth experiments were successful in producing enough algae biomass from aquaponics wastewater to run lactic acid fermentation over the course of a dozen harvests across four ATS lanes. The dilution experiment tested the effect that diluting the aquaponics wastewater with dechlorinated tap water had on the emergence of green filamentous algae from the algal turf community. A half dilution of wastewater and tap water succeeded in creating the conditions for the green filamentous algae to thrive. The filamentous algae produced was high in carbohydrates and low in ash content, making it desirable as a feedstock in lactic acid fermentation.

The lactic acid fermentation experiments using the dilution and production phase algae produced lactic acid yields that were highly promising, while analysis on the various treatments and additions showed a general trend of higher yields when in the presence of additional nutrients and buffering agent. Although not statistically significant, maximum and mean lactic

acid yields were improved with these additions and would be beneficial for use in future experiments. The surprising results of the heat and pressure pretreatment producing lower lactic acid yields than without for the production phase algae biomass was an important finding in maximizing lactic acid yields for the experiment and warrants additional investigations to fully understand.

5.2 Limitations of study

One of the main limitations of this study was that it was performed within a more controlled, indoor environment at a laboratory scale. This setting shields the ATS system and the algae community from harsh conditions and potential disruptions, and to scale to a production scale would require either a large, enclosed space such as a warehouse or greenhouse while potentially introducing additional costs to achieve.

Most ATS systems are placed outdoors and on a much larger scale, which would impact the algae community that would emerge within the system. The ATS would be exposed to more extreme heat profiles, especially during summer months, which has been observed to sharply impact a larger scale outdoor ATS system located near the aquaponics facility in the study. Longer ATS lanes have been noted to form different zones of algal growth down the length of the system, potentially creating new community conditions that would have algae with unknown qualities, necessitating creating and harvesting a system to gauge the suitability of the algae biomass. The outdoor nature of a larger scale system would also allow new algae species to be introduced and potentially shift the community away from an otherwise stable desired algae community, along with the potential introduction of grazers that would consume the algae biomass.

Due to the connection to the aquaponics system, there is the potential for upstream water changes to flow downstream and impact the water quality used in the ATS system. Changes in fish care, a breakdown of one or more components, or the implementation of new vegetable experiments would also cause changes that would vary over time and cause anomalies in the data as a result. Thus, to maintain a stable, known water quality would require additional treatments to reach the baseline of water quality that had been determined to produce the algae biomass.

This study lasted over the course of most of a year, but there is a gap in the winter and the beginning of spring, which may have an impact on the productivity of the algae biomass and would require extending the study to at least the length of a calendar year to ensure data for each season. Although the data analysis suggests that the productivity is not affected by time, it would be beneficial to have data points for the missing time to ensure complete coverage. It would also be benefitted to maintain a strict seven- to ten-day harvest period over time, as longer growth periods cause the effective productivity to decline, although the overall biomass harvested is higher.

5.3 Conclusions

This study indicates that lactic acid can be produced from the fermentation of algae biomass cultivated using the wastewater from an aquaponics system. From these results, several conclusions can be made:

1. Higher algae productivity within the indoor ATS system using aquaponics wastewater was optimized using a half wastewater, half dechlorinated tap water dilution that yielded an average ash free dry weight productivity of 7 g/m^2/day of algae biomass per ATS lane each harvest. The algae biomass this method produced was better than that of other

dilutions tested, both in ash free dry weight productivity and in the quality, that is sugar and ash contents, of the algae cultivated.

2. Two aspects of the algae produced from the dilution and production phases of the growth experiment, the ash free dry weight algal productivity and ash content of the algae biomass, were varyingly significantly influenced by several factors, from the nutrient quality of the wastewater, in terms of nitrate, phosphate, chloride, and N:P ratio, to effects of time and between the individual dilutions and lanes.
3. The lactic acid yields and efficiencies of the various algae biomass feedstocks showed that the production phase algae had the highest potential for desirable results, with the best results resulting from algae biomass that was not pretreated with heat and pressure while in the presence of additional nutrients and buffering agent. These conditions produced a 20g/L lactic acid yield at an 80% conversion efficiency, and while the other fermentations were not as prolific, they were not significantly different from the other results.

5.4 Recommendations for future work

Continued research into optimizing the indoor ATS system for the dominance of green filamentous algae from the algal turf community would be beneficial to further research projects looking to utilize the biomass, given the promising results of the green filamentous algae when used in lactic acid fermentation. Specific factors, such as water chemistry requirements, light levels, and individual algae species compositions would all help lead towards optimized green filamentous algae growth that would produce the most ideal lactic acid yields when fermented. Individual studies, such as a more in-depth dilution experiment or a more strictly maintained

ATS setup and schedule, would help to generate clearer data to better understand the system and the algae it would produce.

Further work would need to be done to determine whether these small-scale algae productivity results could be feasibly scaled up to an outdoor, full size ATS system and whether the algae biomass would continue to be as promising as a lactic acid fermentation source. Such differences as the change between light sources or the heat profile and the dynamics of the nutrient levels in the water over the length of the lane would need to be studied to determine their impact on the algae biomass's sugar and ash contents, and thus their suitability as a carbohydrate source. The exposure of the system to potential grazers and additional competitors for nutrients would need to be monitored to gauge whether the filamentous algae can adapt to the changes while maintaining their desirable qualities.

Additional work could be done to further compare these algae fermentation yields to other carbohydrate and nutrient sources to gauge their effectiveness and appeal. If these results continue to be predominantly favorable towards the algae, it could make the implementation of ATS systems to wastewater generators an appealing prospect. Other potential waste products like vegetable residues and fish wastes would help to reduce overall waste in the system they originate if they also would lend themselves well to the fermentation process, and therefore turn a otherwise constant waste generation into additional investments when paired with the algae studied in this study.

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Appendix A: Algae Productivity Minitab Statistical Output

Appendix A.1:Dilution Phase Algae Productivity Undiluted vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: Undiluted versus Date

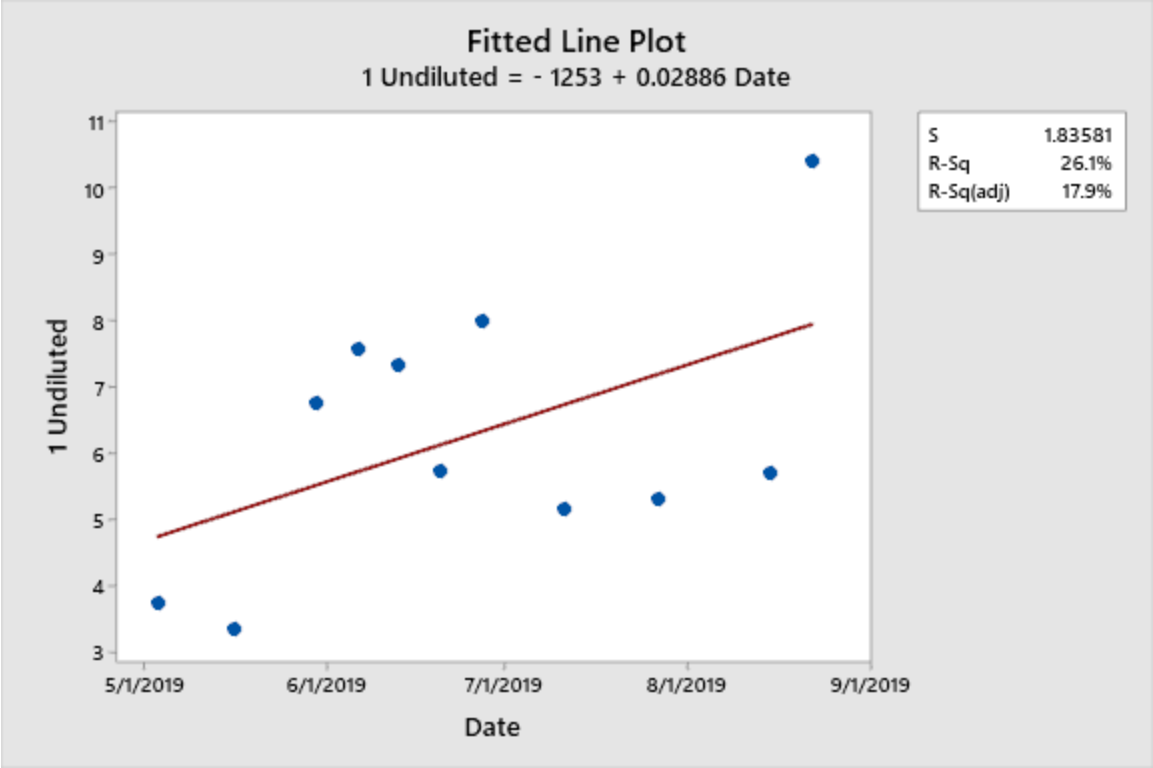
The regression equation is
1 Undiluted = - 1253 + 0.02886 Date

Model Summary

S	R-sq	R-sq(adj)
1.83581	26.10%	17.89%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	10.7116	10.7116	3.18	0.108
Error	9	30.3319	3.3702		
Total	10	41.0435			



Appendix A.2: Dilution Phase Algae Productivity 2x Dilution vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: 1/2 Dilution versus Date

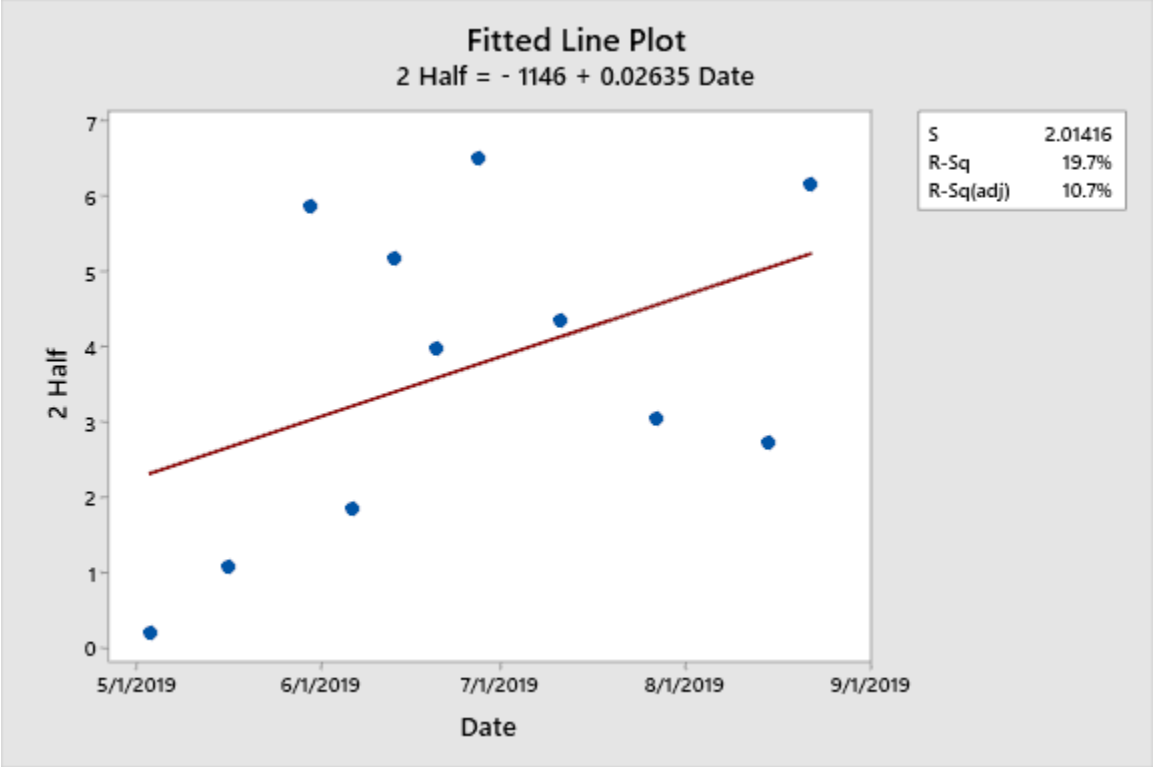
The regression equation is
2 Half = - 1146 + 0.02635 Date

Model Summary

S	R-sq	R-sq(adj)
2.01416	19.65%	10.73%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	8.9316	8.93163	2.20	0.172
Error	9	36.5116	4.05685		
Total	10	45.4432			



Appendix A.3: Dilution Phase Algae Productivity 4x Dilution vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: 1/4 Dilution versus Date

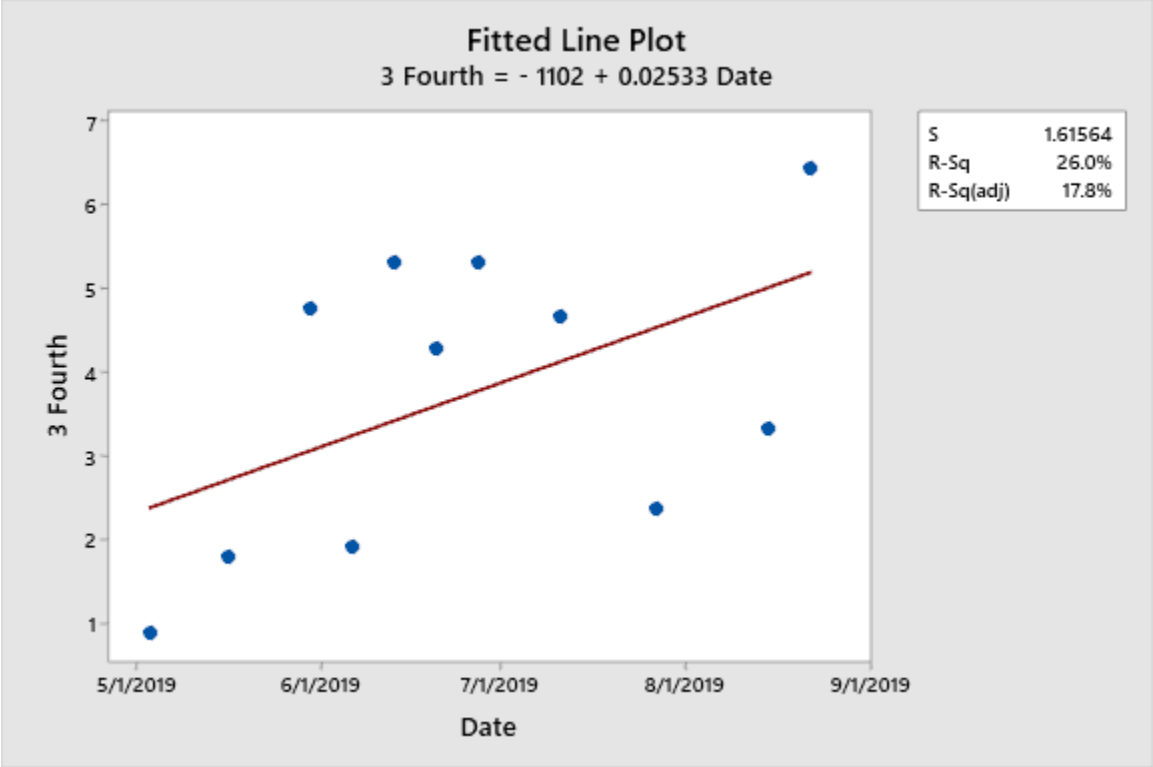
The regression equation is
3 Fourth = - 1102 + 0.02533 Date

Model Summary

S	R-sq	R-sq(adj)
1.61564	26.00%	17.78%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	8.2544	8.25436	3.16	0.109
Error	9	23.4927	2.61030		
Total	10	31.7470			



Appendix A.4: Dilution Phase Algae Productivity 8x Dilution vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: 1/8 Dilution versus Date

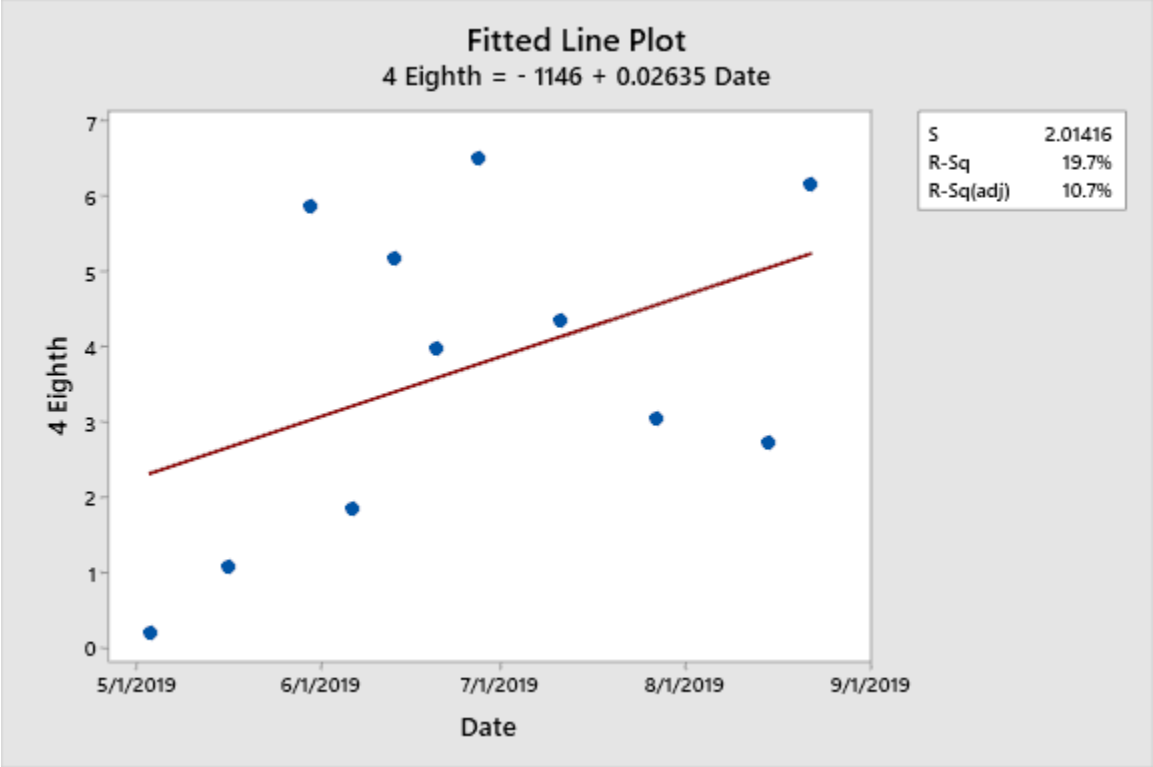
The regression equation is
4 Eighth = - 1146 + 0.02635 Date

Model Summary

S	R-sq	R-sq(adj)
2.01416	19.65%	10.73%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	8.9316	8.93163	2.20	0.172
Error	9	36.5116	4.05685		
Total	10	45.4432			



Appendix A.5: Dilution Phase Algae Productivity vs Dilution

REGRESSION DIL.

One-way ANOVA: Productivity g/m2/day versus Dilution

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Dilution	4	1 Undiluted, 2 Half, 3 Fourth, 4 Eighth

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Dilution	3	85.58	28.525	5.10	0.004
Error	40	223.91	5.598		
Total	43	309.48			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2.36595	27.65%	22.22%	12.46%

Means

Dilution	N	Mean	StDev	95% CI
1 Undiluted	11	6.280	2.026	(4.838, 7.722)
2 Half	11	6.714	3.251	(5.272, 8.156)
3 Fourth	11	3.730	1.782	(2.288, 5.172)
4 Eighth	11	3.720	2.132	(2.278, 5.162)

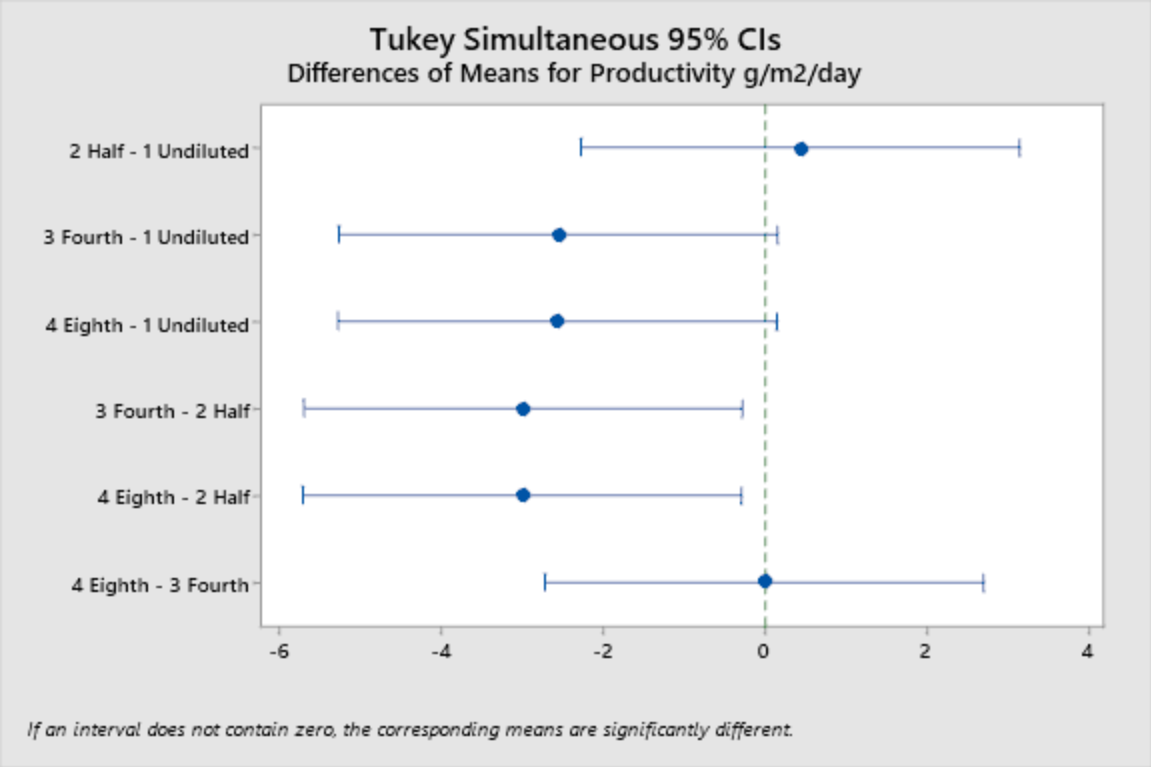
Pooled StDev = 2.36595

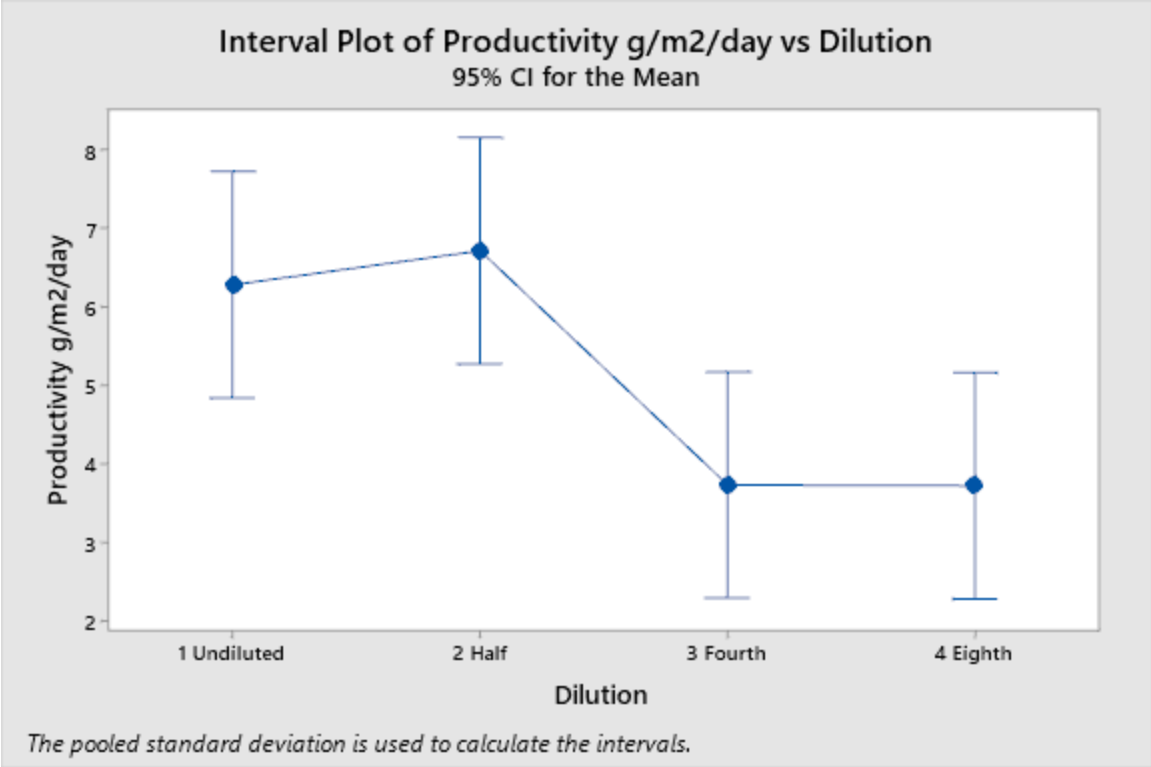
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Dilution	N	Mean	Grouping	
2 Half	11	6.714	A	
1 Undiluted	11	6.280	A	B
3 Fourth	11	3.730		B
4 Eighth	11	3.720		B

Means that do not share a letter are significantly different.


Tukey Simultaneous 95% CIs
Differences of Means for Productivity g/m2/day
2 Half - 1 Undiluted
3 Fourth - 1 Undiluted
4 Eighth - 1 Undiluted
3 Fourth - 2 Half
4 Eighth - 2 Half
4 Eighth - 3 Fourth
-6
-4
-2
0
2
4
If an interval does not contain zero, the corresponding means are significantly different.


Interval Plot of Productivity g/m2/day vs Dilution
95% CI for the Mean
Productivity g/m2/day
8
7
6
5
4
3
2
1 Undiluted
2 Half
3 Fourth
4 Eighth
Dilution
The pooled standard deviation is used to calculate the intervals.

Appendix A.6: Dilution Phase Algae Productivity vs Nitrate

REGRESSION DIL.

Regression Analysis: Productivity g/m2/day versus Nitrate mg/L

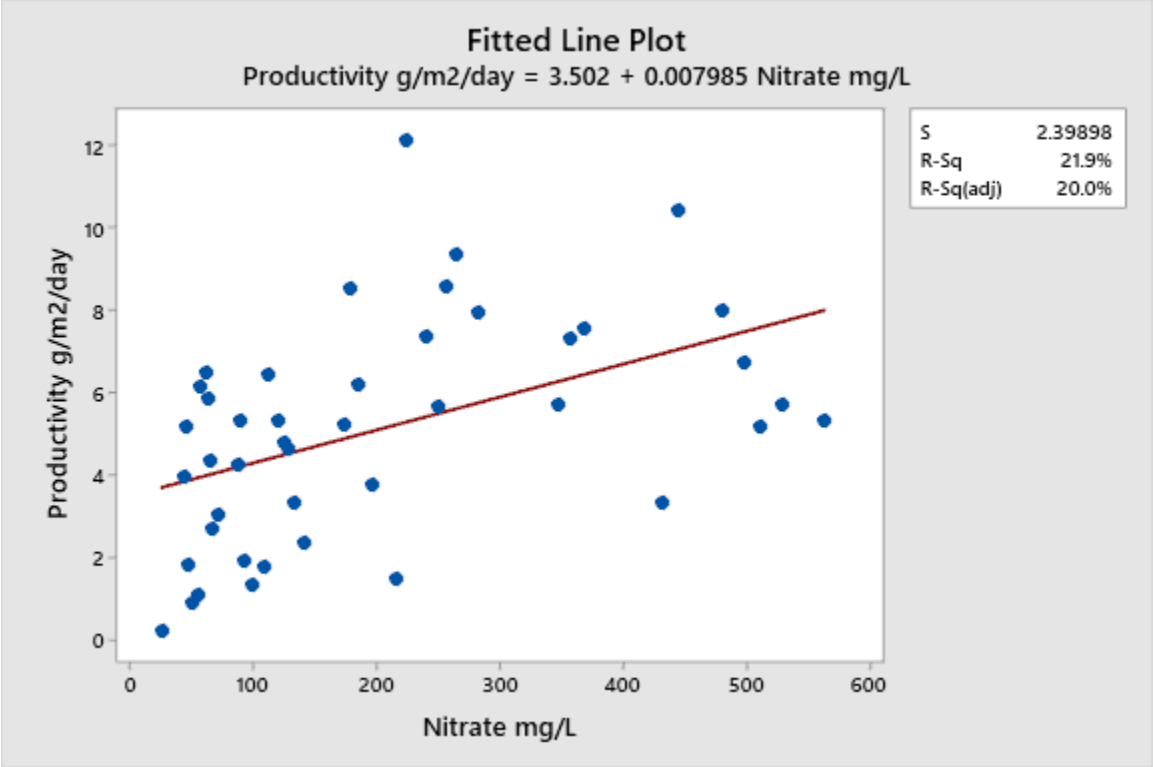
The regression equation is
Productivity g/m2/day = 3.502 + 0.007985 Nitrate mg/L

Model Summary

S	R-sq	R-sq(adj)
2.39898	21.90%	20.04%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	67.770	67.7696	11.78	0.001
Error	42	241.715	5.7551		
Total	43	309.484			



Appendix A.7: Dilution Phase Algae Productivity vs Phosphate

REGRESSION DIL.

Regression Analysis: Productivity g/m2/day versus Phosphate mg/L

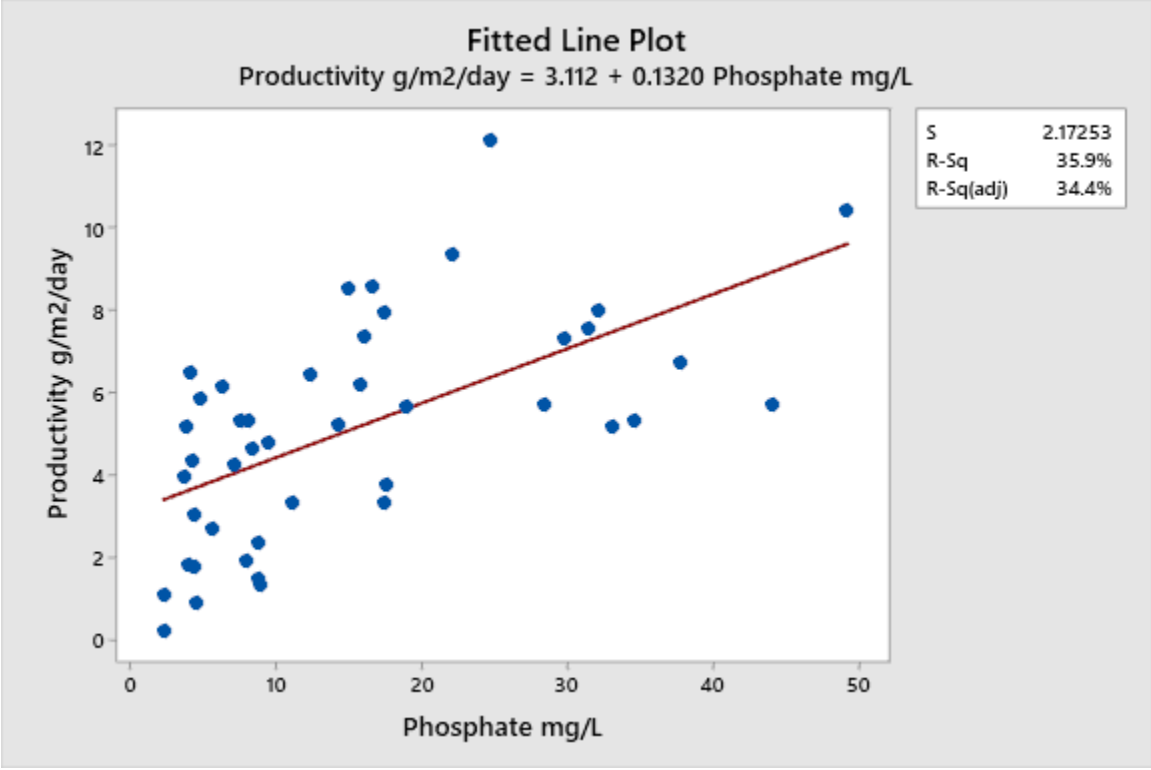
The regression equation is
Productivity g/m2/day = 3.112 + 0.1320 Phosphate mg/L

Model Summary

S	R-sq	R-sq(adj)
2.17253	35.95%	34.42%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	111.249	111.249	23.57	0.000
Error	42	198.235	4.720		
Total	43	309.484			



Appendix A.8: Dilution Phase Algae Productivity vs Chloride

REGRESSION DIL.

Regression Analysis: Productivity g/m2/day versus Chloride mg/L

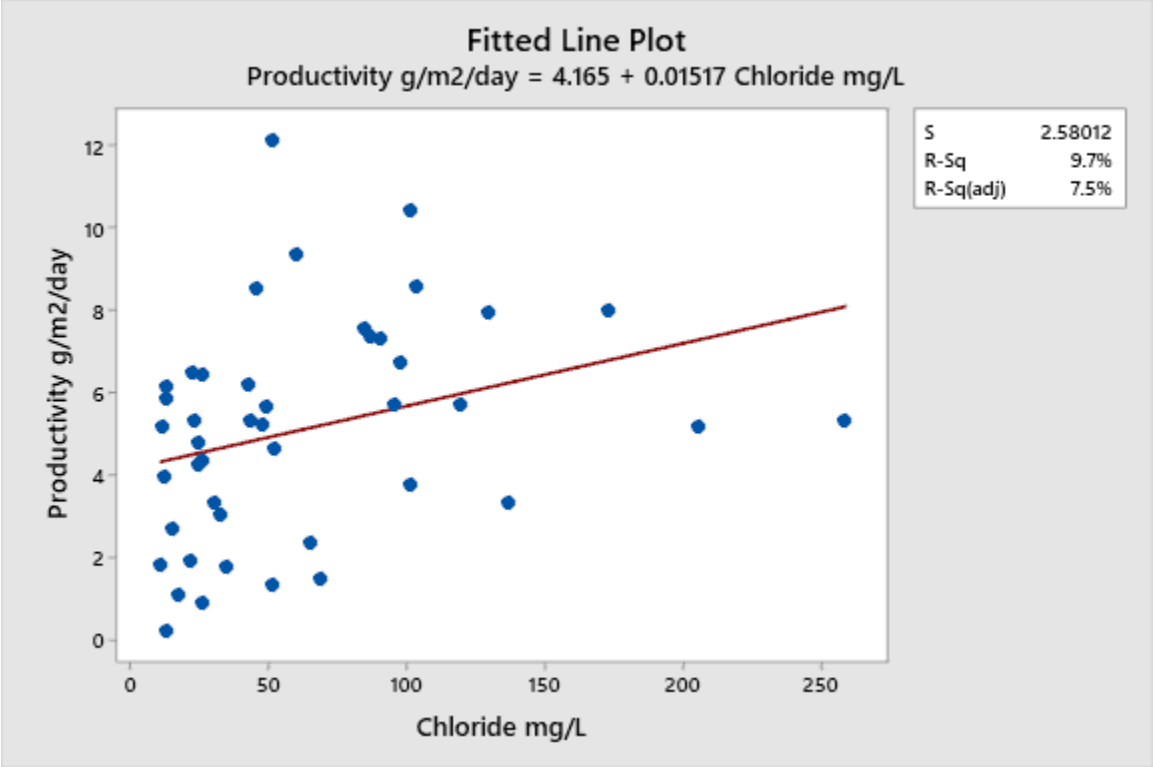
The regression equation is
Productivity g/m2/day = 4.165 + 0.01517 Chloride mg/L

Model Summary

S	R-sq	R-sq(adj)
2.58012	9.66%	7.51%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	29.889	29.8889	4.49	0.040
Error	42	279.595	6.6570		
Total	43	309.484			



Appendix A.9: Dilution Phase Algae Productivity vs N:P Ratio

REGRESSION DIL.

Regression Analysis: Productivity g/m2/day versus N:P

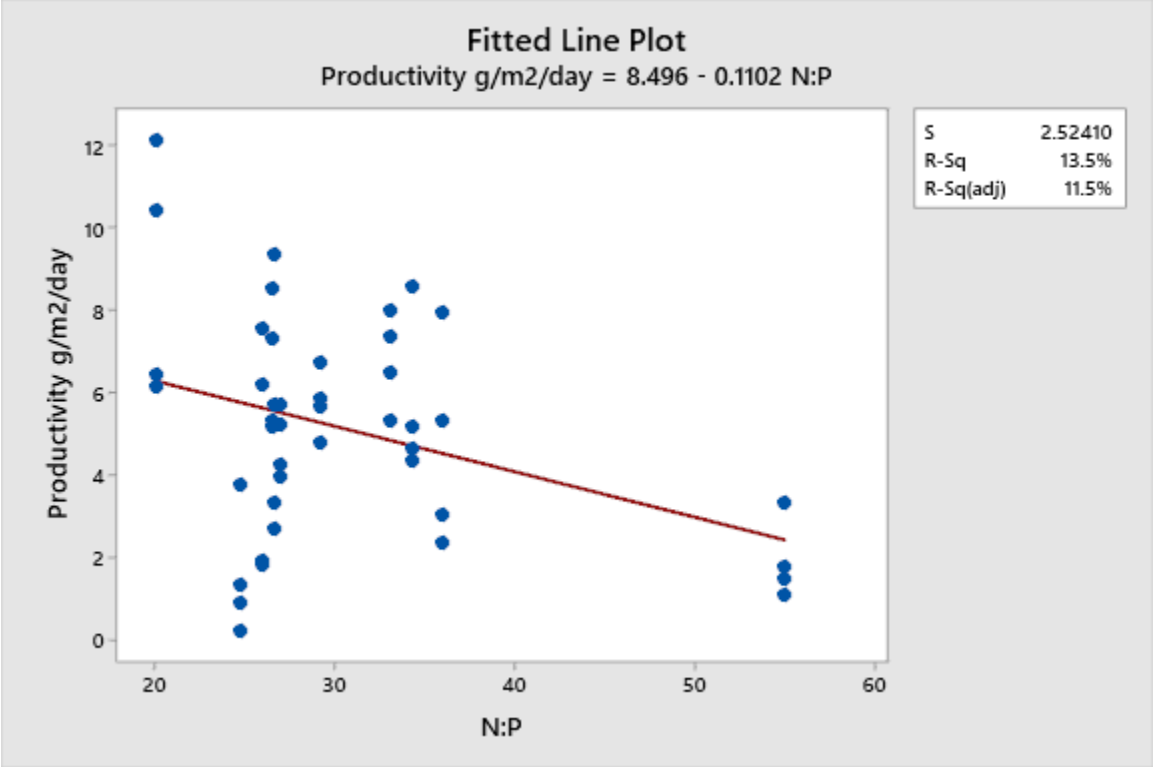
The regression equation is
Productivity g/m2/day = 8.496 - 0.1102 N:P

Model Summary

S	R-sq	R-sq(adj)
2.52410	13.54%	11.48%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	41.898	41.8980	6.58	0.014
Error	42	267.586	6.3711		
Total	43	309.484			



Appendix A.10: Production Phase Algae Productivity Lane 1 vs Time

PRODUCTION PRODUCTIVITY ANALYSIS

Regression Analysis: Lane 1 versus Date

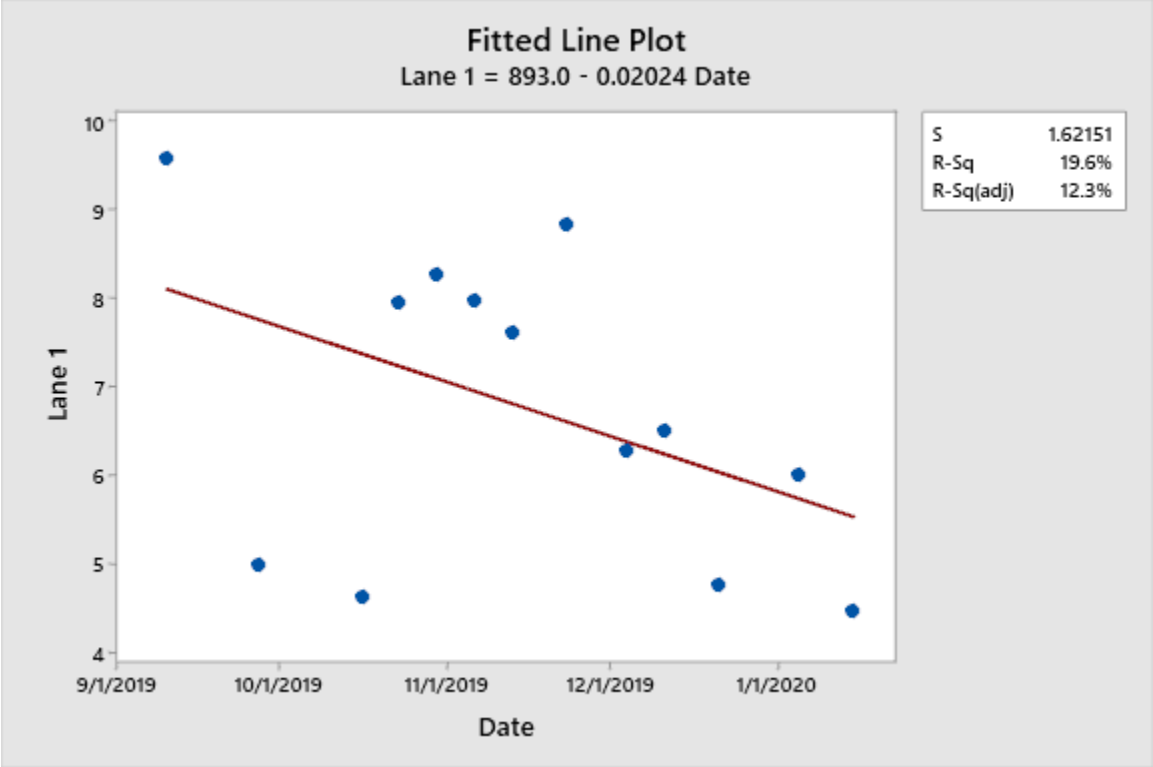
The regression equation is
Lane 1 = 893.0 - 0.02024 Date

Model Summary

S	R-sq	R-sq(adj)
1.62151	19.63%	12.33%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	7.0653	7.06529	2.69	0.129
Error	11	28.9222	2.62929		
Total	12	35.9874			



Appendix A.11: Production Phase Algae Productivity Lane 2 vs Time

PRODUCTION PRODUCTIVITY ANALYSIS

Regression Analysis: Lane 2 versus Date

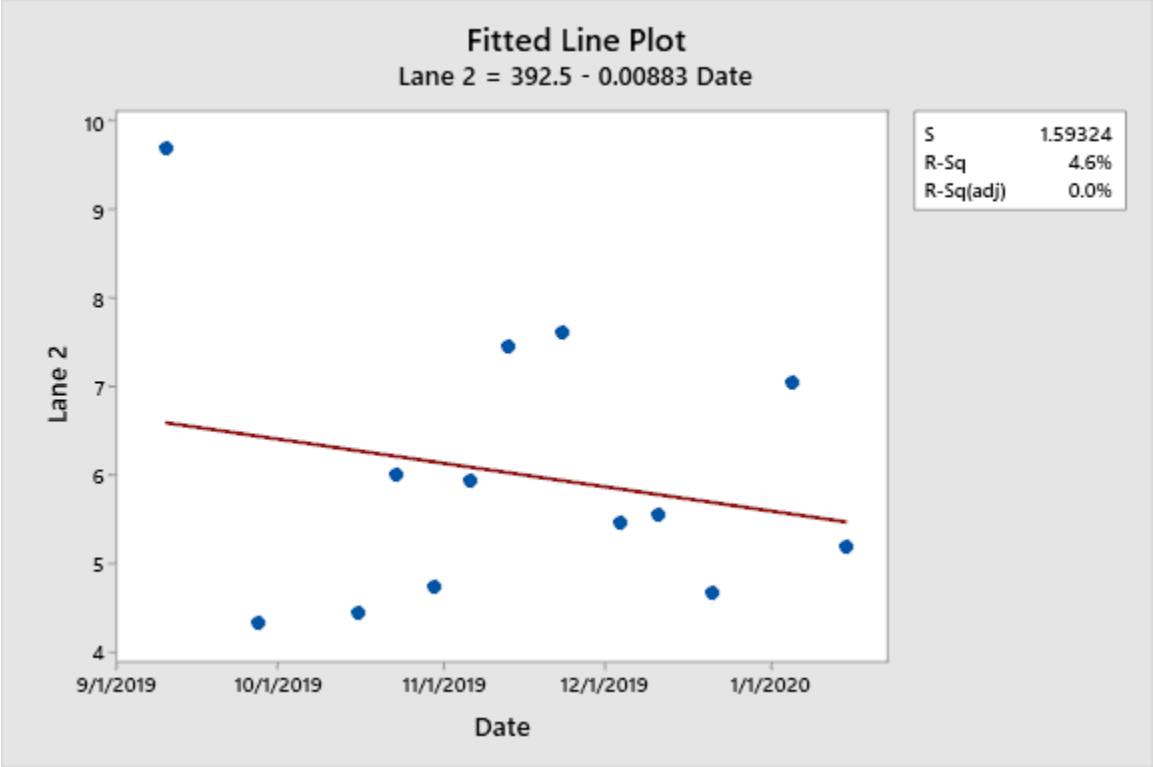
The regression equation is
Lane 2 = 392.5 - 0.00883 Date

Model Summary

S	R-sq	R-sq(adj)
1.59324	4.59%	0.00%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1.3433	1.34335	0.53	0.482
Error	11	27.9225	2.53841		
Total	12	29.2658			



Appendix A.12: Production Phase Algae Productivity Lane 3 vs Time

PRODUCTION PRODUCTIVITY ANALYSIS

Regression Analysis: Lane 3 versus Date

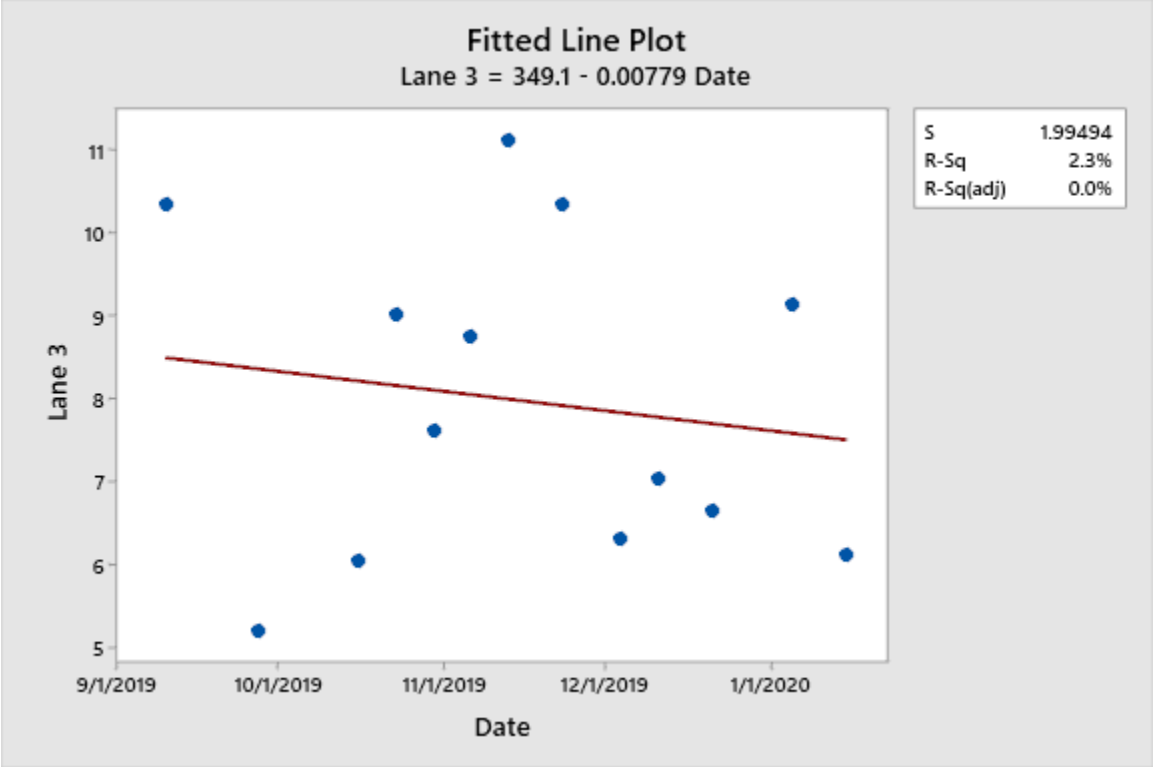
The regression equation is
Lane 3 = 349.1 - 0.00779 Date

Model Summary

S	R-sq	R-sq(adj)
1.99494	2.33%	0.00%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1.0466	1.04663	0.26	0.618
Error	11	43.7775	3.97977		
Total	12	44.8241			



Appendix A.13: Production Phase Algae Productivity Lane 4 vs Time

PRODUCTION PRODUCTIVITY ANALYSIS

Regression Analysis: Lane 4 versus Date

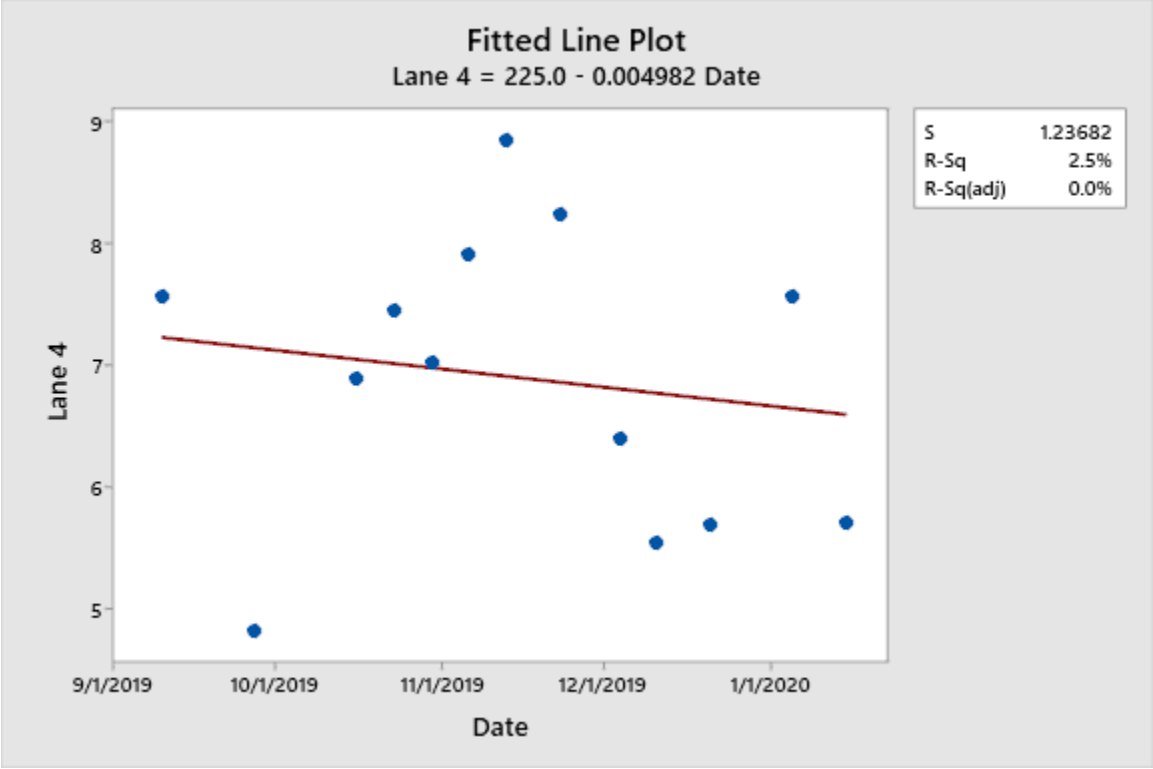
The regression equation is
Lane 4 = 225.0 - 0.004982 Date

Model Summary

S	R-sq	R-sq(adj)
1.23682	2.48%	0.00%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.4280	0.42796	0.28	0.607
Error	11	16.8271	1.52973		
Total	12	17.2550			



Appendix A.14: Production Phase Algae Productivity vs Lane

REGRESSION PRO.

One-way ANOVA: Productivity g/m2/day versus Lane

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Lane	4	1, 2, 3, 4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Lane	3	25.79	8.596	3.24	0.030
Error	48	127.33	2.653		
Total	51	153.12			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.62872	16.84%	11.64%	2.40%

Means

Lane	N	Mean	StDev	95% CI
1	13	6.756	1.732	(5.848, 7.664)
2	13	6.005	1.562	(5.097, 6.914)
3	13	7.978	1.933	(7.070, 8.887)
4	13	6.898	1.199	(5.989, 7.806)

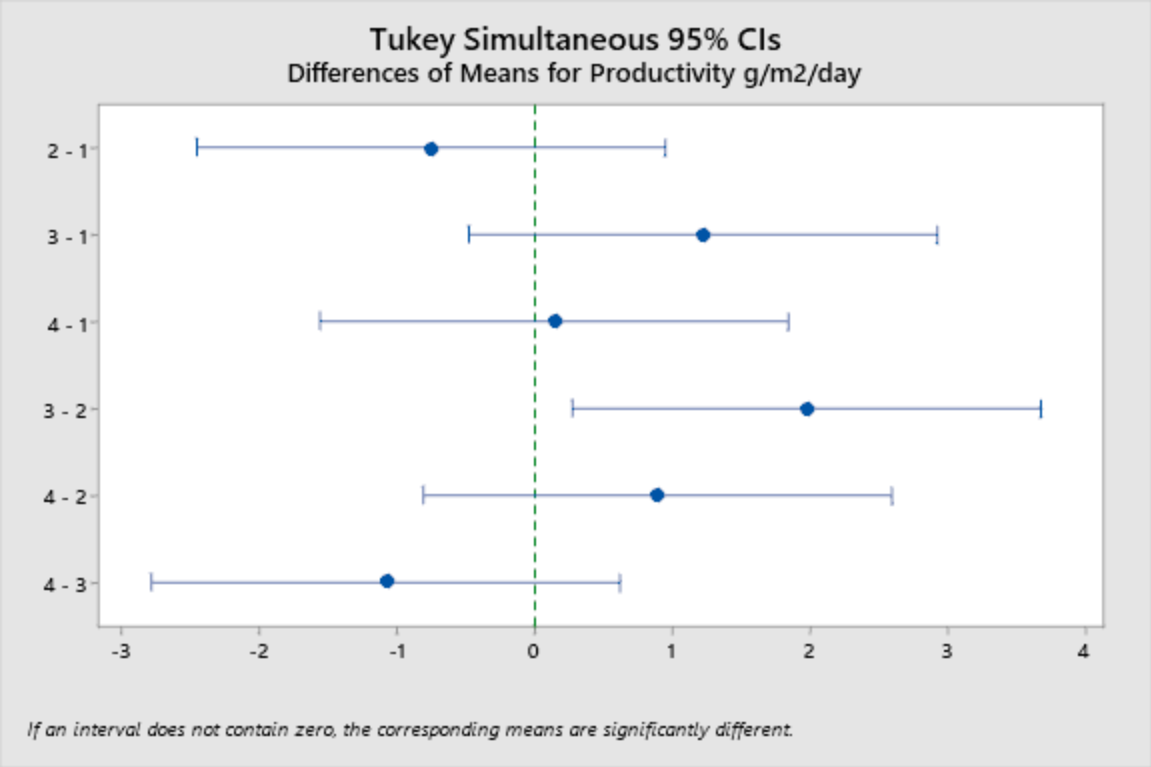
Pooled StDev = 1.62872

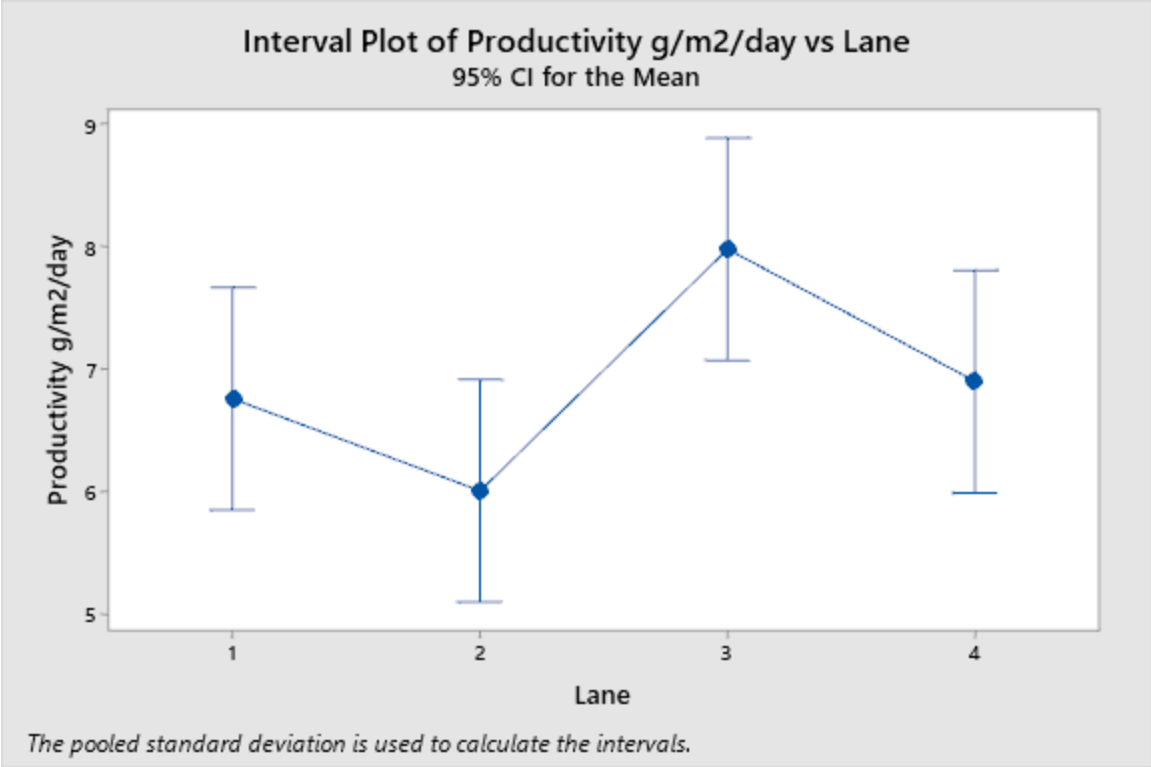
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Lane	N	Mean	Grouping	
3	13	7.978	A	
4	13	6.898	A	B
1	13	6.756	A	B
2	13	6.005		B

Means that do not share a letter are significantly different.


Tukey Simultaneous 95% CIs
Differences of Means for Productivity g/m2/day
2 - 1
3 - 1
4 - 1
3 - 2
4 - 2
4 - 3
-3
-2
-1
0
1
2
3
4
If an interval does not contain zero, the corresponding means are significantly different.


Interval Plot of Productivity g/m2/day vs Lane
95% CI for the Mean
Productivity g/m2/day
9
8
7
6
5
1
2
3
4
Lane
The pooled standard deviation is used to calculate the intervals.

Appendix A.15: Production Phase Algae Productivity vs Nitrate

REGRESSION PRO.

Regression Analysis: Productivity g/m2/day versus Nitrate mg/L

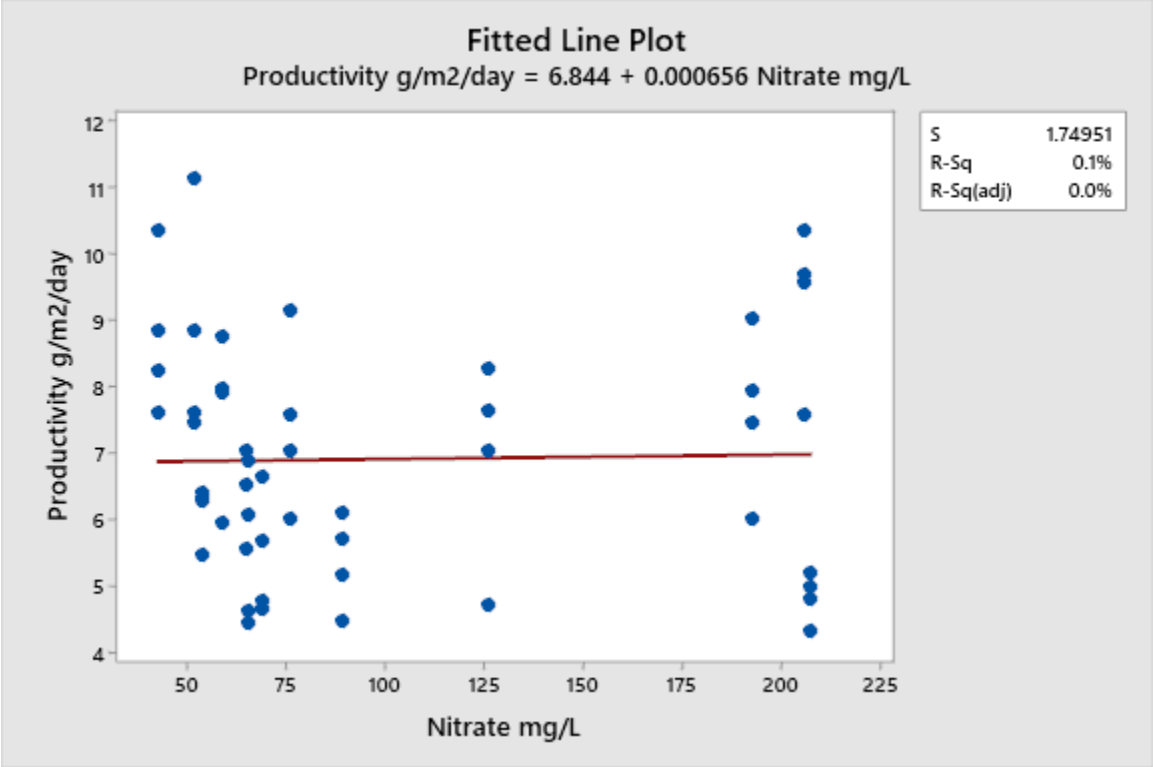
The regression equation is
Productivity g/m2/day = 6.844 + 0.000656 Nitrate mg/L

Model Summary

S	R-sq	R-sq(adj)
1.74951	0.05%	0.00%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.079	0.07892	0.03	0.873
Error	50	153.040	3.06079		
Total	51	153.119			



Appendix A.16: Production Phase Algae Productivity vs Phosphate

REGRESSION PRO.

Regression Analysis: Productivity g/m2/day versus Phosphate mg/L

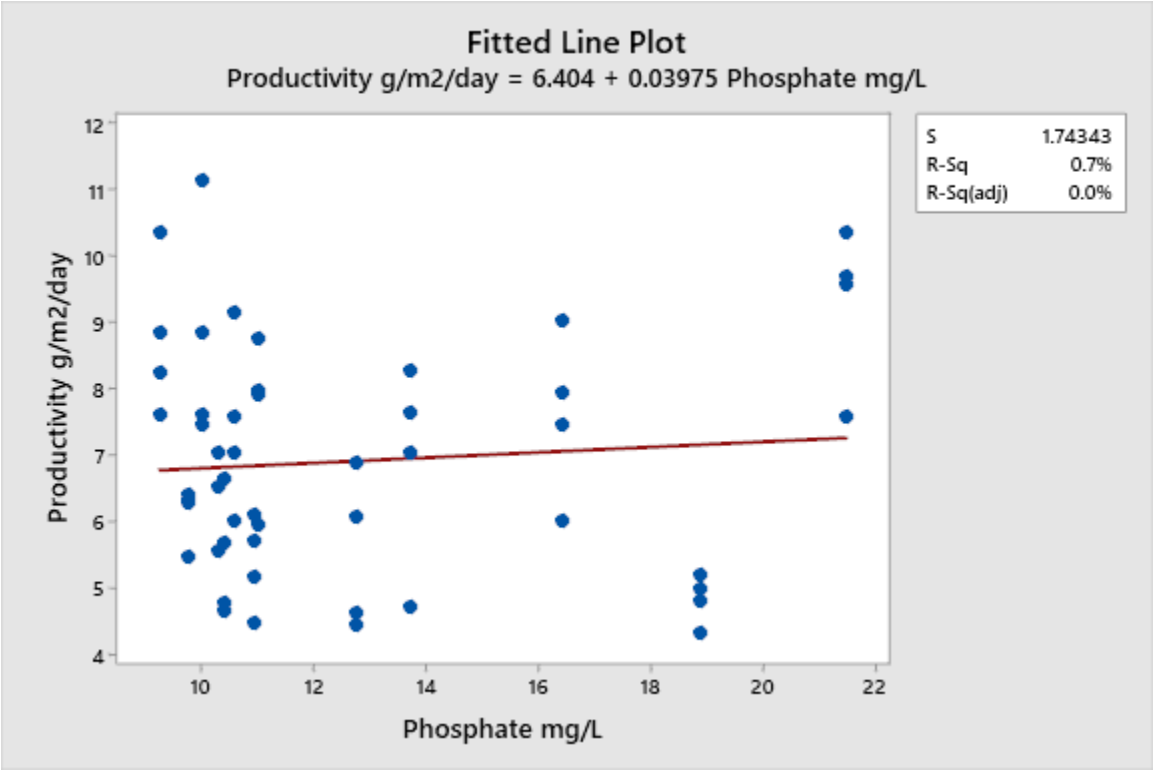
The regression equation is
Productivity g/m2/day = 6.404 + 0.03975 Phosphate mg/L

Model Summary

S	R-sq	R-sq(adj)
1.74343	0.74%	0.00%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1.140	1.14039	0.38	0.543
Error	50	151.978	3.03956		
Total	51	153.119			



Appendix A.17: Production Phase Algae Productivity vs Chloride

REGRESSION PRO.

Regression Analysis: Productivity g/m2/day versus Chloride mg/L

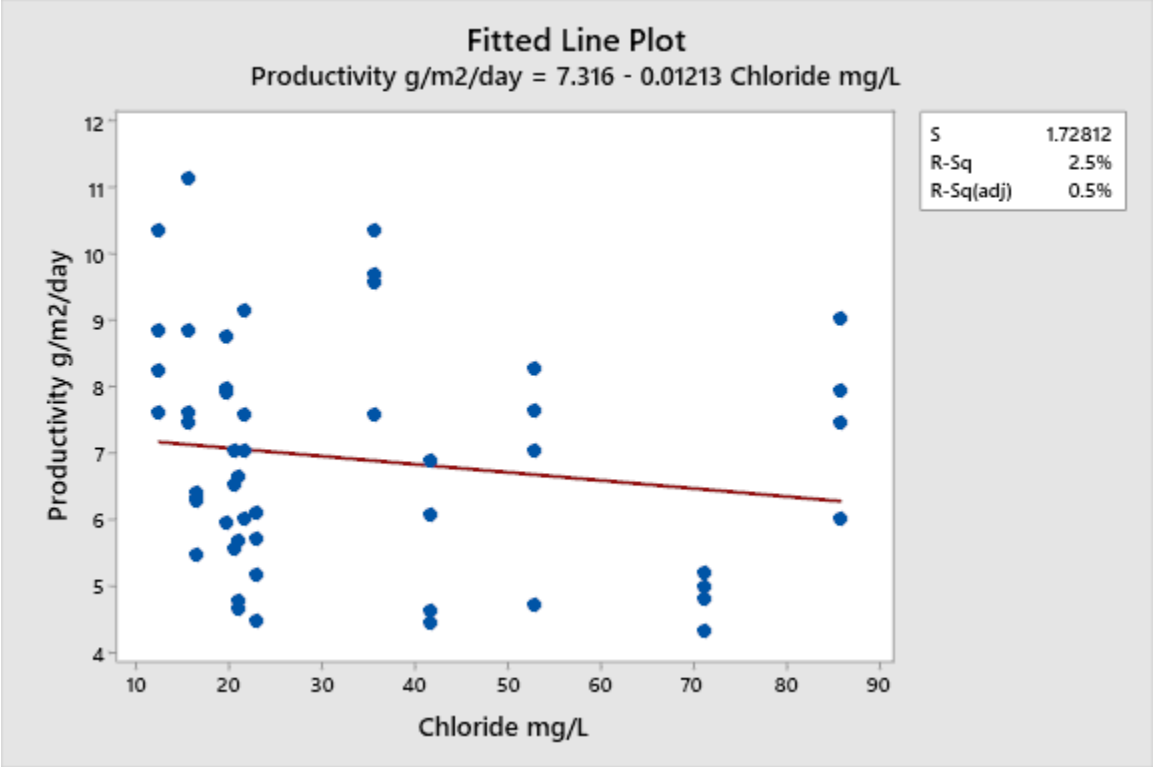
The regression equation is
Productivity g/m2/day = 7.316 - 0.01213 Chloride mg/L

Model Summary

S	R-sq	R-sq(adj)
1.72812	2.48%	0.53%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	3.799	3.79920	1.27	0.265
Error	50	149.319	2.98639		
Total	51	153.119			



Appendix A.18: Production Phase Algae Productivity vs N:P Ratio

REGRESSION PRO.

Regression Analysis: Productivity g/m2/day versus N:P

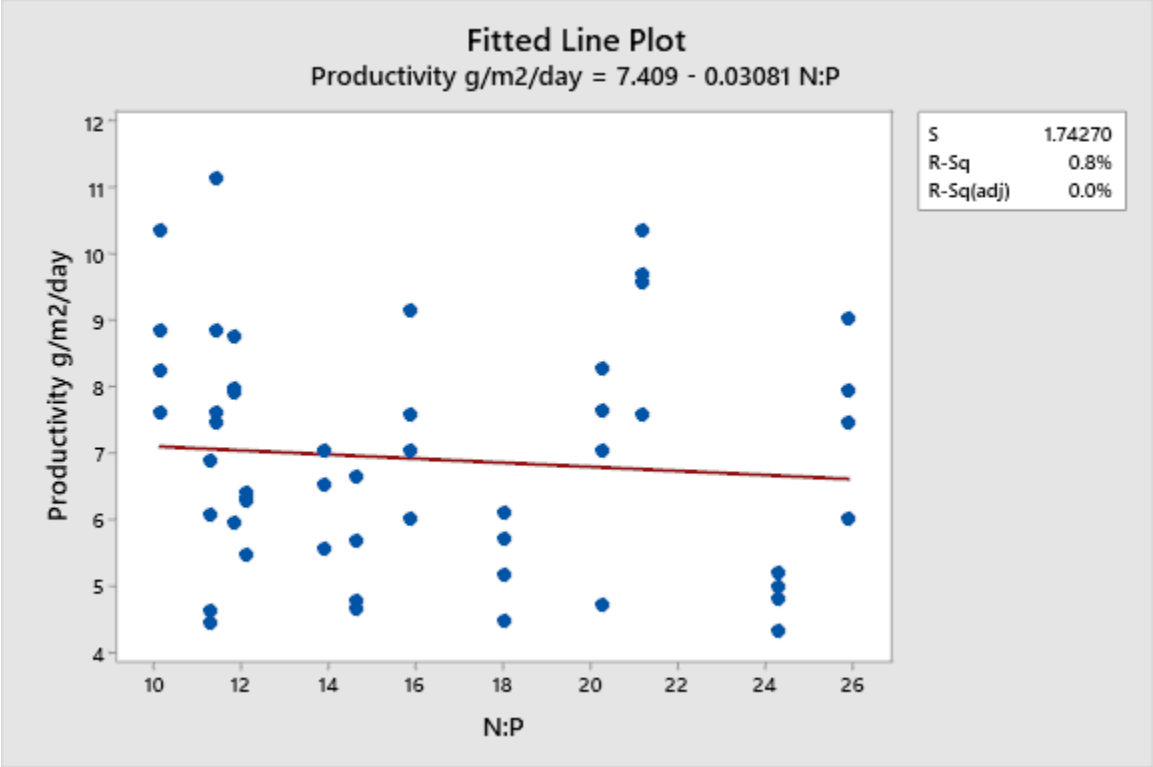
The regression equation is
Productivity g/m2/day = 7.409 - 0.03081 N:P

Model Summary

S	R-sq	R-sq(adj)
1.74270	0.83%	0.00%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1.269	1.26920	0.42	0.521
Error	50	151.849	3.03699		
Total	51	153.119			



Appendix B: Algae Ash% Minitab Statistical Output

Appendix B.1: Dilution Phase Algae Ash% Undiluted vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: Ash% Undiluted versus Date

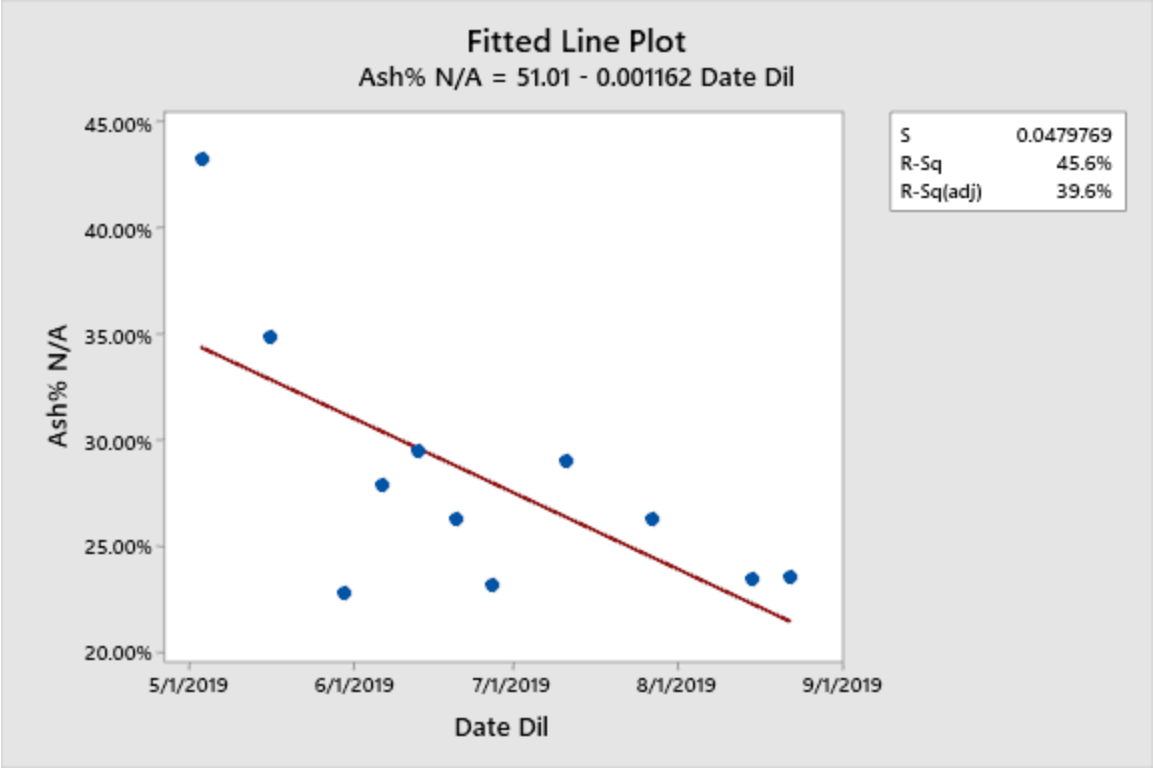
The regression equation is
Ash% N/A = 51.01 - 0.001162 Date Dil

Model Summary

S	R-sq	R-sq(adj)
0.0479769	45.62%	39.58%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0173799	0.0173799	7.55	0.023
Error	9	0.0207160	0.0023018		
Total	10	0.0380959			



Appendix B.2: Dilution Phase Algae Ash% 2x Dilution vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: Ash% 1/2 Dilution versus Date

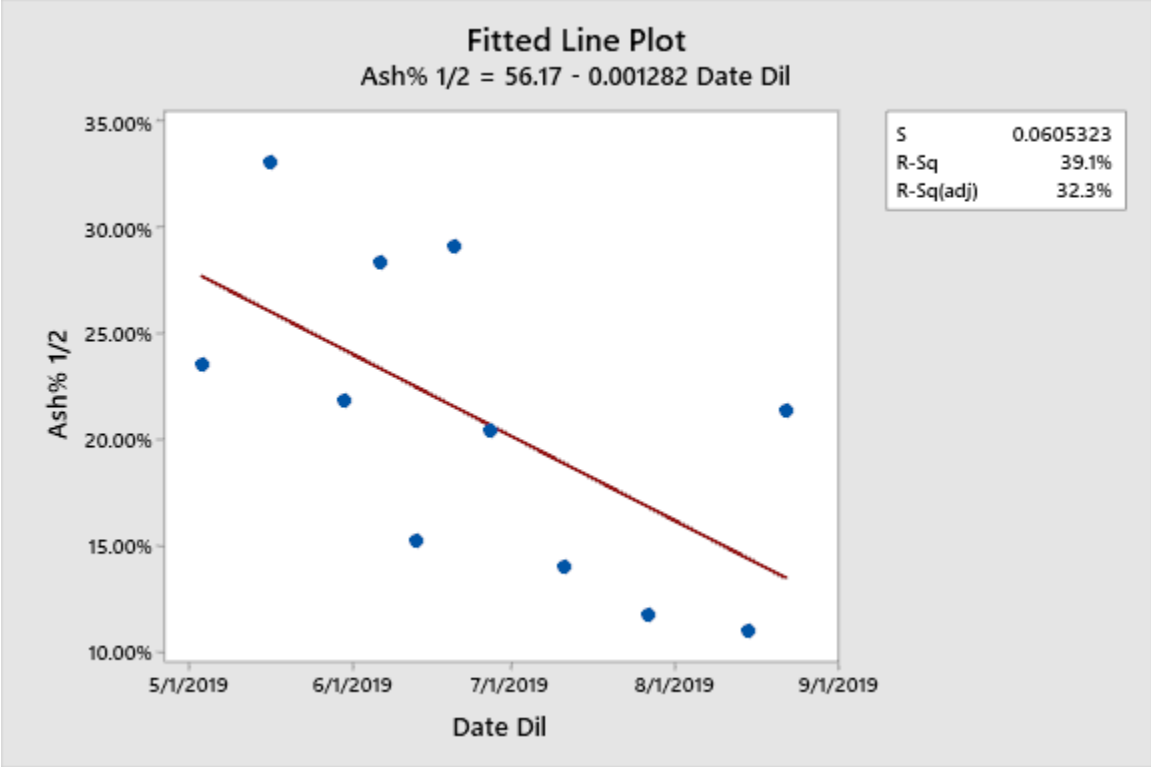
The regression equation is
Ash% 1/2 = 56.17 - 0.001282 Date Dil

Model Summary

S	R-sq	R-sq(adj)
0.0605323	39.07%	32.30%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0211493	0.0211493	5.77	0.040
Error	9	0.0329775	0.0036642		
Total	10	0.0541267			



Appendix B.3: Dilution Phase Algae Ash% 4x Dilution vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: Ash% 1/4 Dilution versus Date

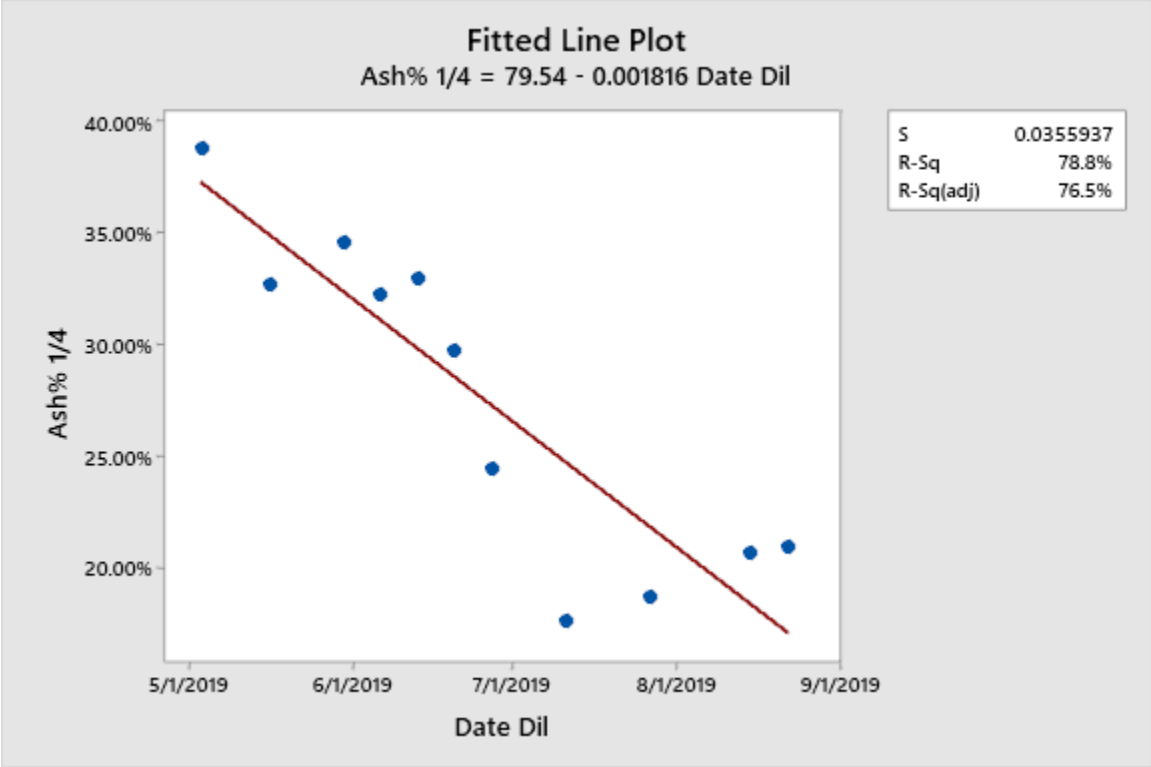
The regression equation is
Ash% 1/4 = 79.54 - 0.001816 Date Dil

Model Summary

S	R-sq	R-sq(adj)
0.0355937	78.82%	76.47%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0424316	0.0424316	33.49	0.000
Error	9	0.0114022	0.0012669		
Total	10	0.0538338			



Appendix B.4: Dilution Phase Algae Ash% 8x Dilution vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: Ash% 1/8 Dilution versus Date

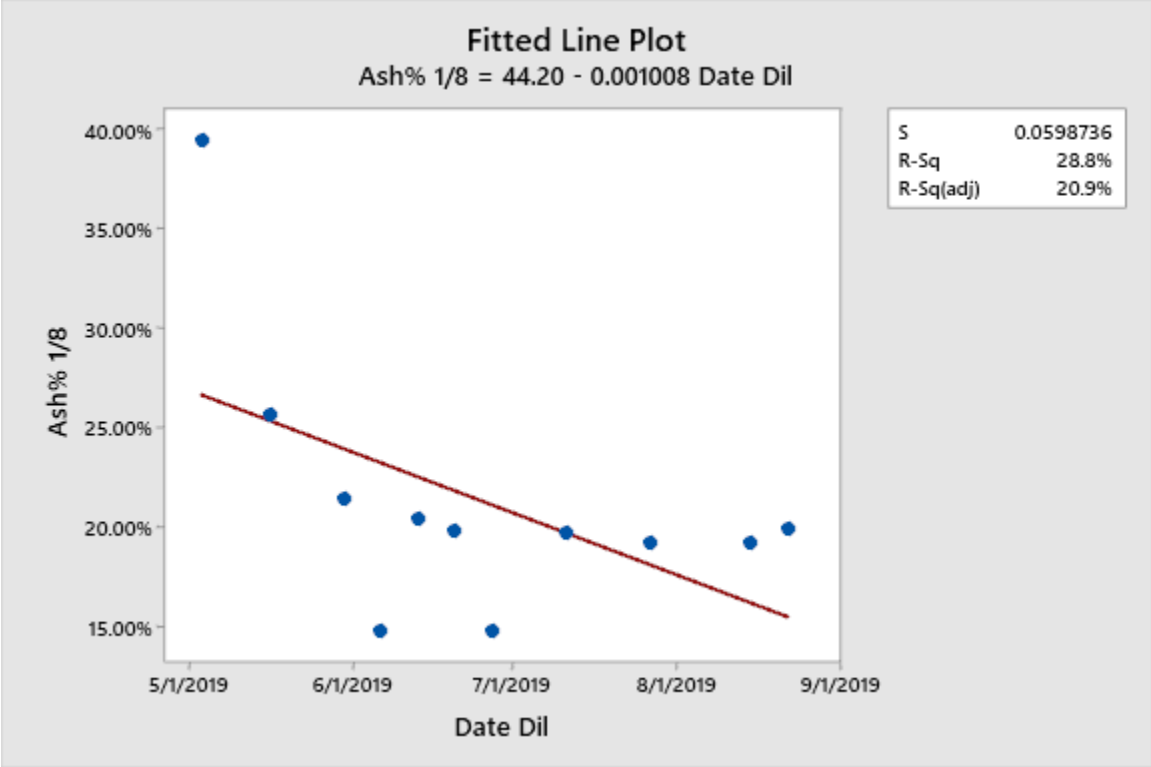
The regression equation is
Ash% 1/8 = 44.20 - 0.001008 Date Dil

Model Summary

S	R-sq	R-sq(adj)
0.0598736	28.83%	20.92%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0130676	0.0130676	3.65	0.089
Error	9	0.0322636	0.0035848		
Total	10	0.0453311			



Appendix B.5: Dilution Phase Algae Ash% vs Dilution

REGRESSION DIL.

One-way ANOVA: Ash% versus Dilution

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Dilution	4	1 Undiluted, 2 Half, 3 Fourth, 4 Eighth

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Dilution	3	0.05109	0.017031	3.56	0.022
Error	40	0.19137	0.004784		
Total	43	0.24246			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0691680	21.07%	15.15%	4.50%

Means

Dilution	N	Mean	StDev	95% CI
1 Undiluted	11	0.2818	0.0617	(0.2396, 0.3239)
2 Half	11	0.2088	0.0736	(0.1667, 0.2510)
3 Fourth	11	0.2759	0.0734	(0.2337, 0.3180)
4 Eighth	11	0.2129	0.0673	(0.1708, 0.2551)

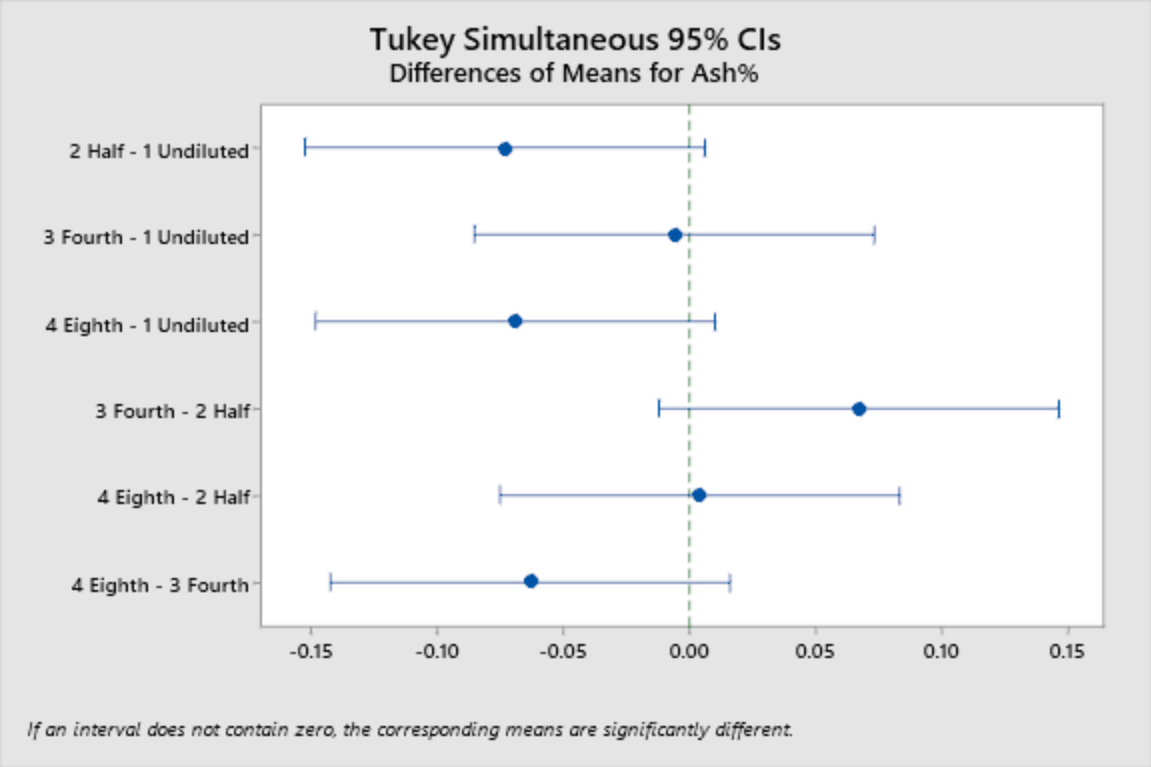
Pooled StDev = 0.0691680

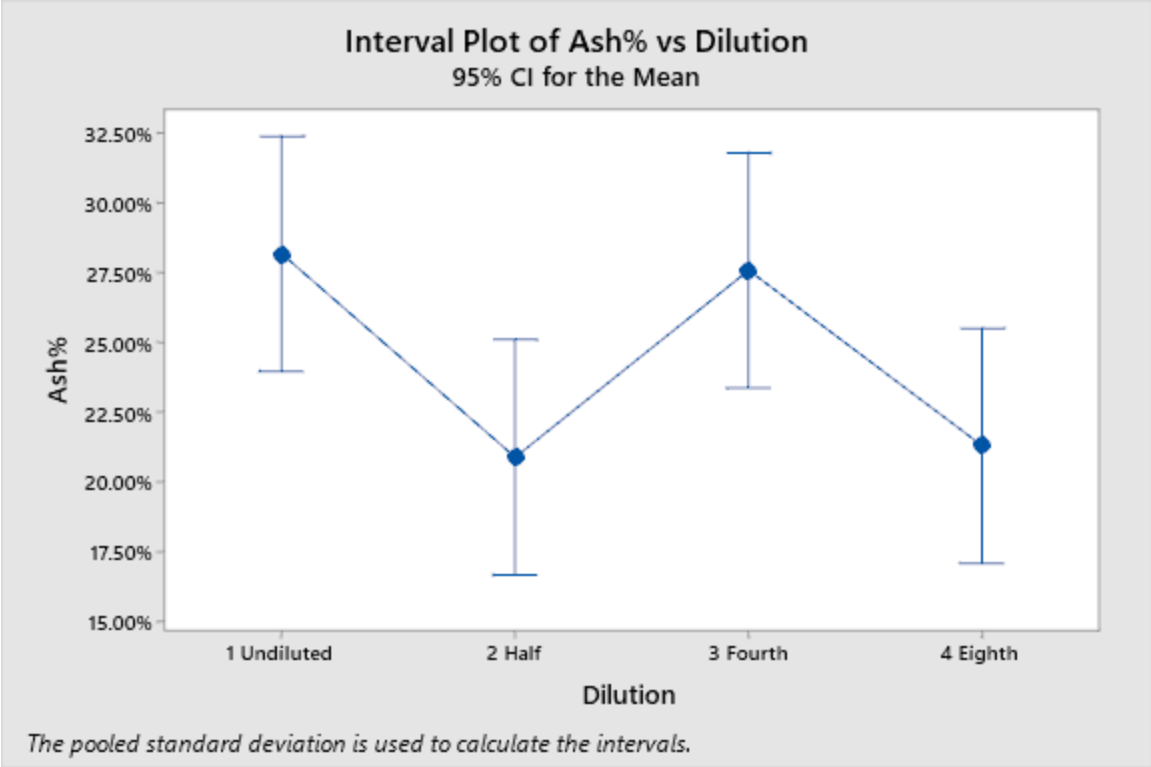
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Dilution	N	Mean	Grouping
1 Undiluted	11	0.2818	A
3 Fourth	11	0.2759	A
4 Eighth	11	0.2129	A
2 Half	11	0.2088	A

Means that do not share a letter are significantly different.


Tukey Simultaneous 95% CIs
Differences of Means for Ash%
2 Half - 1 Undiluted
3 Fourth - 1 Undiluted
4 Eighth - 1 Undiluted
3 Fourth - 2 Half
4 Eighth - 2 Half
4 Eighth - 3 Fourth
-0.15
-0.10
-0.05
0.00
0.05
0.10
0.15
If an interval does not contain zero, the corresponding means are significantly different.


Interval Plot of Ash% vs Dilution
95% CI for the Mean
32.50%
30.00%
27.50%
25.00%
22.50%
20.00%
17.50%
15.00%
Ash%
1 Undiluted
2 Half
3 Fourth
4 Eighth
Dilution
The pooled standard deviation is used to calculate the intervals.

Appendix B.6: Dilution Phase Algae Ash% vs Nitrate

REGRESSION DIL.

Regression Analysis: Ash% versus Nitrate mg/L

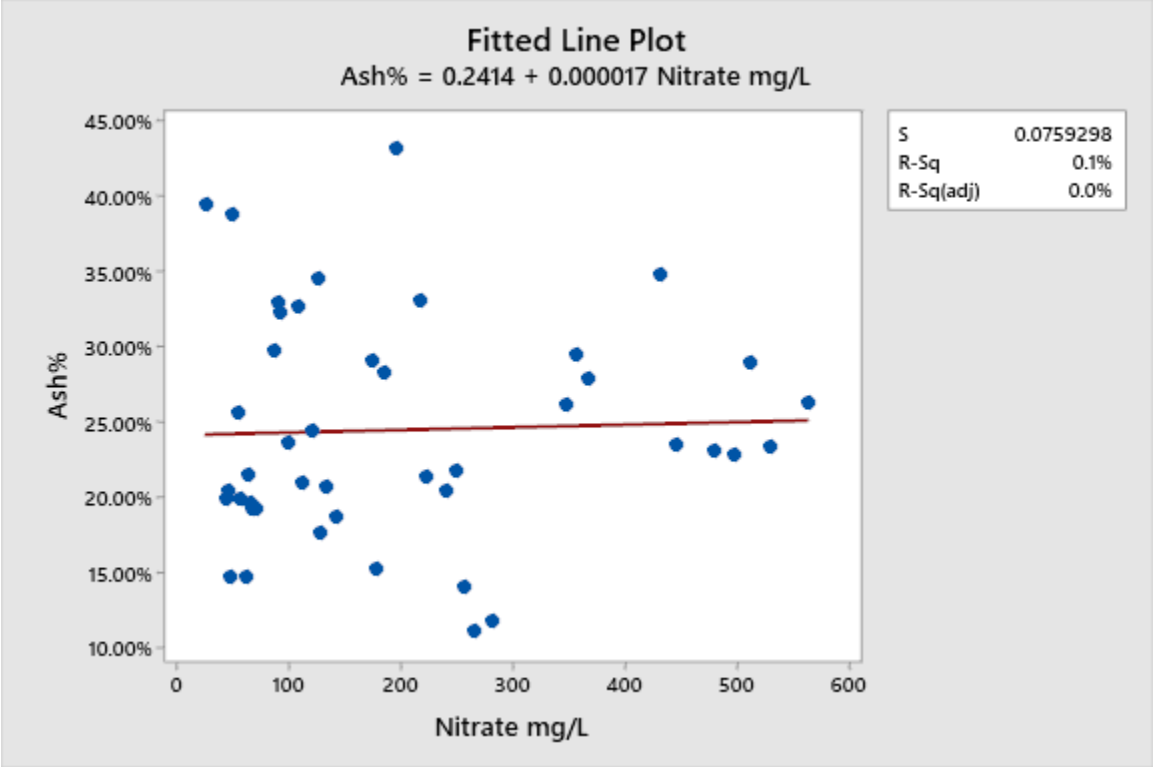
The regression equation is
Ash% = 0.2414 + 0.000017 Nitrate mg/L

Model Summary

S	R-sq	R-sq(adj)
0.0759298	0.13%	0.00%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.000317	0.0003169	0.05	0.816
Error	42	0.242144	0.0057653		
Total	43	0.242461			



Appendix B.7: Dilution Phase Algae Ash% vs Phosphate

REGRESSION DIL.

Regression Analysis: Ash% versus Phosphate mg/L

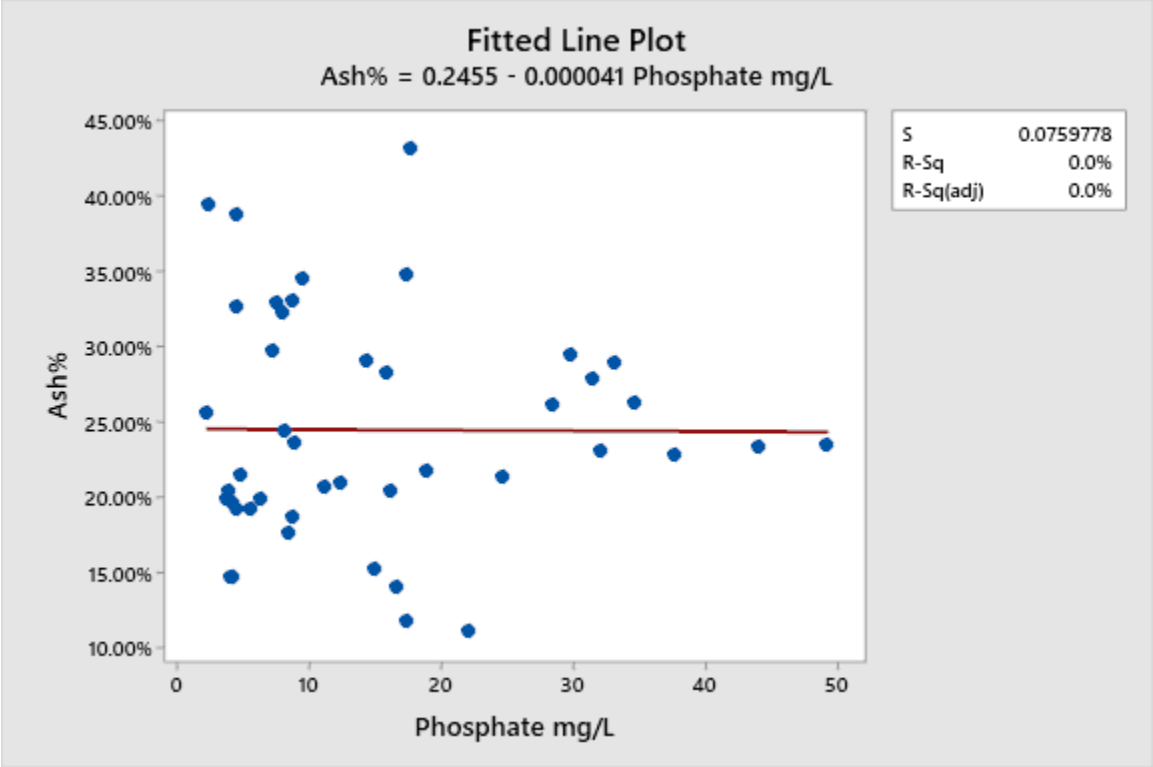
The regression equation is
Ash% = 0.2455 - 0.000041 Phosphate mg/L

Model Summary

S	R-sq	R-sq(adj)
0.0759778	0.00%	0.00%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.000011	0.0000106	0.00	0.966
Error	42	0.242450	0.0057726		
Total	43	0.242461			



Appendix B.8: Dilution Phase Algae Ash% vs Chloride

REGRESSION DIL.

Regression Analysis: Ash% versus Chloride mg/L

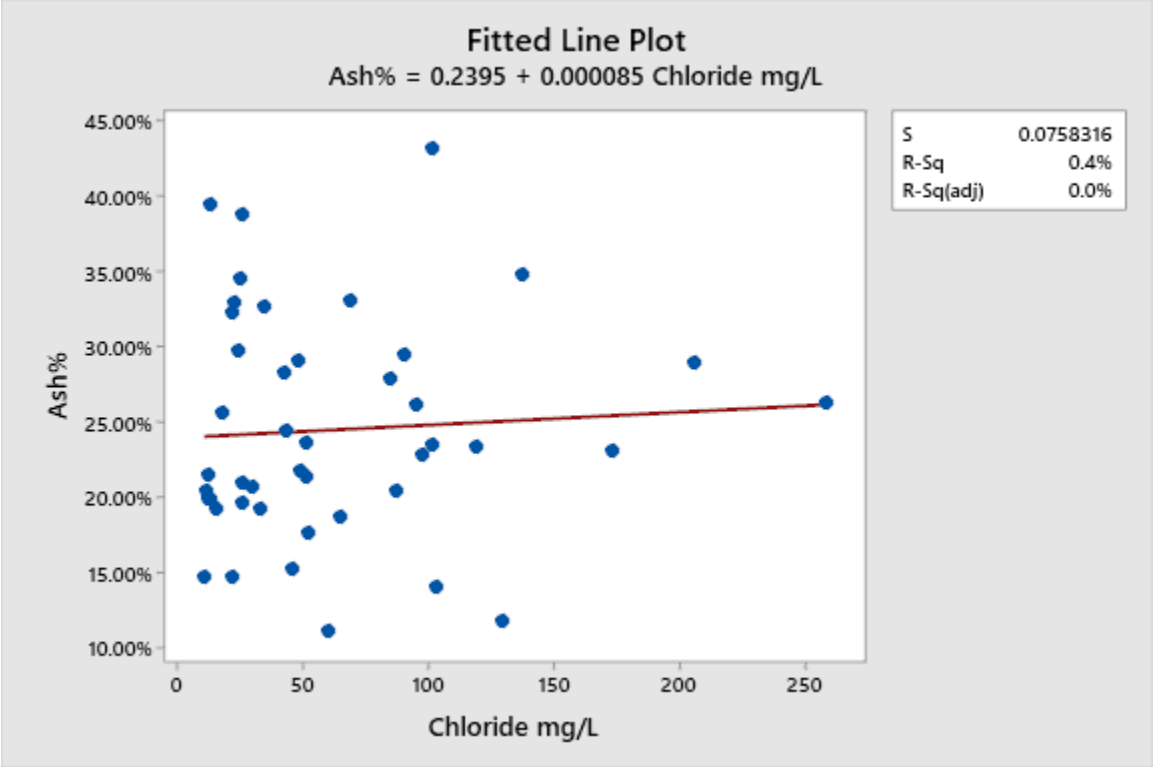
The regression equation is
Ash% = 0.2395 + 0.000085 Chloride mg/L

Model Summary

S	R-sq	R-sq(adj)
0.0758316	0.39%	0.00%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.000943	0.0009429	0.16	0.688
Error	42	0.241518	0.0057504		
Total	43	0.242461			



Appendix B.9: Dilution Phase Algae Ash% vs N:P Ratio

REGRESSION DIL.

Regression Analysis: Ash% versus N:P

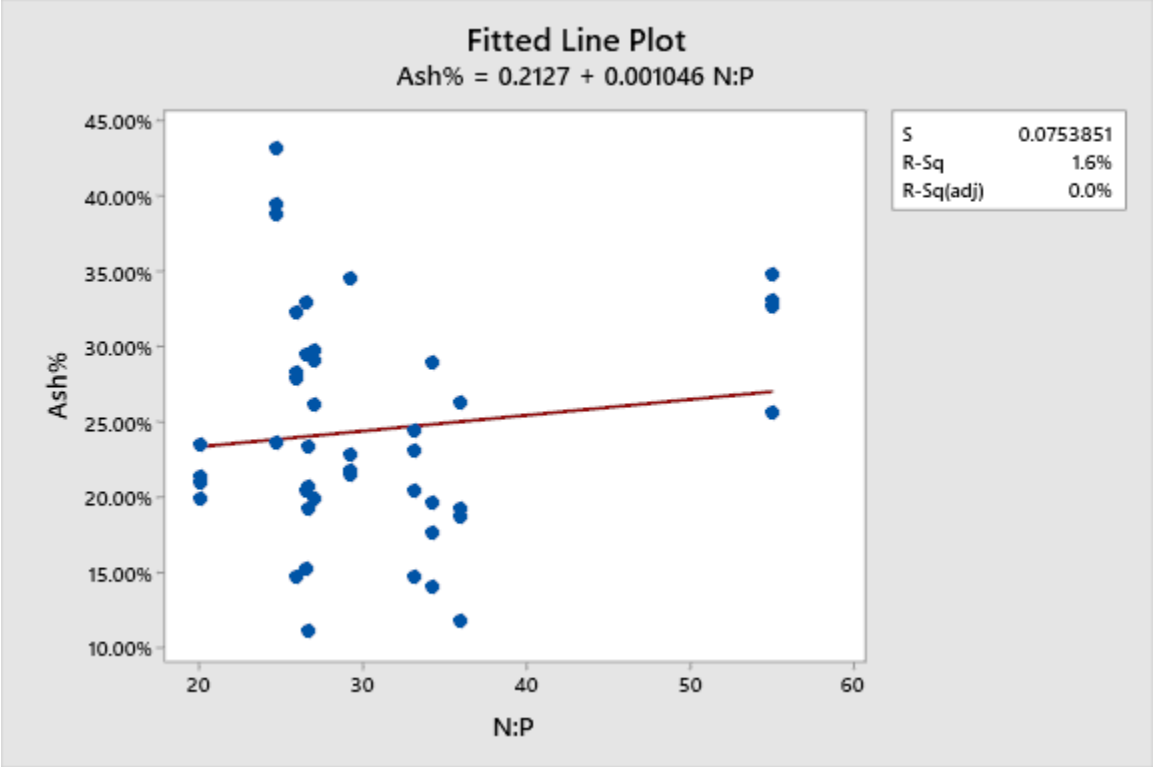
The regression equation is
Ash% = 0.2127 + 0.001046 N:P

Model Summary

S	R-sq	R-sq(adj)
0.0753851	1.56%	0.00%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.003778	0.0037785	0.66	0.419
Error	42	0.238683	0.0056829		
Total	43	0.242461			



Appendix B.10: Production Phase Algae Ash% Lane 1 vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: Ash% Lane 1 versus Date

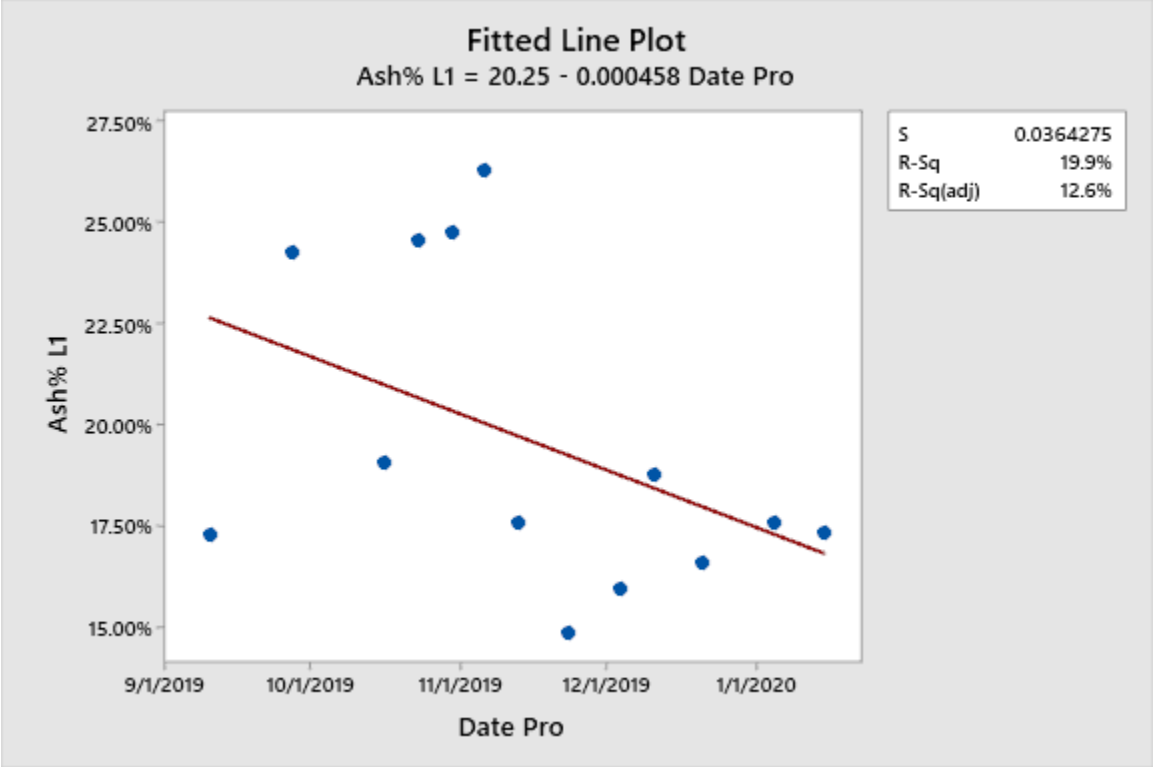
The regression equation is
Ash% L1 = 20.25 - 0.000458 Date Pro

Model Summary

S	R-sq	R-sq(adj)
0.0364275	19.86%	12.57%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0036166	0.0036166	2.73	0.127
Error	11	0.0145966	0.0013270		
Total	12	0.0182132			



Appendix B.11: Production Phase Algae Ash% Lane 2 vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: Ash% Lane 2 versus Date

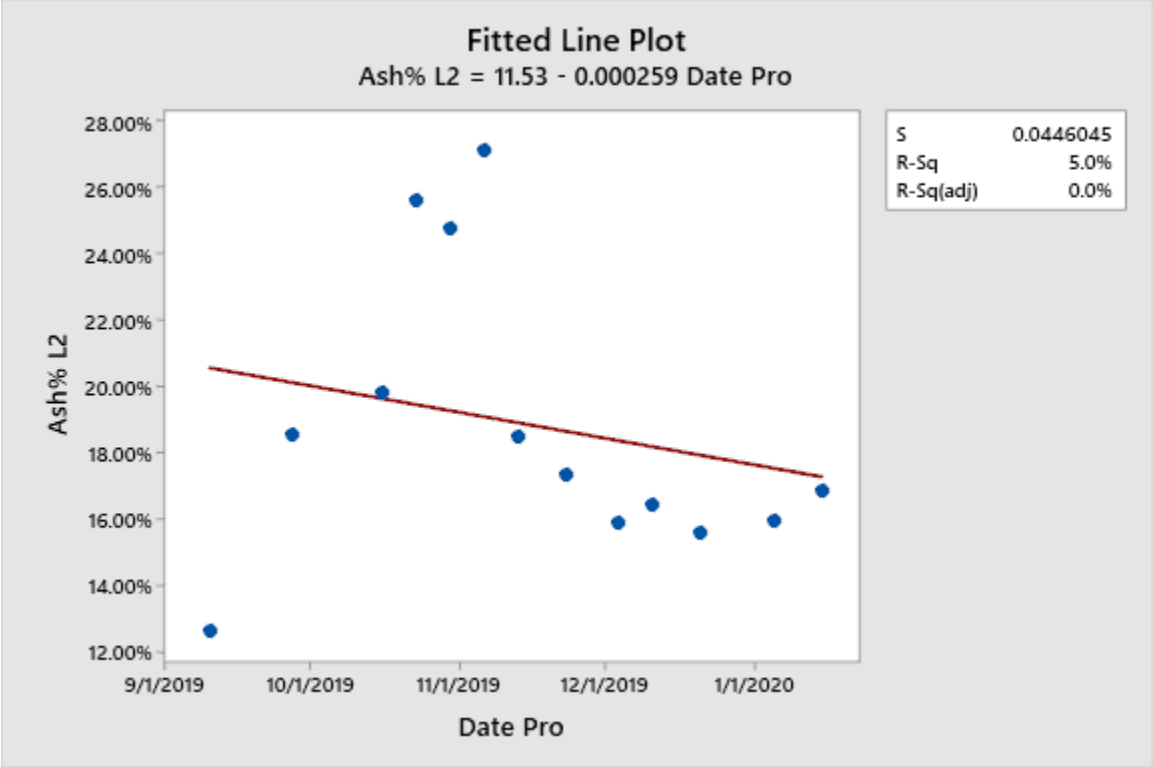
The regression equation is
Ash% L2 = 11.53 - 0.000259 Date Pro

Model Summary

S	R-sq	R-sq(adj)
0.0446045	5.02%	0.00%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0011564	0.0011564	0.58	0.462
Error	11	0.0218852	0.0019896		
Total	12	0.0230416			



Appendix B.12: Production Phase Algae Ash% Lane 3 vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: Ash% Lane 3 versus Date

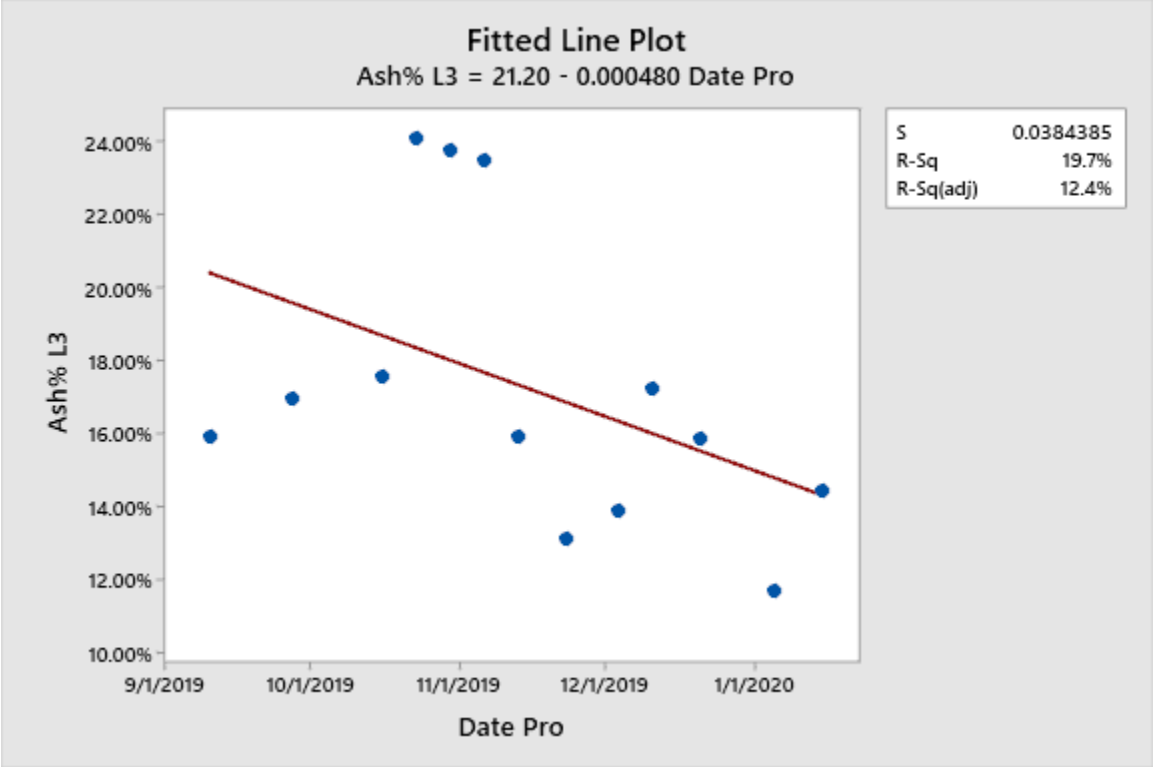
The regression equation is
Ash% L3 = 21.20 - 0.000480 Date Pro

Model Summary

S	R-sq	R-sq(adj)
0.0384385	19.66%	12.36%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0039783	0.0039783	2.69	0.129
Error	11	0.0162527	0.0014775		
Total	12	0.0202310			



Appendix B.13: Production Phase Algae Ash% Lane 4 vs Time

PRODUCTIVITY ANALYSIS

Regression Analysis: Ash% Lane 4 versus Date

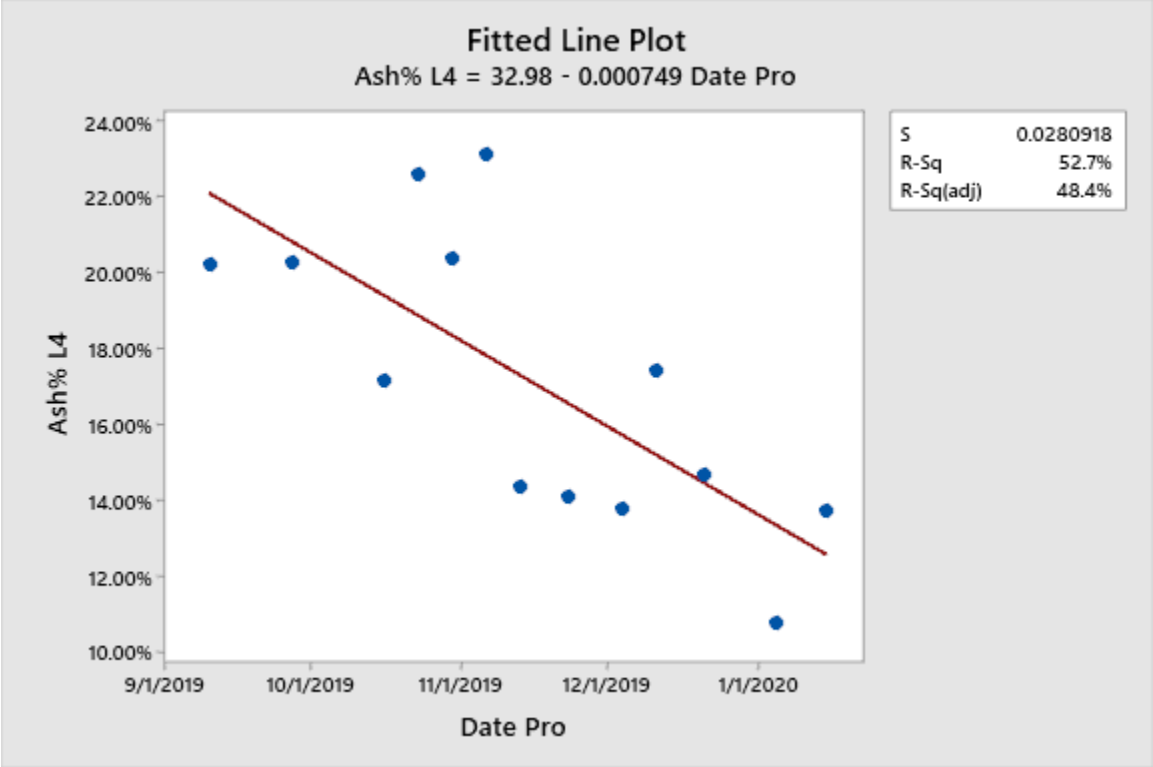
The regression equation is
Ash% L4 = 32.98 - 0.000749 Date Pro

Model Summary

S	R-sq	R-sq(adj)
0.0280918	52.72%	48.42%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0096802	0.0096802	12.27	0.005
Error	11	0.0086806	0.0007891		
Total	12	0.0183608			



Appendix B.14: Production Phase Algae Ash% vs Lane

REGRESSION PRO.

One-way ANOVA: Ash% versus Lane

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Lane	4	1, 2, 3, 4

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Lane	3	0.005837	0.001946	1.17	0.331
Error	48	0.079841	0.001663		
Total	51	0.085678			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0407842	6.81%	0.99%	0.00%

Means

Lane	N	Mean	StDev	95% CI
1	13	0.1960	0.0389	(0.1732, 0.2187)
2	13	0.1885	0.0438	(0.1657, 0.2112)
3	13	0.1722	0.0411	(0.1495, 0.1950)
4	13	0.1712	0.0391	(0.1485, 0.1939)

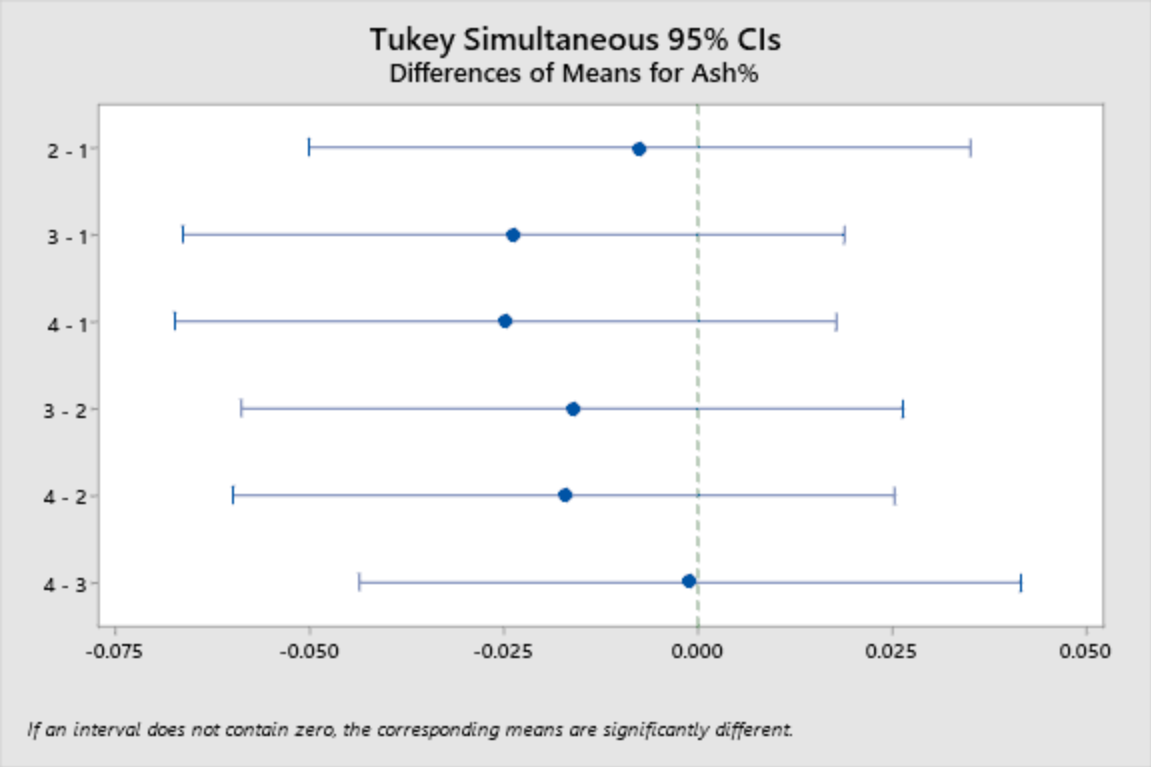
Pooled StDev = 0.0407842

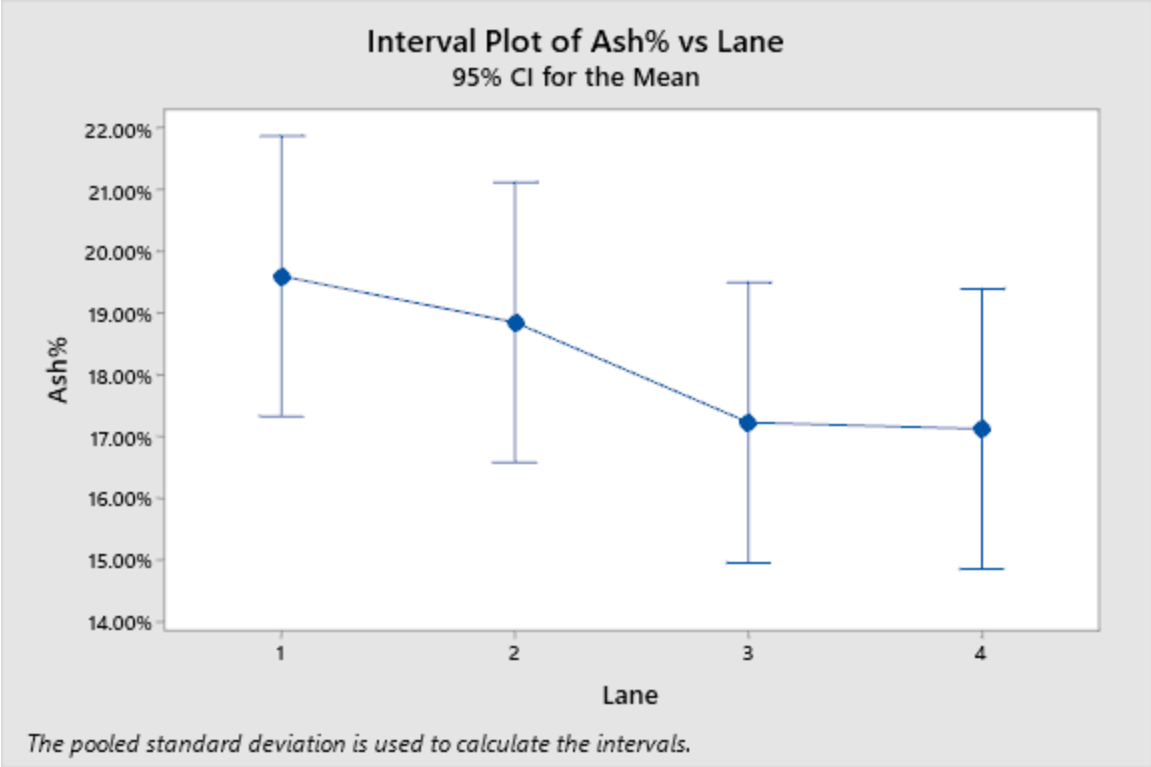
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Lane	N	Mean	Grouping
1	13	0.1960	A
2	13	0.1885	A
3	13	0.1722	A
4	13	0.1712	A

Means that do not share a letter are significantly different.


Tukey Simultaneous 95% CIs
Differences of Means for Ash%
2 - 1
3 - 1
4 - 1
3 - 2
4 - 2
4 - 3
-0.075
-0.050
-0.025
0.000
0.025
0.050
If an interval does not contain zero, the corresponding means are significantly different.


Interval Plot of Ash% vs Lane
95% CI for the Mean
Ash%
22.00%
21.00%
20.00%
19.00%
18.00%
17.00%
16.00%
15.00%
14.00%
1
2
3
4
Lane
The pooled standard deviation is used to calculate the intervals.

Appendix B.15: Production Phase Algae Ash% vs Nitrate

REGRESSION PRO.

Regression Analysis: Ash% versus Nitrate mg/L

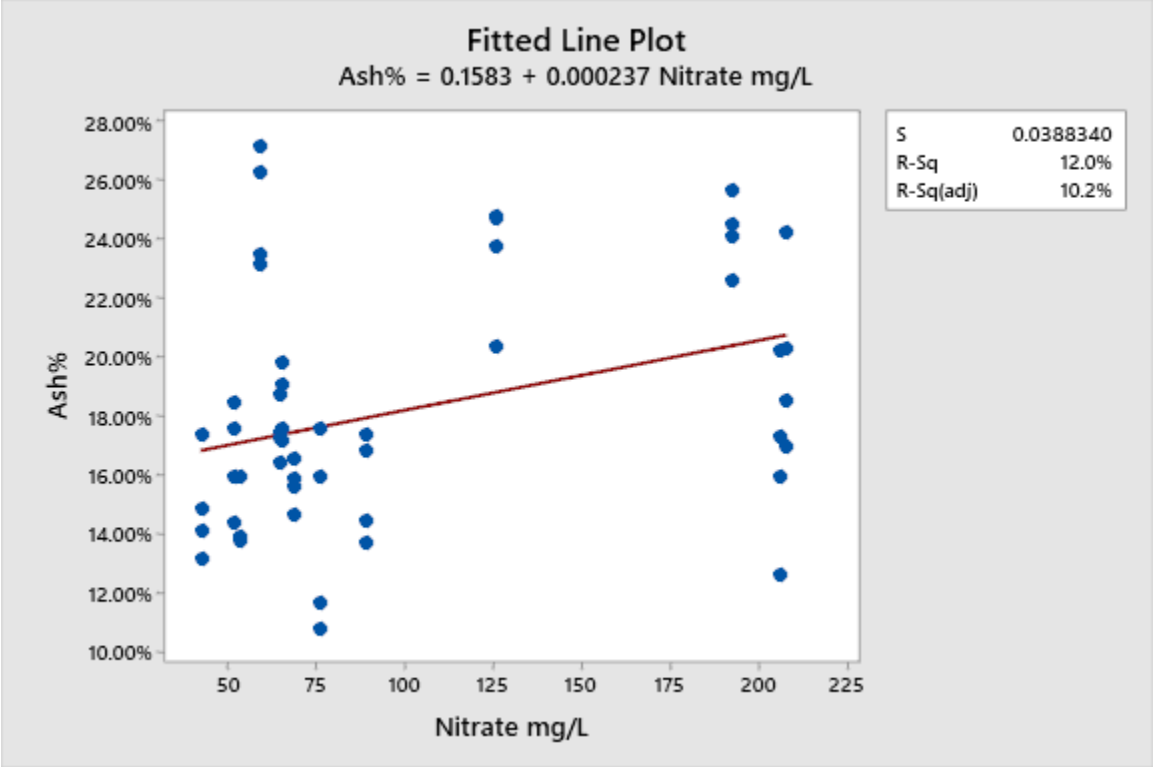
The regression equation is
Ash% = 0.1583 + 0.000237 Nitrate mg/L

Model Summary

S	R-sq	R-sq(adj)
0.0388340	11.99%	10.23%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0102735	0.0102735	6.81	0.012
Error	50	0.0754041	0.0015081		
Total	51	0.0856777			



Appendix B.16: Production Phase Algae Ash% vs Phosphate

REGRESSION PRO.

Regression Analysis: Ash% versus Phosphate mg/L

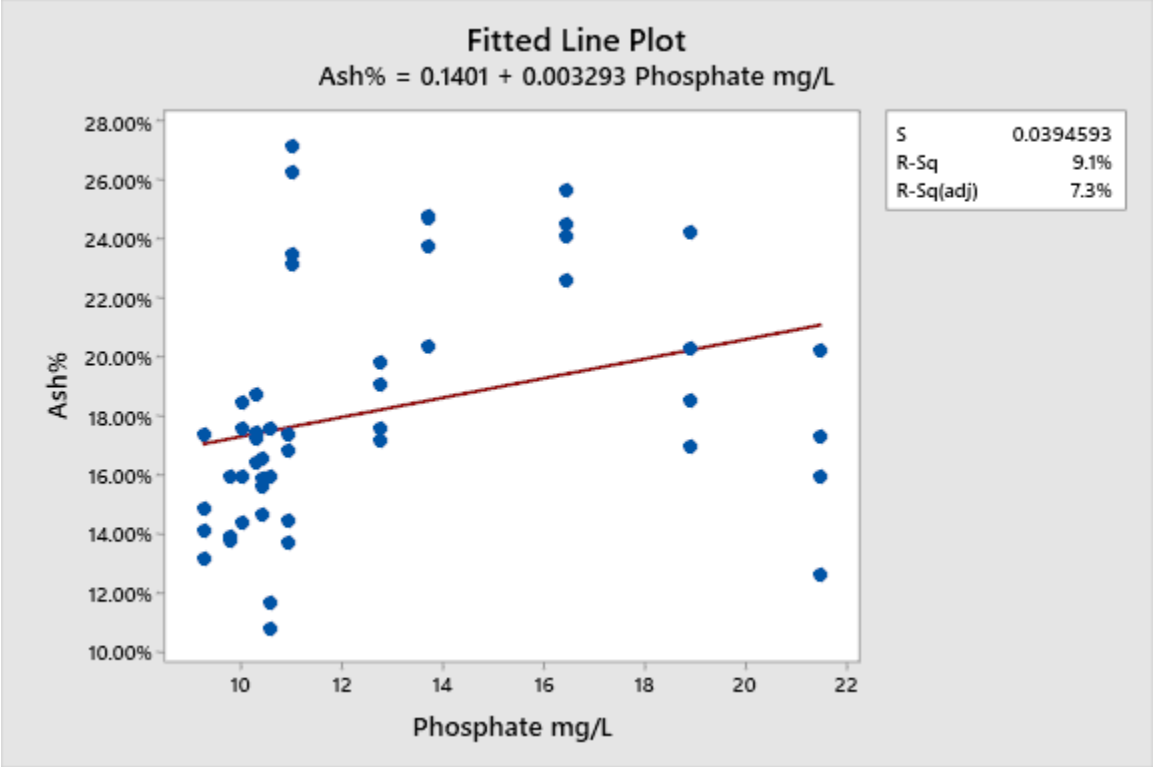
The regression equation is
Ash% = 0.1401 + 0.003293 Phosphate mg/L

Model Summary

S	R-sq	R-sq(adj)
0.0394593	9.13%	7.32%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0078260	0.0078260	5.03	0.029
Error	50	0.0778517	0.0015570		
Total	51	0.0856777			



Appendix B.17: Production Phase Algae Ash% vs Chloride

REGRESSION PRO.

Regression Analysis: Ash% versus Chloride mg/L

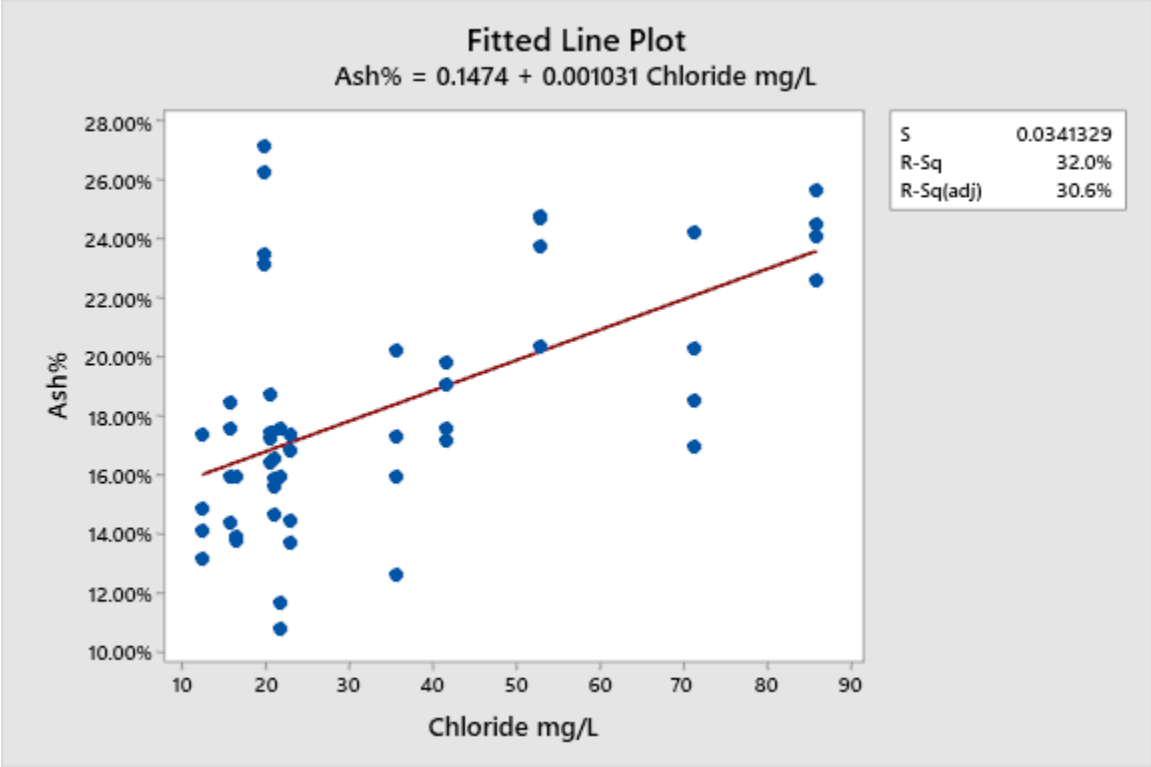
The regression equation is
Ash% = 0.1474 + 0.001031 Chloride mg/L

Model Summary

S	R-sq	R-sq(adj)
0.0341329	32.01%	30.65%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0274248	0.0274248	23.54	0.000
Error	50	0.0582529	0.0011651		
Total	51	0.0856777			



Appendix B.18: Production Phase Algae Ash% vs N:P Ratio

REGRESSION PRO.

Regression Analysis: Ash% versus N:P

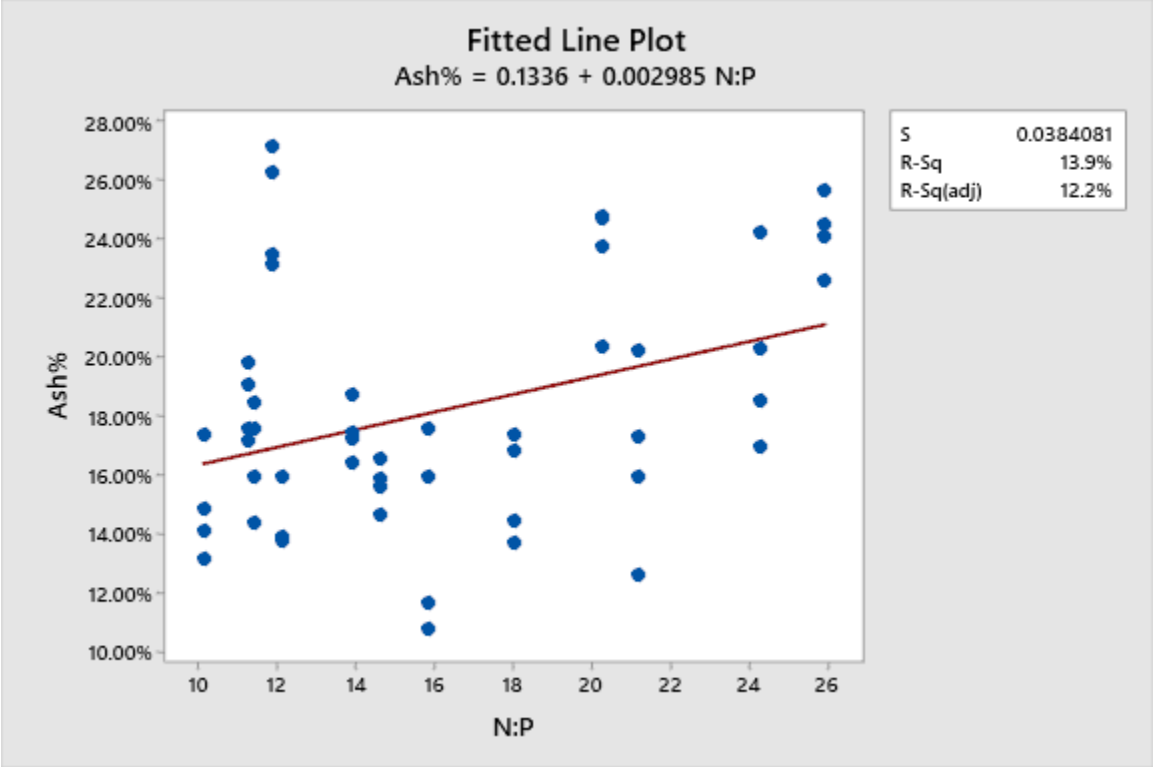
The regression equation is
Ash% = 0.1336 + 0.002985 N:P

Model Summary

S	R-sq	R-sq(adj)
0.0384081	13.91%	12.19%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.0119185	0.0119185	8.08	0.006
Error	50	0.0737592	0.0014752		
Total	51	0.0856777			



Appendix C: Lactic Acid Yield Minitab Statistical Output

Appendix C.1: Dilution Phase Algae Lactic Acid Concentration vs Treatment

ALGAE FERMENTATION ANALYSIS

One-way ANOVA: Concentrations g/L versus Dilution Algae Conditions

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05
Rows unused	4

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Dilution Algae Conditions	4	no YE or Ca, no YE, w/Ca, w/YE and Ca, w/YE, no Ca

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Dilution Algae Conditions	3	16.56	5.521	3.09	0.038
Error	40	71.51	1.788		
Total	43	88.08			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.33710	18.81%	12.72%	1.75%

Means

Dilution Algae Conditions	N	Mean	StDev	95% CI
no YE or Ca	11	12.315	1.497	(11.500, 13.129)
no YE, w/Ca	11	12.382	1.269	(11.567, 13.197)
w/YE and Ca	11	13.444	1.213	(12.629, 14.258)
w/YE, no Ca	11	13.682	1.352	(12.867, 14.497)

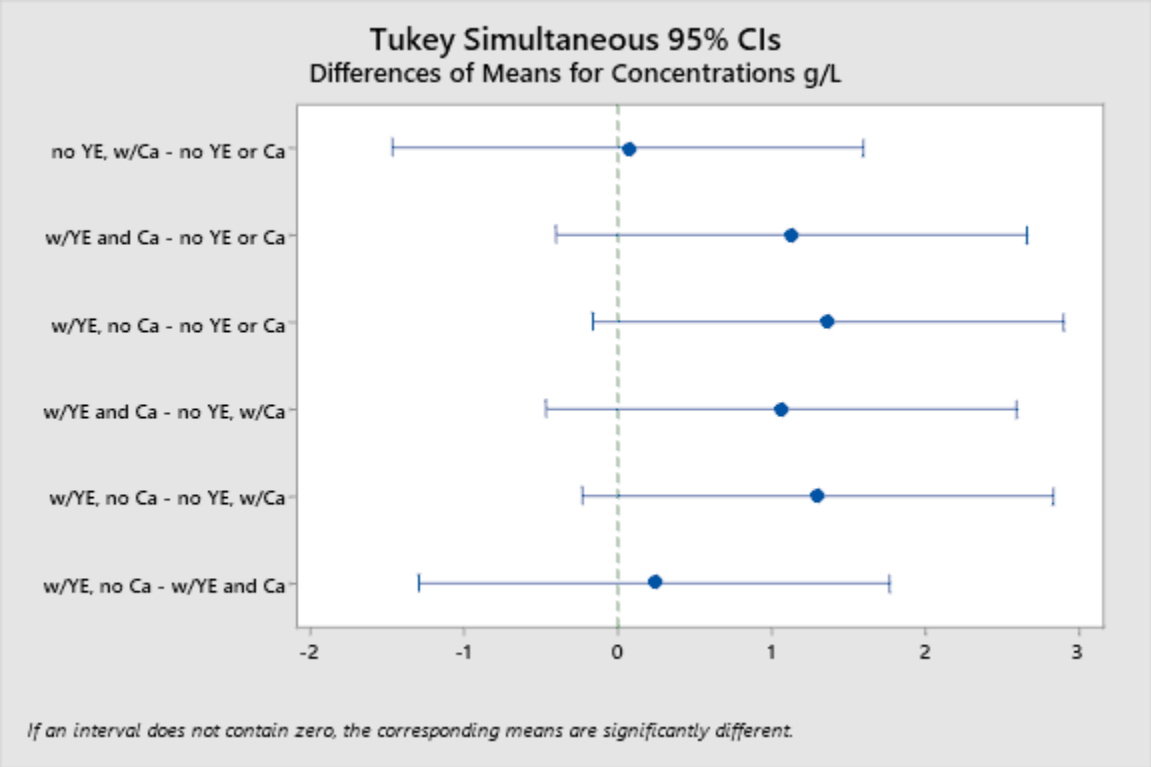
Pooled StDev = 1.33710

Tukey Pairwise Comparisons

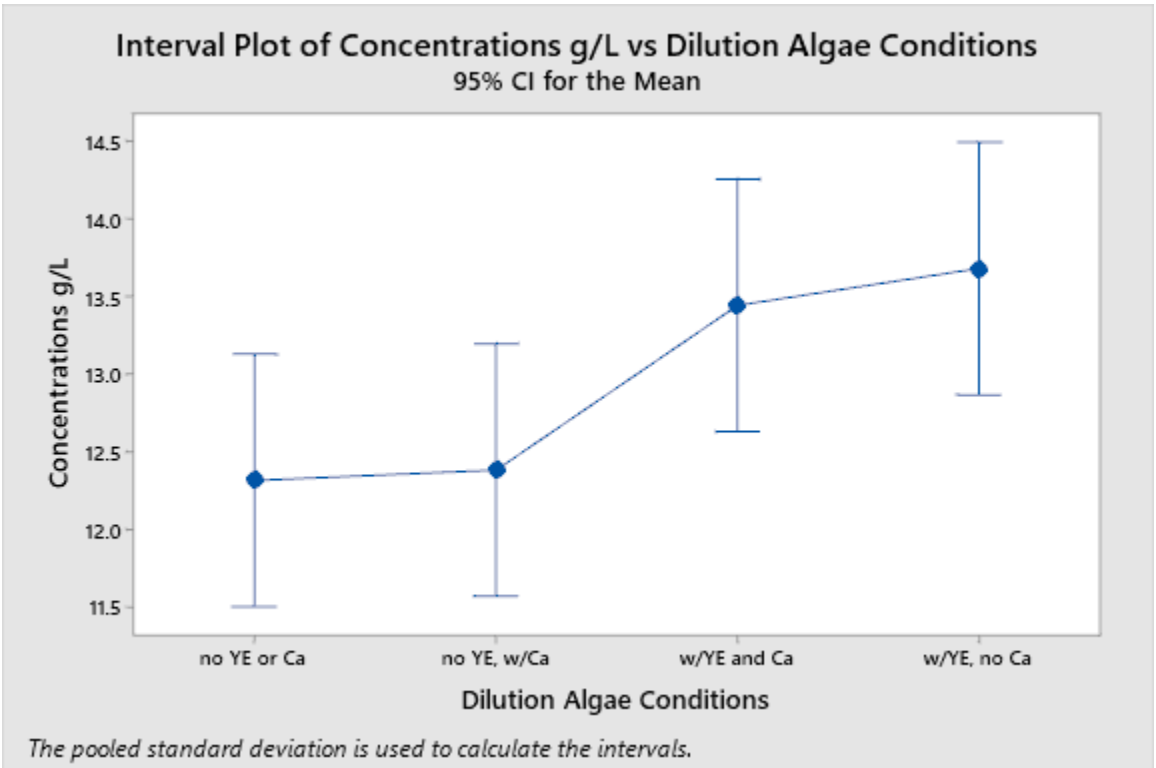
Grouping Information Using the Tukey Method and 95% Confidence

Dilution Algae Conditions	N	Mean	Grouping
w/YE, no Ca	11	13.682	A
w/YE and Ca	11	13.444	A
no YE, w/Ca	11	12.382	A
no YE or Ca	11	12.315	A

Means that do not share a letter are significantly different.



If an interval does not contain zero, the corresponding means are significantly different.



The pooled standard deviation is used to calculate the intervals.

Appendix C.2: Production Phase Pretreated Algae Lactic Acid Concnetration vs Treatment

ALGAE FERMENTATION ANALYSIS

One-way ANOVA: Concentration g/L versus Pretreated Conditions

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05
Rows unused	8

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Pretreated Conditions	5	no YE or Ca, no YE, w/Ca, Unpretreated, w/YE and Ca, w/YE, no Ca

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Pretreated Conditions	4	156.53	39.132	27.78	0.000
Error	35	49.31	1.409		
Total	39	205.84			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.18693	76.04%	73.31%	68.71%

Means

Pretreated Conditions	N	Mean	StDev	95% CI
no YE or Ca	8	13.553	1.130	(12.701, 14.404)
no YE, w/Ca	8	12.420	1.279	(11.568, 13.271)
Unpretreated	8	17.930	1.158	(17.078, 18.782)
w/YE and Ca	8	13.003	1.041	(12.151, 13.855)
w/YE, no Ca	8	15.117	1.305	(14.265, 15.969)

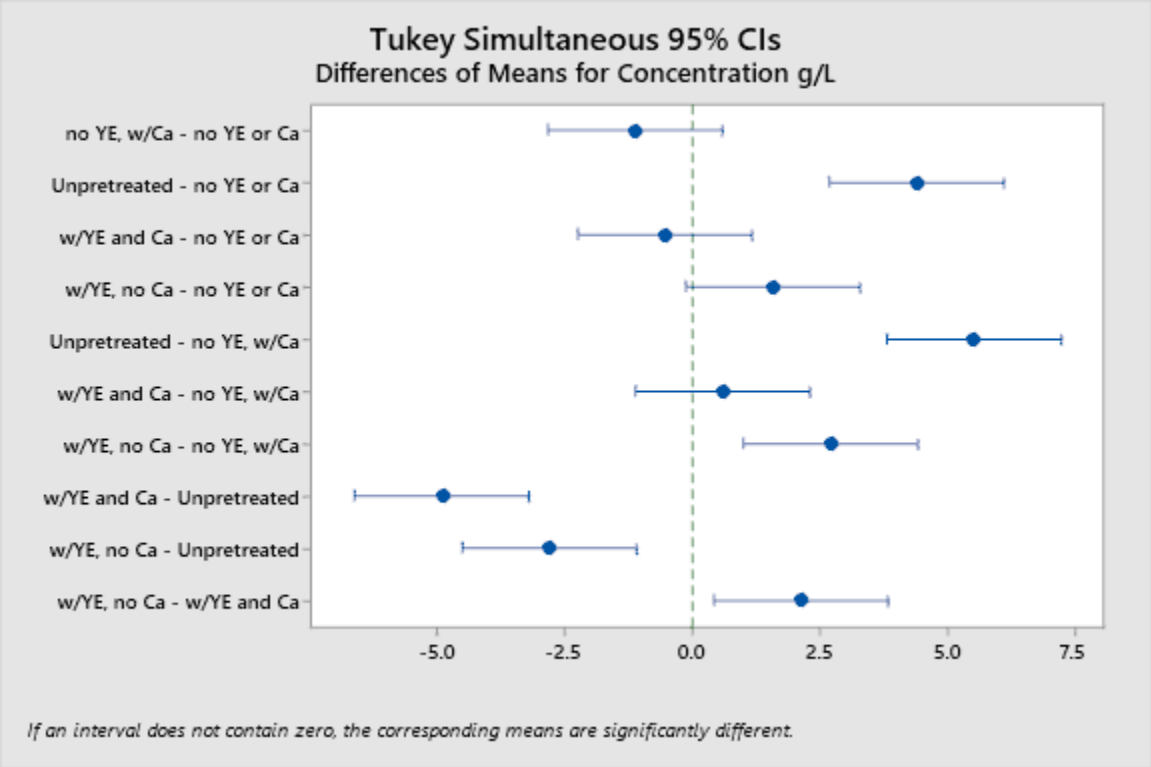
Pooled StDev = 1.18693

Tukey Pairwise Comparisons

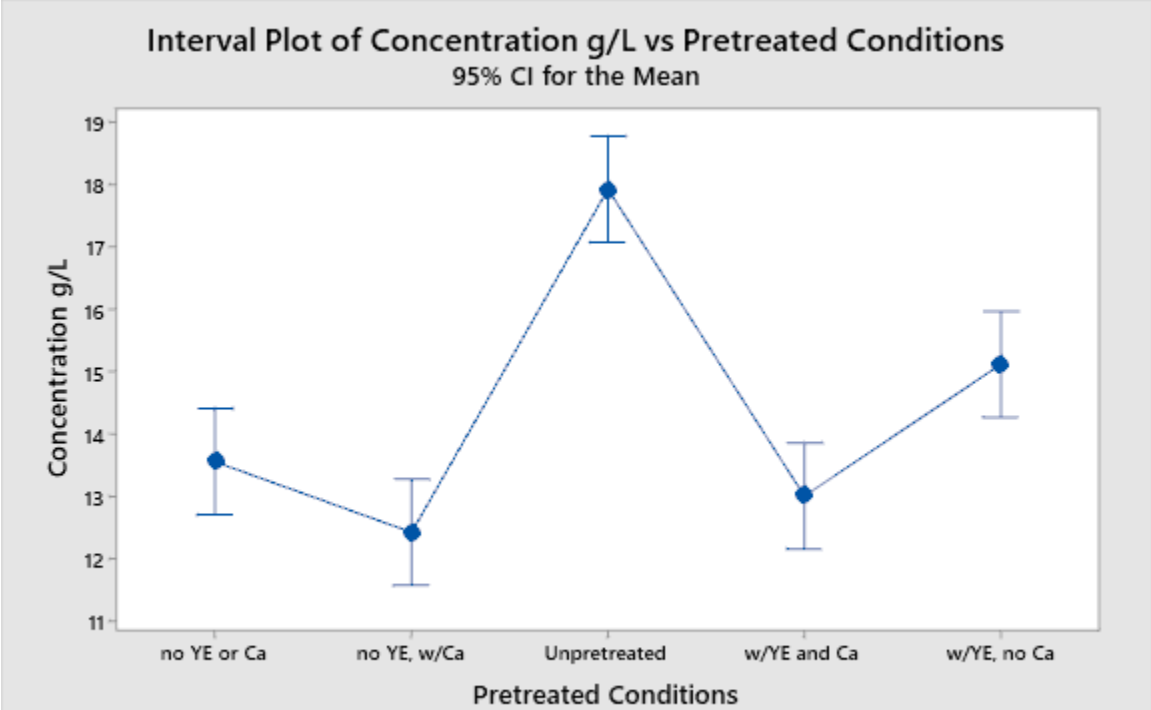
Grouping Information Using the Tukey Method and 95% Confidence

Pretreated Conditions	N	Mean	Grouping		
Unpretreated	8	17.930	A		
w/YE, no Ca	8	15.117		B	
no YE or Ca	8	13.553		B	C
w/YE and Ca	8	13.003			C
no YE, w/Ca	8	12.420			C

Means that do not share a letter are significantly different.



If an interval does not contain zero, the corresponding means are significantly different.



The pooled standard deviation is used to calculate the intervals.

Appendix C.3: Production Phase Untreated Algae Lactic Acid Concentration vs Treatment

ALGAE FERMENTATION ANALYSIS

One-way ANOVA: LA Concentration g/L versus Untreated Conditions

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05
Rows unused	20

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Untreated Conditions	4	no YE or Ca, no YE, w/Ca, w/YE , no Ca, w/YE and Ca

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Untreated Conditions	3	265.47	88.490	38.85	0.000
Error	24	54.66	2.278		
Total	27	320.13			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.50916	82.93%	80.79%	76.76%

Means

Untreated Conditions	N	Mean	StDev	95% CI
no YE or Ca	7	12.149	1.322	(10.971, 13.326)
no YE, w/Ca	7	15.370	1.482	(14.193, 16.547)
w/YE , no Ca	7	13.851	1.227	(12.674, 15.029)
w/YE and Ca	7	20.396	1.913	(19.219, 21.573)

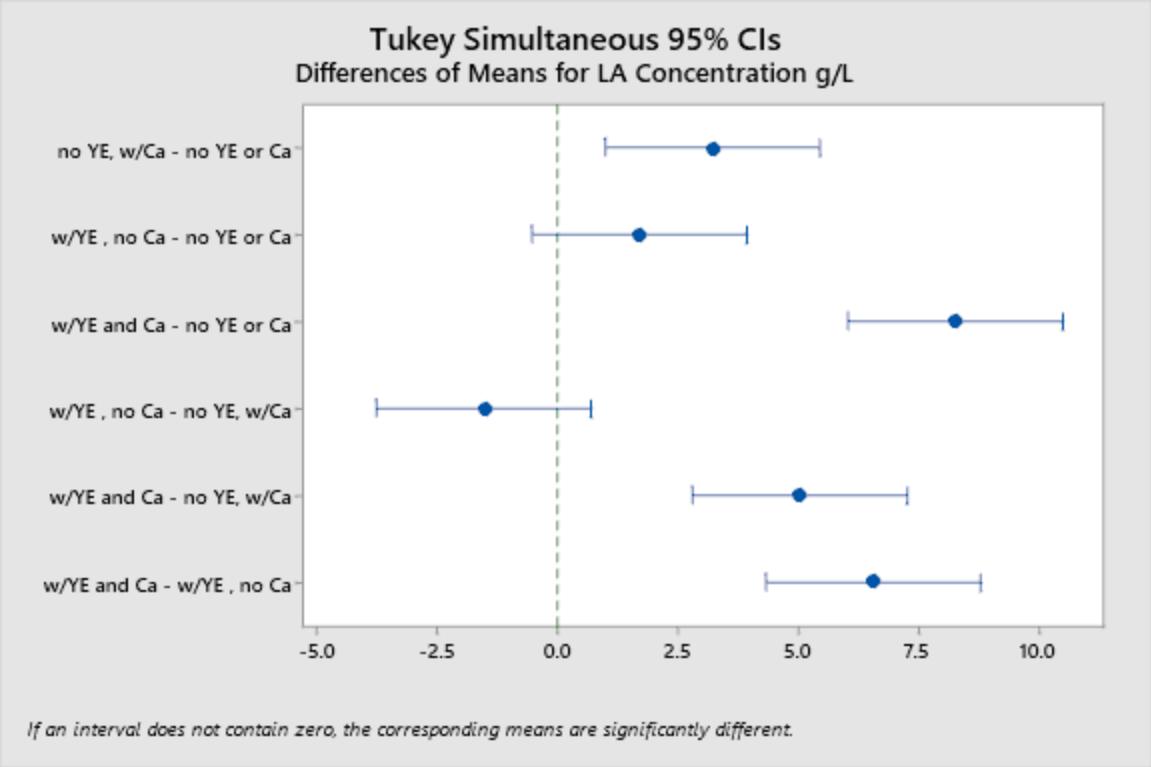
Pooled StDev = 1.50916

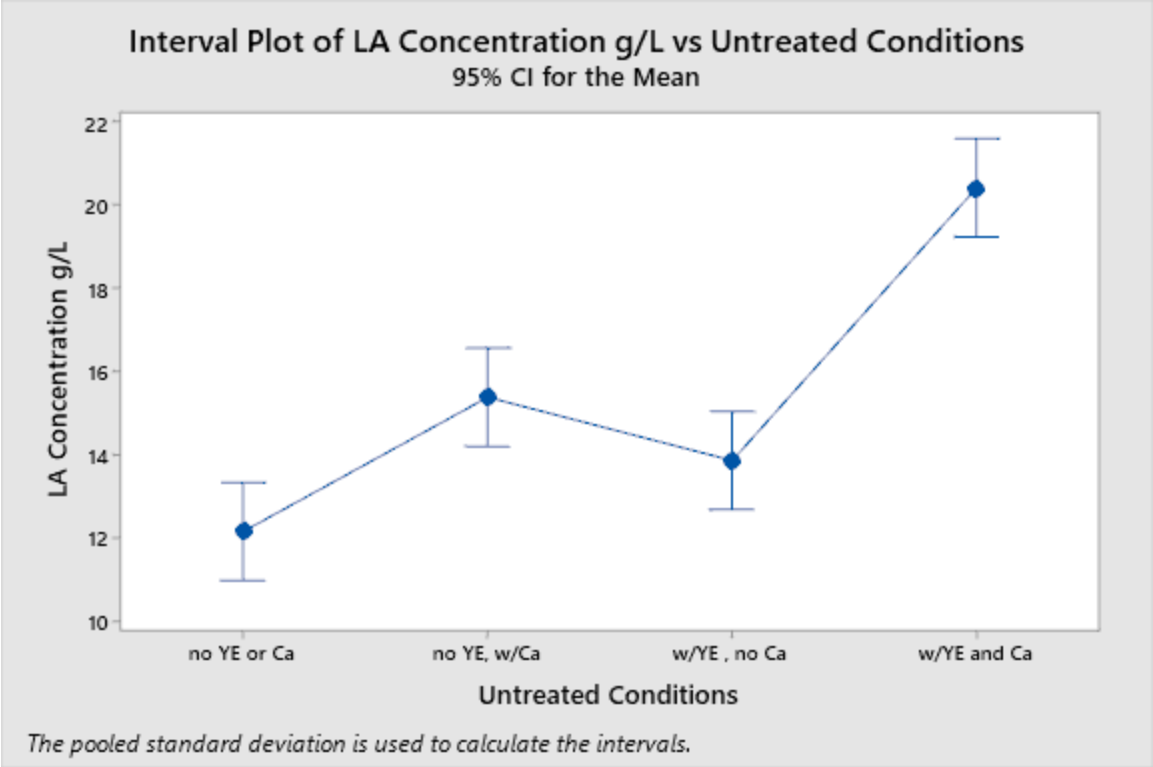
Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Untreated Conditions	N	Mean	Grouping		
w/YE and Ca	7	20.396	A		
no YE, w/Ca	7	15.370		B	
w/YE , no Ca	7	13.851		B	C
no YE or Ca	7	12.149			C

Means that do not share a letter are significantly different.


Tukey Simultaneous 95% CIs
Differences of Means for LA Concentration g/L
no YE, w/Ca - no YE or Ca
w/YE , no Ca - no YE or Ca
w/YE and Ca - no YE or Ca
w/YE , no Ca - no YE, w/Ca
w/YE and Ca - no YE, w/Ca
w/YE and Ca - w/YE , no Ca
-5.0
-2.5
0.0
2.5
5.0
7.5
10.0
If an interval does not contain zero, the corresponding means are significantly different.


Interval Plot of LA Concentration g/L vs Untreated Conditions
95% CI for the Mean
LA Concentration g/L
22
20
18
16
14
12
10
no YE or Ca
no YE, w/Ca
w/YE , no Ca
w/YE and Ca
Untreated Conditions
The pooled standard deviation is used to calculate the intervals.