Spring 5-7-2018

Growth of an Algae Polyculture and Dunaliella Tertiolecta in Oilfield Produced Water at a Range of Salinities and Nutrient Concentrations for Biofuels Production.

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Thesis

Submitted in Partial Fulfillment of the
Requirement for the Degree of

Master of Science

In
Civil Engineering

University of New Mexico
Albuquerque, New Mexico

July, 2018

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Abstract

Growth of algae in saline produced waters (wastewater) from oil and gas production is of interest for potential production of algal biofuels, wastewater treatment and potential reuse or disposal. The objectives of this study were to determine the effects of salt concentrations and nitrogen source (ammonia or nitrate) and concentration on algal growth, lipid production, and nutrient removal to help determine suitability for growth on saline produced waters. *Dunaliella tertiolecta* (a marine algae) and a polyculture (previously discovered growing in PW) were grown on produced water with a range of salinities and varying nitrogen source and concentration, in a series of experiments primarily using triplicate flasks reactors.

Illumina sequencing of 23s RNA genes determined the polyculture was composed largely of *Cyanobacterium aponinum* and *Parachlorella kessleri*. The Polyculture growth was highest from brackish to hypersaline conditions (15 – 60 g TDS/L) of 45 – 50 mg AFDW/L/D. Growth was inhibited in higher ranges and ceased by 120 g TDS/L. The highest polyculture lipid fraction of 40% was achieved at a salinity level of 90 g TDS/L after nutrient stress, while the highest lipid productivity at 60 g TDS/L

(8.5 mg/L/D). Growth rates were higher with ammonium than with nitrate as a nitrogen source (at a 60 g TDS/L), with little ammonia inhibition up to levels of 200 mg NH_4-N/L. The highest biomass productivity was achieved using initial concentrations of 13 mg NH_4-N/L and 1.7 mg PO_4-P/L with a single day increase in biomass of 99 mg AFDW/L. The fatty acid methyl ester (FAME) profile of the polyculture oil was dominated by palmitic and stearic acids, indicating its suitability for transesterification to biodiesel.

D. tertiolecta displayed consistent growth in PW over a broad range of salinities from 30 -210 g TDS/L. A biomass productivity of 16-17 mg AFDW/L/D was maintained up to a salinity of 120 g TDS/L with nitrate as a nitrogen source. Ammonium at the same molar concentration in 120 g TDS/L produced water increased growth to 21.8 mg AFDW/L/D, with higher lipid productivity. *D. tertiolecta* was more susceptible to free ammonia inhibition than was the polyculture. However, free ammonia inhibition was lower at higher salinities, apparently because of a shift in the ammonium species equilibrium away from deprotonation. A higher initial phosphate condition (8 vs. 1.7 mg PO_4-P/L) produced slightly lower growth and oil production. Both biomass and lipid productivity were highest in 120 g TDS/L PW media at concentrations of 13 mg NH_4-N/L and 1.7 PO_4-P/L (21.8 mg AFDW/L/D, 11.2 mg lipid/L/D, and peak lipid content of 44%). FAMEs in *D. tertiolecta* were dominated by palmitic acid and unsaturated C18 chains, with little variation by salinity.

Both cultures demonstrated growth in hypersaline PW at TDS levels not previously published. The reported mixotrophic capabilities of *P. kessleri* should be explored for improving growth. *D. tertiolecta* and other species in the genus could allow

algae cultivation at TDS concentrations in excess of 210 g /L. High carbonate and lower levels of ammonium and phosphate are likely key to optimizing hypersaline media biomass and lipid productivity. These nutrients are commonly already present in PW. Both cultures can uptake bicarbonate in an alkaline media which can be supplied from carbon capture technologies of fossil fuel emissions. The results of this study indicate that algae cultivation in hypersaline produced water is a viable method for biofuel production.

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I would like to thank my wife and family for supporting me while finishing this work. I would also like to thank my advisor Dr. Andrew Schuler and my committee members (Dr. Jose Cerrato, Dr. Kerry Howe, and Dr. J. Sullivan Graham) for all their help. The following people at Santa Fe Community College provided valuable help providing the algae polyculture and a produced water sample: Dr. Stephen Gomez, Luke Spangenberg, and Jonathan Schwilling.

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List of Abbreviations and Chemical Formulas

AFDW	Ash Free Dry Weight	PW	Produced Water
NH_4^+	Ammonium	NH_3	Ammonia
PO_4^{3-}	Phosphate	CO_2	Carbon Dioxide
HCO_3^-	Bicarbonate	CO_3^{2-}	Carbonate
SO_4^{2-}	Sulfate	NO_3^-	Nitrate
H_2S	Hydrogen Sulfide	TDS	Total Dissolved Solids
$NaHCO_3$	Sodium Bicarbonate		
Fame	Fatty Acid Methyl Esters	HRAP	High Rate Algal Pond
GC/MS	Gas Chromatography/Mass spectroscopy		
ICP/OES	Inductively Coupled Plasma/Optical Emission Spectroscopy		
SIM high	Specific Interaction Model (For finding dissolved constituent activity at ionic strength.)		

Chapter 1: Introduction and Background

1.1: Introduction

The need to address climate change in recent years has driven research on carbon neutral fuels that do not increase atmospheric concentrations of greenhouse gases. Renewable agricultural biofuels are one route that has been explored extensively for large-scale production (Mobin & Alam, 2014). Algae are an attractive feedstock because of their rapid growth rates compared to traditional crops (Posada et al., 2016). Growing algae for biofuels on an industrial scale requires a large volume of water, which is a scarce resource in many areas with sufficient sunlight and land for cultivation (Pate et al., 2011). This has the potential to put fuel and food production in direct competition. The limited amount of water in many areas has led researchers to explore using waste water as a medium. This would have the dual benefits of not conflicting with food production, but also the potential to remediate the contaminants.

One potential source of waste water for algae cultivation is from oilfield produced water (PW) (Graham et al., 2017). PW is a natural byproduct of petroleum and natural gas production, including wells drilled with hydraulic fracturing techniques. PW is usually considered as a waste stream that requires disposal. However, it may represent a feasible growth medium source for algae cultivation not claimed by other users (Graham et al., 2017). Since disposal of PW is currently expensive, it is possible that Algae cultivators could be paid to take it providing an additional revenue stream. The chemical nature of PW has historically presented severe challenges for its use. PW can contain potentially toxic constituents such as heavy metals, volatile organic

compounds (VOCs), and radionuclides, and it commonly has salinities well in excess of seawater. This usually makes it unsuitable for agricultural and other applications without further treatment (Fernald et al., 2016). While these constituents can inhibit many species of microalgae others have been demonstrated to grow well in PW [Hodgskiss et al. (2016), Aravinthan & Harrington, (2014), and Graham et al., (2017)]. Also, though PW contains many toxic components that might inhibit algae growth, it also contains a number of nutrients required for cultivation. The crucial nutrient nitrogen is commonly available in the form of ammonium at levels in excess of algae growth media (Harkness et al., 2015). The presence of phosphate has been reported in a number of studies at levels sufficient for algae growth (> 1 mg/L PO_4-P) [Fakhru'l-Razi et al. (2010), Riley et al. (2016, & Pendashteh et al. (2012)]. Lastly, important trace elements such as iron and manganese are present in sufficient quantities to support robust algal growth (Graham et al., 2017).

If large-scale algal cultivation in PW is to be practical, species and cultures need to be identified that can thrive in this challenging medium. Suitable candidates would need to be able to tolerate the very high total dissolved salt content typical of this waste water. The ability to survive osmotic pressure changes is of great importance, because of the great variability in salinity. Therefore, a number of species and strains should be evaluated to find the best match for a particular PW. Resistance to heavy metal toxicity and VOCs would add to robustness. High growth rates and final lipid content would lend to more efficient conversion into biofuel. Being able to efficiently utilize nutrients already present in solution for growth would lower production costs. A wide variety of

potential suitable strains that could meet these criteria would aide in the optimization of biofuel production.

The research objective of this work was to determine the effect of salinity and nutrient levels on biomass and lipid productivity of two different algal cultures in produced water. The first culture is a mixed consortium of at least two different algal species and bacteria discovered to be growing in a produced water sample from the Permian Basin of southeast New Mexico (henceforth, referred to as the Polyculture). Initial observations suggested it has the ability to grow in hypersaline media. The other was the halotolerant algae species *Dunaliella tertiolecta,* a well-studied candidate for biofuel production (Tang et al., 2011). It is known to tolerate salinity up to over 25% by mass, handle significant heavy metal concentrations, and contain high lipid concentrations of up to 70% (Ben-Amotz et al., 2009). Together both cultures represent promising candidates for cultivation in PW. Variations in growth rate, lipid content, and lipid character were measured over a range of salinities from 15 -210 g TDS/L using both nitrate and ammonium as a nitrogen source.

1.2: Background

1.2.1: Basic concept of algae cultivation for biofuel production

The microalgae as referred to in this work are unicellular photosynthetic eukaryotes and prokaryotes (with and without nuclei). Like all photosynthetic organisms they need water, light, and inorganic carbon to synthesize biomolecules containing reduced carbon. In addition, algae need a number of nutrients with nitrogen and phosphorus being limiting in most cases (Munoz, Benoit Guieysse, 2006). Nitrogen,

phosphorus, iron, and other trace metals need to be present in any medium to sustain algae growth. Algae can produce biomass on a given area of land more quickly than plant-based crops which has lead them to be proposed as the third generation of biofuels that can supplant petroleum (John et al., 2011). Though better than plant-based crops, large land areas would be required to produce fuel on a large scale (Pate et al., 2011). Large inputs of carbon, water, and other nutrients are also necessary and are a significant percentage of operational costs (Park et al, 2011).

High rate algal ponds (HRAPs) or outdoor "raceway" ponds (ORPs) are currently considered as the most economically viable way for large scale algae cultivation (Douglas et al., 2015) (Figure 1. 1, Figure 1. 2, & Figure 1. 3). Though a variety of different photo bioreactor designs have been tested at the lab and pilot scale (tubular reactors, plate, rotating drum) (Figure 1. 4), in general, their higher biomass productivity does not offset their increased capital and energy requirements (Sutherland et al., 2015). Algae biomass grown in outdoor raceway ponds has been estimated to have an energy output ratio (energy output/input) of around 10 while engineered photobioreactors less than 1 (Huesemann and Benemann, 2009). Mechanically mixed "Raceway" ponds, usually stirred with a paddle wheel, have been shown to have productivities of about an order of magnitude higher than unmixed ponds (Lundquist et al., 2010). Carbon dioxide is commonly added to maintain pH (between 7.5 and 8) and improve growth. One company, Sapphire Energy, attempted large scale algae cultivation in HRAPs for biocrude in New Mexico using HRAPs and Exxon-Mobil is currently engaged in efforts in California (www.fastcompany.com).

For the purposes of this study, HRAPs are assumed to be the cultivation environment for algae grown in produced water, and suitable candidate species would need to exhibit robust growth in these conditions. These systems being open are obviously subject to evaporation, contamination, abrupt changes in media conditions, predators, and the weather. Initial saline conditions in HRAPs would become increasingly hypersaline over a period of weeks especially in arid dry climates (Ishika et al., 2017). High, warm, and dry wind can greatly increase pan evaporation rates. The pH (despite attempts at control) can switch rapidly due to photosynthetic activity or nutrient uptake [Park et al., (2011) & Eustance et al., (2013)]. The wind can introduce algae grazers, competing algae species, and pathogens to HRAPs. Herbivore protozoa can reduce algae biomass by 90% in two days (Oswald, 1980). Blown in non-optimal (in terms of oil yield) species can take over the pond (Bartley et al., 2013). Pathogenic bacteria and viruses can affect productivity (Park et al., 2011). Despite the challenges many algae species have been successfully cultivated in outdoor HRAPs. Species such as *Spirulina* and *Dunaliella salina* are currently being cultivated in outdoor ponds for the production of food and Vitamin A respectively, and current operational knowledge exists using HRAPs to cultivate algae biomass (Van De Hende et al., 2014).

To sustain high algae growth rates optimal conditions must be maintained. Sunlight is determined by the pond location and dependent on factors such as latitude, season, and cloud cover. Harvesting solar energy with algae for fuel requires a lot of land area because the maximum efficiency of photosynthetic processes is 2.4% of incoming photons (Park et al., 2011). Ponds cannot be very deep (< 1 m) because light

cannot penetrate dense algae biomass (Huesemann & Benemann, 2009). Large scale biofuel production would require significant amounts of water for a growth medium (Rodgers et al., 2014). Rodgers et al. (2014) estimates that 5 billion gallons a year (BGY) of biocrude would require 2160 BGY of water a ratio of 1:432. Graham et al., (2017) suggests a water requirement ratio of approximately 1:232 for biocrude to water. Algae fix carbon, which must be replenished in the medium. Carbon dioxide addition and oxygen dissolution predominantly occurs through gas exchange with the atmosphere in outdoor ponds. CO_2 addition is commonly proposed to increase availability of carbon to boost growth rates by injecting CO_2 from flue gas or flaring petroleum wells (Lundquist et al., 2010). The addition of bicarbonate has also been proposed due to it being easier to transport and handle (Konst et al., 2017). Nitrogen, phosphorus, and other trace elements are required for the algal growth. Nitrogen can be supplied by ammonium, nitrate, or urea with wastewater sources commonly proposed to save cost, though ammonia toxicity might be an issue (Mobin & Alam, 2014). Phosphate is a source of phosphorus which is present in many wastewaters. Other trace elements are usually not the limiting factors and much smaller amounts are required to sustain production. Typically, the recycling of nutrients from biofuel conversion (anaerobic digestion or lipid extraction) is proposed for reducing inputs into the overall production process (Posada et al., 2016). Industrial scale algae biofuel production meeting a significant portion of the economy's fuel needs would require large amounts of water, nutrients, and carbon. PW and the oil/gas production process might be able to provide these inputs in significant quantities.

The economic viability of algae-based biofuels is currently not firmly established, and it is possible that using PW might improve the profitability. Lundquist et al., (2010) states that without major technological breakthroughs algae biofuels will not be cost competitive with fossil fuels. Many of the significant costs of algae-based fuels are related to harvesting, lipid extraction, and conversion to fuel, but the use of PW might improve the economic and energy return. Figure 1. 5 displays a bar graph of different annual operating costs of a HRAP biofuel facility theoretically based in New Mexico from Rodgers et al. (2014). Nutrient addition in the form of DAP (diammonium phosphate) and urea represent significant production costs. As will be discussed PW could reduce or eliminate these costs, due to the fact that many PW sources contain nutrients such as ammonium and phosphate. Based on the values given in this paper a full elimination of these costs could reduce annual operating expenses by almost 25%. Accepting PW from oil and gas operators represents a potential additional revenue stream for an algae biofuel production facility. PW is typically transported and deep well injected at costs that range from $1-$11 a barrel (Boschee, 2012). An HRAP algae cultivation facility could potential reduce PW disposal costs saving money for oil and gas companies. It is possible even that spent growth media could be sold back to the operators for reuse in the hydraulic fracturing process. The creation of an economical algae derived fuel process is an area of much active research, and the incorporation of PW has the potential to greatly improve the economics.

Figure 1. 1: High rate Alga Pond (HRAP).

Source: https://www.niwa.co.nz/freshwater-and-estuaries/freshwater-and-estuaries-update/freshwater-update-62-september-2014/niwa-advances-wastewater-treatment

Figure 1. 2: High Rate Algae Pond or Raceway in Pecos, TX.

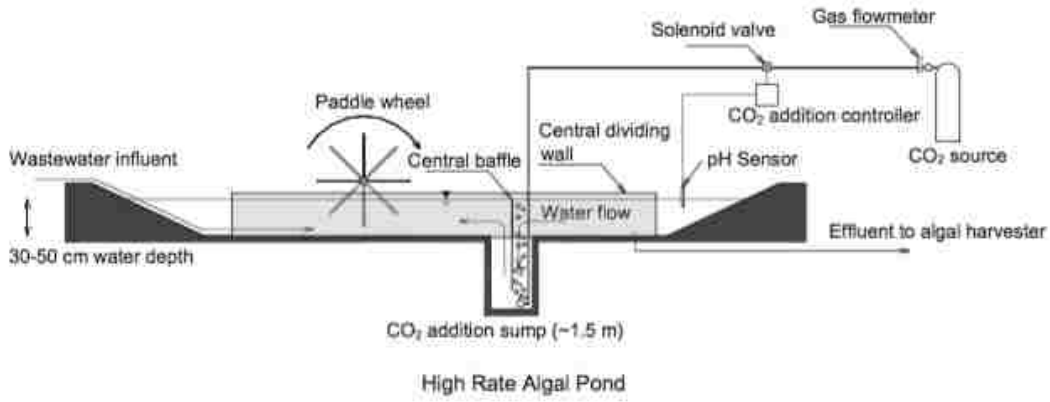

Figure 1. 3: Cross-section of HRAP.

From Park et al., (2011).

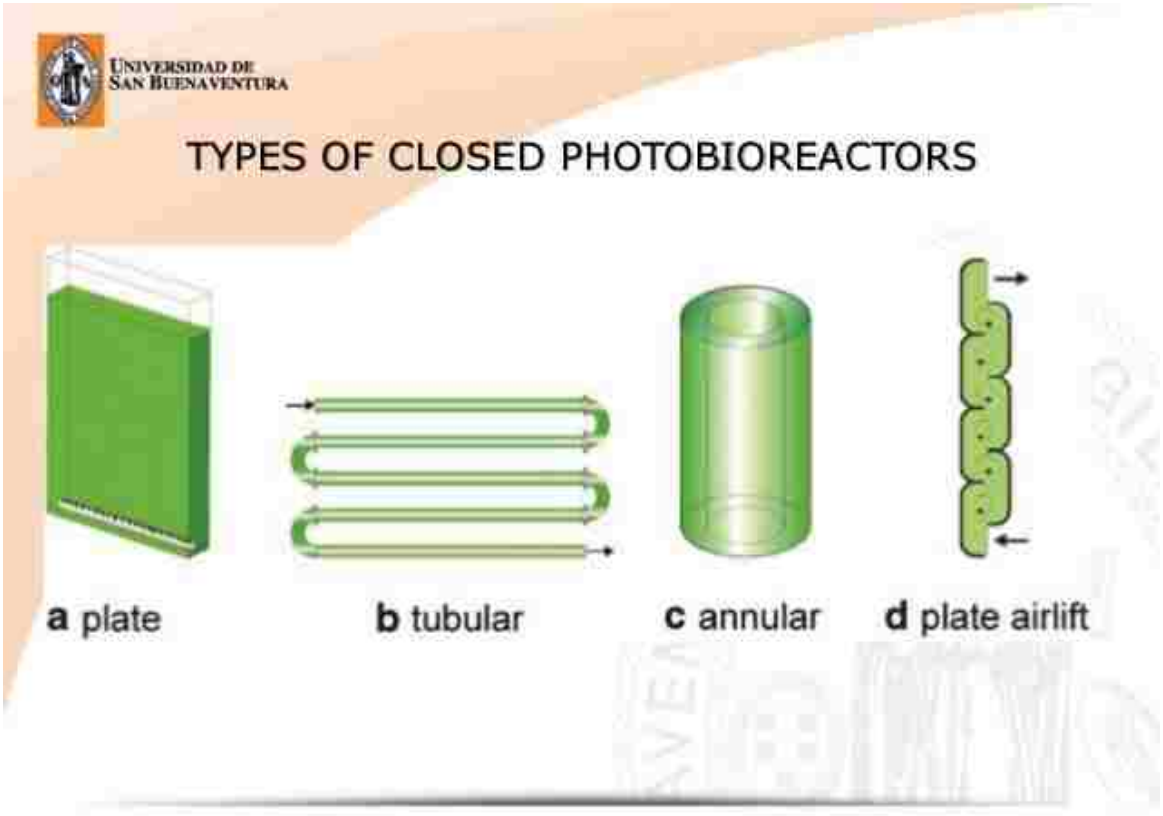

Figure 1. 4: Types of closed algae photo bioreactors.

Source: https://i.pinimg.com/736x/4c/40/cb/4c40cb0553417845f21eab8c157dcef5.jpg

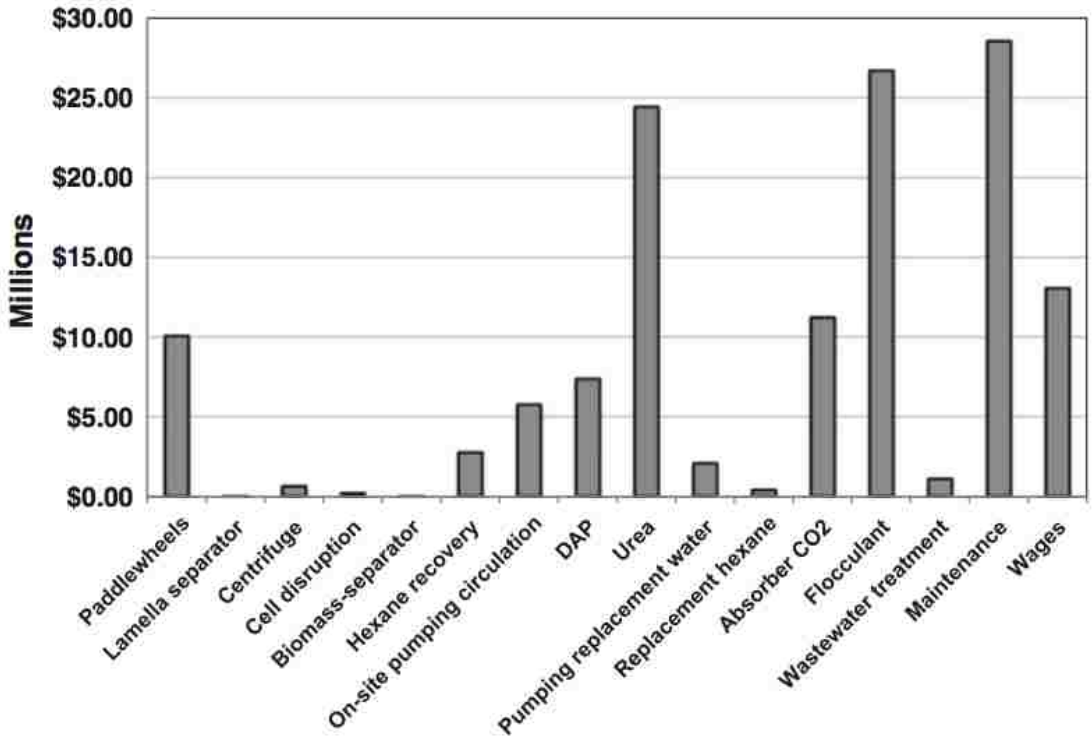

Figure 1. 5: Annual operating cost for a HRAP production facility.

1.2.2: Methods of conversion of algae biomass to biofuels

Algae biomass conversion to fuel is considered promising for their high growth rate and lipid content (Suganya et al. 2016). A variety of potential pathways exist for production that vary in their outputs that can include biodiesel, ethanol, biogas, hydrogen, biocrude, or charcoal. Fuel conversion methods also differ on whether the entire biomass is being utilized or subcomponents (e.g. lipids or carbohydrates). As a result, this can influence the desired composition of the algal biomass. As a rule, higher biomass lipid content increases the amount of extractable energy since lipids contain a higher energy content than carbohydrates or protein. In addition, the nitrogen content of proteins can present problems for the creation of fuel (e.g. ammonia inhibition during anaerobic digestion) (Ward et al., 2014). Lastly, the dewatering of the biomass is a

critical parameter for creating a quality fuel conversion process. Below is a list of potential methods that should be taken into account when designing algal biofuel production systems.

Transesterification of lipids to biodiesel

Fatty acids in the lipid component of the biomass can be converted to biodiesel through transesterification. First, the cell biomass is disrupted, and solvents are used to extract the lipid portion. The target lipid molecules are triglycerides usually C16 or C18 chains, saturated or unsaturated, though polar lipids (e.g. phospholipids) are involved in the process. To reduce viscosity, fatty acid chains are separated from the glycerol backbone with a catalyst (often a strong base) in methanol producing fatty acid methyl esters (FAMEs) the basis of biodiesel. Since triglycerides can be converted to biodiesel, high neutral lipid content is a crucial parameter for the economics of the process (Tang et al., 2011). The phospholipids in the oil extract are less desirable because they can inhibit the transesterification process and lead to insoluble precipitates in the biodiesel (Christian and Van Gerpin, 2017). Overall, saturated C16 and 18 fatty acids are preferred for their higher cetane number (a measure of the combustive speed of the fuel) (Fakhry et al., 2013). Many studies have focused on biodiesel as an end product due to its predominance as a transportation fuel [Tang et al. (2011), Mobin & Alam (2014), & Griffiths and Harrison (2009)]. Numerous algal biofuel studies have focused on maximizing and optimizing lipid content, productivity, and FAME character. Because the transesterification process only utilizes the lipid portion of the biomass, other uses

are proposed for the residual biomass such as fertilizer, animal feed, or fuel production by other means.

Anaerobic Digestion

Anaerobic digestion (AD) has been used extensively at wastewater treatment plants to produce biogas to offset their energy consumption (McCarty, 1964). Diverse microbial communities convert a portion of the raw biomass to methane that can be combusted as fuel. Converting algae to biofuel using AD has been studied extensively and presents benefits and challenges. In general, it is perceived as essential for biofuel economics by improving the energy output ratio (Ward et al., 2014). Commonly, AD is an option to increase the total energy production, because the process is able to utilize the residual biomass post lipid extraction while recycling nutrients (Sialve et al., 2009). In addition, the glycerol byproduct from transesterification can be used as an input. The high nitrogen and/or saline nature of the algae biomass can be inhibiting to the process, though methods exist for overcoming these issues. Also, high lipid content here would negatively affect biogas production because of the formation of volatile fatty acids (Awe et al., 2017). A two-stage process can maintain high methane production or low nitrogen feedstock can be blended in to maintain gas production (Yang et al., 2011). Ward et al. (2015) demonstrated that a halotolerant bacteria community can yield acceptable methane production with a 7% salinity in the algal biomass feed. Other potential challenges and solutions to them for AD exist. Overall, AD would be a necessary process to incorporate in large scale algae biofuel production to increase energy yield and revenue.

Hydrothermal Liquefaction

Hydrothermal Liquefaction (HTL) is a common thermochemical method studied for conversion of algal biomass to fuel. As the name implies, it uses heated, pressurized water at super critical levels to convert all components (lipids, proteins, and carbohydrates) to a biocrude oil (Douglas et al., 2015). Two chief advantages are that the algae biomass does not have to be completely dried for the process, and proteins and carbohydrates are also converted into biocrude. An aqueous and gas phase are also produced that can be processed through further thermochemical conversion to useful products. Nutrients such as nitrogen and phosphorus can be recycled from the process (Elliot et al., 2015). For algae biomass pressures of 2,000–3,000 PSIA and temperatures of 300–350 °C are used (Jones et al., 2014). The oil, aqueous, and gas phases can undergo further thermochemical conversion to useful fuel. In general, a higher oil yield is achieved with a higher lipid content of the algae [Li et al. (2014) & Teri et al. (2014)], however this is not always the case. Compared to petroleum, biocrude typically contains high amounts of oxygen and nitrogen whose fraction would ideally be reduced (Vardon 2012). The energy recovery ratio seems to vary but was stated to be better than slow pyrolysis (around 2:1 rather that 1:1) in Vardon (2012). HTL is at the present time an area of active research and represents a promising alternative to transesterification.

Bioethanol

Algae cells contain carbohydrates such as starch and sugars that can be converted to ethanol by fermentation. Just as for plant-derived carbohydrates, yeast can be used for the process. Commonly, bioethanol production process would use the

residuals from lipid extraction (Lee et al., 2013). Carbohydrates would first be converted to sugars with enzymes, and then used a s a substrate for anaerobic fermentation by organisms such as *Saccharomyces cerevisiae*. Ethanol can be blended with gasoline or burned in its pure form.

Pyrolysis

Algal biomass can be heated in the absence of oxygen to produce a carbon rich bio-oil. This can be done with the whole algae biomass or with the residuals after lipid and carbohydrate extraction (Kim et al., 2015). Bio-oils produced through algae pyrolysis can have undesirable high nitrogen and oxygen attributes, but this method remains capable of converting the full biomass into fuel (Thangalazhy-Gopakumar et al., 2012). Methane and hydrogen gas can also be produced.

Biorefinery

To maximize the economic utility of algae cultivation many papers have proposed and advocated a biorefinery approach. A variety of processes would be incorporated in one industrial facility to extract a variety of fuel and other value-added products such as animal feed, fertilizer, and pharmaceuticals (Suganya et al., 2016). Byproducts could be recycled into the algae cultivation ponds or sold as raw material for other industrial products (Posada et al., 2016). While complex economically viable systems have been proposed, this concept has yet to be demonstrated in practice (Lundquist et al., 2010).

1.2.3: Chemistry of Produced Water

It is estimated that the United States brings 3 million megaliters of PW to the surface every year (Graham et al., 2017), which can vary wildly in composition. PW brought to the surface is commonly stored in tanks or pits (Figure 1. 6), and then predominantly disposed in deep salt water disposal wells. Oilfield waste water, whether from conventional or hydraulic fracturing extraction, contains a number of potential toxic but also some beneficial components. Large variation in quality exists even within a production basin. One consistently cited characteristic of PW is its very high salinity (Pendashteh et al., 2012). Chadhaury et al. (2016) reports variation in total dissolved solids (TDS) from 10 – over 330 g/L in the Permian and Delaware Basins spanning the range from brackish to sodium chloride saturation using the USGS PW data base. Lauer et al., 2016 lists PW values from 3– 400 g TDS/L in the Bakken Region of North Dakota. A similar range was reported in the Marcellus shale oil and gas water in Harkness et al., (2015) with chloride concentrations ranging from brackish (2 g/L) to hypersaline (> 150 g/L). Typically, waste water from Coal Bed Methane is of lower salinity (Brackish) than conventional or hydraulic fractured PW (mostly saline to hypersaline) (Hodgskiss et al., 2016) (Aravinthan & Harrington, 2013). Chadhaury et al. (2016) reports that average TDS concentrations in different formations of the Permian Basin range from 154 – 225 g/L. While certainly saline to hypersaline TDSs for PW are prevalent in many basins, brackish concentrations should not be over looked.

Figure 1. 6: PW treatment and pit storage facility in Southeast New Mexico.

Courtesy J. S. Graham.

The salinity of produced water from the Permian Basin in NM was explored for this thesis using information from the United States Geological Survey PW data base (https://energy.usgs.gov/EnvironmentalAspects/EnvironmentalAspectsofEnergyProductionandUse/ProducedWaters.aspx) and the Petroleum Recovery and Research Center at New Mexico Tech (http://baervan.nmt.edu). One of the few consistently reported parameters in the data is the TDS content of the water. Using ARC GIS and the latitude and longitude of well locations, a map of the spatial distribution of salinity was created (Figure 1. 7). The TDS of the PW samples spans the range of 2.65 – over 300 g TDS/L reported in the literature [Graham et al. (2017) & Harkness et al. (2015)]. Figure 1. 8 displays an interpolated map of TDS weighting for the frequency of well values. From these results, though brackish salinities exist, a blended average is weighted toward saline to hypersaline conditions. Due to the fact that the PW waste water stream is commonly mixed together in pits the higher salinities would probably dominate over brackish conditions for cultivation.

Another common component of PW that is an important consideration for algal cultivation is carbonate. Lauer et al., (2016) lists carbonate concentrations ranging from 35 to 856 mg/L in PW from North Dakota. Papendick et al., (2011) cites bicarbonate

concentrations from 330 – 1550 mg/L in Australia Coal Bed waters. Graham et al., (2017) 's survey of PW in the Southwest reports ranges from 585 – 1417 mg/L bicarbonate, which is also consistent with the USGS database. Significant bicarbonate alkalinity appears common and presumably relates to the carbonate concentration of the source rocks in which the PW is in contact. Figure 1. 9 contains a map of PW samples from the Permian basin using the same data base as for salinity. Well concentrations fall within the same range as the literature cited. Many studies have linked carbonate content with increases growth rates for microalgae [Mokashi et al. (2015), Gardner et al. (2012), Fabregas et al. (1994), & Srinivasan et al. (2015)]. Figure 1. 10 displays that pH measurements in the Permian Basin are mostly neutral with some more acidic and basic values being recorded. Algae can be cultivated in the entire range of PW pH values shown.

Considerable metals concentrations are commonly reported in PW samples that discourage its reuse and could potentially be toxic to algae species. Graham et al. (2017) found metal levels up to 31 ppb (~2.5e-7M) for cadmium (Cd), 3.5 ppm (~1.7e-5 M) for lead (Pb), 17 ppm (~2.6e-4 M) for zinc (Zn), and 366 ppb (~6e-6 M) for copper (Cu) along with a number of other metals present. Pendashteh et al. (2012) found mercury (Hg) levels up to 830 ppb (~4e-6 M). Godfrey (2012) found 60 ppb (~8e-7 M) arsenic (As) in PW samples. Jain et al. (2017) found Cd levels up to 1.21 ppm, As up to 11 PPM, Zn up to 17.4 PPM, and Cu up to 0.539 PPM. Clearly, though there is variation, elevated metal concentrations exist in PW that would exceed groundwater and discharge standards. Any algae cultivated in this medium would need to be able to

tolerate heavy metals and could potentially accumulate significant amounts in the biomass (Crist, et al., 1994) (Hirata et al., 2001).

The presence of nutrients required for algal growth in PW has the potential to improve the economic feasibility of large scale biofuel production. PW has been found to contain significant concentrations of ammonium which is an important nitrogen source for microalgae. Tasaki et al. (2013) list the NH_4^+ content of PW from 8.1 to 19 mg/L (in the range of algae growth media). Harkness et al. (2015) measured NH_4^+ concentrations from around 12 to 400 mg/L in Hydraulic fracturing wastewater in West Virginia and Pennsylvania. Ammonium correlated positively with chloride content. The high NH_4^+ presence was attributed to the reducing conditions and clay-dominated source rocks of the fractured formations. The author has measure high ammonium contents in a produced water (of around 200 mg NH_4-N /L sample from the Permian Basin. Phosphate is often present at levels sufficient for algae growth (> 1 mg/L PO_4 – P) (Fakhru'l-Razi et al., 2010)(Riley et al., 2016)(Godfrey, 2012)(Pendashteh et al., 2012). Pendasteh et al. (2012) cited a value of over 30 mg/L PO_4 which is in excess of many standard algae media. The lower value of around 1 mg/L PO_4-P cited in Riley et al. and Godfrey would be sufficient for algae cultivation. The ideal N to P ratio depends on the algae species but can range from 10-30:1 (Zhang & Hu, 2011). PW media could be adjusted to the optimal levels. Providing nutrients for algae growth is a significant production cost and their presence in the source media could reduce or eliminate it.

While PW chemistry presents challenges to algae that can tolerate the salinity, metabolize the metals, and utilize the nutrients present are candidates for turning this

waste stream into a biofuel medium. There is significant variation in PW chemistry and a full description is beyond the scope of this work. There is evidence the PW characteristics described here can be tolerated by various species of algae. Identifying species that thrive in this medium is still an active area of research to optimize growth rates and lipid production [Hodgskiss et al. (2016), Racharaks et al. (2015), & Graham et al. (2017)].

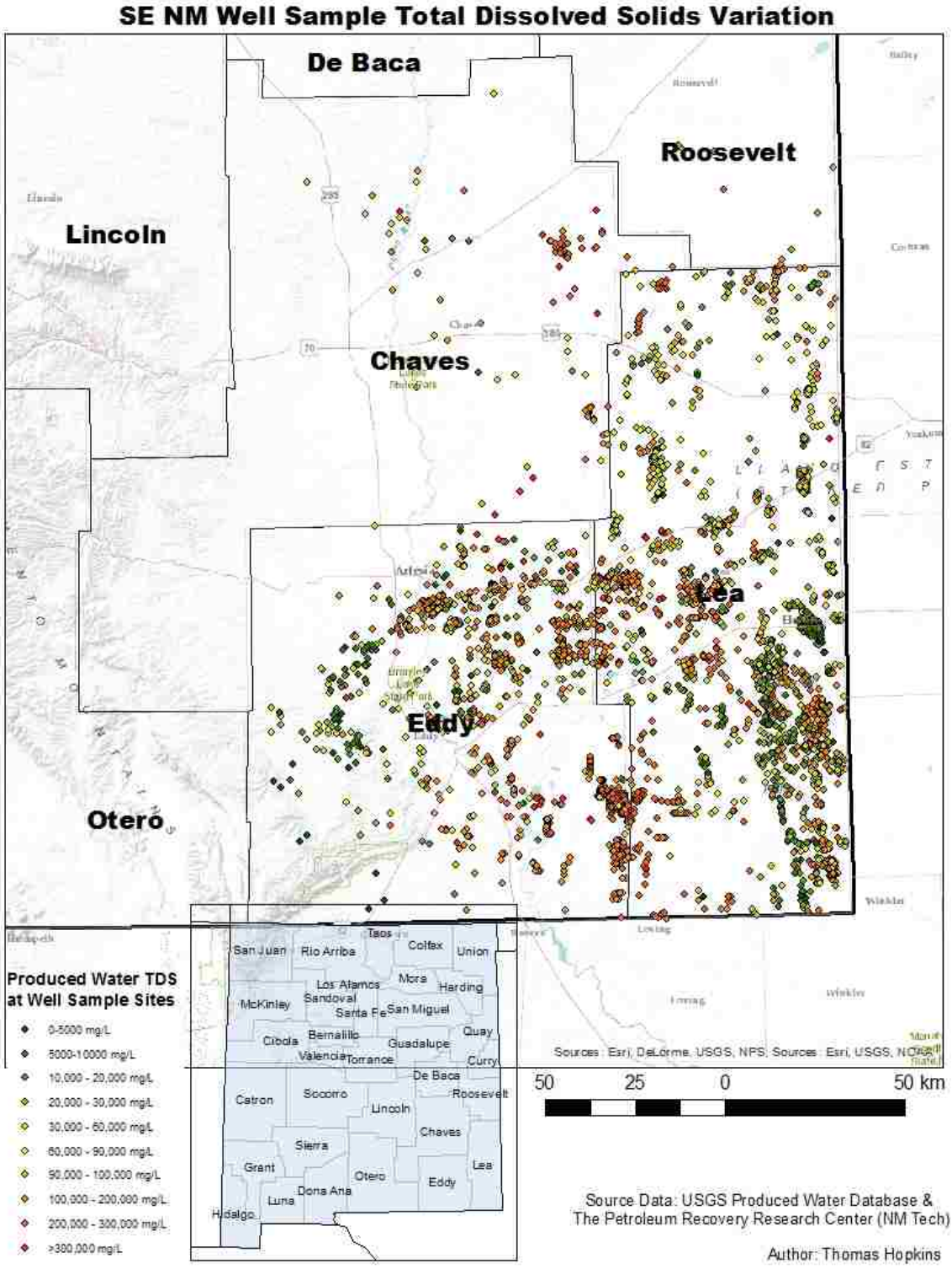

Figure 1. 7: Total Dissolved Solid Concentrations of PW samples in the Permian Basin of SE New Mexico.

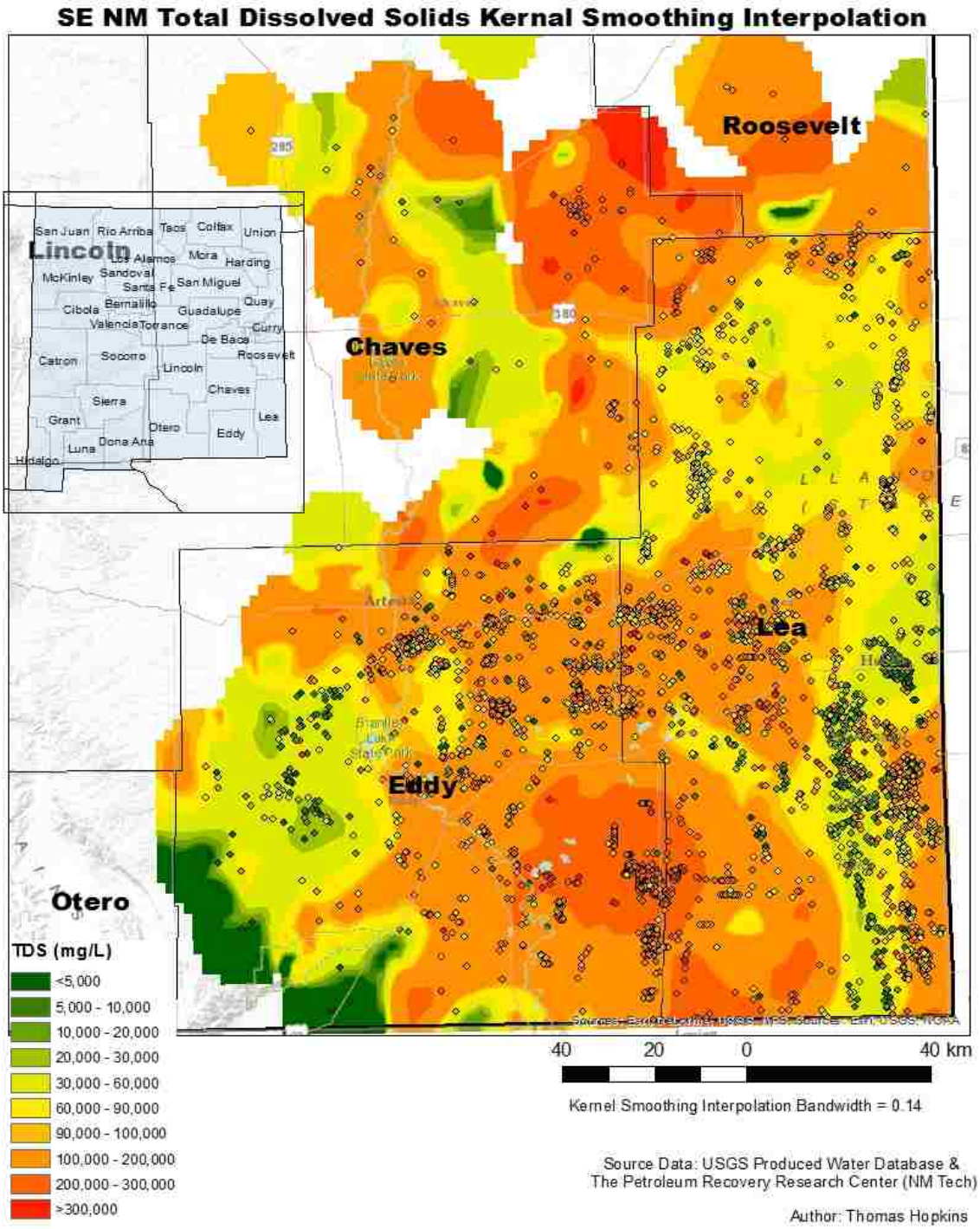

Figure 1. 8: Interpolated map of TDS variation of PW in SE New Mexico.

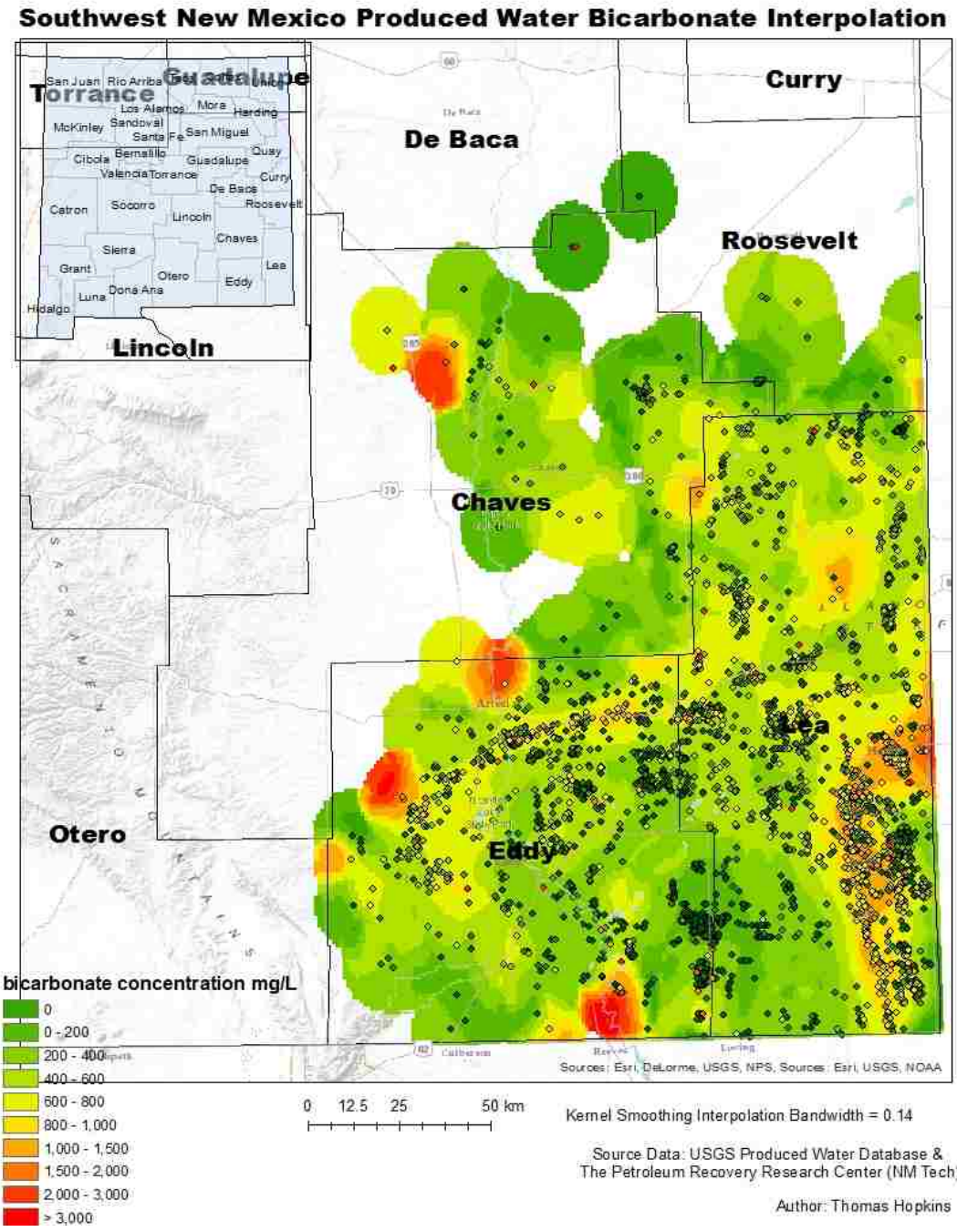

Figure 1. 9: Interpolated map of bicarbonate concentrations of PW from the Permian Basin of SE New Mexico.

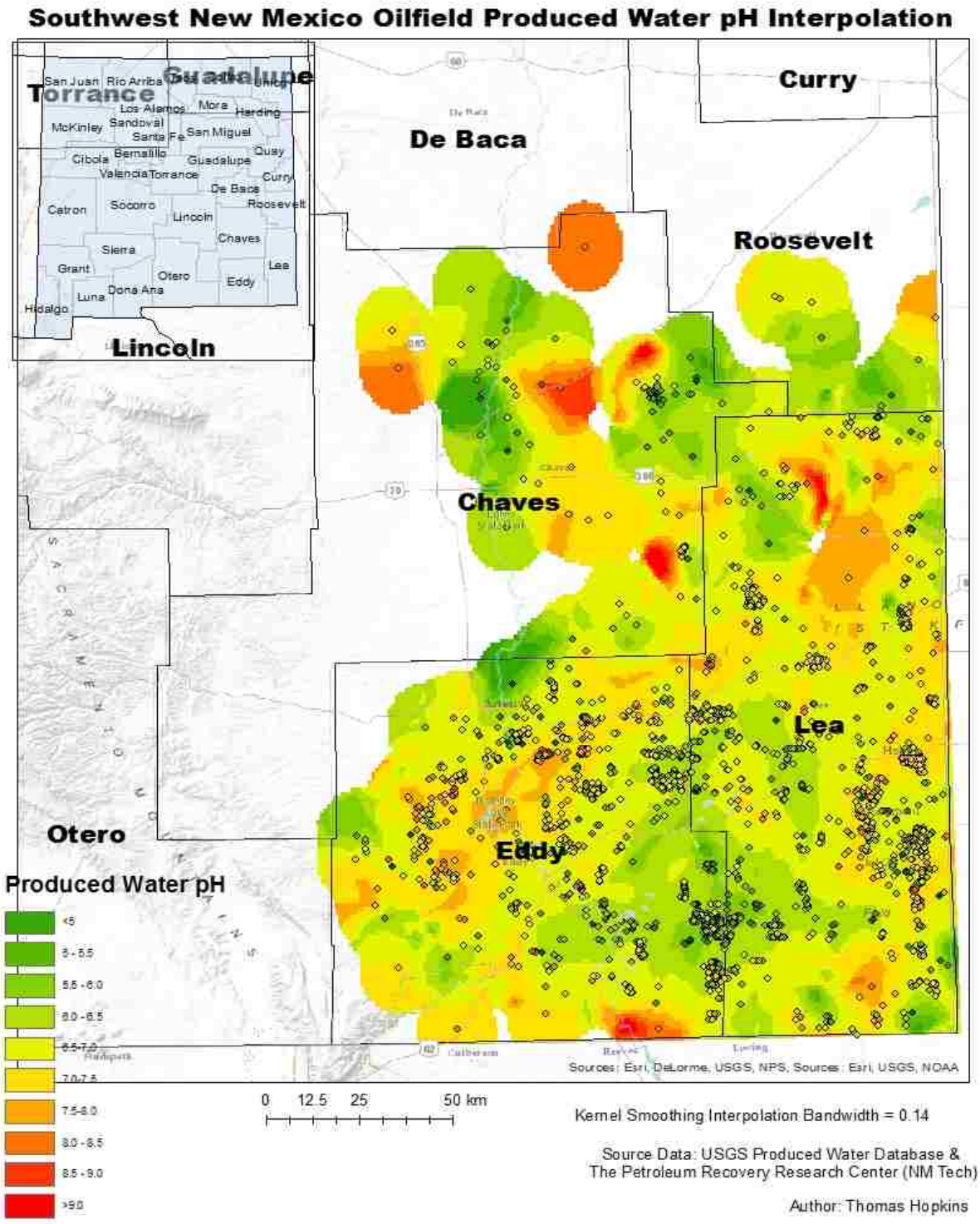

Figure 1. 10: Interpolated map of pH values of PW from the Permian Basin of SE New Mexico.

1.2.4: Previous work on algae growth in produced water

A number of different algal species that could be useful for biofuel have been shown to grow in PW that could be used for biofuel. Graham et al. 2017 reported that a number of promising biofuel algae strains grow in produced water despite its potentially toxic constituents including *Nannochloropsis salina*, members of *Desmodesmus*, and *Chlorellacea*. Ariadia & Abreu (2014) grew *Nannochloropsis occulata* in PW from Brazil at low salinities (2 and 7 g NaCl/L). *Nannochloropsis salina* and *Dunaliella tertiolecta* grew well in shale gas flowback water with a TDS of around 41.7 g/L with growth rates comparable to the commercial F/2 media (Racharaks et al., 2015), which is the highest salinity tested found so far in the existing literature. *D. tertiolecta* is of particular interest due to its halotolerance, high growth rate, and high lipid content (for potential conversion to biodiesel) (Tang et al., 2011). *D. tertiolecta* was also studied at low salinities (10 g/L and below) in coal bed methane waste water (Aravinthan & Harrington, 2013). Hodgskiss et al., 2016 isolated an algae strain from coal bed methane wastewater (brackish) and showed that it could grow well in that wastewater while producing a biomass suitable for fuel production. At the time of this writing studies of algae cultivation in hypersaline (above 41.7 g TDS/L) PW have not been published.

The upper boundary of salinity at which algae cultivation is possible for biofuel has not been well defined in the existing literature. This is an important design parameter since many PW samples have salinities well in excess of seawater. Graham et al., (2017) reports that salinities at or below seawater are best for cultivation, but this might not be true for all species. Ishika et al., (2017) states that the optimal salinity

range depends on the species and some algae can tolerate a broad range and links to studies. This opens the possibility of algae cultivation for biofuels production in hypersaline conditions if suitable species and strains can be found.

The effects of different nitrogen sources and varying levels of nutrients on algae productivity are not well understood yet. Racharaks et al. (2015) used both nitrate and ammonium in their study but did not compare the two separately to one another. Possible differences above salinities of 41.7 g TDS/L were not tested. Chen et al. (2011) did explore ammonium and nitrate separately in a seawater media using *D. tertiolecta*. Ammonium (1mM) did produce the same growth as nitrate at over twice the concentration (2.3 mM). Higher levels of ammonium (10, 20, and 50 mM) produced greatly reduced growth, while nitrate (23 and 46 mM) extremely high. This led them to conclude nitrate is a preferable nitrogen source for *D. tertiolecta.* Higher salinities and intermediate ammonium levels indicative of PW described in Harkness et al. (2015) were not explored. The components PW might produce different effects than those published so far.

All algae research using PW (and most research in general) involves monocultures and exploring a mixed algae culture would be unique. Why monoculture experiments are valuable for understanding a particular species in isolation, different effects might occur in a diverse microbial community. Synergetic and inhibitive relationships might develop between different species. One might out compete the other under different conditions. This might have great relevance for biomass and lipid productivity. Open outdoor ponds will not isolated systems. Algae will be interacting

with a diverse group of microorganisms. A mixed algae culture might prove more resilient to environmental stress than a pure culture.

1.2.5: Algae Cultures used in this Study

In this work two different algae cultures were chosen for their documented and/or suggested potential to grow in saline to hypersaline PW. Important factors are tolerance to salinity, metals, volatile organics, and other inhibiting elements. The two algae strains studied were the species *Dunaliella tertiolecta* and a mixed culture containing algae and bacteria referred to henceforth as the "Polyculture".

Dunaliella tertiolecta

The genus *Dunaliella* is one of the most halotolerant species of algae known and can adapt to virtually the entire range of salt concentrations (Polle, et al, 2009). They can bloom in hypersaline water bodies inhospitable to other algae species such as The Great Salt Lake in Utah (Figure 1. 11). *Dunaliella* are unicellular algae with a potato shape and two flagella for locomotion (Figure 1. 12 & Figure 1. 13). They are approximately 10 microns in length and have been well studied as a candidate species for biofuel production (Tang et al., 2011). Their halotolerance adaptations have also garnered significant research (Fazeli et al., 2006). One key feature of the *Dunaliella* genus is the production of high concentrations of glycerol in the cytoplasm to balance osmotic pressure (up to 7.8M) (Oren, 2005). By using glycerol as a "compatible solute" intracellular ionic strength can be maintained around 0.1 M in salt solutions of >3M. In addition, *Dunaliella* has key membrane protein adaptations that pump Na^+ out of the cell and capture inorganic carbon (bicarbonate uptake is more difficult at high ionic

strength) (Avron, 1992). It can enhance CO_2 acquisition in highly saline media unlike other algae species (Katz et al., 2009). Katz et al. (2009) identified a number of special proteins that stabilize the membrane, sense osmotic changes, and acquire nutrients. *Dunaliella salina* is currently the only species industrially cultivated in hypersaline outdoor ponds for its high ß-carotene content. Clearly, the *Dunaliella* genus possesses key adaptations that might allow it to be cultivated in the entire range of PW salinities.

D. tertiolecta has been previously cultivated in PW and possesses a variety of characteristics that make it a promising candidate for HRAP biofuel production. *D. tertiolecta* was shown to maintain high growth up to at least a salinity of 1M NaCl with a salt stressed induced lipid concentration of 70% (Takagi et al., 2016). It has been shown to grow in media of salinities up to at least 3 M NaCl (Jahnke & White, 2003). It has already been demonstrated to grow in coal gas seam and hydraulic fracturing flowback water (Racharaks et al., 2015) (Aravinthan & Harrington, 2013). The biomass of the species has demonstrated potential to be converted into fuel through transesterification (Tang et al., 2011), pyrolysis (Kim et al., 2015), anaerobic digestion (Lakaniemi et al., 2011), bioethanol fermentation (Lee et al., 2013), and hydrothermal liquefaction (Chen et al., 2016). It is known to produce significant amounts of carotenoids that would shield it from photo inhibition in direct sunlight (Oren, 2005). In addition, it has evolved a significant antioxidant response that protects if from free radicals produced in intense light conditions. *D. tertiolecta* has the ability to tolerate the potential heavy metal toxicity of PW by producing phytochelatins that sequester these cations in vacuoles (Tsuji et al., 2003). Also, it can tolerate the VOC phenol at levels of 100 ppm, a

constituent that is likely to be found in PW (Erturk & Sacan, 2012). It can be effectively harvested by pH induced flocculation/sedimentation in settling tanks improving the economics (Horiuchi et a., 2003). All things considered, *D. tertiolecta* is the ideal candidate for cultivation using PW in HRAPs over a large range in salinity.

Figure 1. 11: Dunaliella bloom (left of the causeway) in the Great Salt Lake, UT.

Source: https://www.deseretnews.com/article/865580841/State-lists-proposed-fixes-to-UP-railroad-causeway-as-issue-of-top-concern.html?pg=all

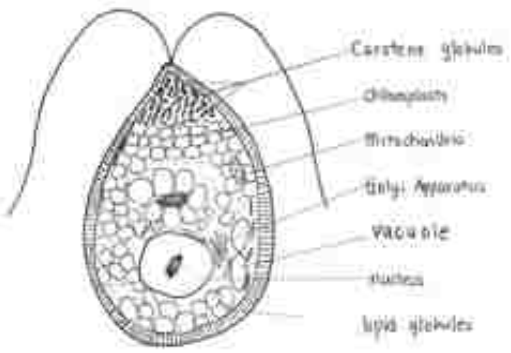

Figure 1. 12: Dunaliella algal cell.

Source: https://microbewiki.kenyon.edu/

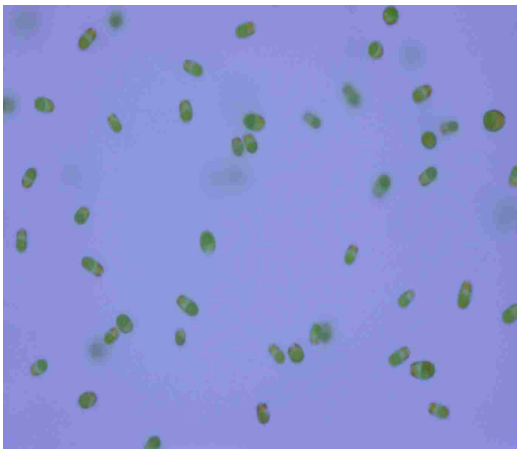

Figure 1. 13: *D. tertiolecta* algae cells (200X).

Taken by author.

The Polyculture

The Polyculture used in this experiment was obtained from Algal Biofuels Laboratory at The Santa Fe Community College, and preliminary observations suggested it might have the ability to grow well in saline to hypersaline PW. It was originally found growing in a PW sample from the Permian Basin of Southeast NM. It maintained biomass in nutrient augmented PW and F/2 media in salinities ranging from seawater (~30 g TDS/L) to hypersaline (~105 g TDS/L). The Polyculture grew well in tubular photo bioreactors under fluorescent light in which it often exhibited a blue hue. Originally, it was thought to be a strain of the algae genus *Nannochloropsis*. Prior to this study no DNA identification was performed. The PW origins of the Polyculture, and its ability to grow and survive highly saline media suggests it as a suitable candidate for cultivation in PW. It is possible its diverse community would provide benefits for algae biofuels cultivation.

1.3: Research Motivations

Produced water can be potentially used as a growth medium for large scale algal biofuel production. In the United States alone large volumes are brought to the surface every year. PW is not typically allocated in a water rights system, and its use for biofuels would not compete with other stakeholders (Graham et al., 2017). The acquisition in areas of oil and gas production might be essentially free. It is even possible that accepting PW might provide an additional revenue stream, since it presents a significant cost to operators for disposal (Sabie et al., 2016). As previously discussed, PW often contains a number of nutrients necessary for algae growth potentially improving the economics. Nutrient addition can consume 45% of the effective energy input of algae cultivation (Greenwell et al., 2009). Open pond cultivation systems are vulnerable to the introduction of algae competitors and predators, but a hypersaline media (>> seawater) would be more resistant due to the fact that few other organisms can survive at such high ionic strengths (Tafreshi and M. Shariati, 2007). PW is available through oil and gas operations that could provide a plentiful supply of CO_2 to boost algal growth. The stimulation of increased lipid content in algae can be accomplished by salinity, heavy metal, or oxidative stress (Sibi et al., 2016), all potential characteristics of PW. Though PW presents its challenges, it seems possible that this waste water source could be utilized for industrial scale biofuel production in arid regions where high evaporation rates are also a concern.

Algae strains that can handle the stresses imposed by PW characteristics (salinity, heavy metals, VOCs) need to be identified to enable the use of PW potential as

an economically feasible growth medium. Also, strains that can thrive on nutrients already present would be ideal. Growth rates and the suitability for conversion to biofuel of candidate species would need to be determined. These characteristics are likely strongly dependent on salinity. The upper limit of the toleration of total dissolved solids is an important parameter for choosing a specific strain (Ishika et al., 2017). The effect of nutrient availability and type might depend on salinity as well. This could also influence the lipid content and character of the biomass. While general trends hold for microalgae many responses are species/strain specific with each required to be studied individually. A diverse mixed algae polyculture might prove more ideal for than a monoculture.

Lipid content and fatty acid chain composition might change with salinity since the ionic strength of the PW might change the physiology of the algae biomass. A number of possibilities exist. Increased salinity stress might lead to a higher lipid content (Talibi et al., 2014)(Takagi et al., 2006). An optimal salinity level might exist for maximum amount of lipid production (Mohan and Devi, 2014). The proportion and type of neutral lipids might vary with salinity, and thus the potential for conversion to biofuel (Bartley et al., 2013). The effect of salinity on lipids should be determined for any suitable candidate for cultivation. The number of way lipid productivity in a polyculture might respond to environmental factors maybe greater and unpredictable. Though high oil content is thought to enhance a biomass' potential for biofuel this is not always the case, and the specific conversion process must be considered.

There are additional reasons to study algae cultivation in saline/hypersaline media beyond reuse of PW for biofuel production. In any open pond cultivation system, the growth medium still contains nutrients after harvesting and reuse improves the economics of the system (Tafreshi and M. Shariati, 2007). Ishika et al., 2017 (based on algae cultivation in Australia) stated that reusing seawater media results in an incremental increase in salinity through evaporation. Reusing media during biofuel production would require a range of different salt tolerant algae species to optimize biomass production at each specific TDS concentration. Their media re-use scenarios reduce nutrient waste by up to 95% with resulting potential significant reduction in cost. Algae that could thrive in the resulting hypersaline media residuals would improve the proposed model. This would maximize the nutrient utilization efficiency.

Halotolerant algae strains could also help remediate saline and hypersaline waste water. For example, oil and gas wastewater containing high amounts of ammonium has been disposed of in local rivers, thus causing eutrophication or the formation of disinfection byproducts downstream (Harkness et al., 2015). Algae growth in the PW could remove the ammonium in the waste water before discharge. Lefebvre et al., (2006) estimates that around 5% of treated waste water might be saline to hypersaline. While salinity typically inhibits aerobic and anaerobic bacteria communities, halotolerant algae could be a good choice for nitrogen and phosphorus removal. Wu et al., (2015) demonstrated just that, by using *D. tertiolecta* (also explored in this study) to remove phosphate, nitrate, and ammonia from brackish municipal waste (salinity around 8 PSU). Belle (2007) also used *D. tertiolecta* to remove N and P as

a tertiary treatment of brackish landfill leachate. Other high salinity wastewaters are produced by a variety of different chemical and food manufacturing processes (Lu et al., 2011), and perhaps halotolerant algae strains could be incorporated with other adapted microorganisms. Identifying potential strains that can grow in high salinity wastewater would expand the engineering options for its remediation.

1.4: Research Objective

The objective of this study was to determine the effects of salinity and nutrients on biomass and lipid productivity with an algal polyculture and *D. tertiolecta* as inoculum using nutrient enriched PW. Both cultures were studied over a range of salinities in a PW medium with identical nutrient concentrations. Nitrogen source and nutrient concentration were further varied to explore their effect on biomass and lipid productivity. The results are discussed in the context algae cultivation using a PW media for conversion to biofuels.

Chapter 2: Materials and Methods

2.1: Analytical methods

2.1.1: Dissolved Constituents

Prior to and during the experiments produced water and algae growth media chemical concentrations of dissolved and undissolved constituents were determined by a variety of methods. Total dissolved solids (TDS) were determined by gravimetric measurements after evaporation of filtered PW. Cation concentrations were measured with inductivity coupled plasma- optical emission spectroscopy (ICP-OES). Non-carbonate anions were determined through ion chromatography (IC) on a Varian machine for the original PW. The pH was determined using an electrical probe with a Denver Instruments data recorder. The alkalinity of the PW was determined through acid titration to a pH of 8.3 (for carbonate) and 4.5 for (for bicarbonate). Spectrophotometry was used for a number of other constituents with commercial kits as per their instructions. Chemical oxygen demand (COD) was determined with Hach test kit 3 – 150 PPM and a Hach spectrophotometer (DR2700). Nitrate concentrations were determined with Millipore's Spectroquant Nitrate Test for Seawater model #14942 which is resistant to interference from other dissolved ions using a custom calibration curve. Phosphate levels were measured with Spectroquant phosphate test kit model #14543. Ammonium was measured using Hach's High Range Ammonia Reagent Set 2606945. Nitrite was measured using Hach's NitriVer Reagent Set 2608345.

2.1.2: Biomass

Biomass density was measured using optical density and Ash Free Dry Weight (AFDW). Optical density was measured at a wavelength of 680 nm (which is the peak of chlorophyll A absorption) using a Hach DR2700 spectrophotometer. AFDW was obtained by filtering 15 – 25 ml of algae culture using Pall glass fiber 0.4 µm filters using vacuum suction. Filters were then dried in an oven at 105º C for 1 hour and the mass was recorded to the tenth of a milligram. Samples were then heated to 550º C for 1 hour and the difference in mass was used to find AFDW in g/L (similar to volatile suspended solids (VSS)). Calibration curves were made to relate OD680 and AFDW. Microscopy was also used to characterize microbial populations and monitor for contamination.

2.1.3: Lipid concentration and content

The lipid concentration of the biomass (defined as the chloroform soluble portion) was determined through solvent extraction. The biomass in 30 ml of media was first concentrated by centrifugation at 8,000 RPM. The supernatant was siphoned off and the green algae "pellet" was then frozen at -23º C. The lipid extraction technique was based on chemical solutions and methods presented in Wang and Benning (2011). Once thawed a solution of methanol, chloroform, and formic acid (20:10:1) was added in a volume proportional to the biomass (usually 2-4 ml). To facilitate cell lysis the mixture was vortexed for approximately five minutes. The solids were observed for pigment bleaching, which would indicate removal of chloroform soluble molecules. The lipid phase separation was accomplished by adding a solution of 0.2 M phosphoric acid and 1 M KCl. This extracted the denser chloroform phase with dissolved lipids and pigments below with a lighter methanol phase with polar components above (Figure 2. 1). The

chloroform phase was removed by pipette and evaporated in glass vials. To further isolate the lipid portion of biomass, 1 ml of pure chloroform was then added to the vials, and then decanted leaving behind salt and protein residue. Additional chloroform rinses did not result in left behind residues. The lipid concentration was then determined gravimetrically, and samples were frozen at -23º C. To determine the lipid content of biomass the lipid concentration was divided by that of the AFDW.

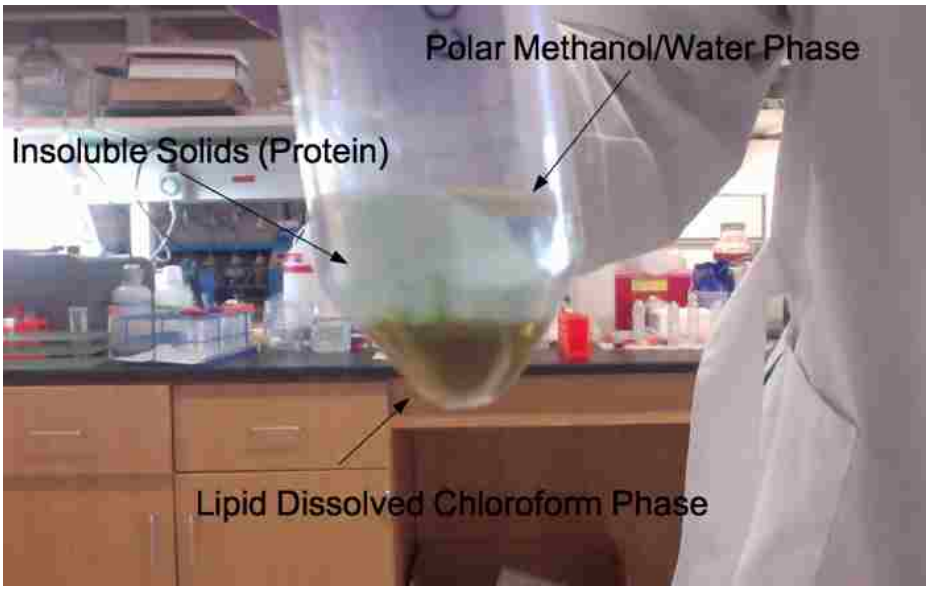

Figure 2. 1: Chloroform Phase separation during lipid extraction.

The lower green chloroform phase contains the lipid component of biomass and is extracted by pipette.

2.1.4: Lipid FAME profile

The relative proportions of fatty acid methyl esters (FAME) derived from lipid extracts were determined by gas chromatography mass spectroscopy (GC/MS). Lipid extracts were first re-dissolved in a 2:1 chloroform methanol solution at a concentration of 1 mg lipid/ml. To accomplish FAME catalysis 300 µL of a 0.6 M KOH methanol solution was added to 250 µL of the dissolved lipid sample. The mixture was placed in a shaker plate oven at 50º C for 30 minutes. After 30 minutes the solution was neutralized

with 100 µL of glacial acetic acid. To extract the converted FAME, 250 µL of hexane (with an internal C23:0 FAME standard) was then added, that resulted in a phase separation. The top FAME containing hexane layer was pipetted into sealed sample vials for analysis.

A Varian GC/MS machine was utilized to perform the analysis. The relationship between peak area and concentration of different saturated and unsaturated carbon chain lengths was calibrated against an external standard solution using a serial dilution. Peak areas were then used to calculate individual FAME concentration, and the relative proportion determined as the percentage of total FAME observed. An internal C23:0 standard was also used to determine FAME concentration.

2.1.5: DNA sequencing of Polyculture

Illumina sequencing of the Polyculture was performed by MRDNA (www.mrdnalab.com, Shallowater, TX, USA). Algae biomass samples were taken from a culture growing in a 50% produced water, 50% f/2 media at a salinity of approximately 75 g TDS/L. A 23s RNA primer was chosen that identifies chloroplast and cyanobacteria to try and characterize the broad range of potential algae species. A 16s universal primer was selected to identify prokaryotic sequences. Samples were centrifuged and frozen before being shipped to concentrate and preserve the biomass before and during shipping.

Samples were then sent to MRDNA Genome Sequencing using Illumina where the following steps were taken. The 23s RNA universal algae primer p23SrV_f1 (5' GGACAGAAAGACCCTATGAA 3') (Sherwood and Presting, 2007) and the 16s RNA universal primer 515F (5'GTGCCAGCMGCCGCGGTAA 3') (Turner et al., 1999) with barcode on the forward primer were used in a 30 cycle PCR (5 cycle used on PCR

products) using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, PCR products are checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples are pooled together (e.g., 100 samples) in equal proportions based on their molecular weight and DNA concentrations. Pooled samples are purified using calibrated Ampure XP beads. Then the pooled and purified PCR product is used to prepare DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). In summary, sequences were joined, depleted of barcodes then sequences <150bp removed, sequences with ambiguous base calls removed. Sequences were denoised, OTUs generated and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using the BLAST tool from the National Center for Biotechnical Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.1.6: Carotenoid Determination of Lipid Extracts

The Carotenoid content of lipid extracts were determined through spectrophotometry. Lipid extracts were re-dissolved in hexane and pipetted to quartz cuvettes. Absorbance was measured on a Cary 50 Conc UV-Visible Spectrophotometer at the wavelengths of 470, 642.2, 660.6, and 750 nm. The absorbance at 750 nm was

subtracted to normalize for scattering. Total carotenoids were determined using the following equations from Lichtenthaler and Buschmann (2001),

Conc. Of Chl. A (µg/ml)= C_a =10.05($A_{660.6}$) – 0.97($A_{642.2}$)

Conc. Of Chl. B (µg/ml)= C_b =16.36($A_{642.2}$) – 2.43($A_{660.6}$)

Total Carotenoids = [1000*A_{470} – (1.43)*C_a – (33.12)*C_b]/205

The carotenoid concentration of the original algae medium was then found using the original sample volume.

2.1.7: Determination of pKa values at different salinities for the NH_3/NH_4 system

The specific interaction model (SIM) was used to estimate the change in the pKa of the ammonium acid/base system in saline and hypersaline media conditions. The following equation was taken from Maeda et al. (1990) as is based on the SIM (also commonly called the Pitzer approach) for the pKa of the disassociation of NH_4^+ at higher ionic strength.

$pKa = pKa^0 + (\ln\gamma_{H+} + \ln\gamma_{NH3} - \ln\gamma_{NH4+})/\ln 10$

Where pKa = 9.245 at standard conditions, γ_{H+} = activity of H^+, γ_{NH3} = activity of NH_3, and γ_{NH4+} = activity of NH_4^+. The activities of the ions H^+ and NH_4^+ where determined from the Pitzer equation (Pitzer, 1979) for a cation M:

$\ln \gamma_{(M)} = f^{\gamma} + 2I(B_{MCl} + IC_{MCl}) + 2m_{Na}\theta_{MNa} + m_{Na}m_{Cl}(B'_{NaCl} + C_{NaCl} + \Psi_{MNaCl})$

Where I = ionic strength, m is the concentration of Na or Cl, (θ_{MNa} and Ψ_{MNaCl} are constants), and,

$f^{\gamma} = -0.392[\sqrt{I}/(1 + 1.2\sqrt{I}) + (2/1.2)\ln(1 + 1.2\sqrt{I})]$

$B_{MCl} = \beta^o_{MCl} + (\beta^1_{MCl}/2I)[1 - \exp(-2\sqrt{I})(1 + 2\sqrt{I})]$

$B'_{MCl} = ((\beta^1_{MCl}/2I^2)[-1 + \exp(-2\sqrt{I})(1 + 2\sqrt{I} + 2I)]$

$C_{MCl} = C^{\phi}_{MCl}/2$

β^o, β^1, C^{ϕ} values were taken from Pitzer and Mayorga (1973) and are as follows.

$\beta^o_{NaCl} = 0.0765$, $\beta^1_{NaCl} = 0.2664$, $C^{\phi}_{NaCl} = 0.00127$, $\beta^o_{NH4} = 0{,}0522$, $\beta^1_{NH4} = 0.1918$, $C^{\phi}_{NH4} = -0.00301$, $\beta^o_{H+} = 0.1775$, $\beta^1_{H+} = 0.2945$, and $C^{\phi}_{H+} = 0.0008$.

θ_{MNa} and Ψ_{MNaCl} in the HCl/NaCl system are constants taken from Pitzer and Kim (1974).

$\theta_{MNa} = 0.036$ and $\Psi_{MNaCl} = -0.004$

θ_{MNH4} and Ψ_{MNH4l} in the NH_4Cl/NaCl system are constant taken from Maeda et al., (1989).

$\theta_{MNH4} = .004$ and $\Psi_{MNH4l} = .0005$

The activity of ammonia was determined by the following equation from Maeda et al. (1990).

$\ln\gamma_{NH3} = 2m_{Na}\lambda_{NH3Na} + 2m_{Cl}\lambda_{NH3Cl}$ where $\lambda_{NH3Na} = 0.0238$ and $\lambda_{NH3Cl} = 0.0064$

This model is assumed to be accurate since sodium and chloride are the most common ions in the media and ammonium, hydrogen, and ammonia concentrations are orders of magnitude lower.

2.2: Growth Media

All experiments were performed using produced water, synthetic brine (modelled on PW described in Harkness et al. (2015), or the commercial F/2 algal media. Both the Polyculture and *D. tertiolecta* were maintained in F/2 media based on a recipe from the University of Texas, Austin (UTEX). 35 g/L of Instant Ocean aquarium salt was

used to create a seawater solution. Next the following nutrients were added, 75 mg/L $NaNO_3$, 5 mg/L $NaH_2PO_4*H_2O$, 30 mg/L Na_2SiO_3, and 1 ml/L trace metal solution. The trace metal solution had the following components in mg/L: 23 $ZnSO_4$-$7H_2O$, 152 $MnSO_4$-H_2O, 7.3 Na_2MoO_4-$2H_2O$, 14 $CoSO_4$-$7H_2O$, 6.8 $CuCl_2$-$2H_2O$, 4.6 Fe(NH4)2(SO4)2-6H2O, and 4.4 Na_2EDTA-$2H_2O$. The pH was then adjusted to 8.1 with hydrochloric acid. The resulting solution was then autoclaved at 121º C for 20 minutes. Once cooled vitamin solutions of B-12, biotin, and thiamine were added as per UTEX directions. The F/2 media was stored at 4º C until use.

Flask experiments used produced water obtained from an oil production facility in the Permian Basin of SE New Mexico. The chemical composition is listed in Table 2.1 as determined by the analytical methods described previously. The overall salinity was in the lower range of PW samples at 6.7 g TDS/L. The dominant anions were chloride, carbonate, and sulfate. The total alkalinity is fairly high at 20 mM about 1000 mg as $CaCO_3$ which is reflected by the pH. The most abundant cation is sodium, followed by magnesium, potassium, and calcium by mass. The low value of 6 mg/L for the chemical oxygen demand suggests a low concentration of organic molecules. Crucial algae nutrients such as ammonium, nitrate, iron, and phosphate are not present in this PW sample. The metals copper and arsenic at levels of potential significance. Overall, the chemical composition falls in the range of low salinity PW presented in Graham et al., (2017). The salinity and nutrient content of this water was adjusted for experiments with the two algae cultures.

Table 2. 1: Chemical composition of PW used in this study.

Component	Value
TDS	6.7 g/L
Sodium	2,023 mg/L
Chloride	2,352 mg/L
Sulfate	843 mg/L
Bicarbonate	976 mg/L
Carbonate	120 mg/L
Potassium	113.8 mg/L
Magnesium	167 mg/L
Calcium	18.0 mg/L
Lithium	10.5 mg/L
Copper	2.42 mg/L
Iron	Below detection limits
Arsenic	0.386 mg/L
Ammonium	0.1 mg NH_4-N /L
Phosphate	Below detection limits
pH	9.1
COD	6 mg/L

In tubular photobioreactor experiments a synthetic media was used that attempted to mimic the composition of hydraulic fracturing flowback wastewater as described in (Harkness et al., 2015). The composition is listed in Table 2.2. It is characterized by high concentrations of Na^+, Cl^-, Br^-, Ca^{2+}, Mg^{2+}, and SO_4^{2-}.

Table 2. 2: Dissolved constituents of the synthetic brine growth media used in the tubular photobioreactors.

Component	Concentration
Instant Ocean Commercial Sea Salt	35 g/L
NaCl	25 g/L
$MgSO_4$	1 g/L
$CaCl_2$	1 g/L

NaBr	0.322 g/L
$NaH_2PO_4*H_2O$	22.3 mg/L
F/2 trace metal solution	6 ml/L

2.3: Experimental Methods

In this study 6 different flask experiments were performed and 1 tubular photobioreactor experiment with varying salinity and nutrient concentration for the two cultures. Table 2.3 provides a list of all experiments performed (3 flask Polyculture, 3 flask *D. tertiolecta*, and 1 Polyculture tubular).

The following conditions were kept constant for all flask experiments. PW media was enriched with F/2 media trace metal solution (UTEX recipe) to provide iron (and perhaps other necessary trace metals). The pH of the media was lowered to 8.1 to match seawater and the F/2 media. Temperature was maintained at 24 ºC in a growth chamber under a fluorescent grow lamp illumination of 100 µmol/m^2/sec. 500 mL glass flasks were used with a 250mL media volume tested in triplicate for each condition (Figure 2. 2). To facilitate gas exchange, flasks were placed on a shaker table at 140 RPM (Figure 2. 3 & Figure 2. 4). Flask position was randomly alternated to account for light intensity variations on the shaker table. A 16-hour light vs. 8-hour dark cycle was kept constant for all experiments. The experiment was continued into the stationary phase of growth with the total time ranging from 17 to 40 days. Periodically, media samples were removed for biomass and media measurements. Late experiment flasks were combined once volumes fell below 100 ml (not before day 16).

All experiments were inoculated in an identical manner. Cultures were acclimatized (grown at that salinity) prior to the experiment at the salinity to be tested. Algae cultures were used that were in the linear growth phase. Algae inoculant was separated from the previous media by centrifugation at 3500 RPM prior to addition to the media. Initial biomass levels were set to 0.04-0.05 g AFDW/L. Cultures were check microscopically for live cells and contamination before and for the duration of each experiment.

Figure 2. 2: 500 ml Erlenmeyer flask triplicate

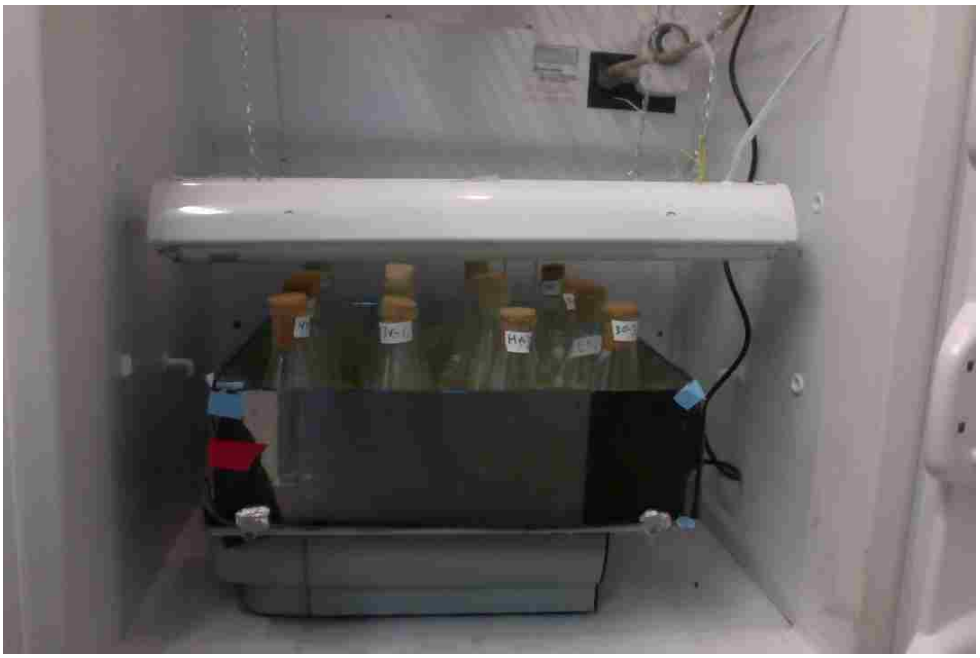

Figure 2. 3: Flask shaker table setup in temperature-controlled chamber.

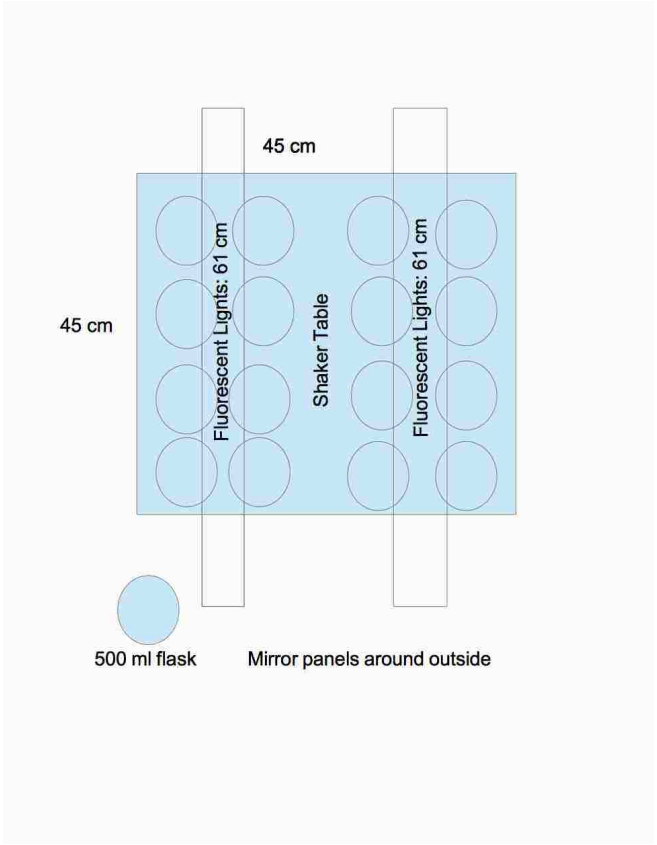

Figure 2. 4: Schematic of flask experiment layout on shaker table.

2.3.1: Polyculture Flask Experiments (P1, P2, & P3)

Experiment P1 varied salinity (from 15-150 g TDS/L) in a PW medium with nutrient levels identical to the commercial F/2 media to determine its effect on biomass and lipid productivity (Table 2.4). Salinity as in all experiments was increased with sodium chloride the dominant salt in PW analyses [Graham et al. (2016) & Chadhaury et al. (2016)]. Based on the results of this experiment in #P2 salinity was again varied between 60 and 120 g TDS/L to better define the effects in this range (Table 2.5). In experiment P3, ammonium was used as a nitrogen source at concentrations of 13, 30, 50, and 70 mg NH_4-N/L (Table 2.6). The purpose was to compare growth for two different nitrogen sources and to determine whether increased ammonium encouraged or inhibited productivity. The F/2 media was also tested to compare growth between a common commercial media and PW.

Table 2. 3: Algae experiments

Number	Inoculate	Media	Salinity(s) g TDS/L	Initial N Conc.(s) mg N/L	Init. PO_4 Conc.(s) mg PO_4-P/L	Experimental Variables
Poly culture Flask Experiments (P)						
P1	Polyculture	PW	15, 30, 60, 120, 150	13 (NO_3)	1.7	Salinity (15-150 g TDS/L)
P2	Polyculture	PW	60, 75, 90, 105, 120	13 (NO_3)	1.7	Salinity (60-120 g TDS/L)
P3	Polyculture	PW & F/2	60 & 30 (F/2)	13, 30, 50, 70 (NH_4) & 13 (NO_3) (F/2)	1.7 & 8	Nitrogen source, NH_4 level, P Conc., & media type
D. tertiolecta Flask Experiments (D)						
D1	*D. tertiolecta*	PW	30, 60, 120, 180, & 210	13 (NO_3)	1.7	Salinity (30 – 210 g TDS/L)

D2	*D. tertiolecta*	PW,F/2	120, 180, 30 (F/2)	13 (NO_3) 13, 70 (NH_4)	1.7	Retest 120 & 180, N source, NH_4 Conc., & media type
D3	*D. tertiolecta*	PW	30 & 120	13, 26, 39, 52 (NH_4)	1.7 & 8	NH_4 & P Conc., NH_3 at high low salinity
Polyculture Tubular Photobioreactor Experiment (T)						
T1	Polyculture	Synthetic Brine	60	30 & 200 (NH_4)	4.8	NH_4 Conc., Inorganic carbon addition, and light/dark

Table 2. 4: Polyculture PW media flask experiment #P1 (varying salinity 15-150 g TDS/L)

Objective: To measure how salinity influences growth and lipid productivity.					
Flask Condition Triplicate	#1 brackish	#2 saline	#3 hyper	#4 hyper	#5 hyper
Salinity g TDS/L	15	30	60	120	150
N Source mg N/L	13 NO_3	13 NO_3	13 NO_3	13 NO_3	13 NO_3
Phosphate mg PO_4-P/L	1.7	1.7	1.7	1.7	1.7

Table 2. 5: Polyculture PW media flask experiment #P2 (varying salinity 60-120 g TDS/L)

Objective: To better define the upper limit of max biomass productivity between 60 and 120 g TDS/L.					
Flask Condition Triplicate	#1	#2	#3	#4	#5
Salinity g TDS/L	60	75	90	105	120
N Source mg N/L	13 NO_3	13 NO_3	13 NO_3	13 NO_3	13 NO_3

Phosphate mg PO_4-P/L	1.7	1.7	1.7	1.7	1.7

Table 2. 6: Polyculture PW media flask experiment #P3 (varying NH_4 and PO_4 conc.)

Objectives: To investigate the effect of ammonium concentration and surplus phosphate on growth and lipid productivity.					
Flask Condition Triplicate	#1 Low N	#2	#3	#4	#5 High P
Salinity g TDS/L	60	60	60	60	60
N Source mg N/L	13 NH_4	30 NH_4	50 NH_4	70 NH_4	13 NH_4
Phosphate mg PO_4-P/L	1.7	1.7	1.7	1.7	8

2.3.2: D. Tertiolecta Flask Experiments (D1, D2, & D3)

Similar flask experiments were conducted with *D. tertiolecta* in PW. In experiment D1 salinity was varied at 30, 60, 120, 180, 210 g TDS/L in PW at 13 NO_3-N and 1.7 PO_4-P/L (Table 2.7). Once again, these nutrient levels are identical to the F/2 commercial media and the purpose was to determine the effect of salinity on growth and lipid content. The next experiment D2 retested the 120 and 180 g TDS/L conditions from #D1 (Table 2.8). An initial low ammonium concentration (13 mg NH_4-N/L) was tested to compare against nitrate at the same salinity (120 g TDS/L). A high ammonium concentration (70 mg NH_4-N/L) was incorporated to determine whether growth would increase or be inhibited by ammonia toxicity. The F/2 media was tested for comparison of PW with a conventional media. The final *D. tertiolecta* experiment (#D3) explored varying NH_4 concentration, salinity effects of ammonium, and a higher phosphate concentration (Table 2.9). Initial results in experiment #D2 displayed high growth at 13 mg NH_4-N/L and culture death at 70 in 120 g TDS/L PW. Ammonium levels were varied in between to determine at what level a switch from encouraging to inhibiting growth

occurred. A lower salinity of 30 g TDS/L was tested to see whether growth improved in saline conditions over the hypersaline. Increased phosphate was tested to see its effect on growth and lipid productivity.

Table 2. 7: *D. tertiolecta* PW media flask experiment #D1 (salinity 30–210 g TDS/L)

Objective: To investigate how salinity influences growth and lipid productivity.					
Flask Condition Triplicate	#1	#2	#3	#4	#5
Salinity g TDS/L	30	60	120	180	210
N Source mg N/L	13 NO_3	13 NO_3	13 NO_3	13 NO_3	13 NO_3
Phosphate mg PO_4-P/L	1.7	1.7	1.7	1.7	1.7

Table 2. 8: *D. tertiolecta* PW media flask experiment #D2 (varying N source and concentration with salinity)

Objectives: To investigate the effect of nitrogen source and ammonium concentration on growth and lipid productivity. F/2 media for comparison. Retest 120 and 180 g TDS/L conditions.					
Flask Condition Triplicate	#1	#2	#3	#4	#5 F/2
Salinity g TDS/L	120	120	120	180	30
N Source mg N/L	13 NH_4	70 NH_4	13 NO_3	13 NO_3	13 NO_3
Phosphate mg PO_4-P/L	1.7	1.7	1.7	1.7	1.7

Table 2. 9: *D. tertiolecta* PW media flask experiment #D3 (varying NH_4 & P Conc. and salinity)

Objectives: To investigate the effect of ammonium concentration, surplus phosphate, and ammonium in saline and hypersaline conditions on growth and lipid productivity.					
Flask Condition Triplicate	#1 High P	#2 low TDS	#3	#4	#5
Salinity g TDS/L	120	30	120	120	120
N Source mg N/L	13 NH_4	13 NH_4	26 NH_4	39 NH_4	52 NH_4
Phosphate mg PO_4-P/L	8	1.7	1.7	1.7	1.7

2.3.3: Tubular Photobioreactor Experiment

In Experiment #T1 the Polyculture was grown in four tubular photobioreactors to test growth at higher illumination, aeration, with ammonium as a nitrogen source. (Figure 2. 5). The synthetic media described previously was used as an analogue for a PW brine as described in Harkness et al. (2015). An LED illumination of around 1000 µmol/m^2/s was maintained for a 16-hour light: 8-hour dark ratio. One reactor was kept in the dark as a control to see if nitrification was occurring by non-photosynthetic metabolism (Table 2.10). The reactors were continuously aerated at 0.5 L/min/L of media. The room temperature was maintained at approximately 25º C. Ammonium levels were varied between low (30 mg NH_4-N/L) and high (200 mg NH_4-N/L). In Condition #3 Carbon dioxide gas and sodium bicarbonate to prevent basic and acidic conditions respectively. Biomass, nutrient levels, and lipid concentrations were determined by methods described previously. Inoculate cultures were acclimatized to a salinity of 60 g TDS/L, spun to separate from the previous growth media, and added to a level of 0.04 g AFDW/L.

Table 2. 10: Experimental matrix for tubular reactor experiment.

Name	Condition #1(L30)	Condition #2(200)	Condition #3(CO_2)	Condition #4(D30)
Light	yes	Yes	yes	No
Init. NH_4 Conc. (mg NH_4-N/L)	30 (Low)	200 (High)	200 (High)	30 (Low)
CO_2 and $NaHCO_3$ addition	No	No	Yes	no

Figure 2. 5: Tubular Photobioreactor

Chapter 3: Results and Discussion

3.1: Calibration of OD680 and AFDW Measurements of Biomass

The biomass density was determined by both optical density at 680 nm (OD680) and ash free dry weight (AFDW). OD680 and AFDW measurements exhibited a strong correlation for both the polyculture and *D. tertiolecta* in experiments. A second order polynomial fit matched the biomass measurements well for the Polyculture flask experiments in a PW Media ($R^2 \geq 0.969$) (Figure 3.1. 1, Figure 3.1. 2, & Figure 3.1. 3). This suggests that AFDW concentration was increases faster than the absorbance at chlorophyll A's maximum. Polyculture growth in 60 g TDS/L PW media at initial concentrations of 13 NH_4-N/L and 8 mg PO_4-P/L followed a different trend than other conditions. Scatter does increase with increasing biomass in flask conditions. Polyculture biomass measurements in the tubular reactor experiment exhibited an opposite bend in the curve over a larger range in biomass concentration (Figure 3.1. 4). A second order curve fit the data trend to an even higher degree (R^2=0.992). *D. tertiolecta* experimental data also followed a second order trend (Figure 3.1. 5). The R^2 value of the second order polynomial trend lines was improved when the salinity ranges 30 and 60, 120 & 150, and 180 & 210 were fit separately. The lowest R^2 value is 0.971 for the 180 and 210 conditions. For *D. tertiolecta* higher salinity correlates with a larger AFDW at a given OD680 possibly due to increase in glycerol content at higher salinity to maintain a balance in osmotic pressure (Zidan et al., 1987). Scatter seems to increase in older cultures which could be linked to changes in chlorophyll content and cell lysis. Due to the closeness of the fit between AFDW and optical density, the OD680

measurements were used with the polynomial fits to estimate AFDW in much of the analyses.

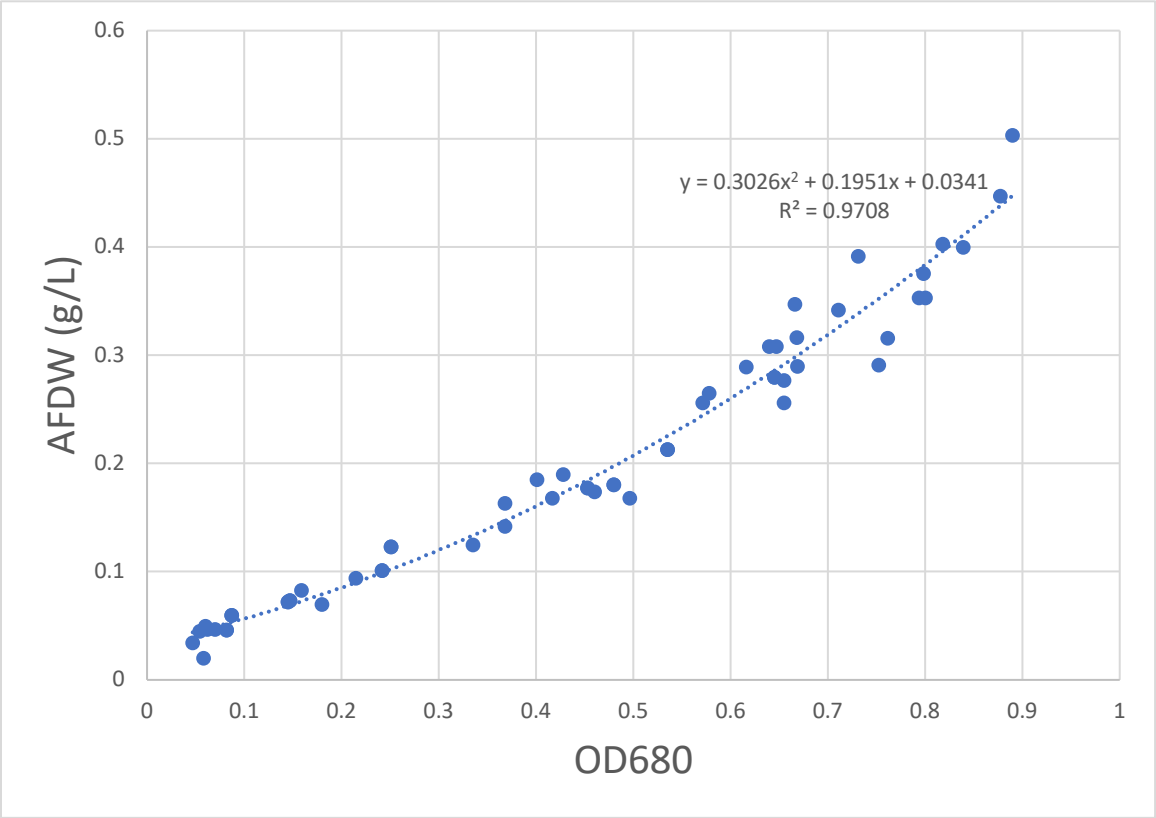

Figure 3.1. 1: Polyculture flask experiments (P1 & P2) ash free dry weight vs. OD680. second order trend line included.

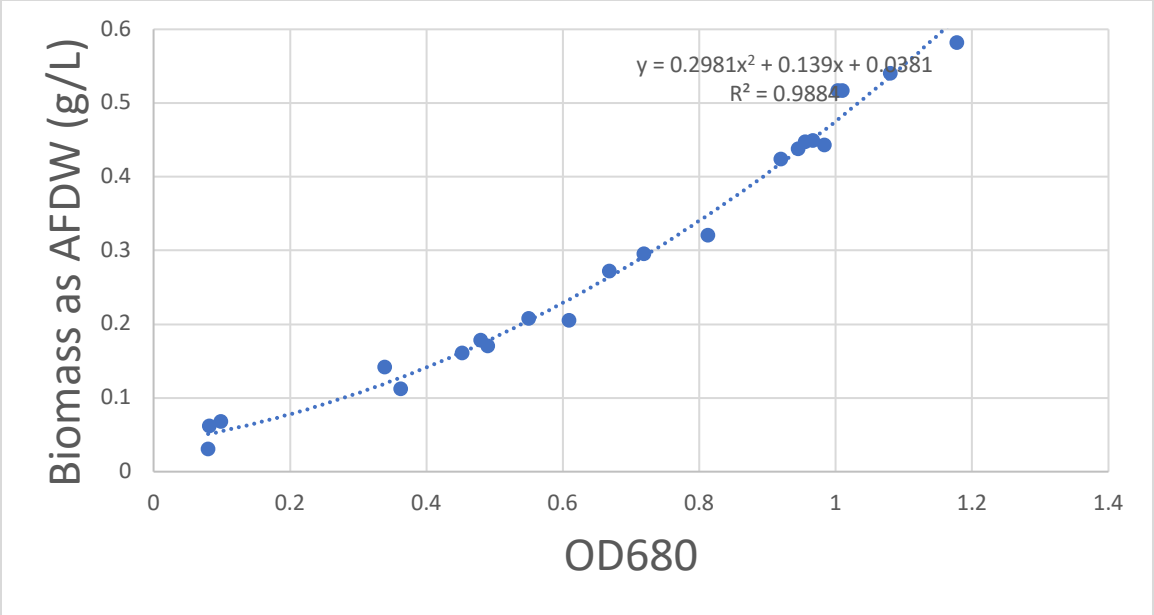

Figure 3.1. 2: Polyculture flask experiment (P3) ash free dry weight vs. OD680.

second order trend line included.

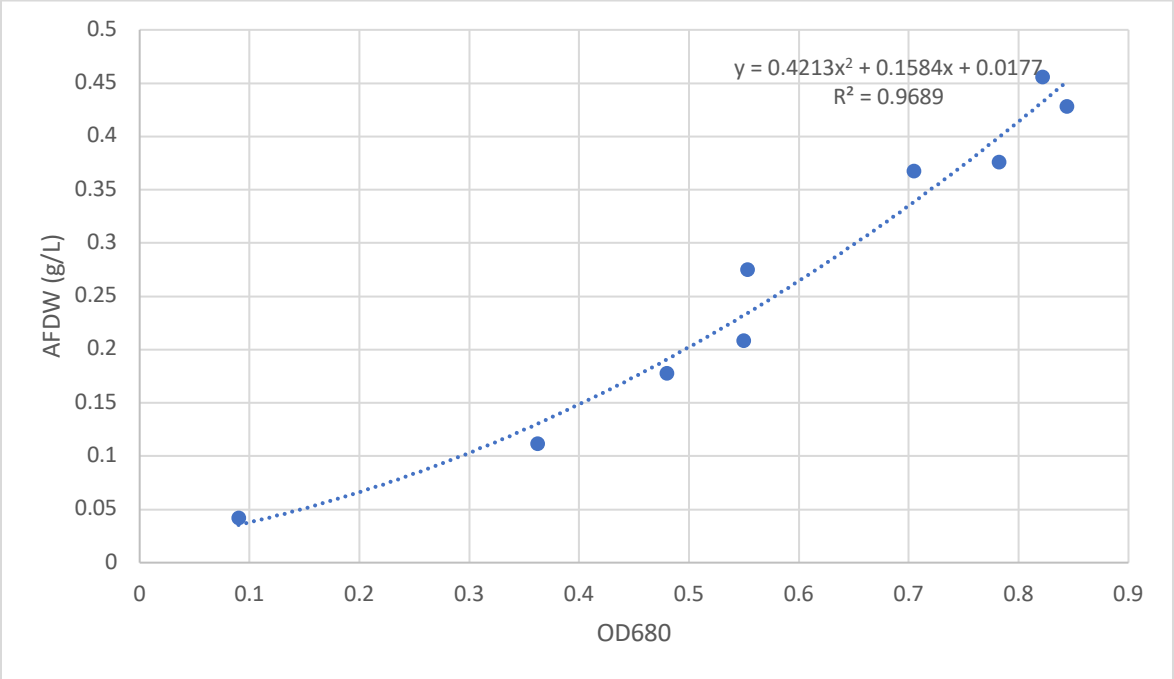

Figure 3.1. 3: Polyculture flask experiment (P3) high phosphate (8 mg PO_4-P/L) condition ash free dry weight vs. OD680.

Second order trend line included.

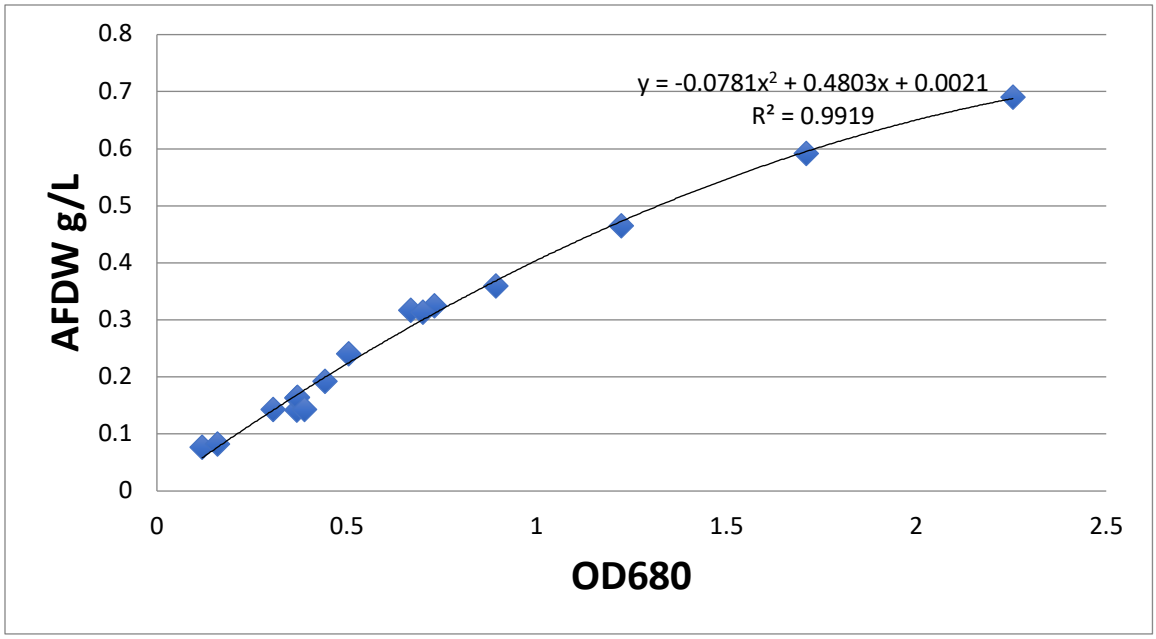

Figure 3.1. 4: Polyculture tubular photobioreactor experiment (T1) ash free dry weight vs. OD680.

Second order trend line included.

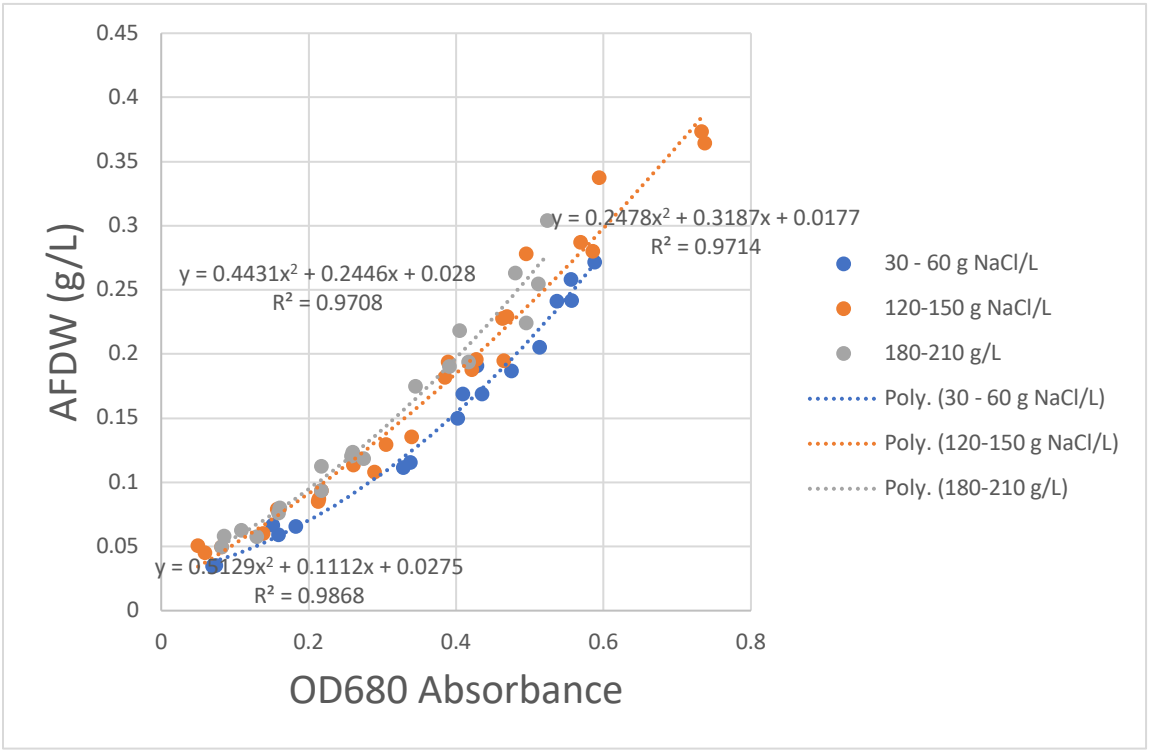

Figure 3.1. 5: Calibration curves for *D. tertiolecta* flask experiments (D1, D2, & D3).

Separate 2nd order polynomial fits were made based on salinity.

3.2: Microscopic and Illumina Sequencing identification of the Polyculture

Microscopy of the Polyculture identified at least two distinct microalgae forms. One form was predominantly spherical ranging from 1 to 3 µm in diameter (Figure 3.2. 1, Figure 3.2. 2, Figure 3.2. 3, & Figure 3.2. 4). Higher magnification images show distinct organelles and a nucleus indicating it to be eukaryotic. Commonly the chloroplasts appear as a crescent shape inside the cell. Light colored bodies are observed in the cytoplasm along with clear vacuoles. Usually cells are solitary but occasionally they form clusters of a few to dozens, more commonly in older nutrient deprived cultures and the high ammonium and phosphate growth conditions. This could be linked to cell stress. These algae cells are clearly eukaryotic with nuclei, chloroplasts, and large vacuoles. The other distinct algae form was typically rod to oval shaped (cocci to bacilli) (Figure 3.2. 5). These cells are approximately 1 µm wide and up to 2 µm long. The shape of the rods can vary from more pickle to oval. The majority of rod, oval cells in the culture exhibit a diplo form whether during active growth or stationary phase. They are colored commonly by a more bluish green pigment that seems confined to the cell membrane with no chloroplast bodies. While some intracellular bodies are present no clear nucleus has been observed. Light and bluish granules are commonly evident under high magnification. These phonologic characteristics suggest these cells are cyanobacteria due to the absence of a nucleus, chlorophyll confined to the outer membrane, and blue pigmentation (probably phycocyanin only synthesized by cyanobacteria (i.e. blue-green algae)).

The relative proportion of each microalgae form varies between cultures and over time. Cultures with a bluish hue have a higher proportion of the rod algae form. A wide variety of prokaryotic cells less than 0.5 µm are observed in the culture. Their morphology covers the large range seen in bacteria from cocci, bacilli, and spirilla. Algae and bacteria have been observed tethered to one another hinting at a symbiotic relationship (Figure 3.2. 5). The Polyculture is clearly a diverse community consisting of at least one eukaryotic algae species, cyanobacterium, and many non-photosynthetic prokaryotes.

The illumina 23s universal algal primer sequencing results (designed to identify both eukaryotic and prokaryotic algae) were dominated (>99%) by three different sequences (Figure 3.2. 8). *Cyanobacterium aponinum* was the most common OTU at 63.2%. The second most numerous hit is an uncultured clone. The paper referencing this clone did not link it to a species, but stated it was found with cyanobacteria in thermal spring in France. The third most common hit was a eukaryotic green algae *Parachlorella kessleri*. A large variety of other algae species made up the less than 1%. These probably represent contamination from the diverse algae grown at Santa Fe Community College. For the purposes of describing the algal make-up of the Polyculture, spherical eukaryotic algae will be identified as *P. kessleri*. Diplo, rod, oval, and pickle shaped cyanobacteria will be named *C. aponinum*. These are the two categories of algae morphology observed in the Polyculture throughout this study.

A similar cell shape for C. aponinum has been reported to that of the diplo rods and ovals observed in the Polyculture. Microscope images from Moro et al. (2007)

clearly display rod-shaped diplo cells, which are similar to this study (Figure 3.2. 9). *C. aponinum* was originally isolated from thermal springs in Italy and is able to grow in fresh and salt water at temperatures up to 45º C (Moro et al., 2007). It was also found at a water treatment plant for PW in Oman and shown to have a constant growth rate up to 70 g NaCl/L (Winckelmann et al., 2015). Ertugul and Donmez (2011) suggested *C. aponinum* is a strong candidate for biofuel, due to its measure lipid content from 25-45% with a high proportion of saturated fatty acids. The *C. aponinum* cells in the Polyculture may be of a different genetic variety than those so far studied, thus having different attributes. The uncultured clone was sequenced in a sample of a cyanobacterial mat in and has not been unidentified (Milferstedt et al., 2017). *C. aponinum's* reported tolerance to a range of salinities, high temperatures, high growth rate, oil content, and growth in PW recommend it as a study subject for this study.

Parachlorella kessleri is closely related to the common and well-studied green algae genus *Chlorella*. *P. kessleri* has a spherical morphology with a cell size similar to those found in the polyculture (Figure 3.2. 10). *Parachlorella* is commonly viewed as a marine genus with a high growth rate (Přibyl, et al., 2012). Li et al., (2012) identified it as a highly efficient lipid producer that could be induced to have a content of 60% under a variety of stress conditions. It is also reported tolerant to high temperatures (Fernandes et al., 2013). The closely related species *Chlorella kessleri* can grow heterotrophically and mixotrophically on glucose and glycerol (Wang et al., 2012). All these characteristics could make it a promising candidate for biofuel production in saline PW.

The 16s universal prokaryotic primer identified a number of halotolerant bacteria species and *Cyanobacterium aponinum* (Figure 3.2. 11). *Salinarimonas rosea* was the top hit which according to Wikipedia was isolated first from a salt mine in China (Liu et al., 2010). *Cyanobacterium aponinum* turned up as the second most abundant OTU which agrees with the algal 23s primer results. *Rhodobacteraceae bacterium* was the third most common, which is known as a chemoorganotroph and photoheterotroph (Simon et al., 2017). The Fourth *Oceanicola sp*. strain B17 was identified in a marine environment with phytoplankton (Gutierrez et al., 2017). *Bacteroidetes bacterium* ends the top five which inhabits evaporative basins (Dorador et al., 2009). Together these five constitute over 85% of the 16s hits while the remaining 15% is made up of over 20 other species. Overall, the Polyculture contains a diverse collection of prokaryotes.

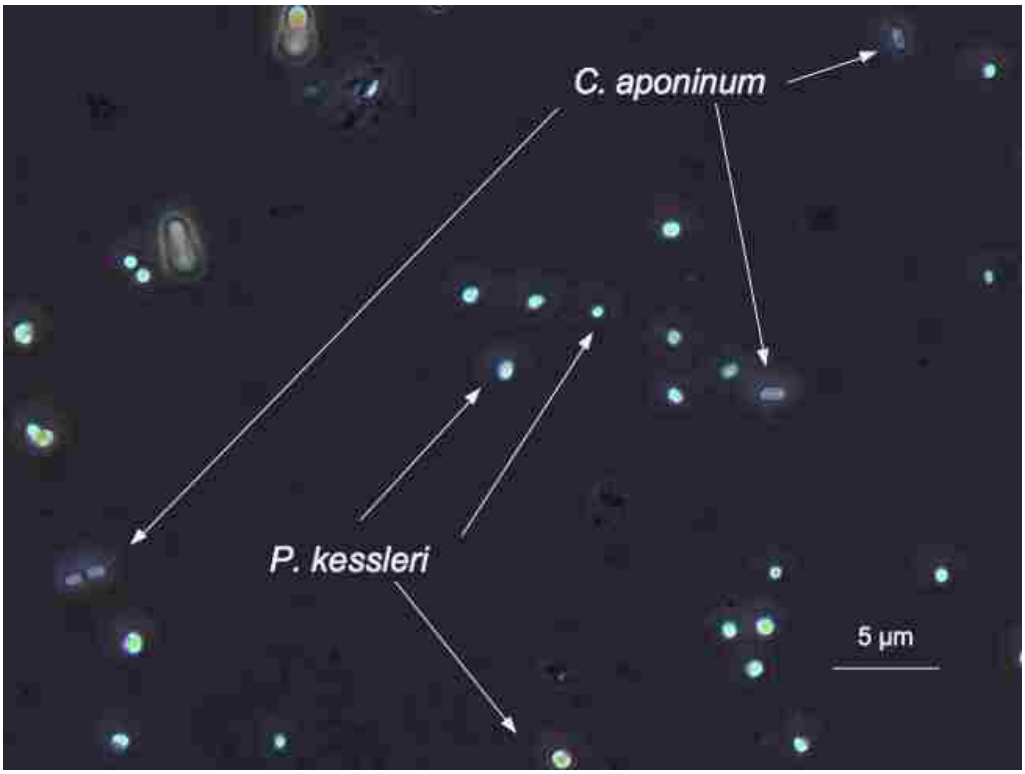

Figure 3.2. 1: Polyculture under phase contrast at 800X.

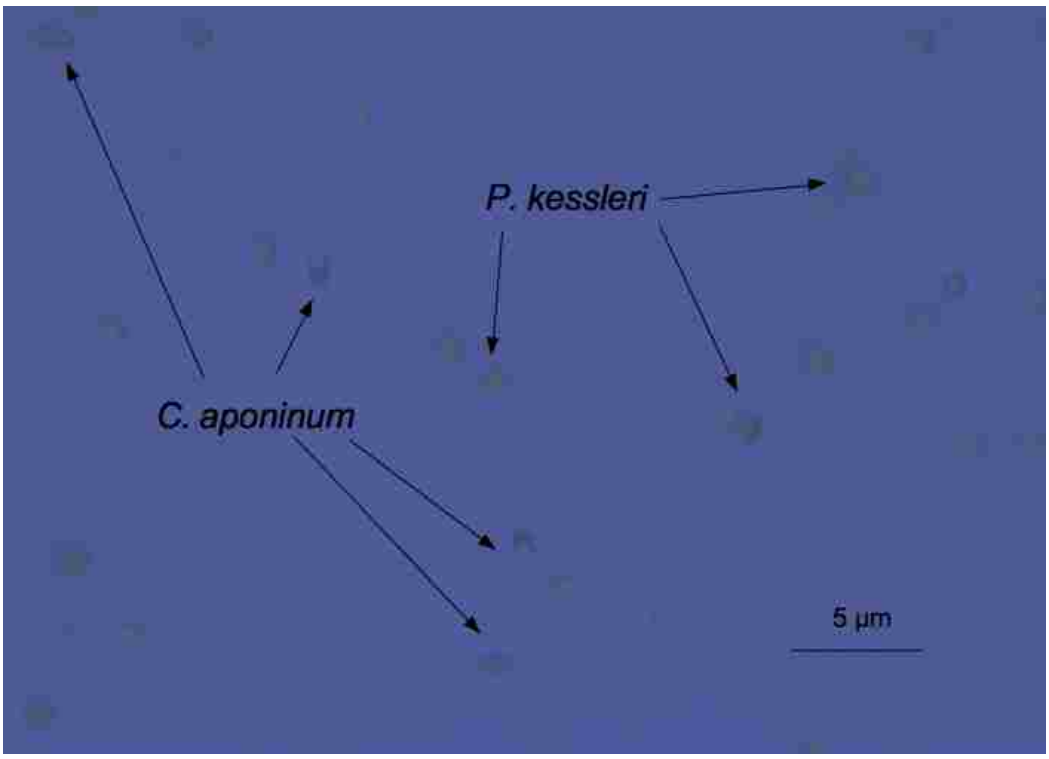

Figure 3.2. 2: Polyculture with no phase contrast (800X).

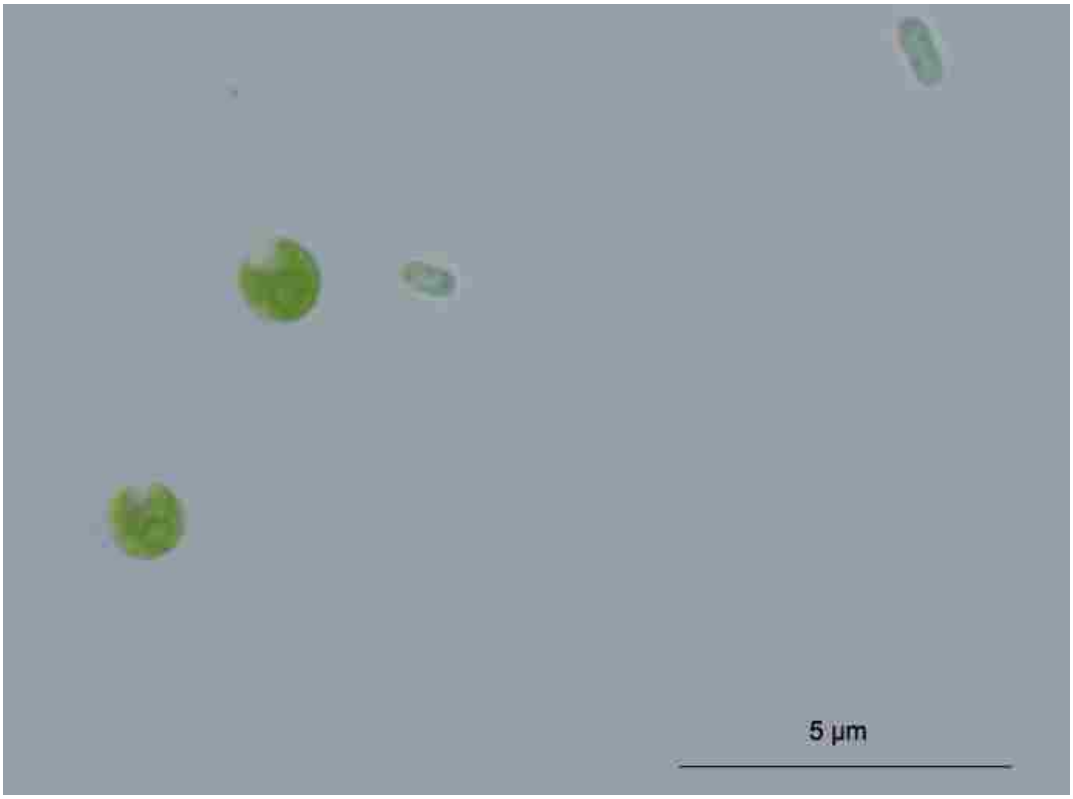

Figure 3.2. 3: Polyculture Spheres and rod-shaped algae with no contrast (2000X).

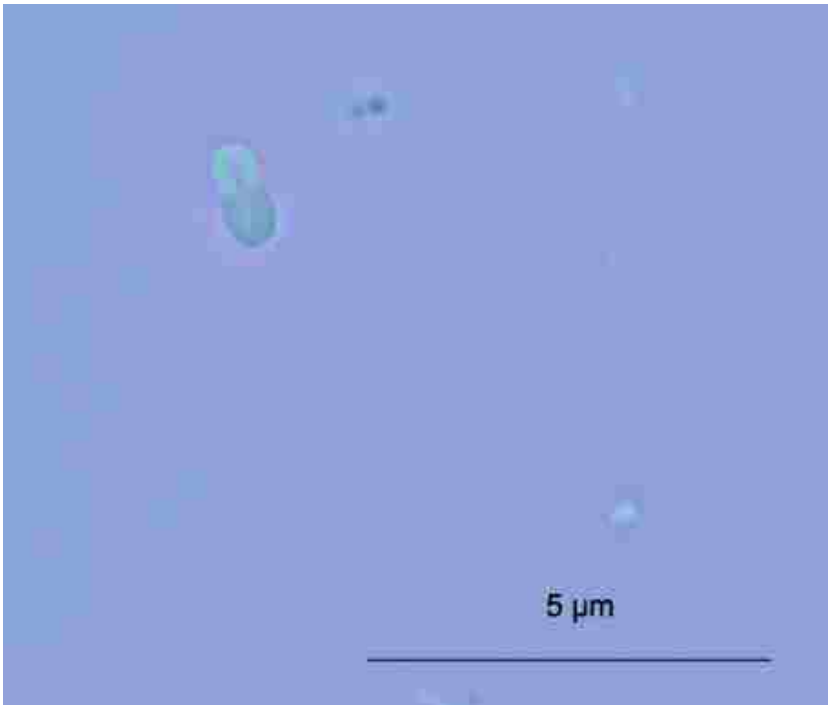

Figure 3.2. 4: Polyculture double rod cyanobacteria (2000X).

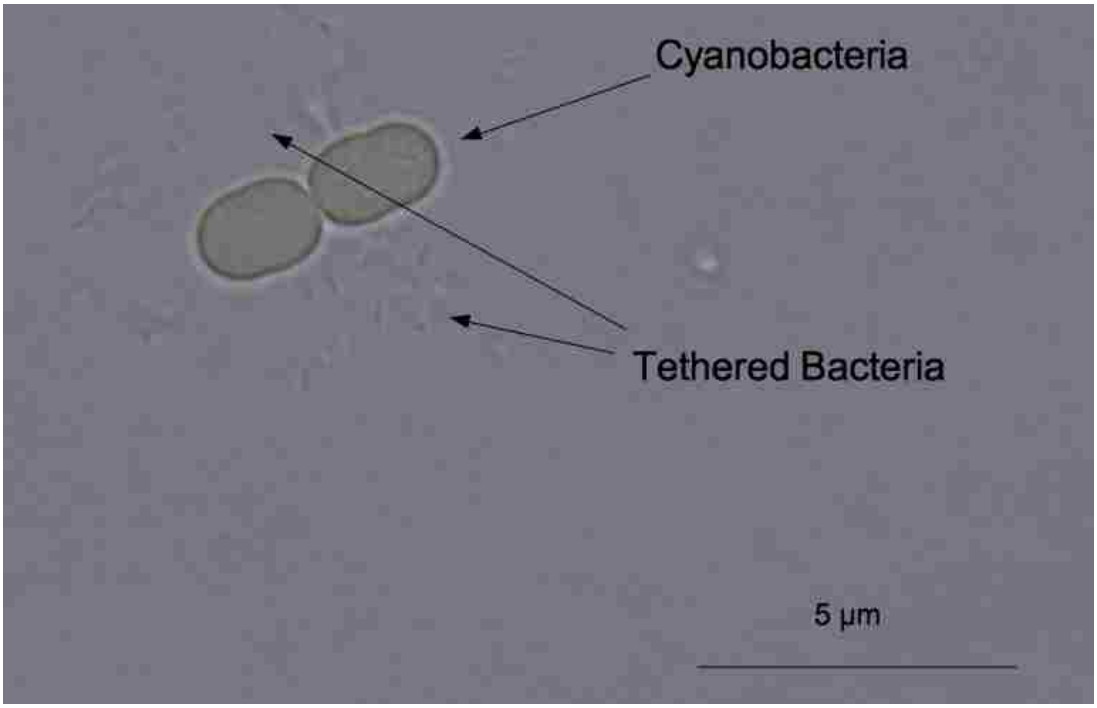

Figure 3.2. 5: Polyculture image of a diplo cyanobacteria tethered to bacteria in a possible symbiotic relationship (2000X).

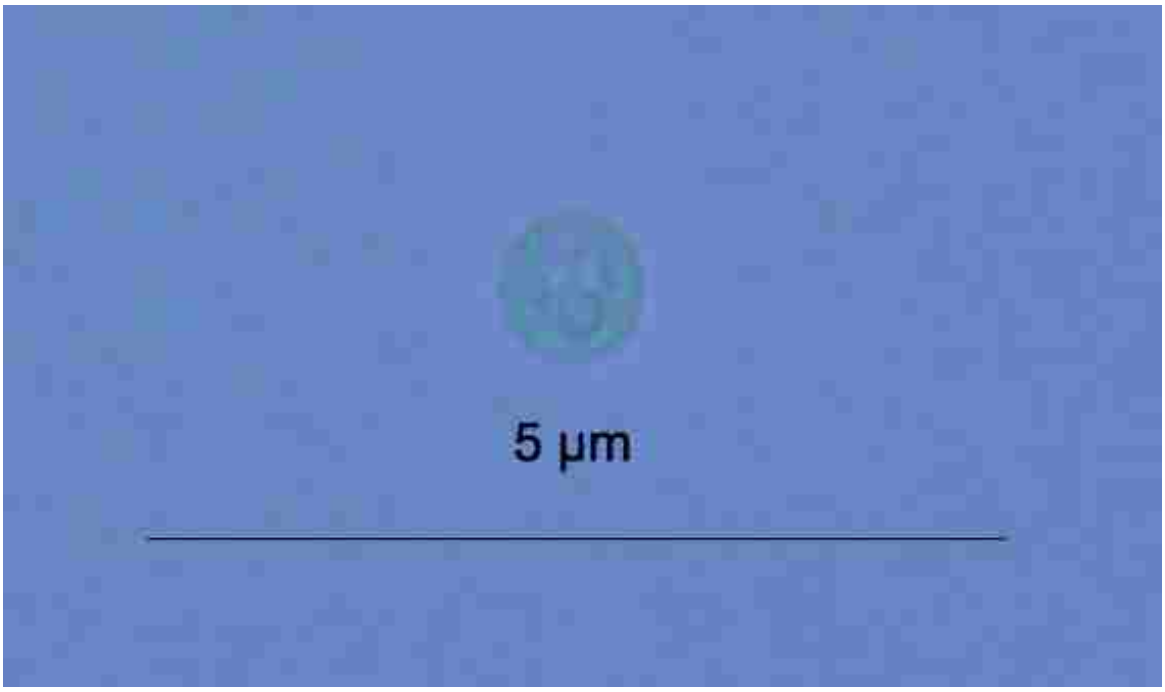

Figure 3.2. 6: Polyculture Spherical algal cell identified as *P. kessleri* (2000X).

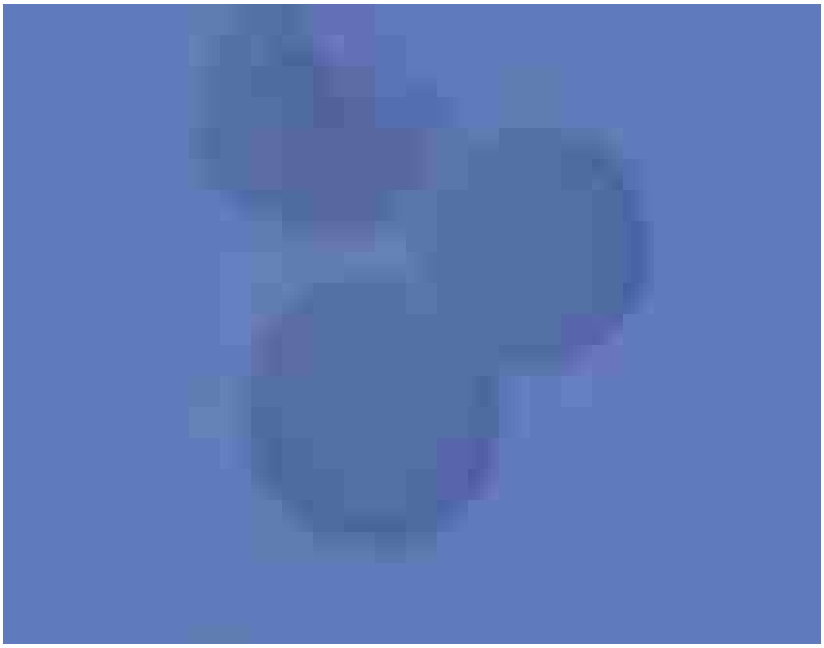
Figure 3.2. 7: Polyculture Diplo-ovals cell(s) identified as *C. aponinum* (2000X).

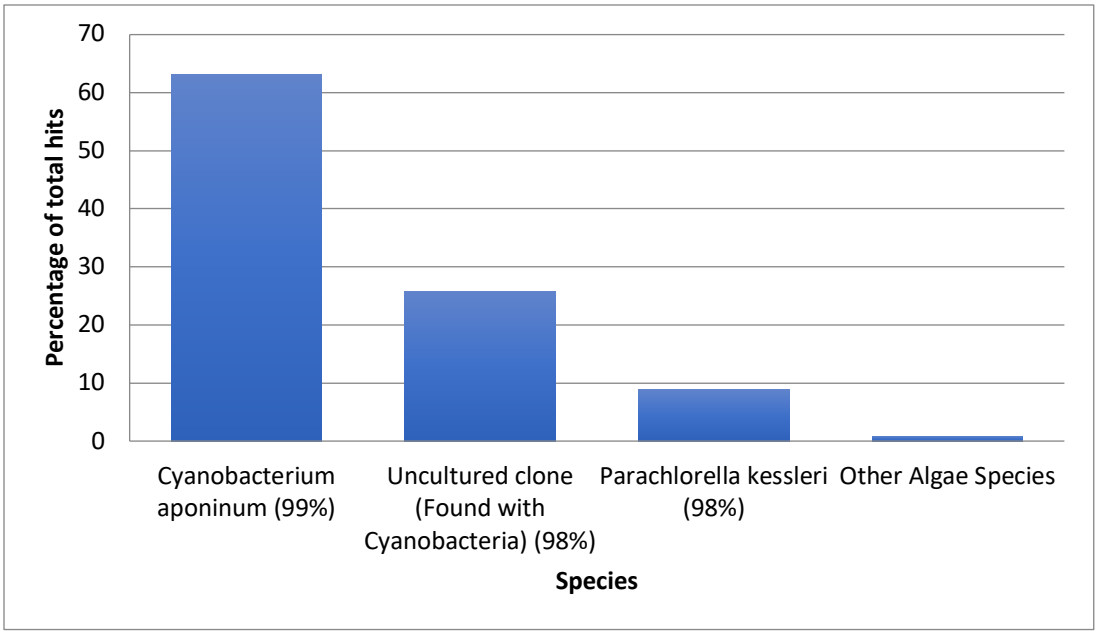

Figure 3.2. 8: Polyculture Illumina DNA sequencing results. A 23s primer targeting chloroplasts and cyanobacteria was used. Percent homology in parentheses.

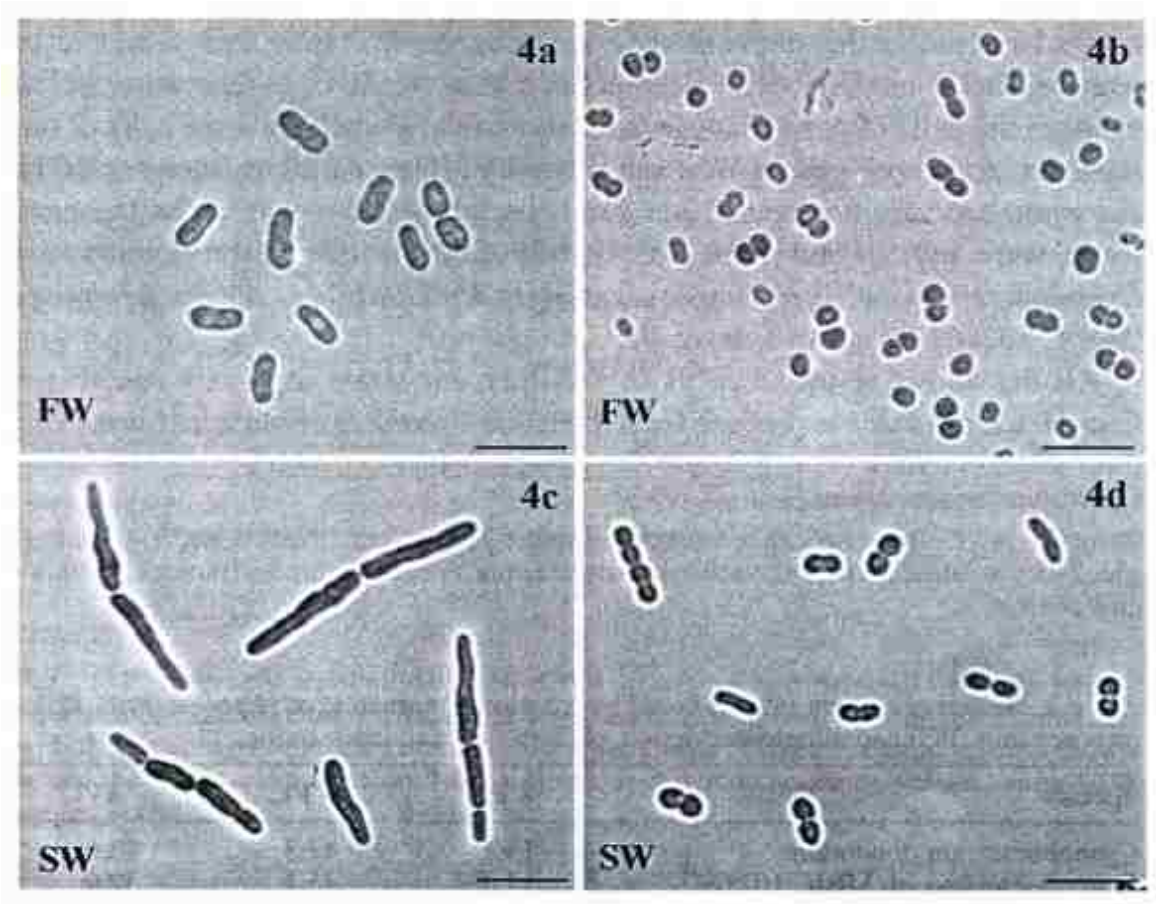

Figure 3.2. 9: Images of *Cyanobacterium* aponinum (4a and 4c) and *Cyanobacterium sp.* from Moro et al. (2007).

Figure 3.2. 10: Microscope images of *Parachlorella kessleri*.

Source: https://botany.natur.cuni.cz/algo/images/CAUP/H1901_1.JPG

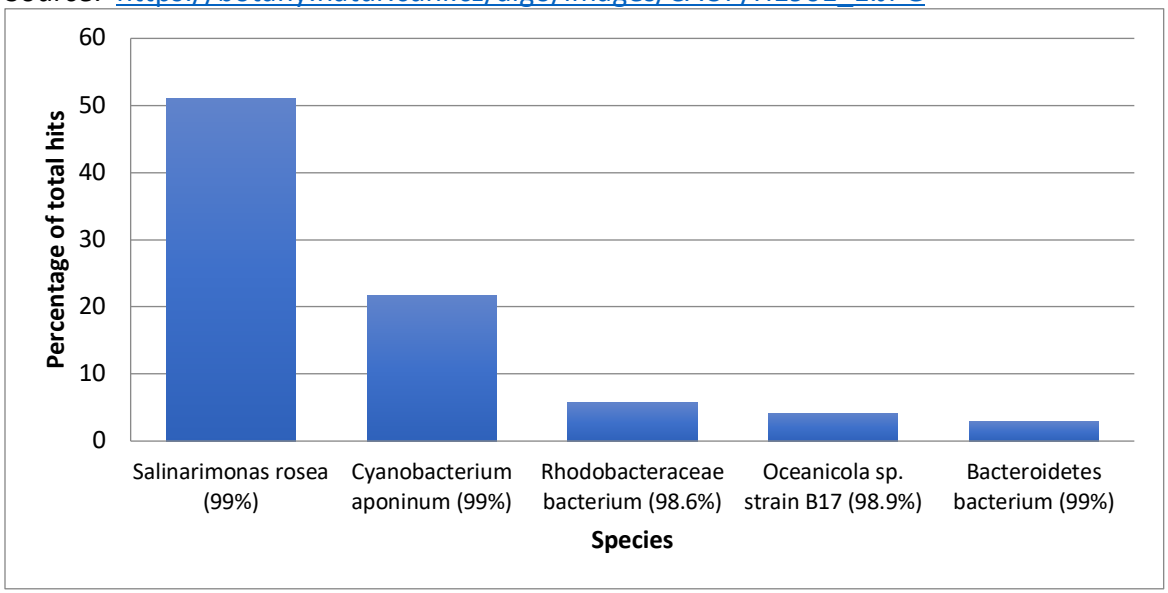

Figure 3.2. 11: Polyculture Illumina DNA sequencing results using a 16s primer. This primer targets prokaryotes. Percent homology in parentheses.

3.3: Polyculture Flask Experiments (#1 & #2) in PW from brackish to hypersaline conditions

3.3.1: Polyculture growth varying salinity in PW (Experiment P1 & P2)

The polyculture exhibited robust growth up to 60 g TDS/L in the PW medium. Experiments P1 and P2 were run to determine the effects of salinity on the Polyculture growth, with P1 run in the range 15 to 160 g TDs/L and P2 run in the range 60 to 120 g TDS/L (to supplement the P1 results). Growth rates were similar in the 15, 30, and 60 g TDS/L conditions, but at 75 g/L and above growth was inhibited (Figure 3.3. 1). Growth rates were greatest between day 2 and 7 for salinities 60 g TDs/L and lower (exponential growth phase). At around day 7 conditions the rate of increase slowed but did not cease for the duration of the experiment (linear growth phase). Final biomass concentrations ranged from 0.41-0.48 g AFDW/L. Growth in the 75 g TDS/L condition was clearly reduced compared to 15, 30, and 60 with a lower rate and final biomass concentration (about 0.3 g AFDW/L). The 90 g TDS/L condition exhibited even lower growth rates with total biomass density not even reaching 0.2 g AFDW/L. In the 105 g TDS/L condition growth was observed to be almost negligible, while in the 120 and 150 conditions the biomass density decreased slightly. Overall variation in growth within the triplicate set is small with a standard deviation of 0.02 g AFDW/L being the greatest.

Although cell counting was not performed, visual inspection using microscopy did not detect obvious changes in the algal forms present at different salinities. Figure 3.3. 2 for the conditions 15, 30, and 60 shows a biomass containing predominantly spherical cells (*Parachlorella kessleri*) and diplo rods (*Cyanobacterium aponinum*). This distribution did not change significantly over the course of the experiment (images not shown). At the higher salinities of 75 and 90 g TDS/L *P. kessleri* was more common and cyanobacteria cells displayed an oval morphology. At the highest salinities (where

growth did not occur) dormant spherical algae forms can be seen in the media that appear to have a smaller volume than at lower salt concentrations, which would be consistent with dehydration under saline conditions. In all cultures, numerous smaller prokaryotes were observed whose abundance correlates with the algae biomass.

The results suggest the upper limit of salinity for optimal growth appears to be at or just above 60 g TDS/L. Growth rates were plotted vs. TDS from day 2 to 7 (the period of highest growth after the lag phase) (Figure 3.3. 3). The growth rates at 15, 30 and 60 g TDS/L were between 45 and 50 mg AFDW/L/D. The 60 g TDS/L condition had a slightly lower growth rate which was within the standard deviation of the measurements. At greater salinity growth was much reduced. In the 75 g TDS/L condition biomass was added at about 1/3 the rate of the 60. At 90 g TDS/L the growth rate fell to around 12% of the 60. At 105 g TDS/L growth was negligible and completely absent at 120 and 150.

TDS effects on nutrient uptake were consistent with effects on algal growth. In the 15, 30, and 60 g TDS/L conditions nitrate was rapidly removed from solution once strong growth commenced after Day 2 (Figure 3.3. 4). By day 5 nitrate was absent in the media. In the 75 and 90 g TDS/L conditions nitrate uptake was slower with complete removal occurring by day 7 and 9 respectively. In the conditions that did not display significant algal growth (105, 120, and 150 g TDS/L), nitrate was also taken up possibly, due to denitrification by bacteria. Phosphate uptake was similar to nitrate removal with the rate being linked to an increase in biomass density (Figure 3.3. 5). In the 15, 30 and 60 g TDS/L flasks, phosphate levels quickly declined and was absent from the media by day 5. Lesser uptake occurred in the 75 and 90 g TDS/L conditions. At the highest

salinities phosphate levels did not consistently drop as did nitrate. This could be due to the fact that phosphate is not used as a terminal electron acceptor as is nitrate in prokaryotic metabolism. This suggested that bacteria in the 120 and 150 g TDs/L media may have been engaging in denitrification. The Polyculture was capable of growth and nutrient removal of at least several mg NO_3-N/L/D and 1 mg PO_4-P/L/D in hypersaline conditions up to 60 g TDS/L.

Biomass growth after the depletion of the crucial nutrients nitrate and phosphate suggests luxury uptake (storage for later growth) and nitrification may have occurred. Both cyanobacteria and photoheterotrophs can fix nitrogen (Bentzon-Tilia et al., 2014). Nutrient depletion in the media did not abruptly terminate the early exponential growth phase. Figure 3.3. 6 shows AFDW and nitrate concentrations for the 15, 30, and 60 g TDs/L conditions. While nitrate was removed from the media on day 5 high growth continued until day 7. A similar pattern is seen with phosphate. Figure 3.3. 7 shows the AFDW and phosphate measurements. Phosphate is largely absent in the media on day 5 while the growth rate did not slow until around day 7. The fact that biomass increases for the length of the experiment hints that perhaps some amount of excess nitrogen is being introduced through fixation. Nutrient recycling may have also been occurring in the diverse culture and individual cells may be adding biomass based on stored nutrients and carbon fixation. It might be possible that a diverse mixed culture might produce higher growth than a pure algae culture through recycling and fixation of nitrogen. This likely could result in the extended linear phase of growth seen in the biomass measurements, which may be relevant for biofuel production.

Algal culture growth was simultaneous to increased pH values of the media. Figure 3.3. 8 shows pH measurements made on samples taken during the light phase of the diurnal cycle. pH increases from that of seawater (around 8.1) to between 9.5 and 10 by day 5 in the highest growth conditions 15, 30, and 60 g TDS/L. This pH was maintained until around day 10, after which there is a downward trend until the end of the experiment. In the higher salinity cultures that displayed growth (75 and 90 g TDS/L) a lesser increase in pH was observed. In the highest salinities tested there was only a slight trend toward more alkaline conditions. Photosynthetic activity increases pH due to the uptake of CO_2 or bicarbonate [Fabregas et al. (1994) & Chi et al. (2011)]. Compared to the F/2 media (30 g TDS/L and 13 mg NO_3-N) the higher growth salinities (15, 30, 60 g TDS/L) exhibited a higher pH increase (Figure 3.3. 9). This is likely a result of the increased amount of dissolved carbonate in the PW media. Uptake of bicarbonate (to H_2CO_3 and then CO_2 gas at photosynthetic centers) consumes aqueous protons thus increasing pH. The Polyculture in the PW media had a higher growth than the commercial F/2 media rate indicating greater uptake of inorganic carbon.

While growth was negligible at 120 g TDS/L, algae survived for three weeks at this salinity and were able to grow again after dilution to 60 g TDS/L. The triplicate 120 g TDS/L flasks displayed consistently decreasing biomass, with average OD680 decreasing from day 0 (0.067) to Day 19 (0.024). One flask was diluted to 60 g TDS/L on day 19 and OD680 increased to 0.190 two days later (Figure 3.3. 10). Dilution of the 150 g TDS/L condition did not restore growth. Prior to dilution of this flask, algae cells were viewed under a microscope with a decreased volume compared to the growing cultures.

After dilution, spherical algae cells became more numerous and green in the culture (Figure 3.3. 11). This result is consistent with the spherical algae cells (probably *P. kessleri*) being dormant but not dead at these high salinities. This observation might have relevance for biofuel production using PW as a medium. Spikes in salinity at or below 120 g TDS/L would not immediately destroy the culture and could possibly be used to kill undesirable microorganisms.

Overall, the results of the flask experiments confirmed the Polyculture as a potential candidate for biofuel production in a produced water media. The range of salinity from 15 – 60 g TDS/L covers the values of a large amount of PW. Mixing PW with low and high salinity could create an algae media that falls in this range. Growth does not decrease significantly until the 75 g TDS/L level. This is higher than the established threshold of 35 g salt/L stated for a commonly research species, *Nannochloropsis* 1776 (Graham et al., 2017). The potentially toxic metals copper and arsenic did not inhibit growth relative to the F/2 commercial media. The nutrient concentration of nitrate tested could be supplied from agricultural municipal waste potentially at reducing production costs. The phosphate concentration tested may already be present in many PW sources [Fakhru'l-Razi et al. (2010), Riley et al. (2016), Godfrey (2012), & Pendashteh et al. (2012)]. Algal growth occurred in alkaline conditions up to a pH of 10.2. Pilot scale testing of the Polyculture using a PW media in HRAPs would further define its potential.

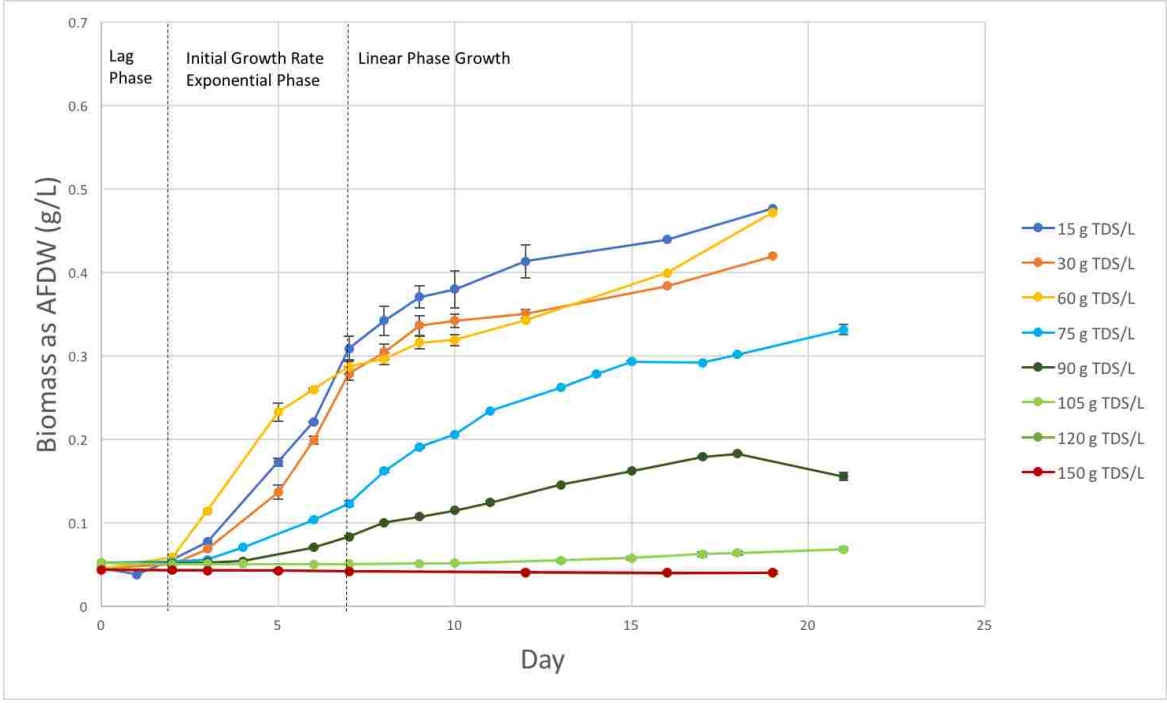

Figure 3.3. 1: Polyculture biomass measurements in flask experiments P1 & P2 (PW) at different salinities.

Initial nutrient levels were 13 mg NO_3-N/L and 1.7 mg PO_4-P/L.

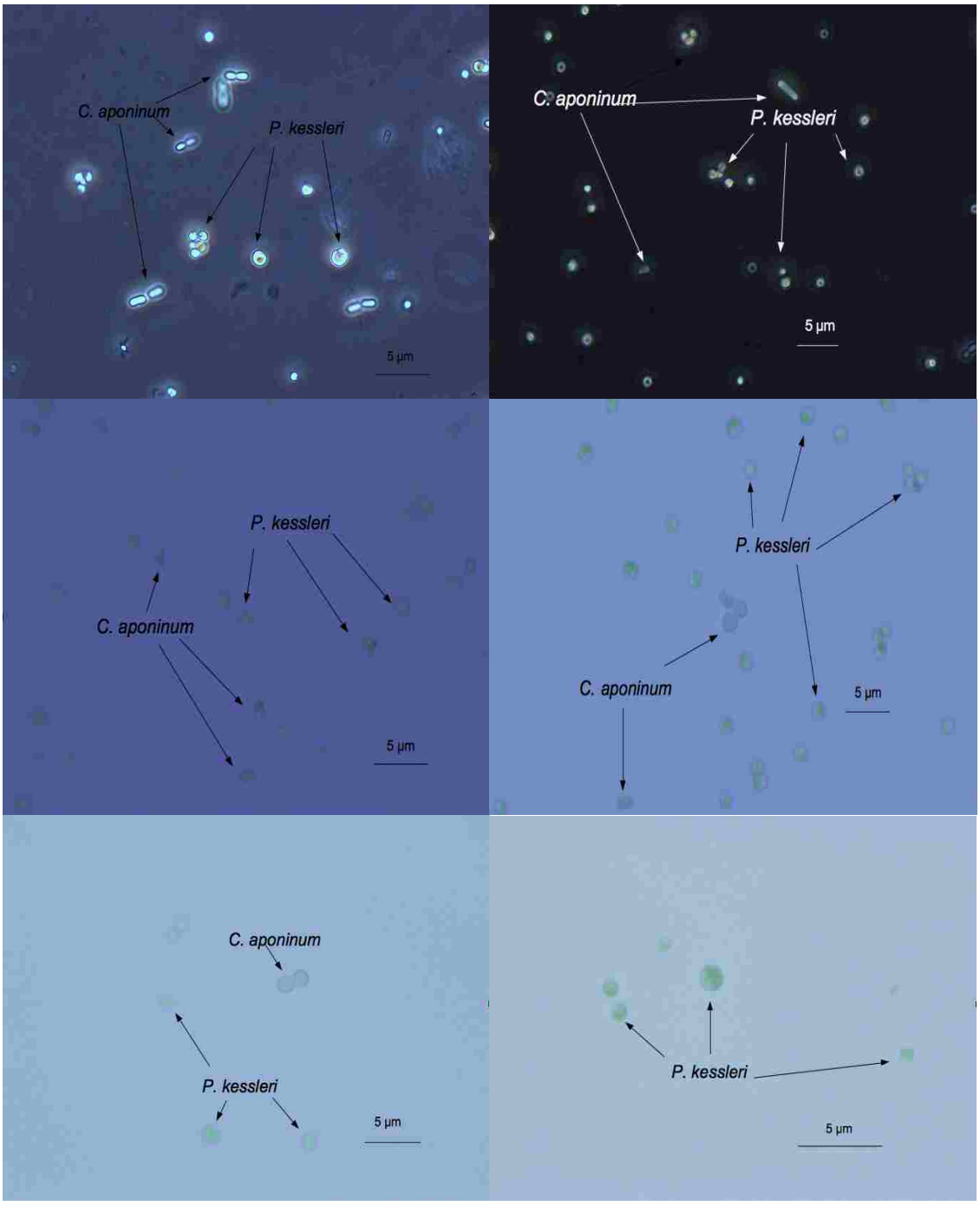

Figure 3.3. 2: Microscopic images of the Polyculture at 400X.

Images taken at the following salinities: Top Left (15 g TDS/L), Top Right (30 g TDS/L), Middle Left (60 g TDS/L), Middle Right (75 g TDS/L), Lower Left (90 g TDS/L), and Lower Right (105 g TDs/L). Cell labels tentative identifications.

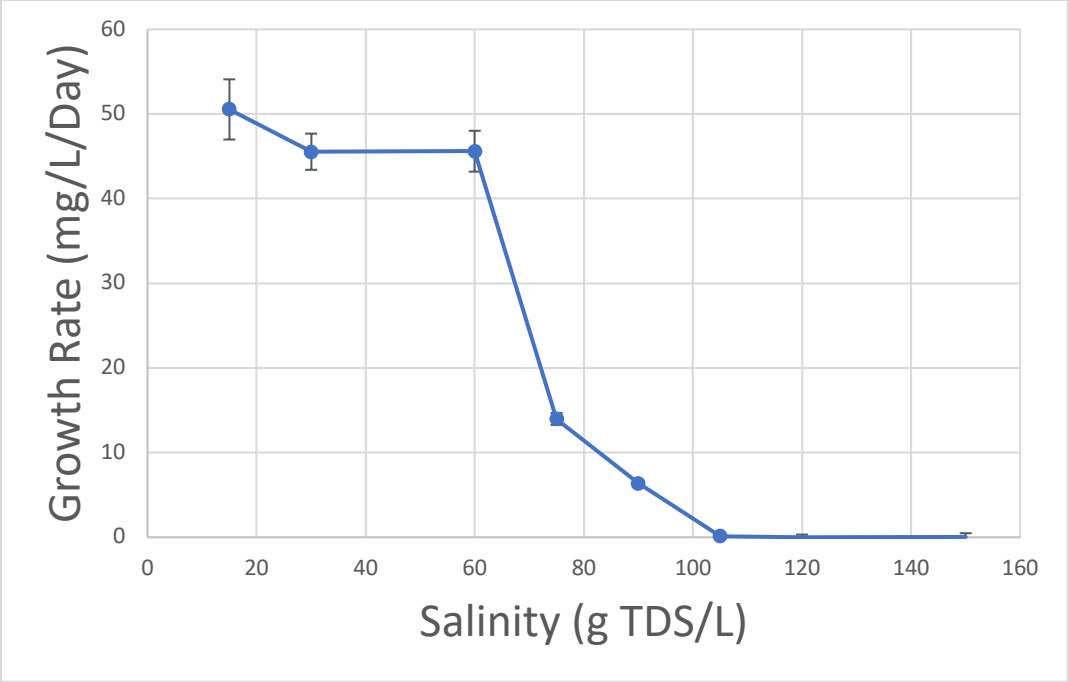

Figure 3.3. 3: Average Growth rate for the Polyculture in PW flask Experiments (#1 & #2).

Measured between days 2 and 7 for the Polyculture in PW flask Experiments.

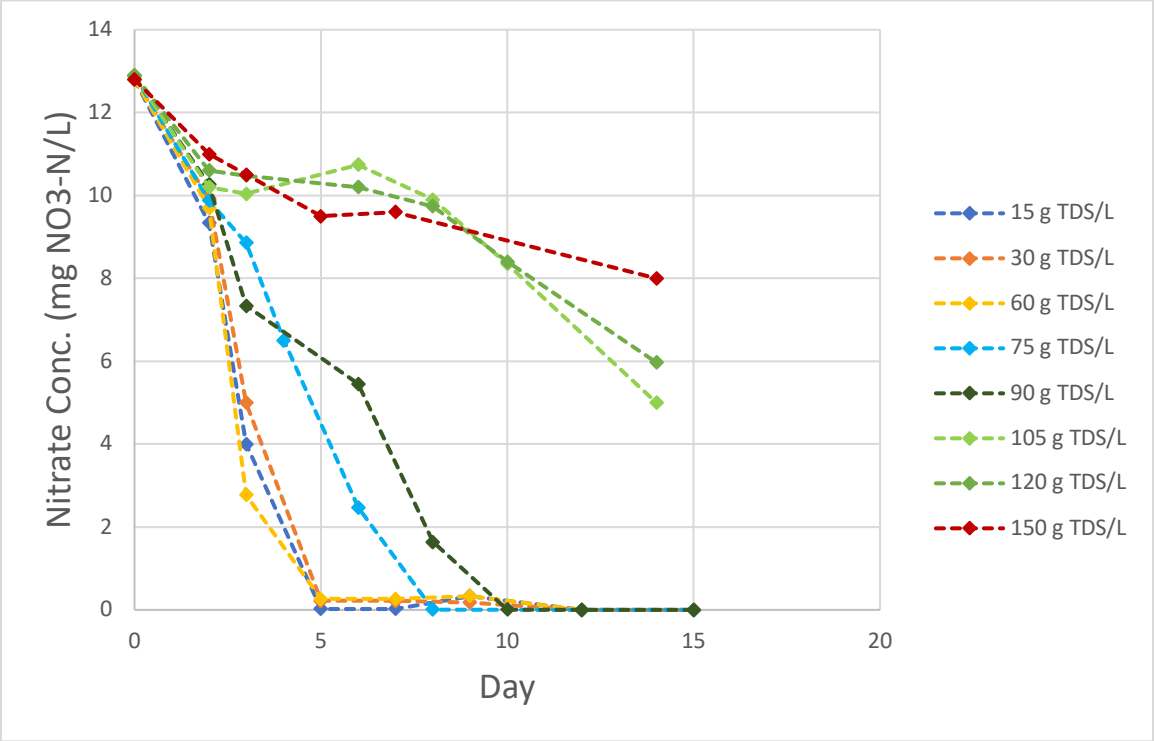

Figure 3.3. 4: Polyculture PW media flask experiment (#1 & #2) nitrate measurements.

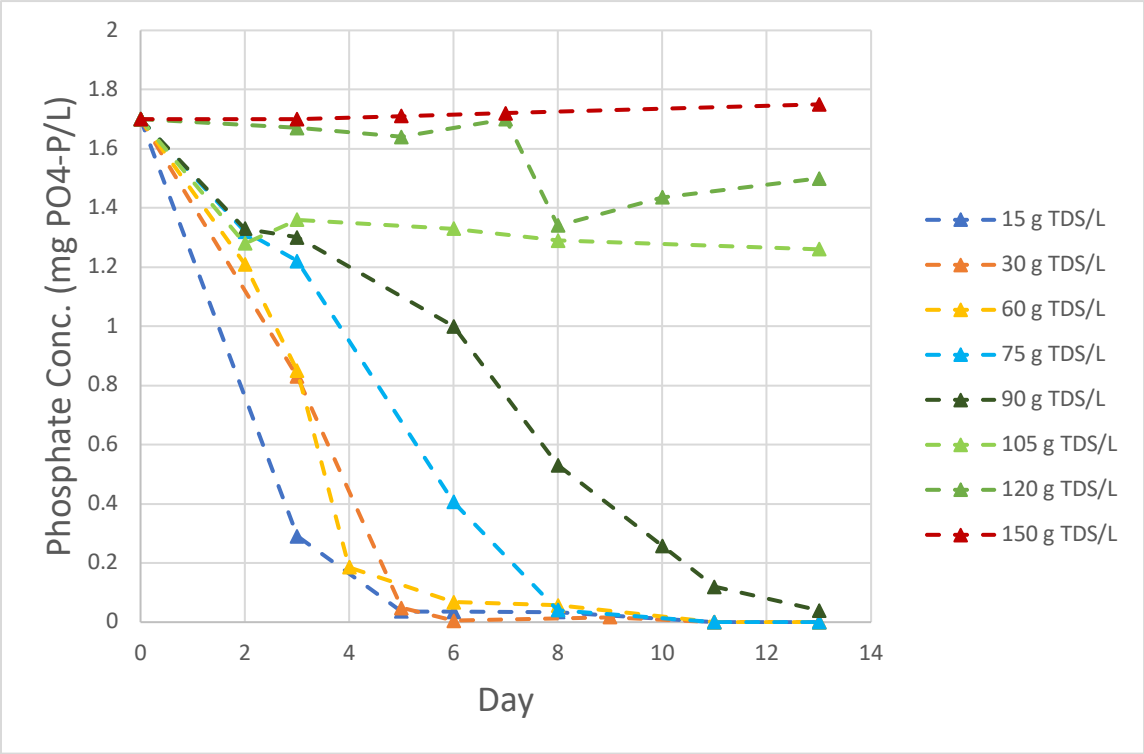

Figure 3.3. 5: Polyculture PW media flask experiment (#1 & #2) phosphate measurements.

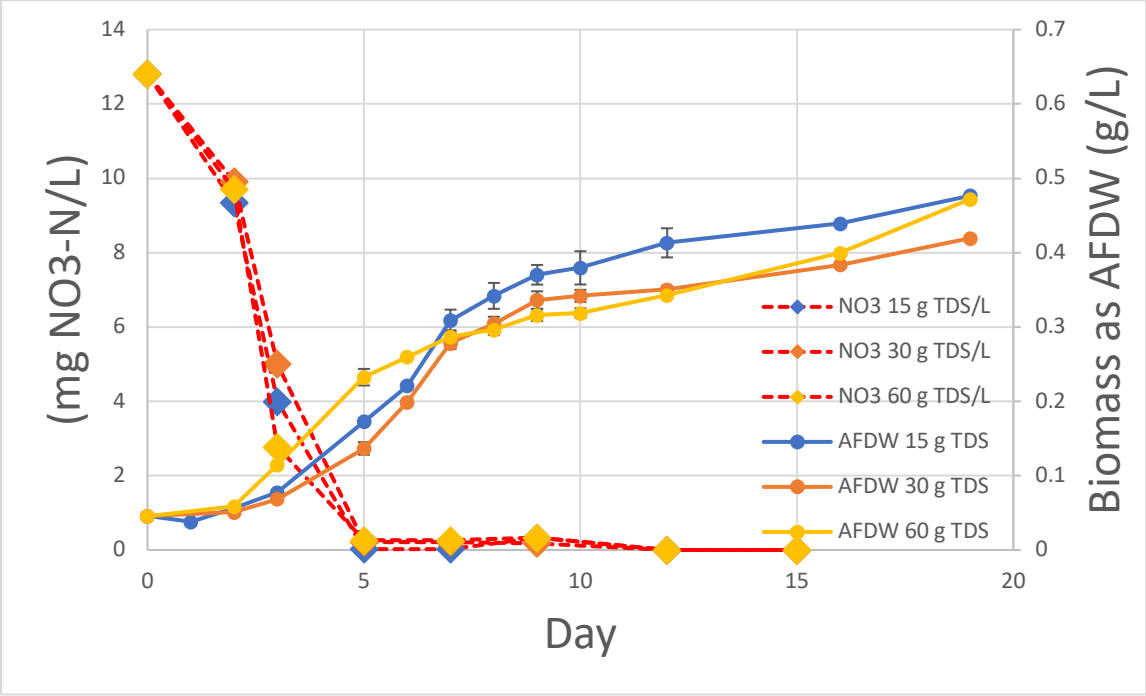

Figure 3.3. 6: Polyculture PW media flask experiment biomass and nitrate concentrations for 15, 30, and 60 g TDS/L.

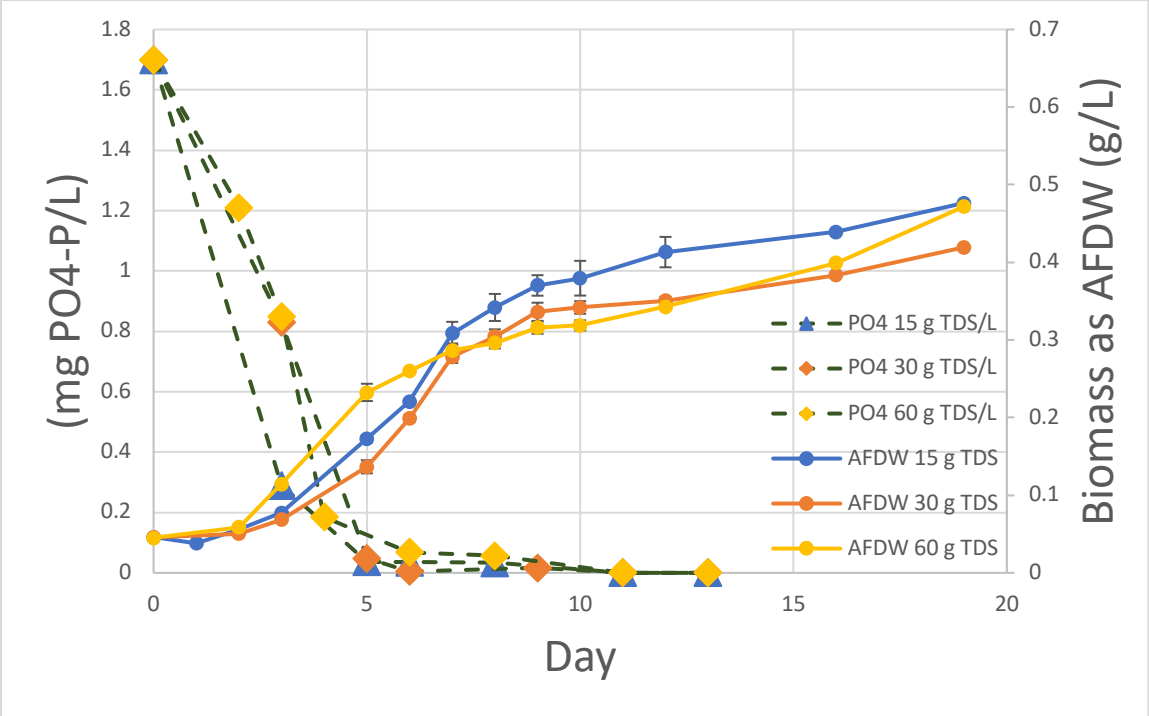

Figure 3.3. 7: Polyculture PW media flask experiment biomass and phosphate concentrations.

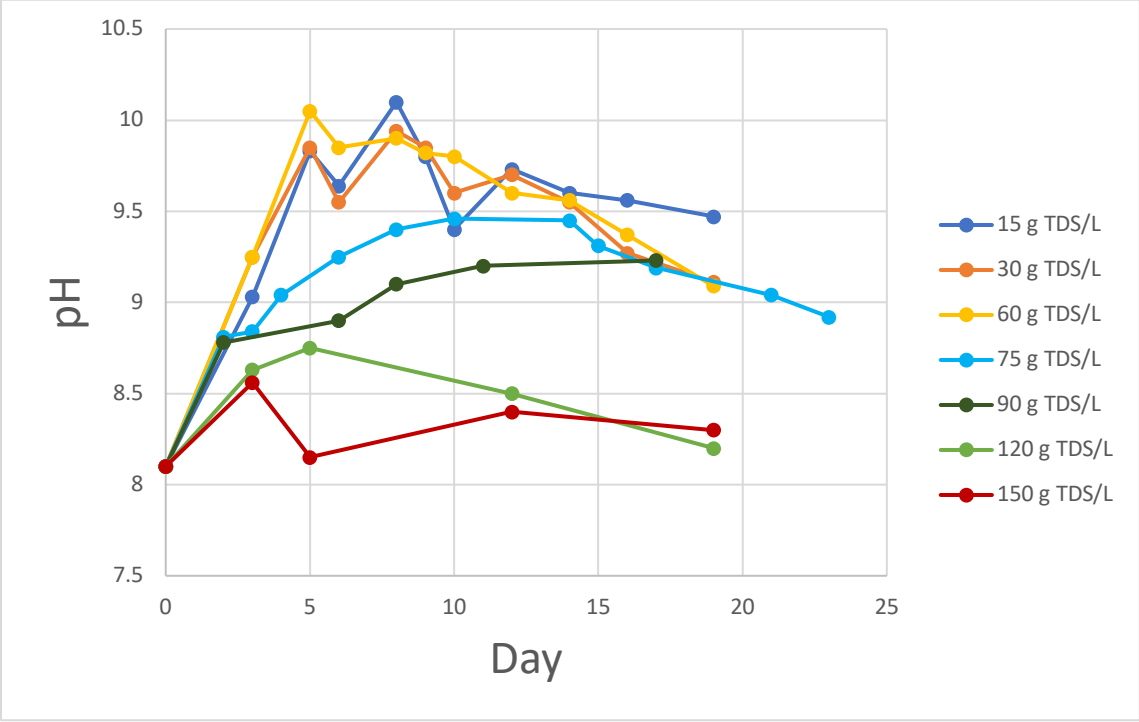

Figure 3.3. 8: Polyculture PW media flask experiment (#1 & #2) pH measurements at different salinities.

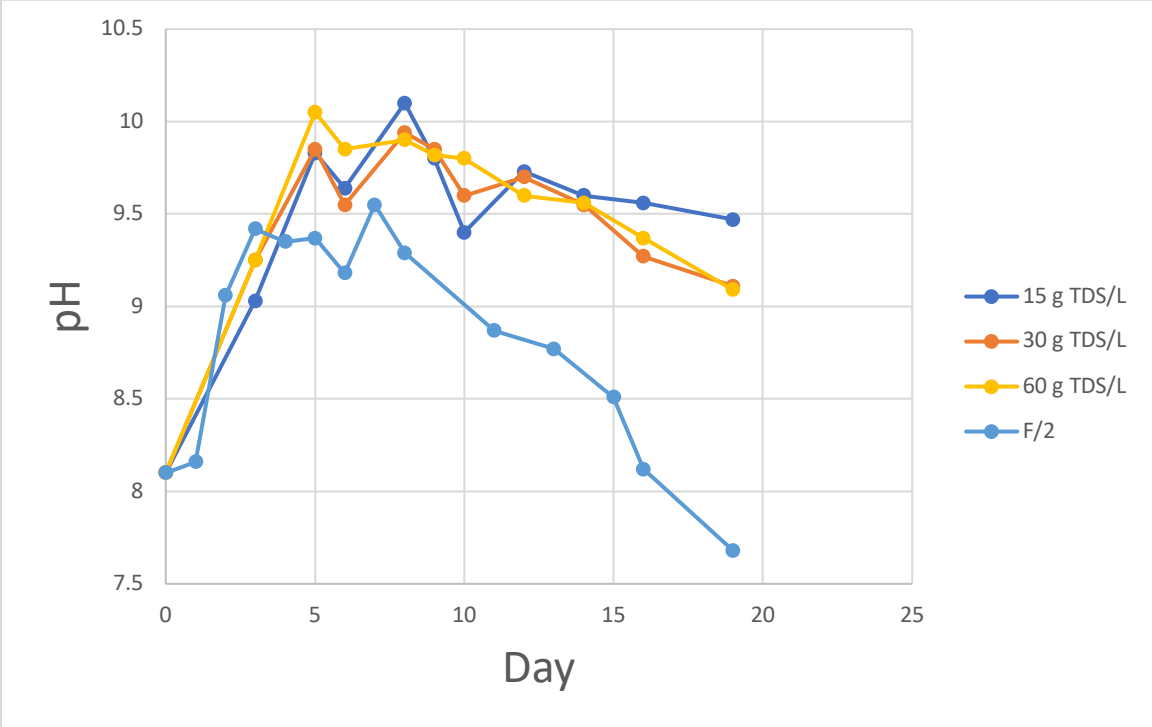

Figure 3.3. 9: Polyculture PW media flask experiment pH measurements at the highest growth salinities with the F/2 media for comparison.

Figure 3.3. 10: Higher salinity condition flasks 120 (left) and 150 (right) g TDS/L) diluted to 60 g TDS/L.

Only the 120 g TDS/L condition displayed growth.

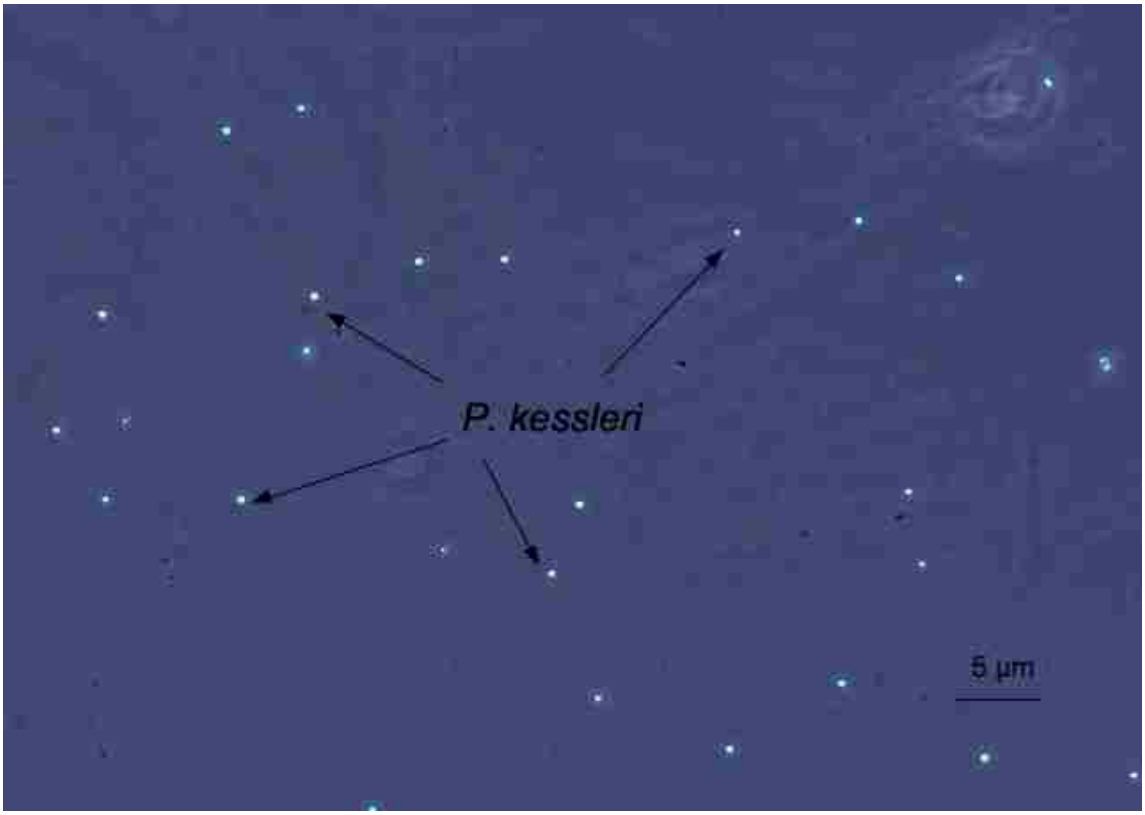

Figure 3.3. 11: Spherical green cells growing in PW media after dilution from 120 to 60 g TDS/L.

Cells are possibly *P. kessleri* (200X).

3.3.2: Polyculture Biomass Lipid Measurements in PW Media with Nitrate (Exp.: P1 & P2)

The lipid content of the Polyculture also varied with salinity. The 15 g TDS/L condition was measured to contain a lipid concentration of 0.03 to 0.06 g/L with some variation over the course of the experiment (Figure 3.3. 12). The polyculture in the 30 g TDS/L had a slightly higher lipid productivity, reaching a concentration of around 0.065 g/L by day 16. After day 8 the flasks growing at a salinity of 60 g TDS/L had a higher lipid concentration than the lower salt conditions reaching 0.123 g/L on day 16. Early in the

experiment (Days 5 and 8) the lipid fraction of the biomass for the 15, 30, and 60 g TDS/L conditions were similar, ranging from 15-18% (**Error! Reference source not found.**). After day 8 only the 60 g TDS/L condition displayed a lipid enrichment, eventually reaching over 30%. This lipid enrichment clearly occurred after nutrient depletion (NO_3 & PO_4) in the media (Figure 3.3. 13). The enhancement of oil productivity through nutrient starvation was not apparent in the 15 or 30 g TDS/L PW media. As a result, while total biomass production of the Polyculture was similar at 15, 30, and 60 g TDS/L, lipid productivity was approximately double in the 60 g TDS/L condition.

At 75 and 90 g TDS/L higher lipid concentrations were obtained over a longer time frame. The Polyculture at 75 and 90 g TDS/L displayed a similar trend to one another in lipid concentration and content (Figure 3.3. 14 & **Error! Reference source not found.**). The percent lipid content was relatively constant at 15-20% until day 28 for the 75 g TDS/L condition. The 75 g TDS/L flasks became enriched in lipids (34-38%) after day 28 resulting in a doubling in concentration from 0.068 to 0.124 g/L by day 32. At 90 g TDS/L there was a similar lipid enrichment effect where content increased from 20 to 40% between days 21 and 28. As a result, lipid concentration doubled from 0.034 to 0.069 g/L. In both cases enrichment came after nutrient depletion, albeit offset by over a week (Figure 3.3. 15). It seems likely that nutrient starved algae are increasing the lipid proportion of their biomass.

The Polyculture lipid production rate at 60 g TDS/L was much higher than other salinities tested (Figure 3.3. 16). This is due to the higher growth rate and lipid

enrichment that begins after nitrate depletion. While biomass production rates were similar for the 15, 30, and 60 g TDS/L growth conditions lipid enrichment at the 60 g TDS/L condition led around an 80% productivity increase over the next highest condition (30 g TDS/L). This suggests that approximately 60 g TDS/L may be the optimal balance of growth and lipid content in the Polyculture. The higher growth rates of the 15 and 30 g TDS/L flasks yielded higher lipid productivity despite lower content when compared to the 75 and 90 conditions due to faster algal growth. A technique to increase lipid yield in saline to brackish PW might be to induce salinity stress once the culture achieves a stationary phase under nutrient starved conditions. Though not tested in this study, salinity stress has been shown to increase lipid content and improve suitability for biodiesel for *Chlorella* (Pandit et al., 2017).

GC/MS analyses of the Fatty Acid Methyl Esters (FAME) formed from the lipid extract of the polyculture yielded consistent results for the 15, 30, and 60 g TDS/L salinities over time. Figure 3.3. 17 displays FAME profiles that are dominated by C16:0 (palmitic acid) which ranges from 52-74%. Stearic acid (C18:0) was the second most abundant FAME at 10-30%. Other C16 C18 unsaturated fatty acid chains make up 0-15% on the analyses. C14:0 (myristic acid) was found in trace amounts. The FAME composition was reasonably consistent at the different salinities. Palmitic acid seems more common in the 60 g TDS/L condition. Of the three salinities shown, only the 60 g TDS/L condition exhibited lipid enrichment after nutrient depletion on day 5. This may have increased the proportion of C16:0 and C18:2 while decreasing C18:0 and C18:3

(Figure 3.3. 18). Overall, the consistent character of the Polyculture FAME was desirable for conversion to biodiesel.

The FAME profile should produce a quality biodiesel. The dominance of palmitic and stearic acid should ensure a high cetane number for the fuel. The cetane number is a measure of the ease of ignition and higher values improve the quality of the fuel and decrease nitrous oxide emissions (Sivaramakrishnan and Ravikumar, 2012). The unsaturated C16 and C18 FAMEs have the highest cetane values of those detected and would produce a biodiesel well above the minimum cited of 47 for biodiesel (Racharaks et al., 2015). The slight change in the FAME profile during lipid enrichment of the 60 g TDS/L condition would not change its Cetane value significantly. The viscosity of the detected FAMEs falls well within the desired range of 1.9-6 mm^2/s (Fakhry and Maghraby, 2013). It is possible that the dominance of saturated C16 and 18 could reduce engine performance in cold conditions because of an increase in fuel viscosity. This could be mitigated through blending with sources of unsaturated FAME. Overall, transesterification of the Polyculture lipids should create a quality biodiesel.

Both *C. aponinum* and *P. kessleri* are known to accumulate a high proportion of lipids in their cells. Both species accumulate a high proportion of lipids in their cells. Li et al., (2013) reported lipid content of 5-60 % for *P.* kessleri depending on the age of the culture, and whether lipid production was stimulated by nutrient deficiency. Karatey and Donmez, (2011) measured values of 20 - 45% for *C. aponinum* at various pHs and incubation periods. Both species exhibit lipid enrichment under nitrogen depleted conditions. Microscope images of possible *P. kessleri* in this experiment from nitrogen

deficient, lipid enriched flasks appear to show large triglyceride filled vacuoles (Figure 3.3. 19). These images are similar to those found in McConnell et al., (2015). Salt stress has been shown to also stimulate lipid production in a genus related to *Parachlorella*, *Chlorella vulgaris* (Zhu et al., 2016). Cultivation in PW at a salinity near 60 g TDS/L followed by salt stress and nitrogen starvation might increase lipid productivity further. Salinity and time of harvest are parameters that can be varied to optimize the biomass character for the desired fuel conversion process, and more work could tailor growth conditions to that end.

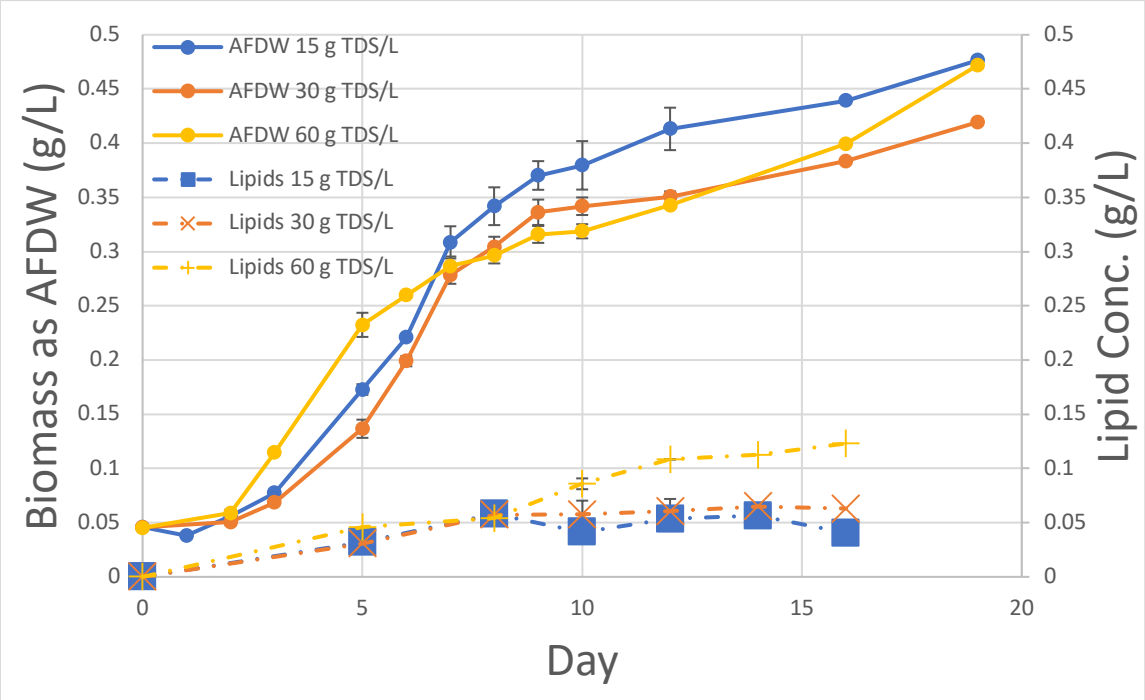

Figure 3.3. 12: Polyculture Flask Experiment (P1 & P2) biomass and lipid concentration at 15, 30, and 60 g TDS/L.

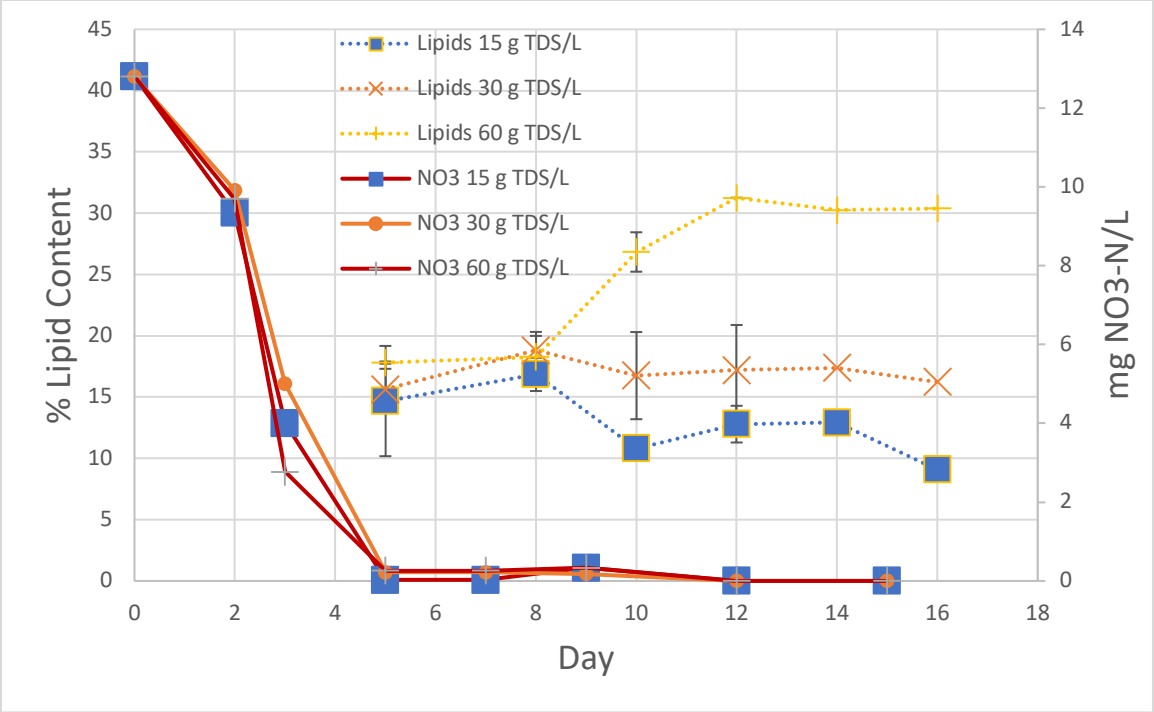

Figure 3.3. 13: Polyculture flask experiment (P1 & P2) lipid content and nitrate concentration in the media for the salinity conditions 15, 30, and 60 g TDS/L.

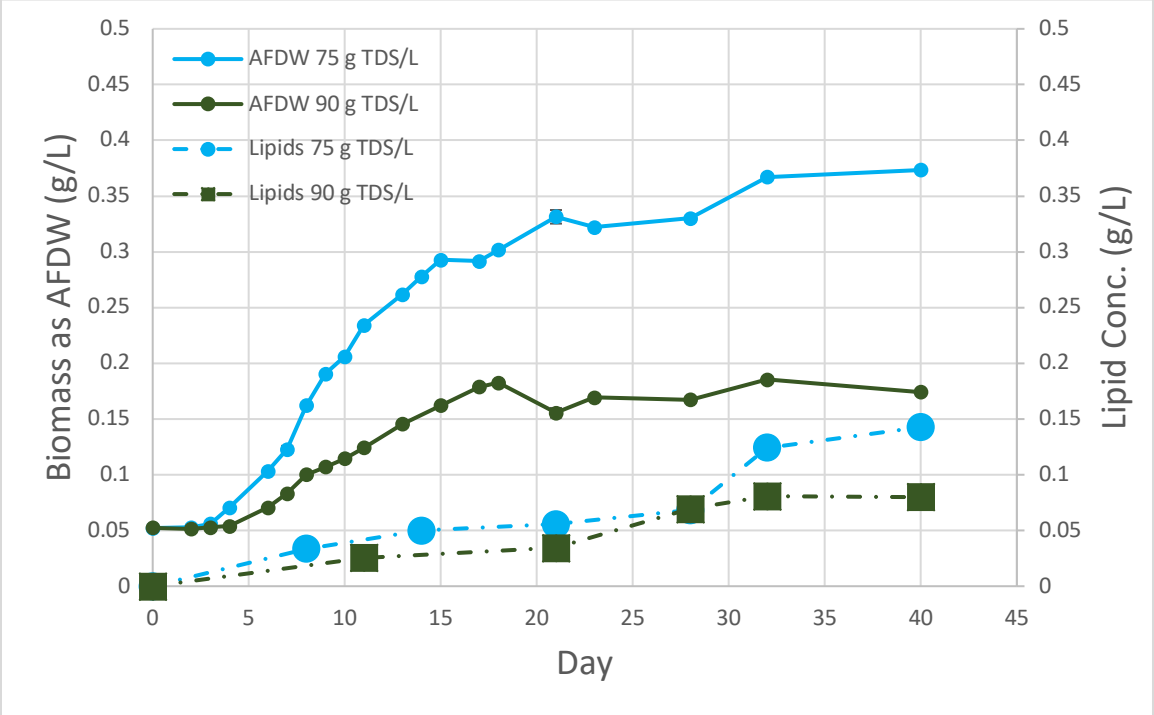

Figure 3.3. 14: Polyculture Flask Experiment lipid and biomass concentrations at 75 and 90 g TDS/L.

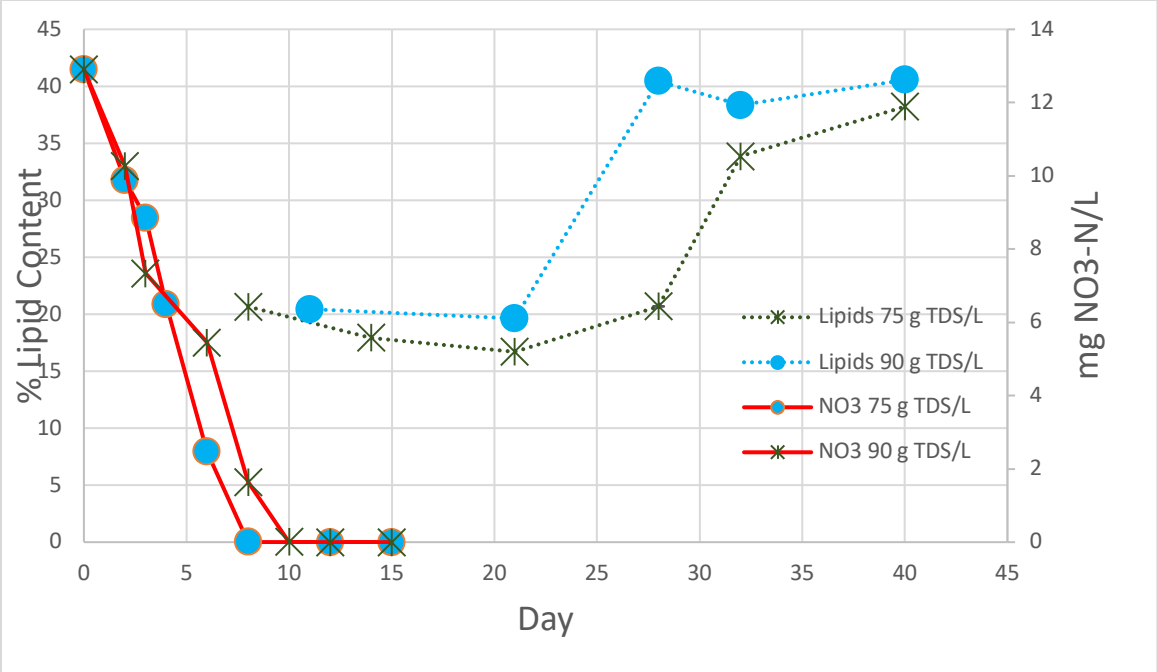

Figure 3.3. 15: Polyculture flask experiment lipid content and nitrate concentration in the media for the salinity conditions 75 and 90 g TDS/L.

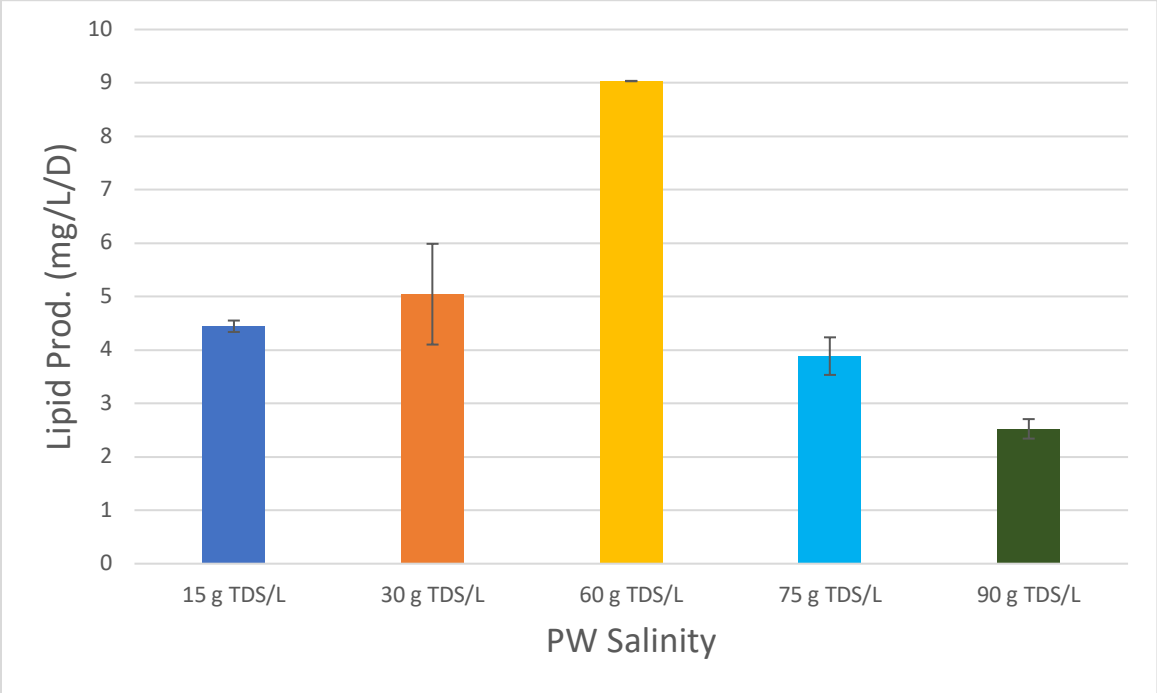

Figure 3.3. 16: Initial lipid production rates for the first 12 days (15, 30, and 60 g TDS/L), 28 days (75 g TDS/L), and 21 days (90 g TDS/L).

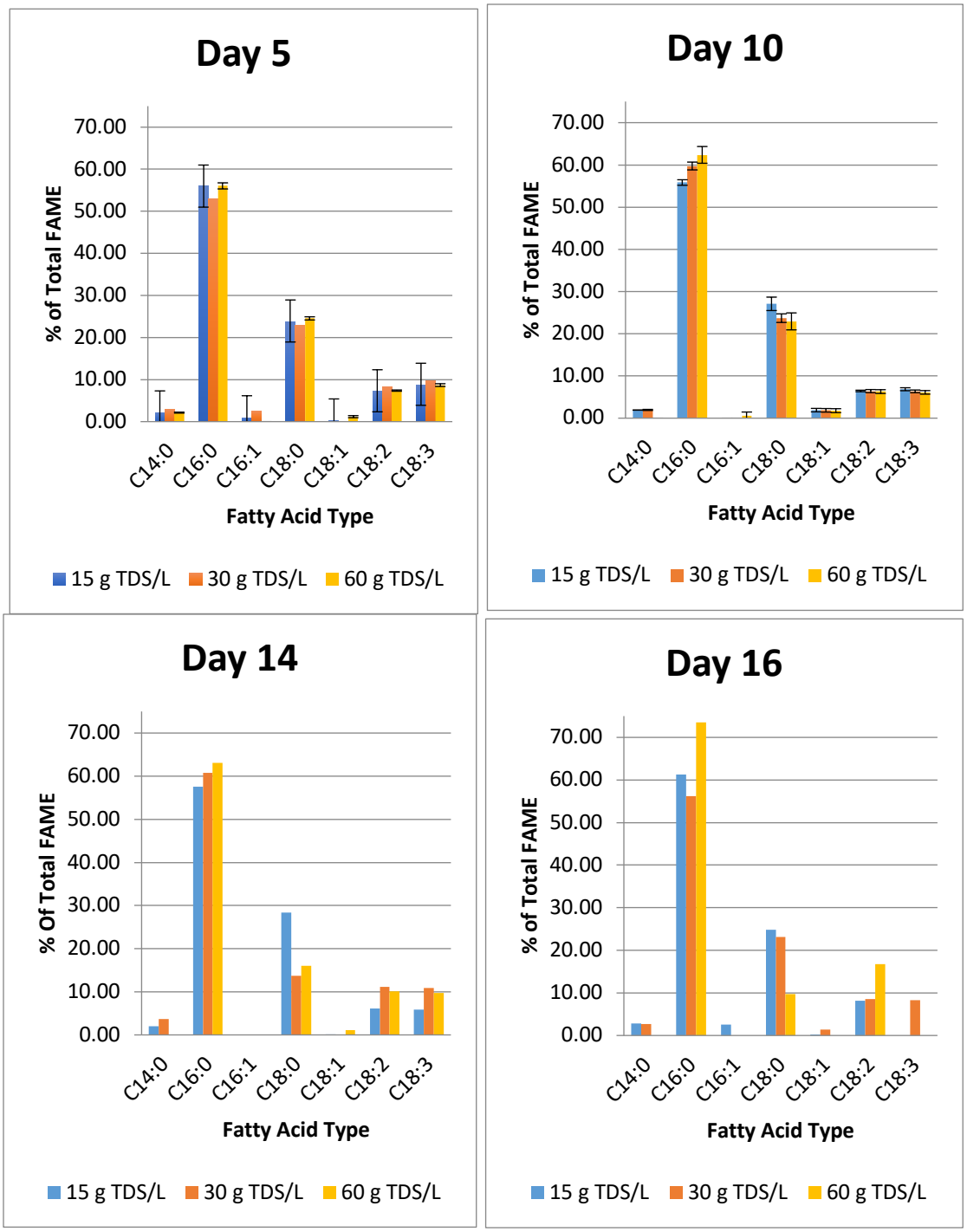

Figure 3.3. 17: GC/MS relative abundances of fatty acid methyl esters (FAME) in Polyculture lipid extracts at three different salinities at different times during the experiment.

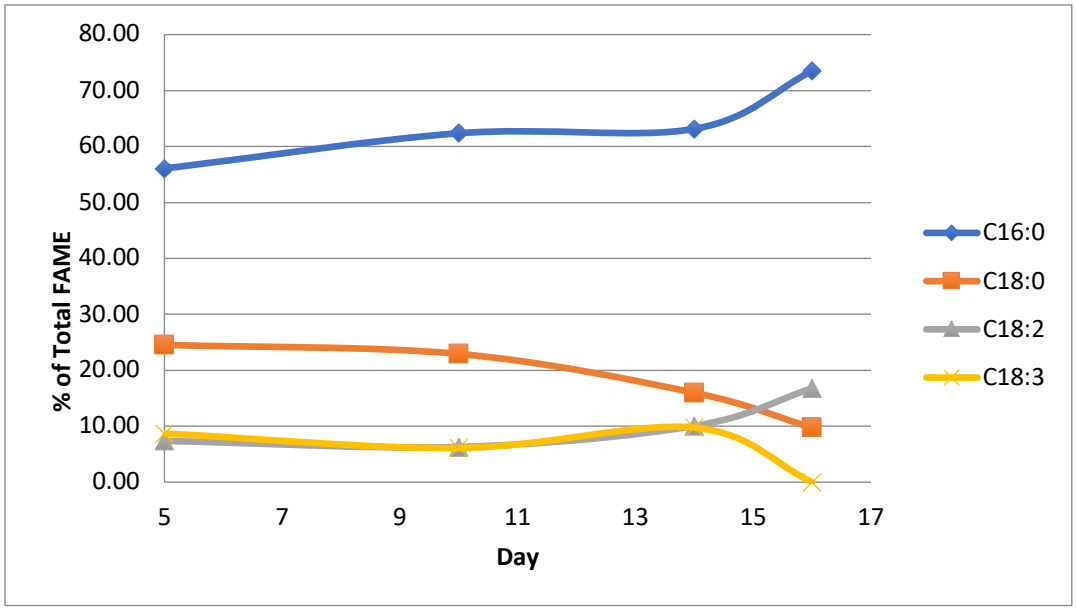

Figure 3.3. 18: FAME profile of lipid extracts of the Polyculture grown in 60 g TDS/L PW media over time.

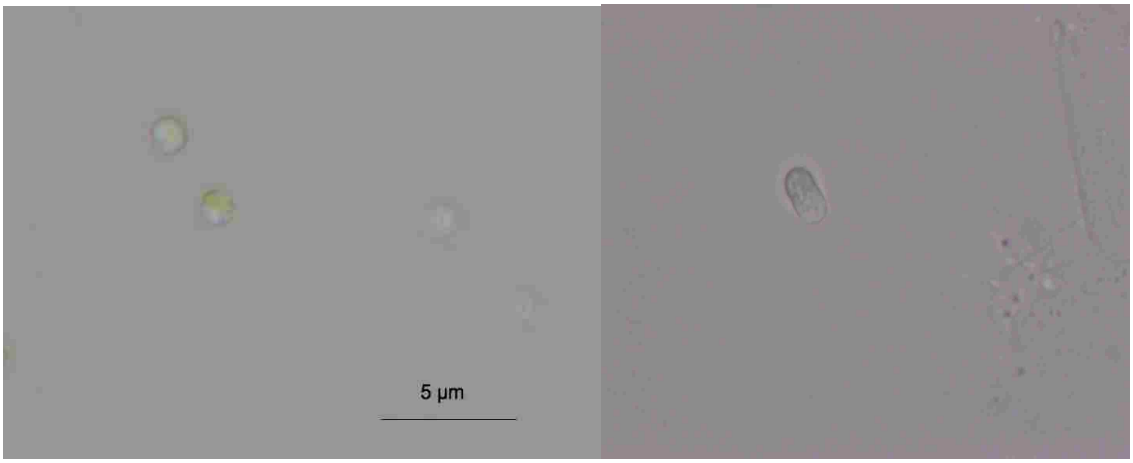

Figure 3.3. 19: *P. kessleri* (left) and *C. aponinum* (right) in PW media at 60 g TDS/L with an enriched lipid content (2000X).

Both cells contain possible lipid filled vacuoles.

3.3.3: Polyculture with ammonium in 60 g TDS/L PW Media (Exp.: P3)

In experiment P3 the Polyculture was grown at a range of ammonium concentrations in produced water with the TDS held constant at 60 g/L to evaluate potential stimulation or inhibition of growth by ammonia, and for comparison with nitrate-based media from experiments in P1 and P2. Ammonium has been detected in

PW samples far more frequently than nitrate and growth on this nitrogen source would improve the economics of PW as algae medium. The Polyculture displayed robust growth utilizing ammonium as a nitrogen source with possible evidence of free ammonia inhibition (Figure 3.3. 20). The lowest initial concentration of 13 mg NH_4-N/L had the greatest growth rate up to day 7. During days 8-10 growth in the higher ammonium concentration flasks matched and then exceeded the lowest NH_4 concentration by over 0.1 g AFDW/L. Final biomass concentrations were in excess of 0.61 g AFDW/L, except for the lowest initial ammonium concentration which was approximately 0.47 g AFDW/L. Final biomass densities were greater than those measured on 13 mg NO_3-N/L in 60 g TDS/L PW media (0.47 vs. 0.49-0.61 g AFDW/L). Biomass increased until day 16 when all conditions appeared to enter a stationary phase. The lower growth rates at initial ammonium levels above 13 mg NH_4-N/L may have been the result of free ammonia inhibition, which has been previously demonstrated (Gutierrez et al., 2016).

The Polyculture growth rapidly removed ammonium and phosphate from the media. Once flasks entered the growth phase, ammonium was removed at rates of 8 – 20 mg NH_4-N/L/D (Figure 3.3. 21). The 13 mg NH_4-N/L condition depleted all nitrogen from the media by day 4, which may be related to the lower growth rate exhibited by this culture after day 5 relative to the higher ammonium conditions tested. Higher concentration took longer, but also were able to uptake the starting ammonium levels. Phosphate was removed from the media in all conditions by day 4 (Figure 3.3. 22). Phosphate and ammonium depletion in the media does not appear to have had an

immediate effect on Polyculture growth. Figure 3.3. 23 shows biomass and nutrient concentrations for the lower initial ammonium levels of 13 and 30 mg NH_4-N/L. The highest beginning growth rates of the 13 mg NH_4-N/L condition decreased a day after ammonium depletion. The 30 mg NH_4-N/L growth rate was relatively constant from days 2 to 16. The final biomass concentrations are greater in the PW media with higher ammonium levels. Likely, nitrogen, not phosphorus, was the limiting nutrient at these concentrations for increased nitrogen concentration resulted in a higher final AFDW. In the 50 and 70 mg NH_4-N/L flasks growth and ammonium depletion rates were constant and similar to each other (Figure 3.3. 24). The low level of phosphate required (1.7 mg PO_4-P/L) to achieve a biomass density typical of HRAP (0.6 g/L) improves the economics of cultivation.

The pH initially increased for the Polyculture grown in the PW media for all conditions tested. The pH increased from 8.1 to above 9 by day 2 in all flasks (Figure 3.3. 25). After day 2 higher growth rate correlates with a higher pH. The 13 mg NH_4-N/L reach a pH of 10.35 on day 3 and the 30 and 50 mg/L conditions reached matched this value a couple days later. The 70 mg NH_4-N/L flasks had a smaller pH increase, while the F/2 media had a generally lower pH never rising above 9.5. In all conditions, the pH gradually decreased by the end of the experiment. The initial increase in pH may have been linked to photosynthetic activity, as algae uptake of bicarbonate consumes H^+. The F/2 media has a much lower amount of dissolved carbonate (18 vs. around 2 mM), and as a result a lower growth rate that did not produce as elevated pH values. Ammonium uptake releases protons into solution [Goldman (1980) & Eustance et al.

(2012)]. The general increase in the pH suggested pH effects were dominated by the uptake of inorganic carbon. The pH increases initially observed in the experiment would shift the NH_4/NH_3 system toward increased free ammonia. Free ammonia has been reported to inhibit photosynthetic activity (Gutierrez et al., 2016), and this might have slowed the pH rise in the higher initial 70 mg NH_4-N/L concentration (Figure 3.3. 20). Nitrate uptake is thought to consume H^+ ions, which is opposite of ammonium (Chen, et al., 2011). Nevertheless, the initial pH rise though was smaller in the F/2 media and in the PW media using nitrate. This is another indication that inorganic carbon uptake dominates pH changes. Overall, pH can be assumed to be a rough qualitative measure of photosynthetic activity of the algae in the PW media exerting a much larger effect than other processes.

Microscopy imagery suggested some variation in the algae composition of the cultures based on ammonium concentration and nitrogen source. The 13 mg NH_4-N/L was dominated by spherical cells (possibly *P. kessleri*) with smaller numbers of diploids (possibly *C. aponinum*) (Figure 3.3. 26). At higher initial ammonium concentrations, the possible *P. kessleri* appeared to make up almost the entire biomass though a small number of possible *C. aponinum* could be found. The possible *P. kessleri* cells formed larger floc and cluster morphologies were observed in higher ammonium concentrations. The Polyculture grown in the F/2 media (around 30 g TDS/L and 13 mg NO_3-N/L) had a higher proportion of the possible *C. aponinum*. Based on these results it is possible that the suspected *P. kessleri* has a greater ability to utilize ammonium for growth and is less susceptible ammonia inhibition.

Biomass increased faster on ammonium compared to nitrate at the same salinity (Figure 3.3. 27). The trends for growth are closely matched for both conditions. Ammonium is removed from the media on day 4 while nitrate on day 5, indicating a Polyculture preference for ammonia (Figure 3.3. 6 & Figure 3.3. 21). Nitrogen in ammonium has an oxidation number of -3, and algae cells must reduce nitrate in order to utilize it (Chen et al., 2011) (Beardall and Giordano, 2009). This results in a higher energy requirement for utilization of nitrogen from nitrate over ammonium. The author hypothesizes that nitrate reduction diverts energy from growth causing a lower rate of biomass increase.

The effect of phosphorus concentration was evaluated by comparing growth with initial concentrations of 1.7 and 8 mg PO_4-P/L (Figure 3.3. 28). Up to day 3 both high and low P conditions were closely matched. After this time, Low P had a higher growth rate and final biomass density. Algae are known to store phosphorus primarily as polyphosphate in the cell for later anabolism and energy (Solovchenko et al., 2016), which requires energy (Lippman, 1941). It is probable this also diverts energy of from growth decreasing the rate of biomass accumulation. The algae cells slow their division rate to sequester a crucial nutrient and extra energy. The practical implications are nutrient levels of 13 mg NH_4-N/L and low phosphate concentrations of 1-2 mg PO_4-P/L may be ideal for Polyculture cultivation in PW.

To compare early phase growth, biomass accumulation rates were calculated between day 2 and 5 for a variety of conditions at the salinity of 60 g TDS/L in PW and the F/2 media (30 g TDS/L) (Figure 3.3. 29). During this time, the lowest ammonium

concentration of 13 mg NH_4-N/L exhibited the highest growth rate of 57.3 mg/L/Day. The initial growth rate decreased with higher ammonium levels possibly due to ammonia inhibition. The high phosphate condition exhibited a rate of 37.5 mg/L/Day, which as previously stated might be linked to energy diversion for phosphate storage. All growth rates in ammonia were higher than that for nitrate at the same salinity and much higher than the F/2 commercial media. The biomass increase of 99 mg/L between day 2-3 of the 13 mg NH_4-N/L, low P condition is the highest measured for the Polyculture. The early growth rates suggest again that a low ammonium and phosphate concentration is optimal for maximum Polyculture growth.

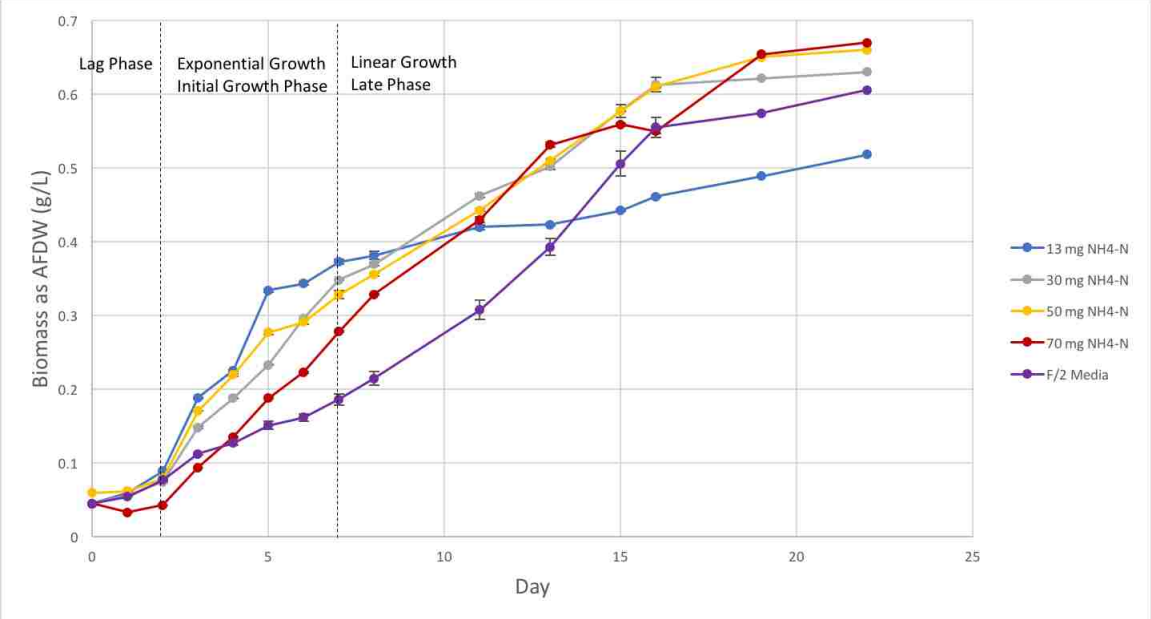

Figure 3.3. 20: Polyculture biomass measurements in 60 g TDS/L PW media at a range of initial ammonium concentrations (Exp.: P3).

The F/2 commercial growth media is displayed for comparison.

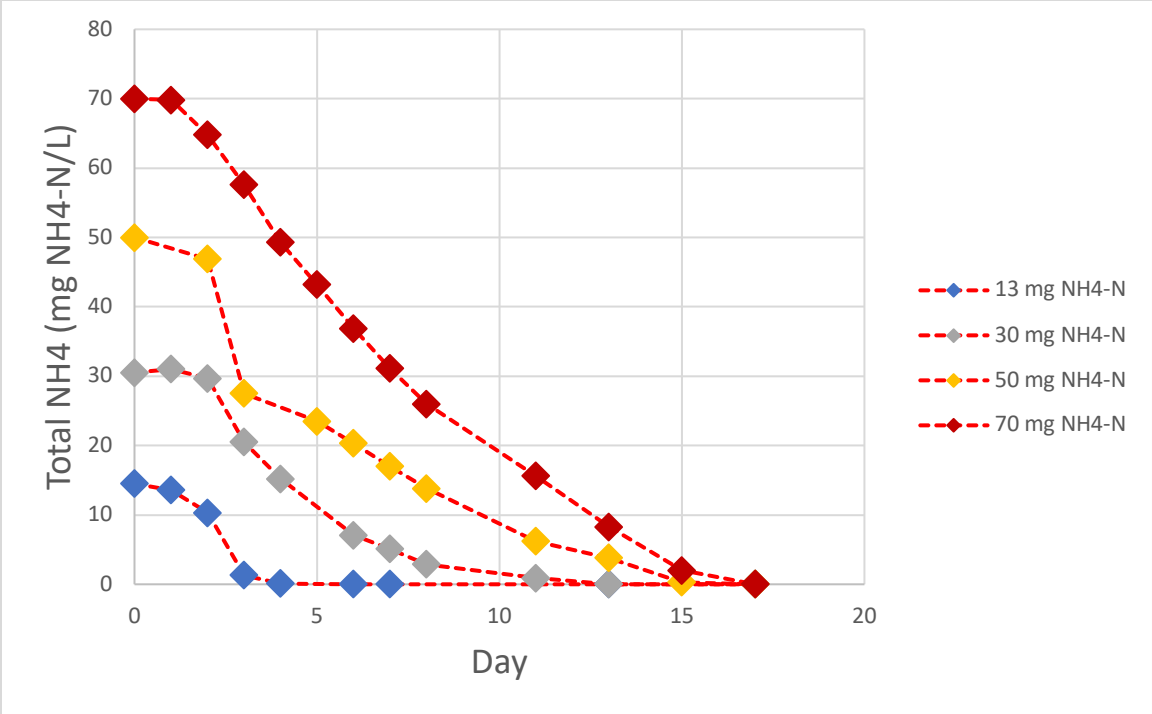

Figure 3.3. 21: Polyculture total ammonium measurements in 60 g TDS/L PW media at 4 different initial concentrations (Exp.: P3).

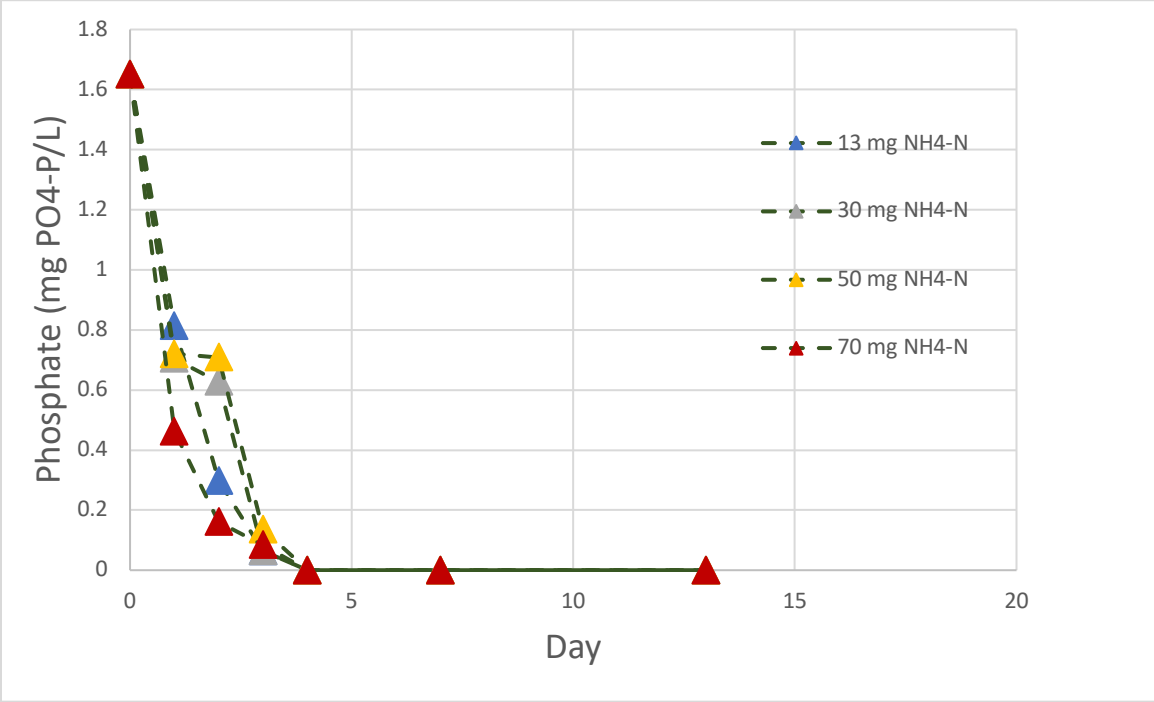

Figure 3.3. 22: Polyculture phosphate measurements in 60 g TDS/L PW media at 4 different initial ammonium concentrations (Exp. P3).

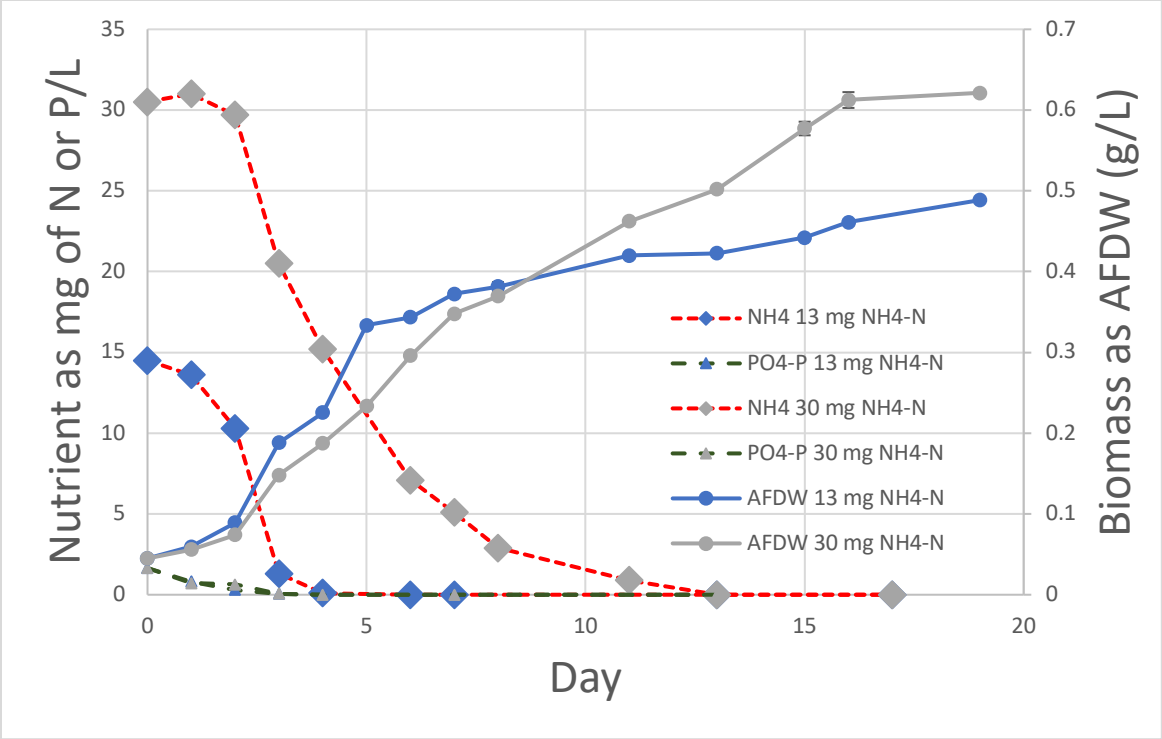

Figure 3.3. 23: Polyculture biomass and nutrient measurements in PW at 60 g TDS/L at initial concentrations of 13 and 30 mg NH_4-N/L (Exp.: P3).

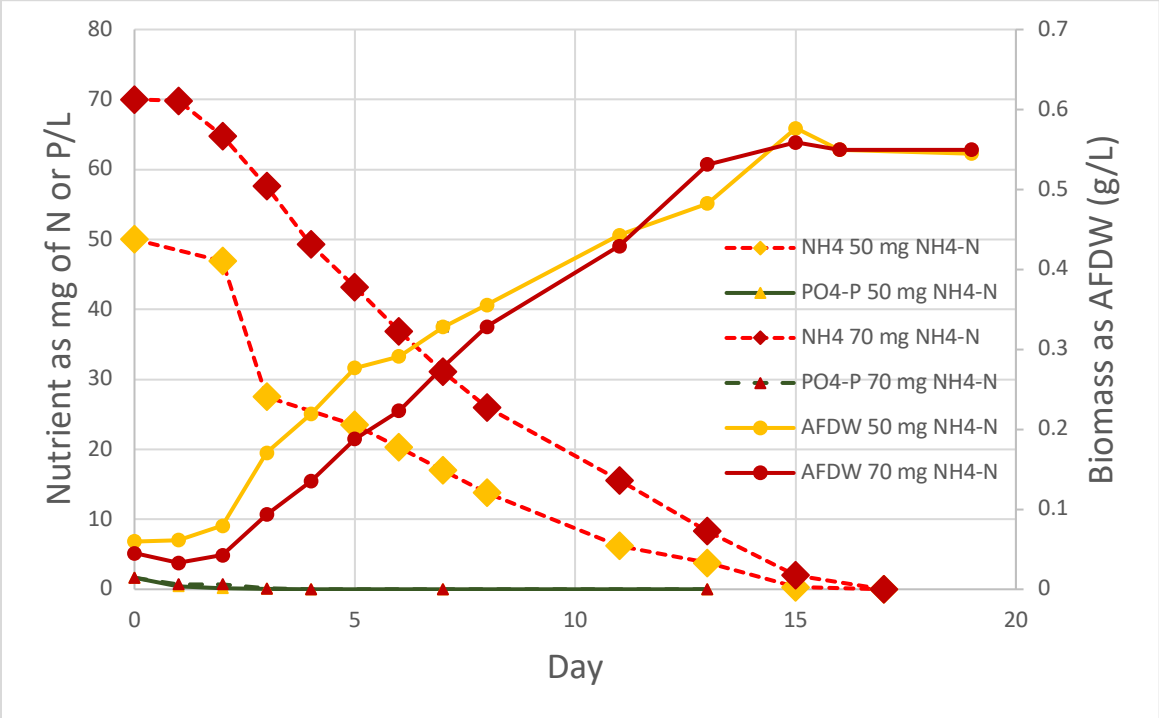

Figure 3.3. 24: Polyculture biomass and nutrient measurements in PW at 60 g TDS/L at initial concentrations of 50 and 70 mg NH_4-N/L (Exp.: P3).

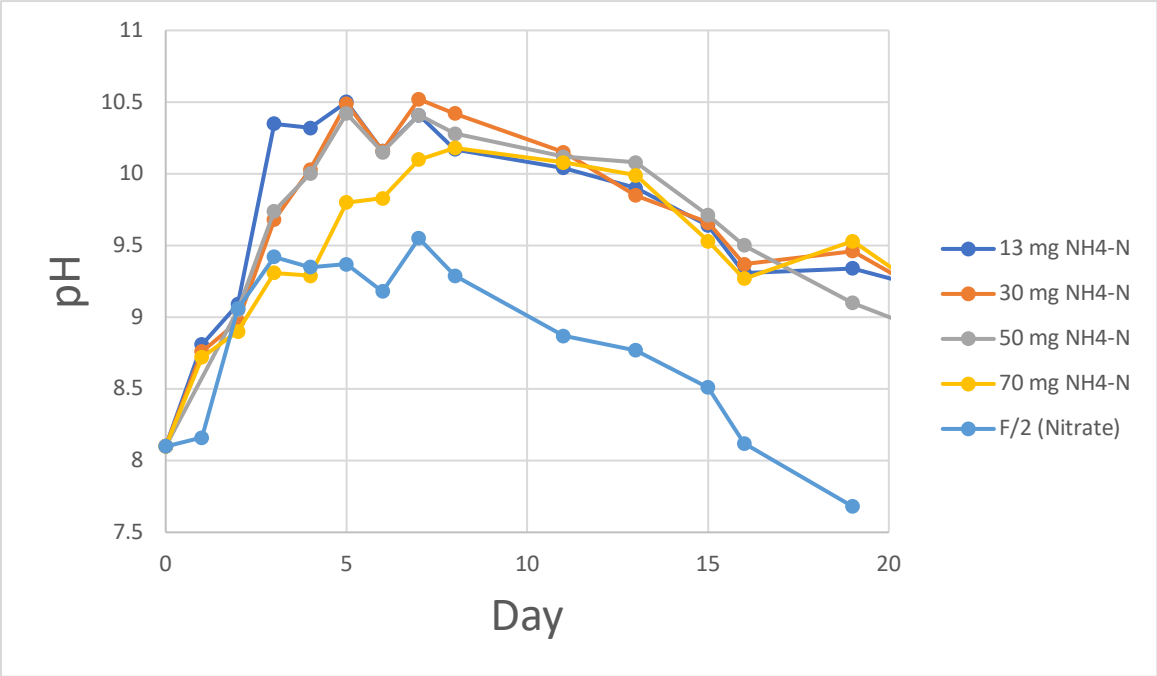

Figure 3.3. 25: Polyculture pH measurements in 60 g TDS/L PW media at a range of initial ammonium concentrations (Exp.: P3).

The F/2 commercial growth media is displayed for comparison.

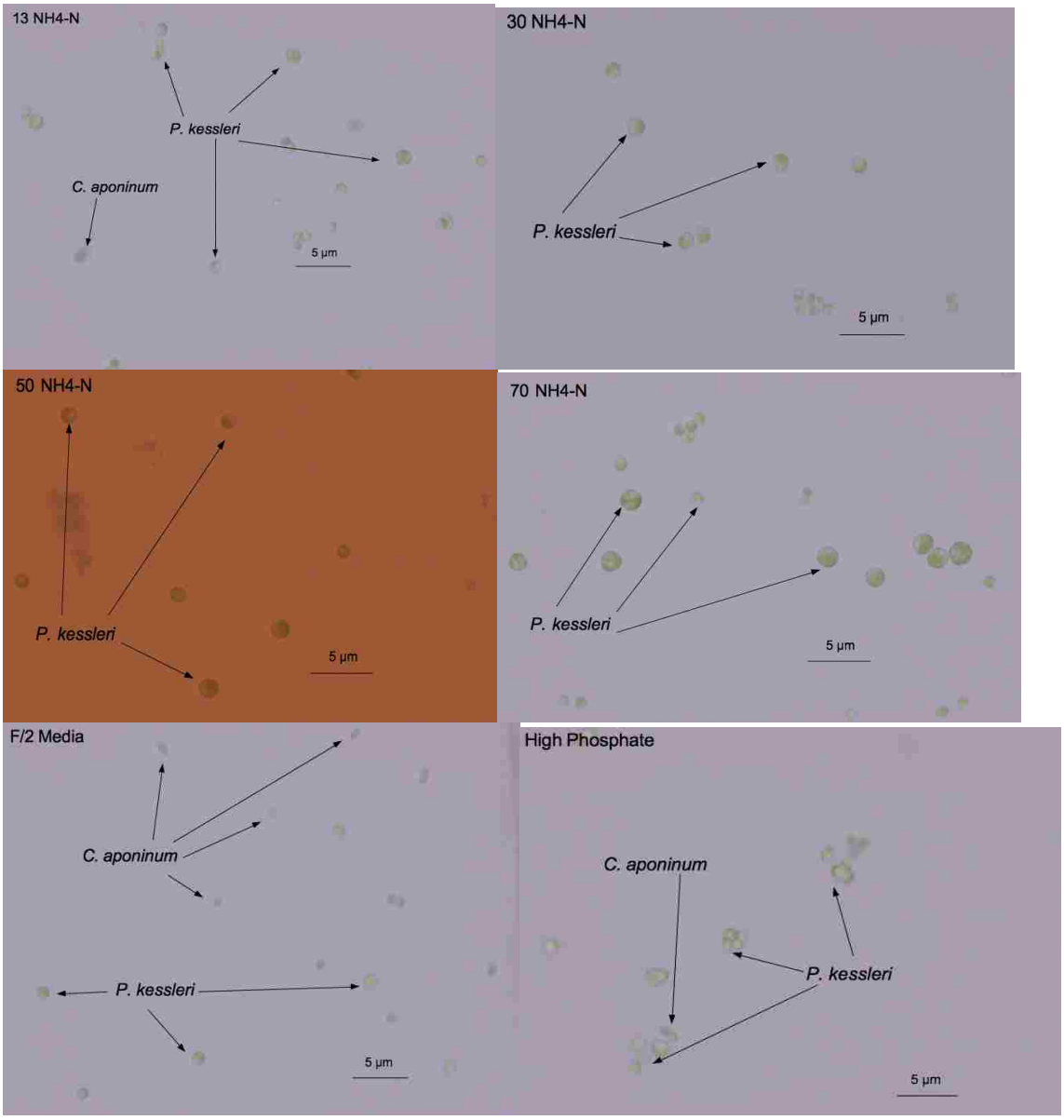

Figure 3.3. 26: Microscopic images of the Polyculture growing at different initial ammonium concentrations in 60 g TDS/L PW media on Day 13 (Exp.: P3).

Initial concentrations in the high phosphate condition were 13 mg NH_4-N and 8 PO_4-P/L.

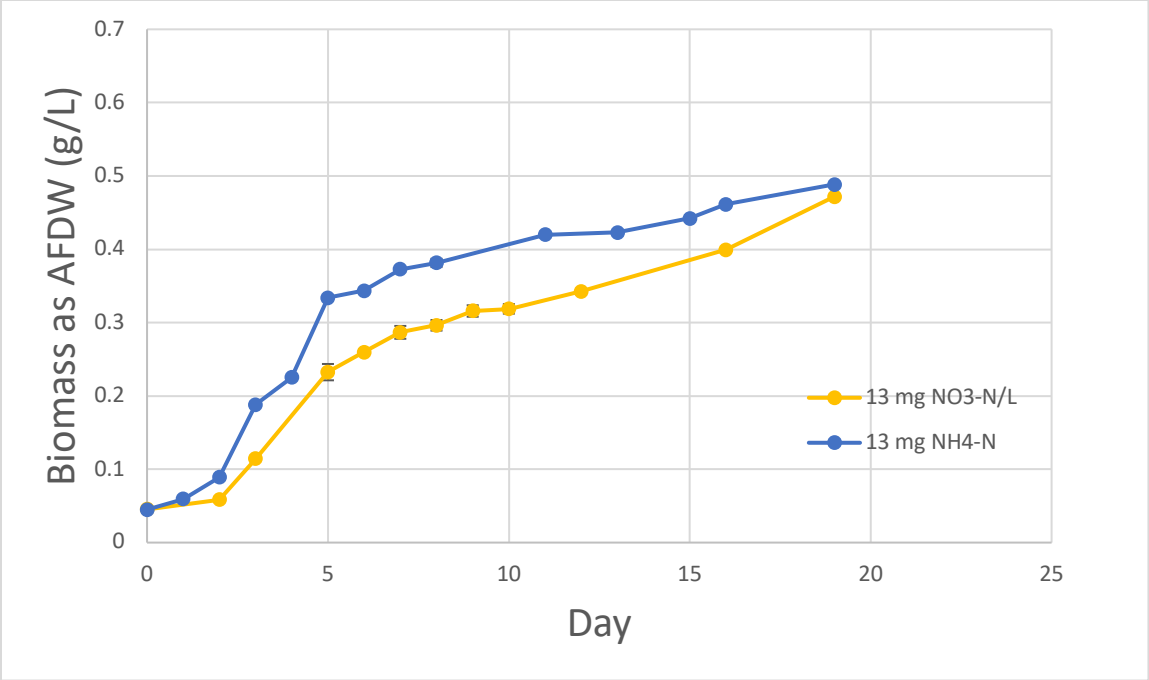

Figure 3.3. 27: Polyculture biomass measurements in 60 g TDS/L PW media with NH_4 or NO_3 mg N/L (Exp.: P1, P2, & P3).

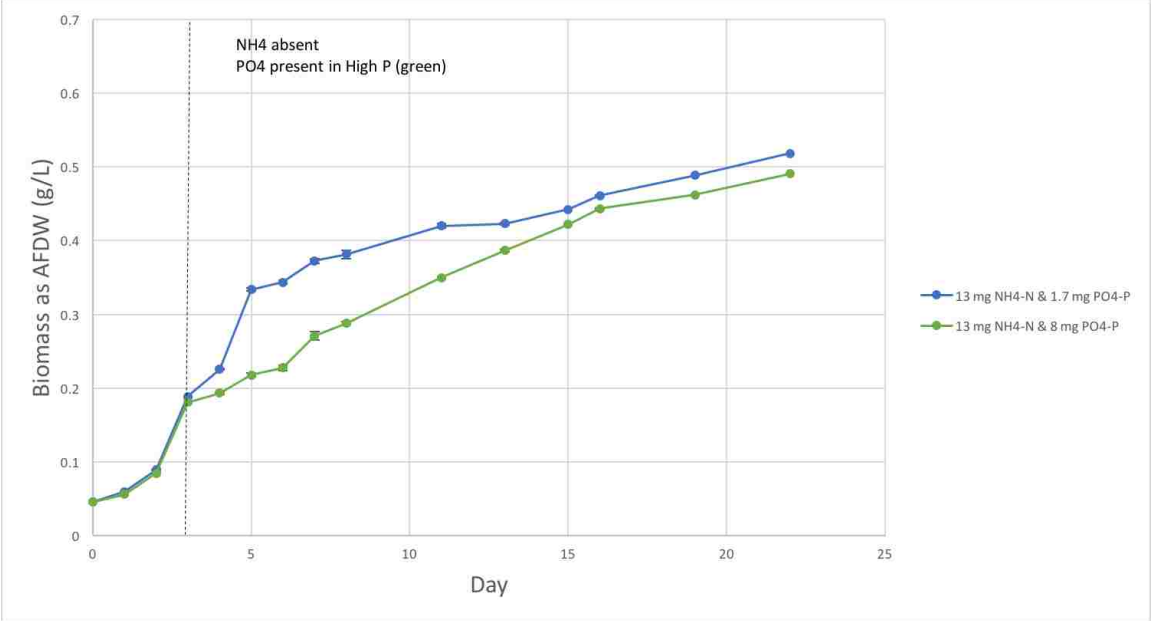

Figure 3.3. 28: Polyculture biomass measurements in 60 g TDS/L PW media (Exp.: P3) at two different initial phosphate concentrations at the same ammonium level.

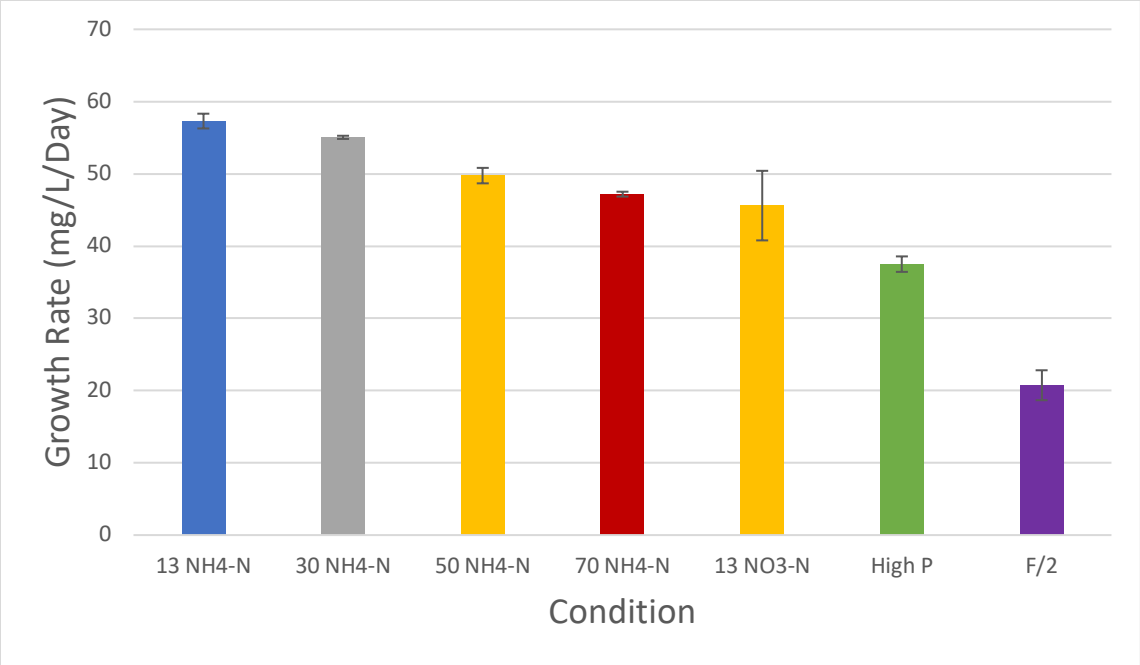

Figure 3.3. 29: Growth rate between day 2 and 5 for the Polyculture in 60 g TDS/L PW media (Exp.: P1, P2, & P3).

All initial phosphate concentrations are 1.7 mg PO_4-P/L except high P which is 8 mg PO_4-P/L. The F/2 commercial media is for comparison.

3.3.4: Polyculture growth using Ammonium in Tubular Photobioreactors (Exp.: T1)

Experiments were conducted with the Polyculture in cylindrical photobioreactors (not flasks) with a synthetic brine media (not PW) modeled on PW analyses in Harkness et al. (2015). The objective of the experiment was to determine whether Polyculture biomass productivity would increase on higher illumination and aeration. High (200 mg NH_4-N/L) and low (30 mg NH_4-N/L) initial levels of ammonium were tested along with addition of inorganic carbon through pure CO_2 aeration and bicarbonate. It was hypothesized that higher biomass productivities would be achieved under these conditions on a low ammonium concentration. The higher initial ammonium concentration was expected to cause toxicity. The ammonium rich synthetic brine is

characteristic of many PW samples particularly those from hydraulically fractured operations (Vengosh et al., 2017)

Initial low (30 mg NH4-N/L) and high (200 NH4-N/L) ammonium concentrations resulted in similar biomass measurements up to day 17 (Figure 3.3. 30). Generally, the lower ammonium concentration had higher biomass measurements up to day 17 at which point the high ammonium condition exceeded it. There was a significant drop in pH to around 3.5 in both ammonium conditions (Figure 3.3. 31 & Figure 3.3. 32), which coincided with a leveling off of growth. These drops in pH were the opposite of what was observed in flask tests with ammonium as a nitrogen source (Figure 3.3. 25). At this acidic pH, growth ceased, but biomass levels were maintained indicating that no mass mortality of algae cells. On day nine pH levels were manually adjusted to neutral with NaOH to test whether culture growth would resume. For both ammonium conditions biomass levels began to increase. The higher ammonium condition ended with a higher biomass density at the end of the experiment, and while removing approximately 45% of the initial ammonium (Figure 3.3. 33 & Figure 3.3. 34). The low ammonium condition removed NH_4^+ by day 14 of the experiment.

The lipid contents were similar to those in the flask experiments, and again demonstrated enrichment with nitrogen depletion. The low and high ammonium conditions produced lipid levels of 158 mg/L and 97 mg/L respectively, and production rates of 6.9 and 4.2 mg/L/Day. The higher lipid content of the low ammonium condition occurred during the nitrogen limiting conditions after day 15 (Figure 3.3. 35). Though phosphorus was depleted from both media, lipid enrichment did not occur in the high

ammonium condition (Figure 3.3. 36). These may due to the higher initial phosphate concentration of 4.6 mg PO_4-P/L (vs. 1.7) preventing as significant phosphorus stress. At this higher initial phosphate level algae cells may have sufficient stores of intracellular phosphorus. A longer experiment would have likely shown a lipid enrichment effect shown in flask experiments.

The results illustrate the challenge of growing algae in ammonium rich PW media due to acidification of the media. The drop in pH in the media clearly halted growth on about day 5. Ammonium uptake by algae releases protons in solution in a 1:1 ratio (Goldman et al., 1980). Eustance et al., (2012) documented a significant pH drop when algae use ammonium as a nitrogen source in media with low alkalinity. This matches the results obtained in this study. The synthetic brine used in the tubular reactors had a low alkalinity. The instant ocean salt used in the media has a total CO_2 concentration of 1.9 mMol./kg (Atkinson and Bingman, 1982). Added phosphate was at a level of 0.150 mM and was being removed by growing algae further reducing buffering capacity. Aeration would further reduce pH by bringing the media in equilibrium with the atmosphere which is close to 5.65. Inorganic carbon absorbed from CO_2 dissolved in the media does not raise pH for there is no net release of protons in solution. In the absence of a dominant pH effect caused by inorganic carbon uptake, ammonium utilization by the algae causes a decrease in pH. Acidification caused by algae uptake of ammonium/ammonia could also occur in CO_2 aerated HRAP ponds in media with low alkalinity and would need to be mitigated to maintain biomass productivity.

Adding sodium bicarbonate (1 g/L to prevent acidification) and aerating with CO_2 (to prevent alkaline conditions that would increase free ammonia) produced the highest multi-day growth rates measured in the experiment. The pH (despite a small dip) was maintained close to neutral for the entire length of the experiment with continual growth (Figure 3.3. 37). Growth between Day 5 and Day 10 (the period of the highest growth rate for the experiment) was approximately 97.5 mg AFDW/L/Day, with ammonium removal of close to 12 mg NH4-N/L/Day (Figure 3.3. 38). Phosphate was removed from the media by day 6, which interestingly did not affect the growth rate (Figure 3.3. 39). During the course of the experiment the biomass density continued to increase. A lipid concentration of 283 mg/L (+/- 4.7) was reached on Day 14 which yields a lipid productivity of 20.2 mg/L/Day (+/- 0.337). These experimental conditions under inorganic carbon and nitrogen saturation may represent the upper end of growth rate for the Polyculture. It is likely a nitrogen starvation phase would have yielded a higher lipid content, but that was not tested. This experiment resulted in the highest lipid productivity of any conducted in this study.

Strong growth after depletion of the vital nutrient phosphate suggests storage in algal cells and is linked to an increase in the relative amount of *C. aponinum* in the culture. The amount of phosphorus in the biomass (Removed PO_4-P/AFDW density) decreased from a high of 3.2% to 0.3% by Day 20 (Figure 3.3. 40). Both *P. kessleri* and *C. aponinum* have been shown to accumulate polyphosphate [Ota, S. and Kawano, S. (2017) & Moro et al. (2007)]. Nessier staining which highlights polyphosphate was used on algae cell slides. Figure 3.3. 41displays purple stained inclusions in the diplo *C.*

aponinum. Most stained *P. kessleri* cells also contained purple inclusions (Figure 3.3. 42). It appears likely *C. aponinum* is capable of superior growth over *P. kessleri* in phosphorus depleted conditions. Cell counts indicated an increasing abundance of diplo rod cells toward the end of the experiment (Figure 3.3. 43). This could be solely caused by the lack of phosphate in the media, but other factors such as light density or ammonium levels cannot be ruled out.

The results of the tubular reactor experiment are relevant to using this culture to produce biofuels in PW media. The Polyculture can grow in hypersaline conditions mimicking those found in PW using ammonium as a nitrogen source. The synthetic media is similar to PW from hydraulic fracturing in the Marcellus Shale region of Pennsylvania as described in Harkness et al., (2015). While many varieties of PW have significant alkalinity, this might not be true in all cases as is evident in the Permian Basin from the USGS database. Hydraulically fractured shale formations might have particularly low alkalinities due to the source rocks being dominated by clay minerals. While acidification can happen in PW media with low alkalinity, it could be counteracted by adding bicarbonate, a carbon source that also stimulates algae growth. The phosphate concentration used to produce biomass (4.6 mg PO_4-P/L) is not incredibly high and is commonly found in PW (See previous references in background). In summary, the Polyculture demonstrated growth using ammonium in a hypersaline brine, but acidification is a concern.

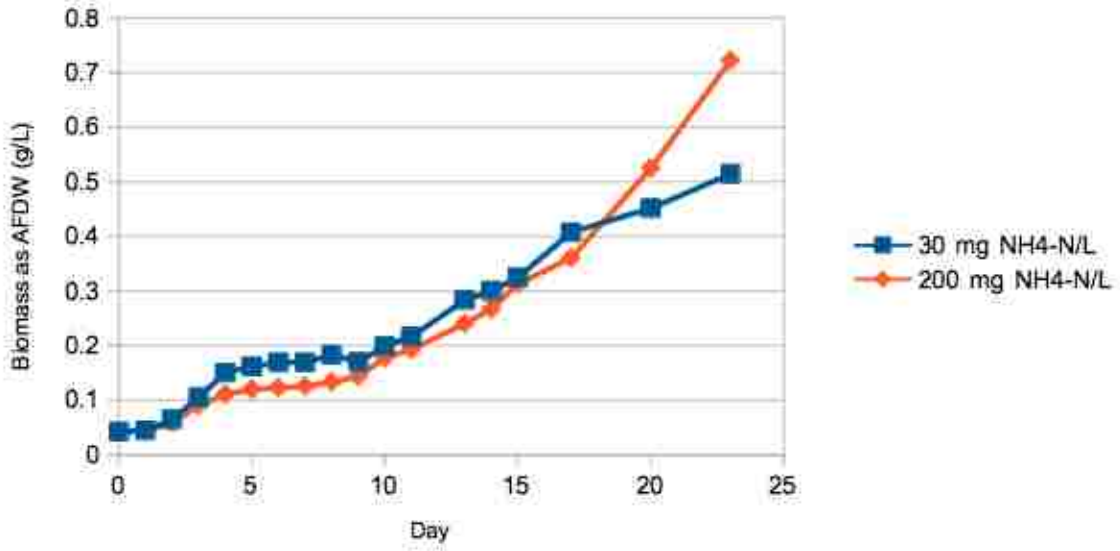

Figure 3.3. 30: Polyculture growth in Tubular reactors at 30 and 200 mg NH4-N/L initial concentrations.

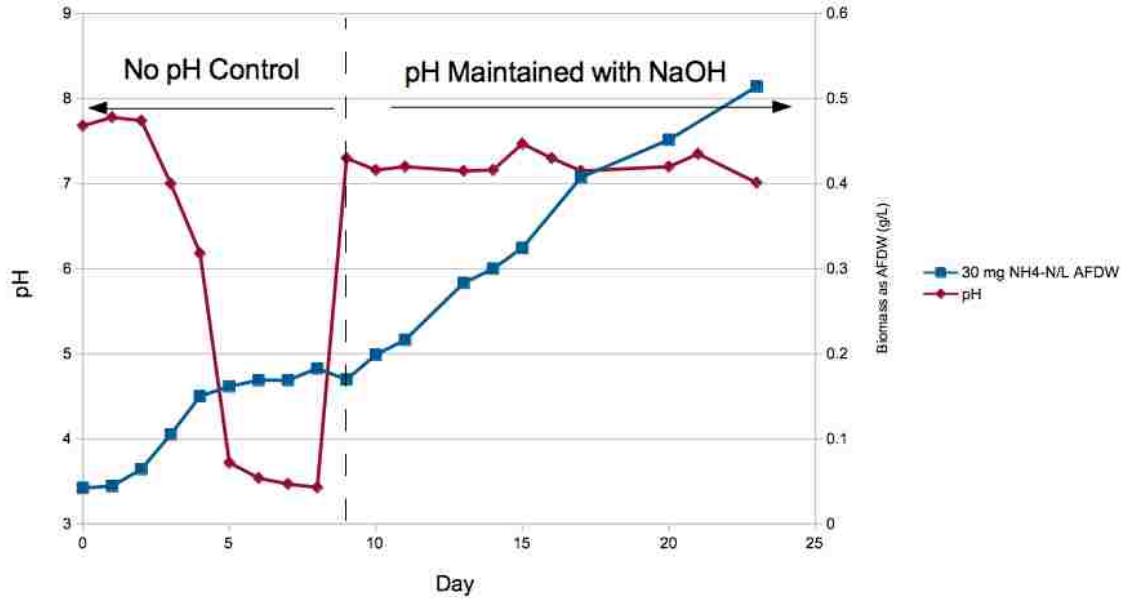

Figure 3.3. 31: Polyculture biomass and pH measurements for tubular reactor with initial Ammonium of 30 mg NH4-N/L.

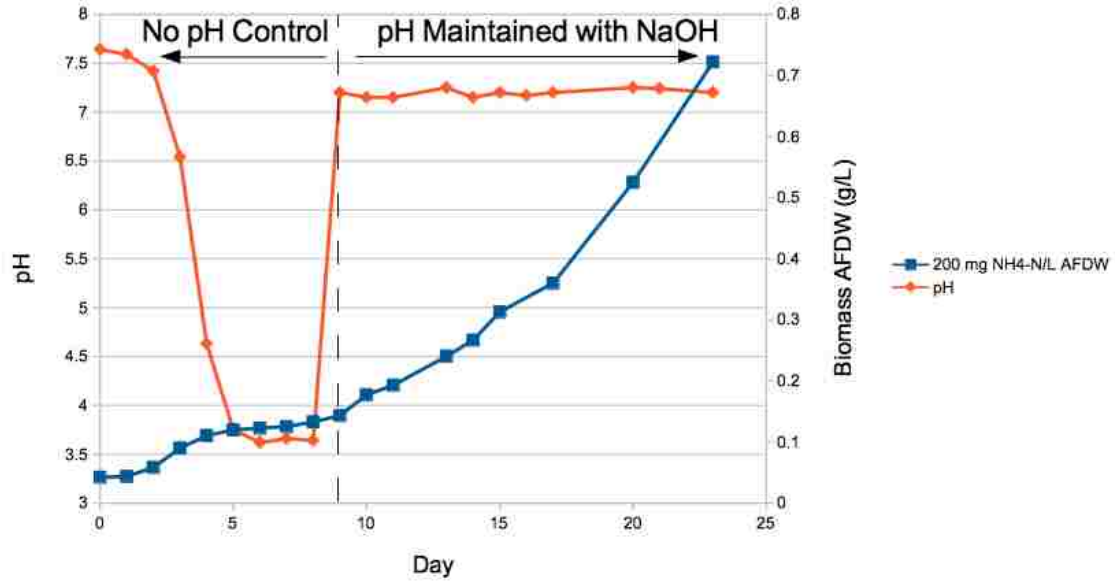

Figure 3.3. 32: Polyculture biomass and pH measurements for tubular reactor with initial Ammonium of 200 mg NH4-N/L.

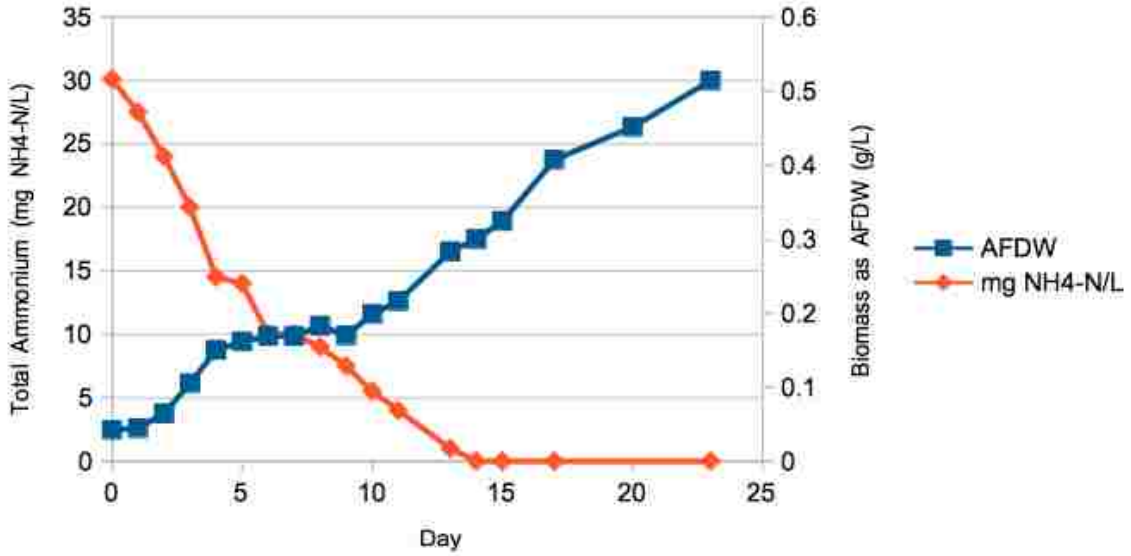

Figure 3.3. 33: Polyculture biomass and ammonium measurements for tubular reactor with initial Ammonium of 30 mg NH4-N/L.

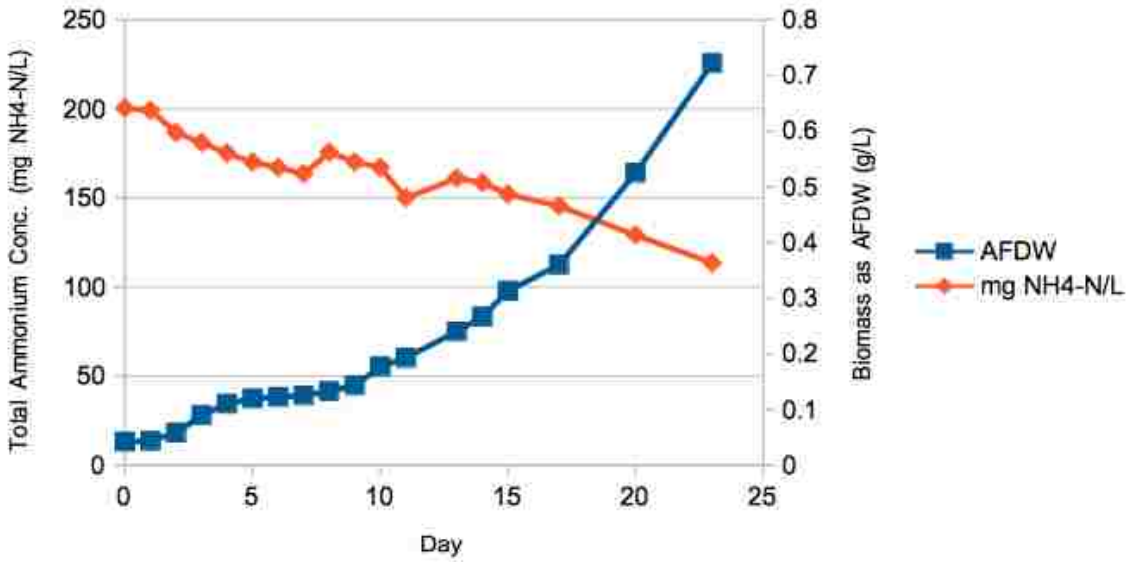

Figure 3.3. 34: Polyculture biomass and ammonium measurements for tubular reactor with initial Ammonium of 200 mg NH4-N/L.

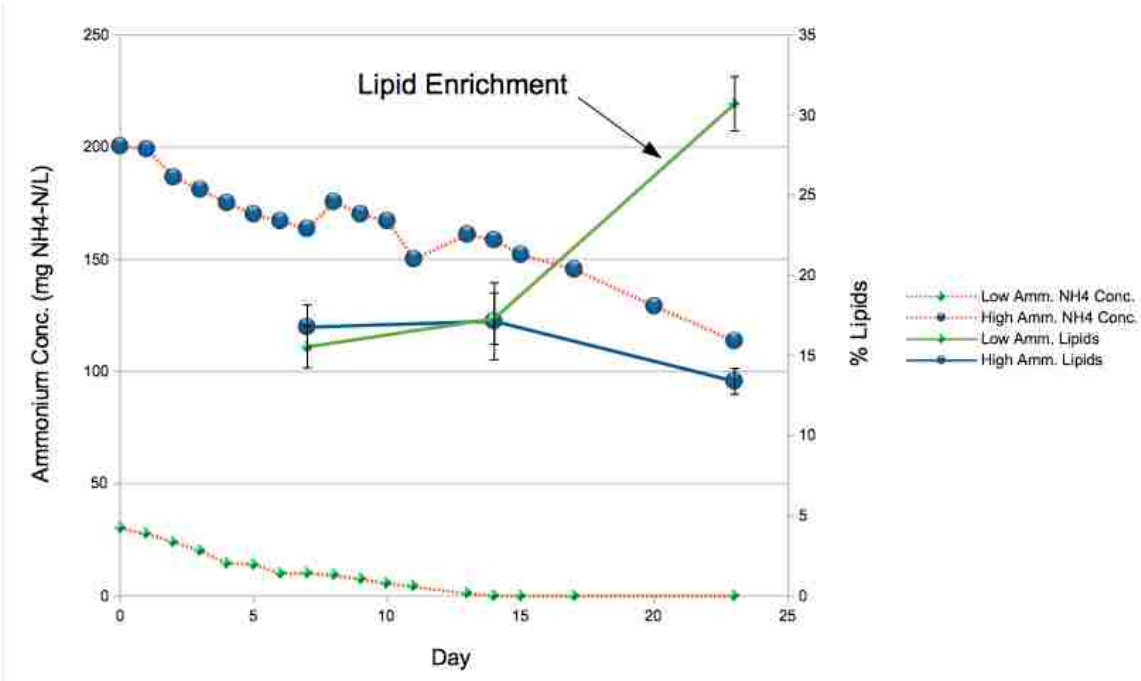

Figure 3.3. 35: Polyculture lipid content and ammonium measurements for tubular reactor with initial Ammonium of 30 and 200 mg NH4-N/L.

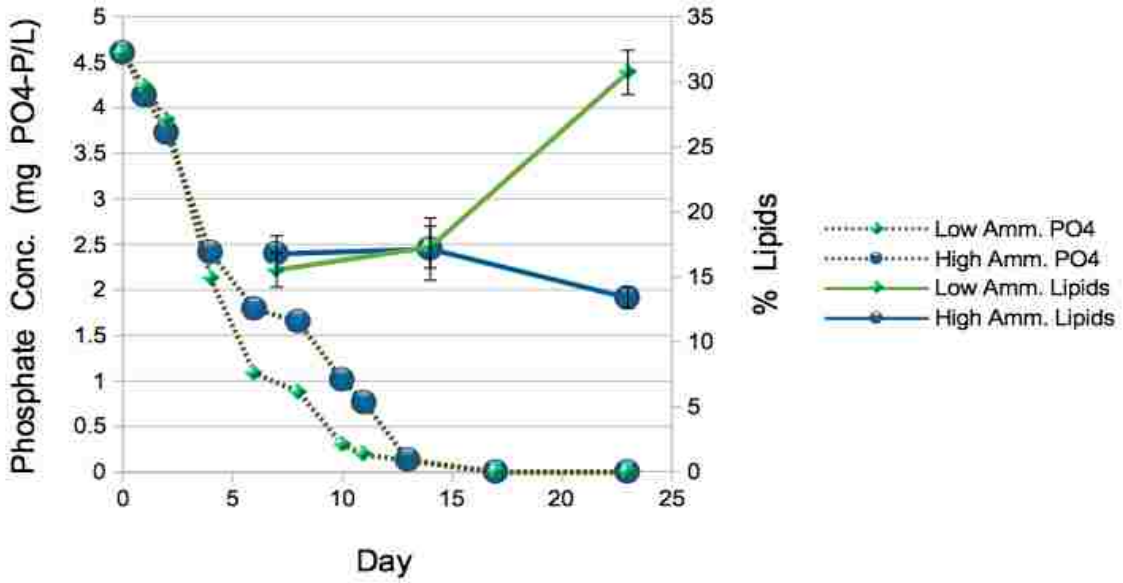

Figure 3.3. 36: Polyculture lipid content and phosphate measurements for tubular reactor with initial ammonium of 30 and 200 mg NH4-N/L.

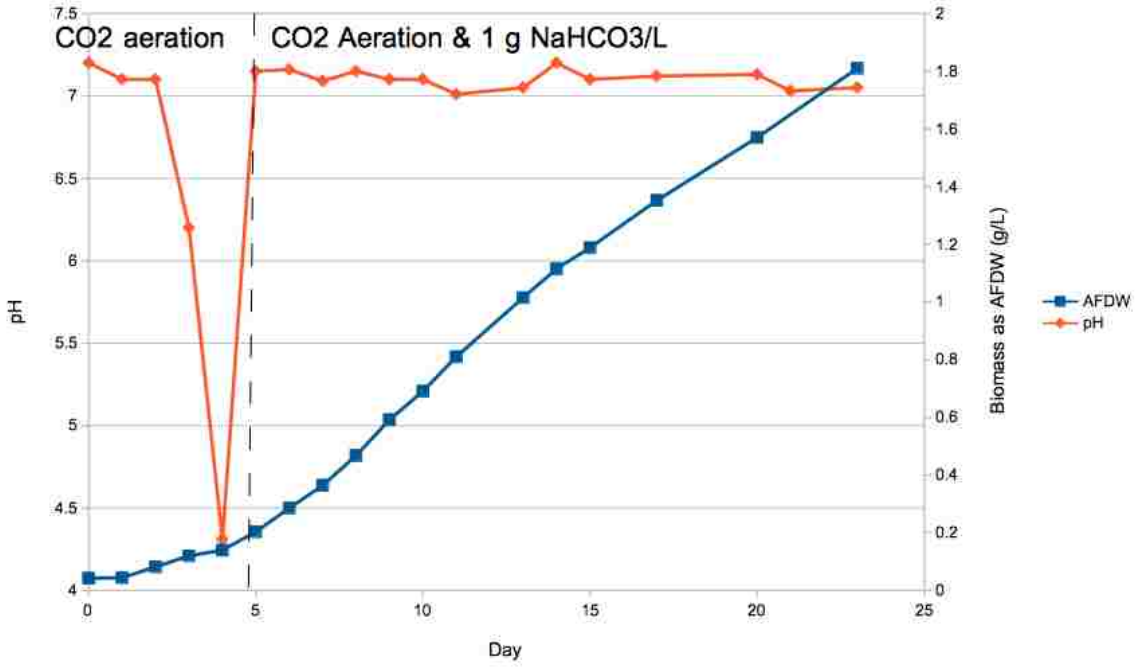

Figure 3.3. 37: Polyculture biomass and pH measurements for tubular reactor with initial Ammonium of 200 mg NH4-N/L, 1 g $NaHCO_3$/L, and CO_2 addition.

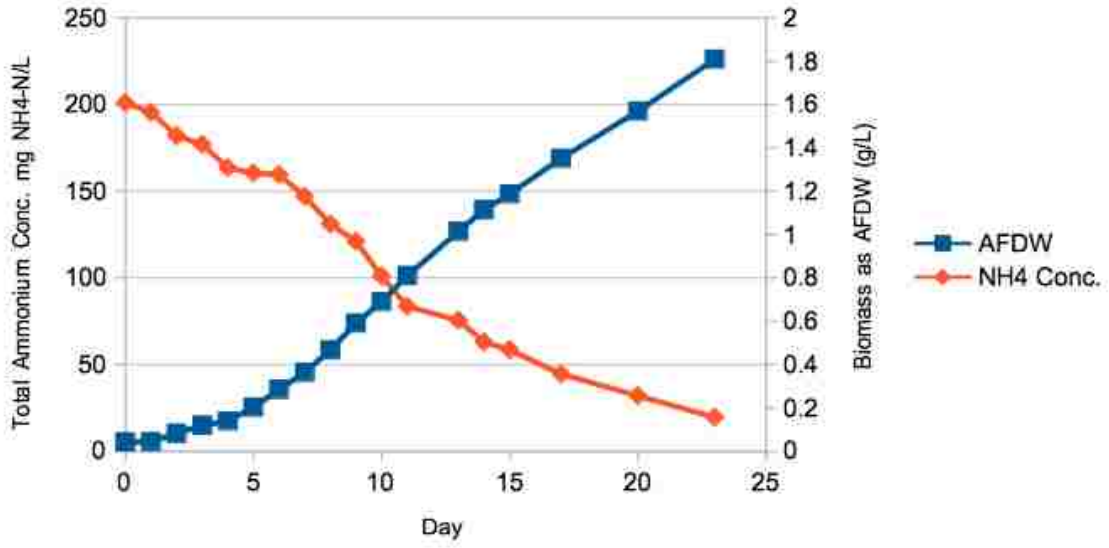

Figure 3.3. 38: Polyculture biomass and total NH_4 concentration measurements for tubular reactor with initial Ammonium of 200 mg NH4-N/L, 1 g $NaHCO_3$/L, and CO_2 addition.

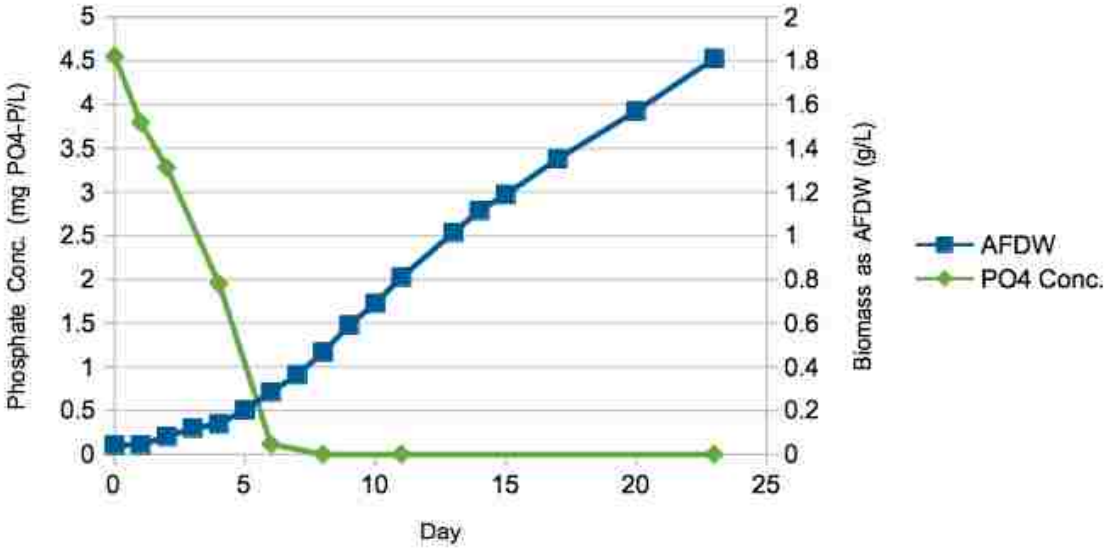

Figure 3.3. 39: Polyculture biomass and total PO_4 concentration measurements for tubular reactor with initial Ammonium of 200 mg NH4-N/L, 1 g $NaHCO_3$/L, and CO_2 addition.

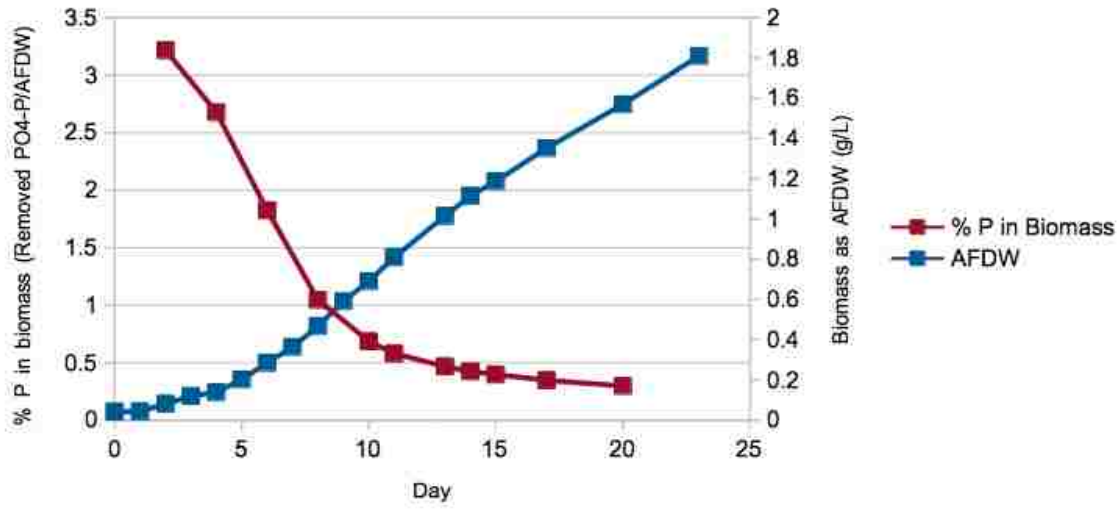

Figure 3.3. 40: Polyculture biomass and % P concentration for tubular reactor with initial Ammonium of 200 mg NH4-N/L, 2 g $NaHCO_3$/L, and CO_2 addition.

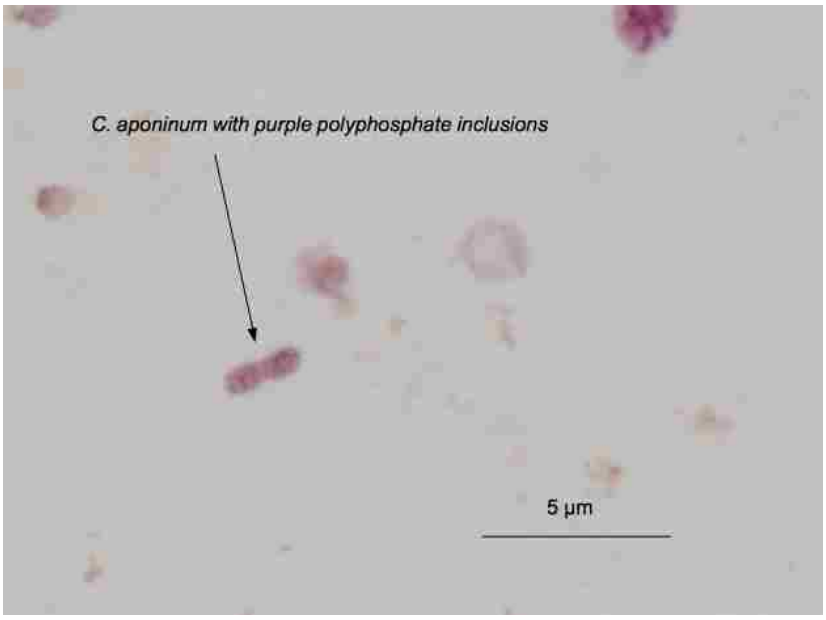

Figure 3.3. 41: Possible *C. aponinum* with purple Nessier stained polyphosphate (2000X).

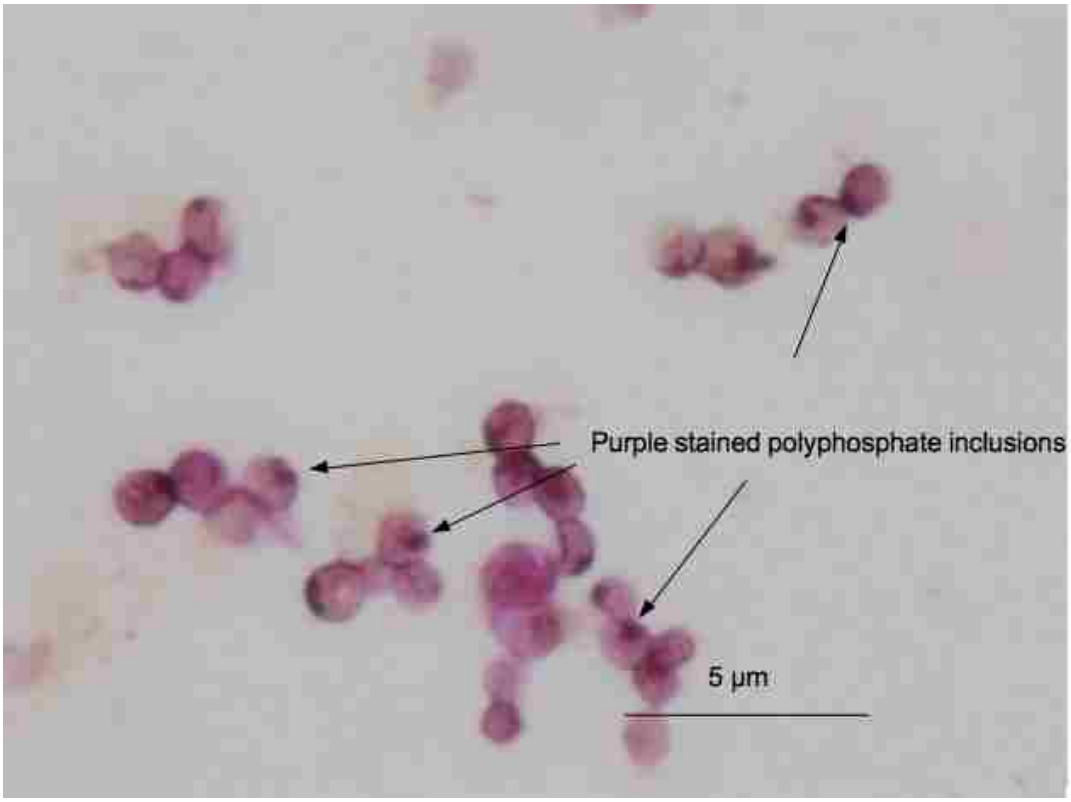

Figure 3.3. 42: Possible *P. kessleri* with purple Nessier stained polyphosphate (2000X).

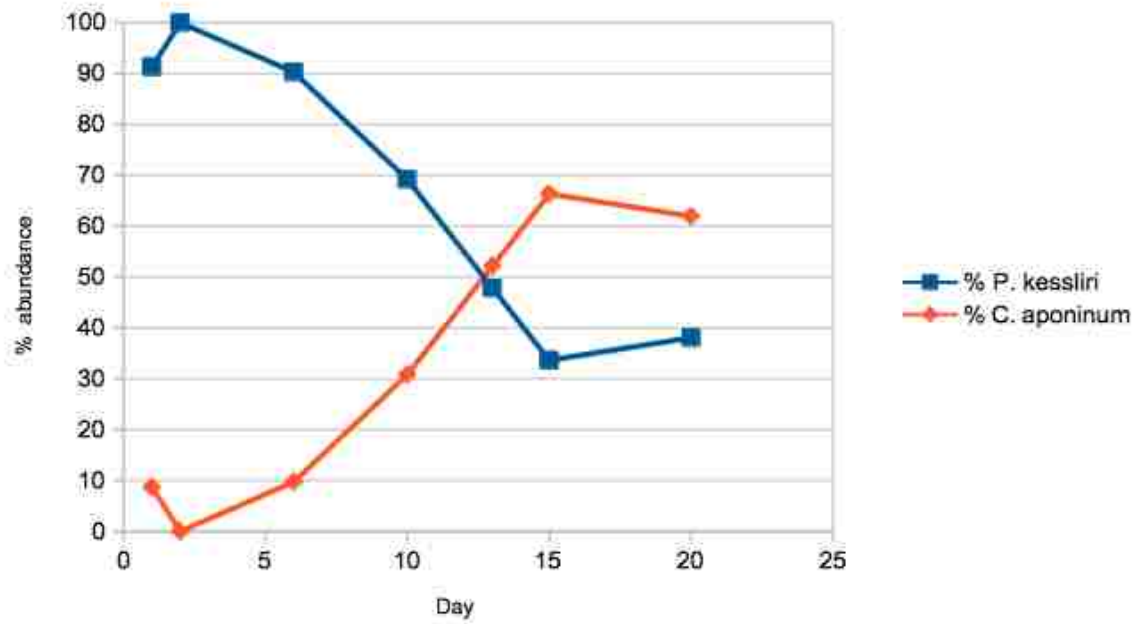

Figure 3.3. 43: Relative abundance of the possible *P. kessleri* and *C. aponinum* in the tubular reactor experiment with time under $NaHCO_3$ and CO_2 addition conditions.

3.4: *D. tertiolecta* Flask Experiments in PW (Exp. D1, D2, & D3)

3.4.1: *D. tertiolecta* flask experiment (D1) at a range of salinities in PW

D. tertiolecta was able to grow in PW at the full range of salinities tested (30 – 210 g TDS/L). At all salinity levels, flasks became yellowish green with growth (Figure 3.4. 1). After an initial lag phase of 1-2 days, growth was linear up to the stationary phase around Day 18-20 (Figure 3.4. 2). Larger biomass densities accumulated in the PW than the F/2 commercial media until day 16, at which point the F/2 caught up with the 210 g TDS/L salinity. The maximum AFDW concentration of the cultures was around 0.275 g AFDW/L for the salinity range from 30 – 180 g TDS/L. The 210 g TDS/L condition reached a lower peak biomass of 0.210 g AFDW/L. The reduced biomass productivity of the highest salinity condition could be due to greater nutrient and energy requirements for growth at almost 6X seawater ionic strength. Biomass growth rates were compared for days 2 to 10 (Figure 3.4. 3). The growth rates were similar from 30-120 g TDS/L at 16-17 mg/L/Day. The rate of biomass increase clearly is reduced for the 180 and 210 TDS/L conditions (13.7 and 8.8 mg/L/Day respectively) indicating salinity inhibition. Flasks became more yellow for the 30, 60, and 120 g TDS/L conditions and orange for 180 and 210 g TDS/L in the late phase of the experiment (Figure 3.4. 4). In general, the triplicate biomass had very small standard deviations (0.0121 g/L, or 6%, being the greatest).

The salinity threshold at which growth rate was inhibited was higher than previously reported for D. tertiolecta. Takagi et al. (2006) measured significant decreases in biomass concentration above 1 M NaCl (Figure 3.4. 5). Cell concentration

decreased by almost a factor of 10 between 58 g (1 M) and 116 (2 M) g TDS/L, while in this study little change was seen (between 60 and 120 g TDS/L). Jahnke and White (2003) also reported a significant decrease in growth (divisions per day) at a salinity lower than 120 g TDS/L of 58 g NaCl/L (1 M). Cell division rates decreased by around 30% between 0.5 M (about 30 g TDS/L) and 2 M (around 120 g TDS/L) salinities. Gonzalez et al., (2009) presented data for two different strains of *D. tertiolecta* showing the peak division rate at seawater salinity (3.5%), which decreases linearly with increasing NaCl concentration. A possible reason for the difference is that the PW in this experiment had a higher concentration of bicarbonate (20 mM) than the seawater values (~ 2 mM) that are commonly studied. Carbon dioxide is less soluble at higher salinity, which would have an adverse effect on *D. tertiolecta's* growth rate. As the ionic strength of the medium increases, *Dunaliella* produces carbonic anhydrase membrane proteins that can capture HCO_3^- ions (Fisher et al., 1996). A significant proportion of carbon uptake is used to synthesize glycerol to maintain osmotic pressure, one key to maintaining cellular metabolism at high ionic strength (Oren, 2005). The presence of bicarbonate in the PW media in this study might also explain the higher growth rate than the standard F/2 media where bicarbonate is approximately 1.9 mM (alkalinity of the instant ocean salt). Ginzburg & Ginzburg (1981) also came to the conclusion that bicarbonate increases growth rate at high ionic strength, after studying a number of species in *Dunaliella*. A bicarbonate rich media may be the key to maintaining *Dunaliella's* growth rate in hypersaline PW for biofuel production.

Nutrient removal from the media was linked to growth as expected. The faster growing 30, 60, and 120 g TDS/L conditions removed nitrate by day 8 to 9, while at the higher salinity conditions this occurred at around Day 11 (Figure 3.4. 6). Phosphate was removed 1 or 2 days earlier in each salinity condition (Figure 3.4. 7). Depletion of nitrate and phosphate did not immediately change the growth rate at any salinity (Figure 3.4. 8, Figure 3.4. 9, Figure 3.4. 10, & Figure 3.4. 11). The growth rate remained relatively steady for all conditions until approximately day 15, when growth slowed with the onset of a stationary phase. As suggested in the Polyculture experiment discussion previously, this may have indicated nutrient storage that did not slow biomass productivity for several days after the absence of N and P nutrients in the media. This is different than the results reported in Takagi et al. (2006), where nitrate removal coincided with a plateau in optical density. The amount of nutrients consumed to reach the biomass concentration at stationary growth (Day 15-18) was 0.0473 g NO_3-N/g AFDW and 0.00625 g PO_4-P/g AFDW approximately (for the salinity conditions 30 – 180 g TDS/L). The 210 g TDS/L condition had a higher nutrient requirement of 0.0619 g NO_3-N/g AFDW and 0.00833 g PO_4-P/g AFDW. It can be inferred that higher salinity growth will not be as productive on available nitrogen and phosphorus nutrients. Overall, *D. tertiolecta* growth effectively removed nutrients from the hypersaline PW media.

The pH (measured during the light phase) increased for all cultures up to around Day 6, at which point it leveled off and then decreased after Day 12 (

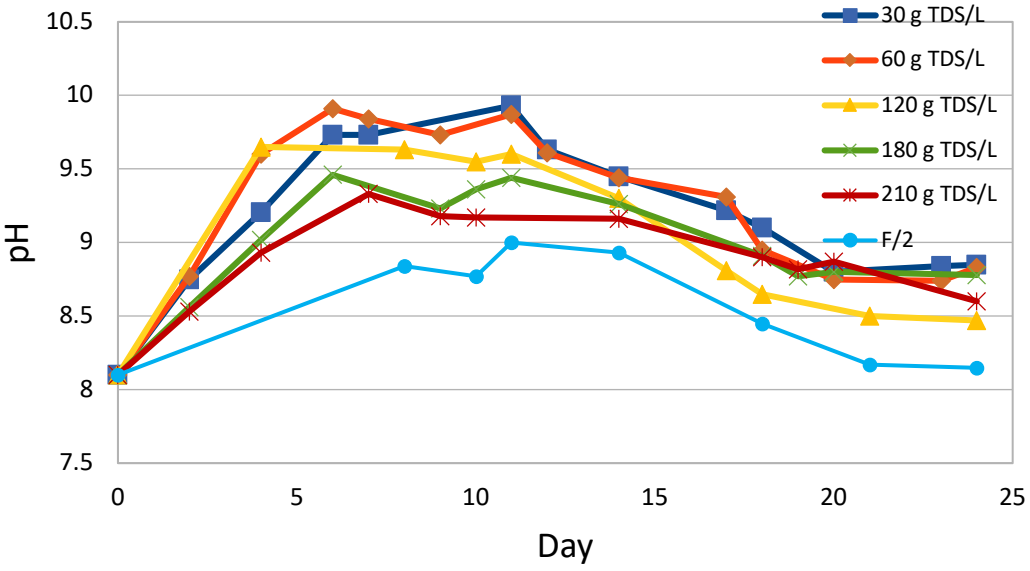

Figure 3.4. 12). The maximum pH reached was higher in the lower salinity 30 and 60 g TDS/L flasks at between 9.8 and 9.6. At higher salinity maximum pH was somewhat lower at 9.6 9.4 and 9.2 in 120, 180, and 210 g TDS/L respectively. It is possible that this reflects less carbon uptake at higher salinities. Inorganic carbon in the cell is mostly in the form of HCO_3^- (Raven, 2009). Enzymes in the chloroplasts convert HCO_3^- into CO_2 gas to increase concentrations so that the enzyme RUBISCO can fix carbon into sugar as part of the Calvin cycle. Carbonic anhydrase in the cell membrane of *Dunaliella* also removes bicarbonate from solution while synporting H^+ ions into the cytoplasm (Oren, 2005). The end result is an increase in pH linked to photosynthetic activity and growth (Aravinthan and Harrington, 2014). Another potential reason for the lower pH at higher salinity is the reduced activity of released H^+ ions in solution due to the increased ionic strength. The decrease in the late stage of the experiment is likely linked to decreased

photosynthetic activity of the stressed algae and is associated with a less (chlorophyll) more carotenoid (to shield the light reaction centers) appearance of the cultures. Photosynthetic activity and growth are also linked to nitrate uptake which consumes H^+ further (Goldman et al. 1980), though this effect is less significant since peak pH occurs well after the days with the highest nitrate uptake. The pH increase might encourage growth further, in that atmospheric CO_2 would be more soluble in alkaline conditions. Alkaline conditions up to a pH of 10 do not appear to inhibit growth and might encourage it because total dissolved inorganic carbon increases with pH.

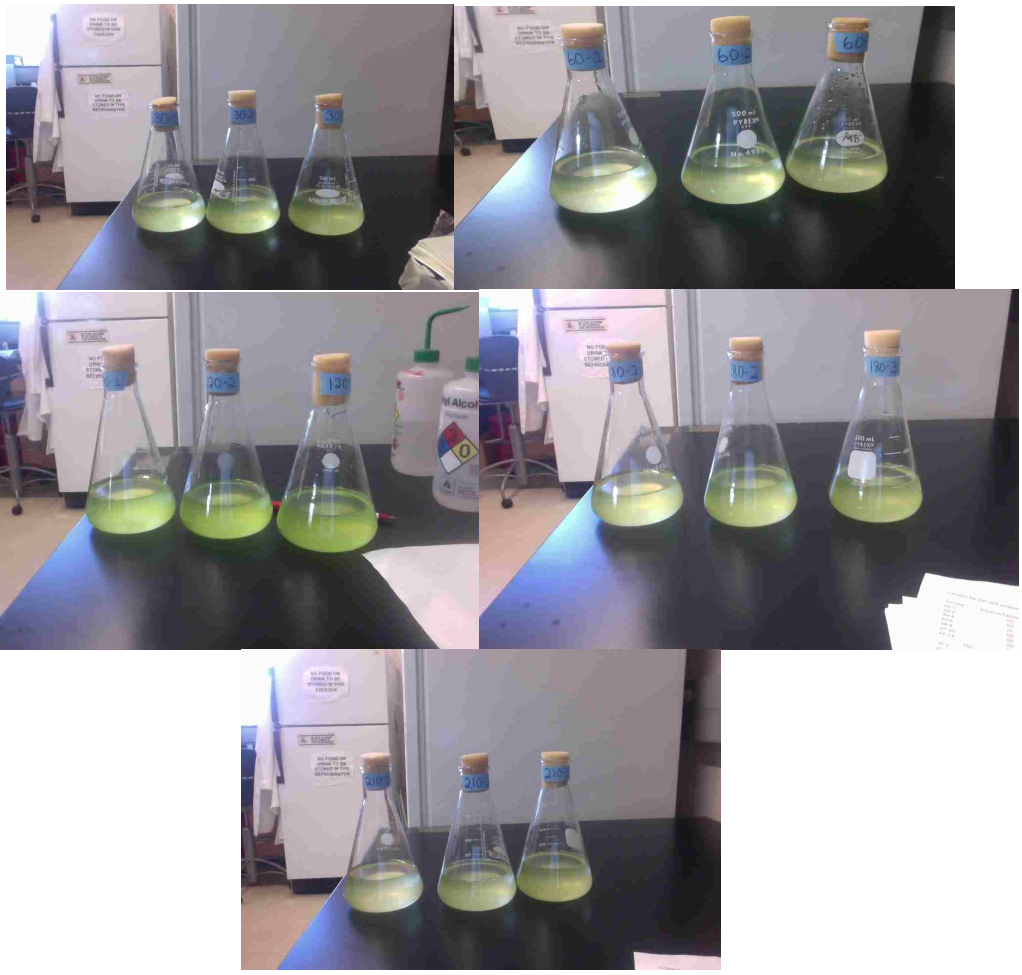

Figure 3.4. 1: *D. tertiolecta* growing in PW at (left to right and top to bottom) 30, 60, 120, 180, and 210 g TDS/L (Exp. D1).

Yellowish green typical of early cultures at all salinities.

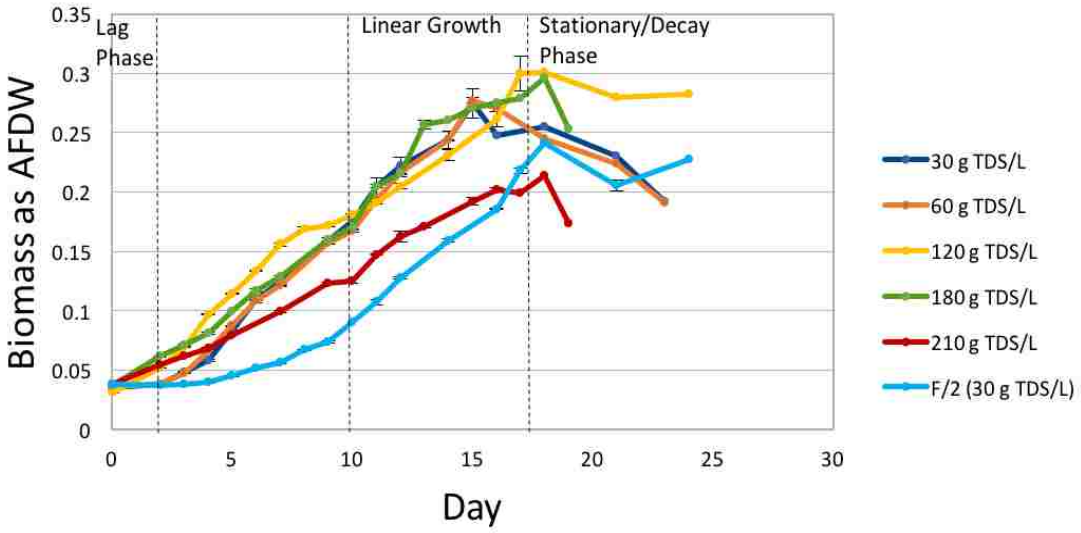

Figure 3.4. 2: *D. tertiolecta* biomass measurements at a range of salinities in a PW medium (Exp. D1).

F/2 commercial media is included for comparison and nutrient levels (nitrate, phosphate, and trace metals are equal). (Note: as flask volume is decreased triplicates are combined in the late stage of growth, thus no error bars)

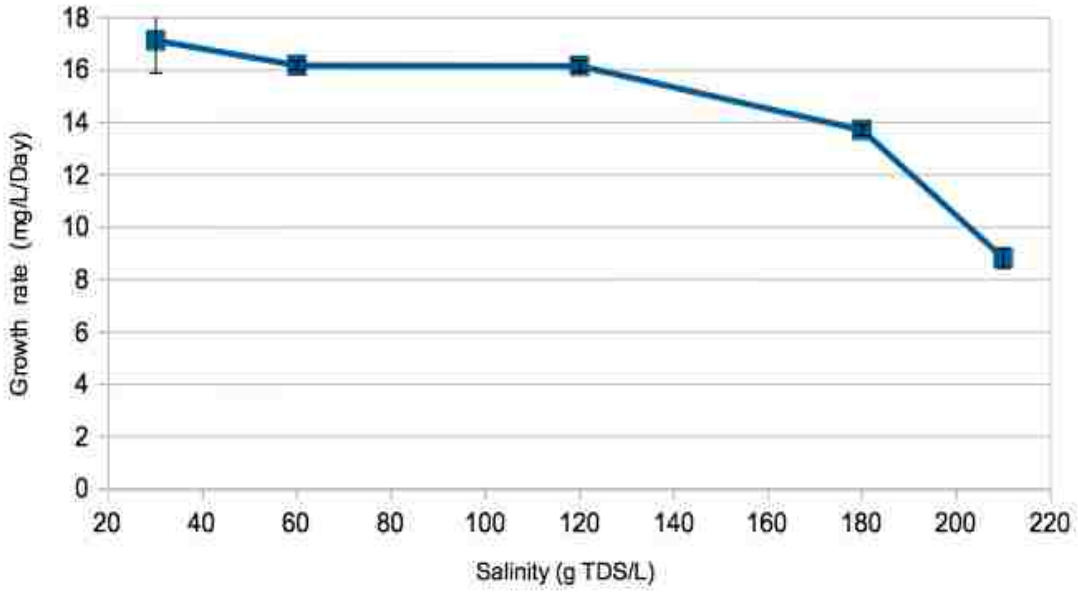

Figure 3.4. 3: *D. tertiolecta* growth rates (based on the biomass increase from Day 2 to 10) in PW at a range of salinities (Exp. D1).

Figure 3.4. 4: *D. tertiolecta* flasks in stationary phase Day 23 (Exp. D1).

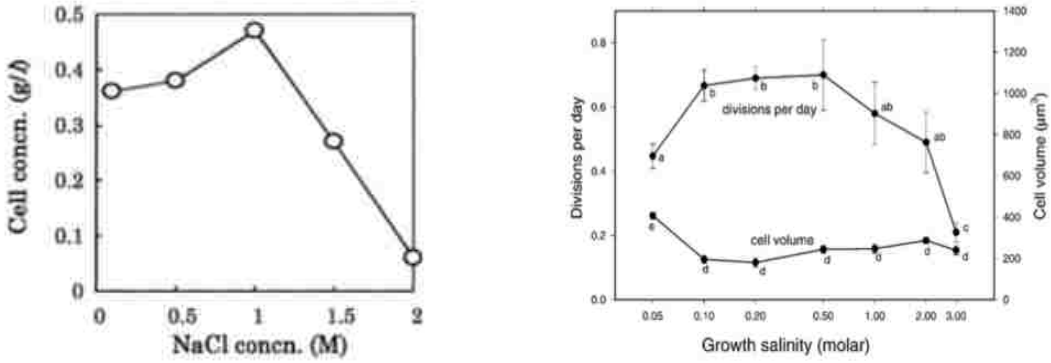

Figure 3.4. 5: Cell Concentration vs. NaCl concentration.

From Takagi et al., (2006) (left) and Divisions per Day vs. Salinity from Jahnke and White (2003) (right).

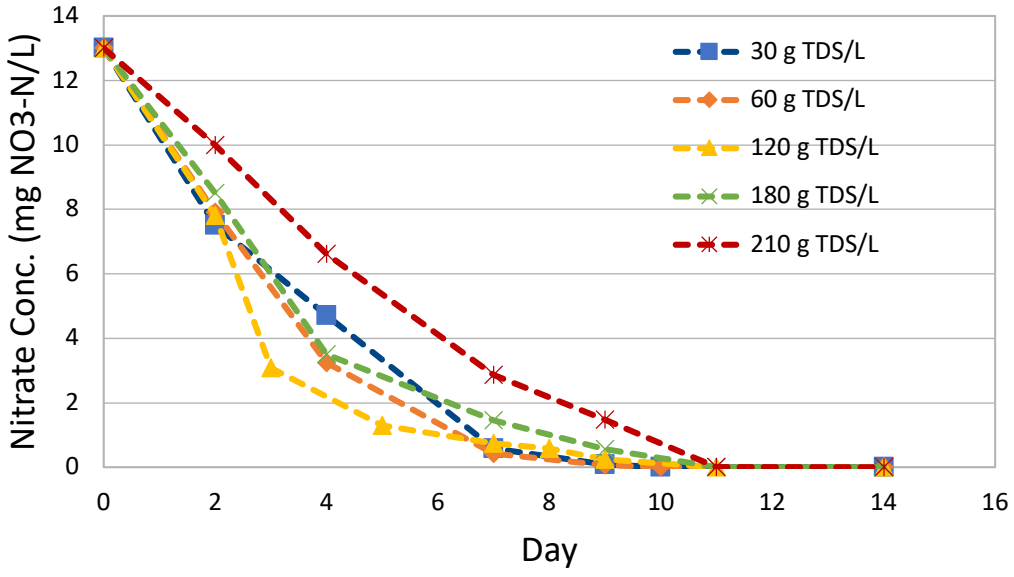

Figure 3.4. 6: Nitrate concentrations during *D. tertiolecta* growth in PW at a range of salinities (Exp. D1).

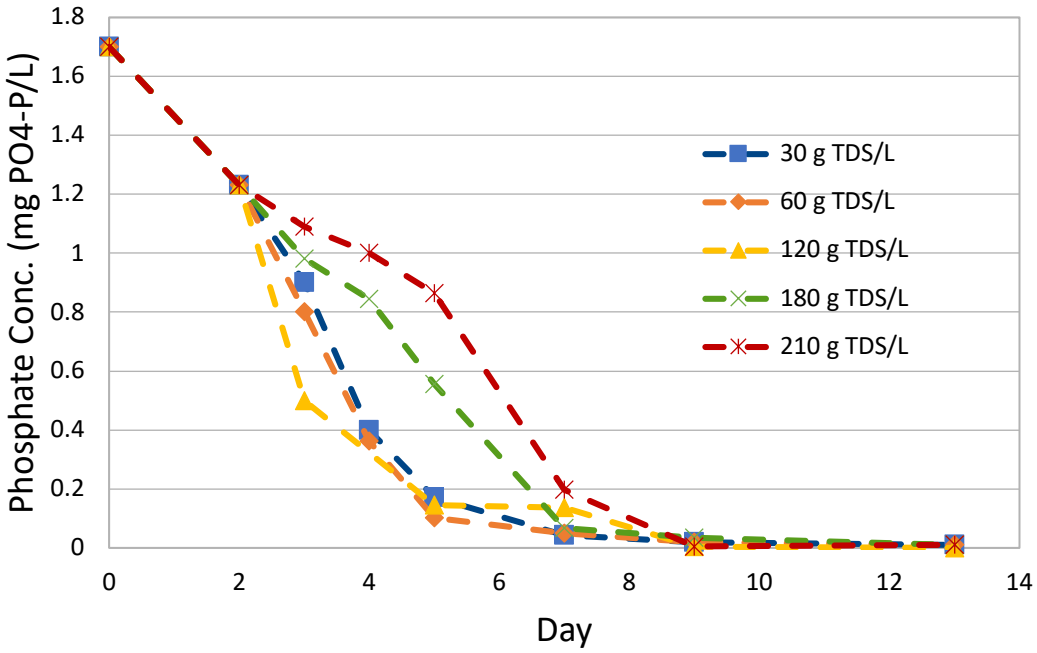

Figure 3.4. 7: Phosphate concentrations during *D. tertiolecta* growth in PW at a range of salinities (Exp. D1).

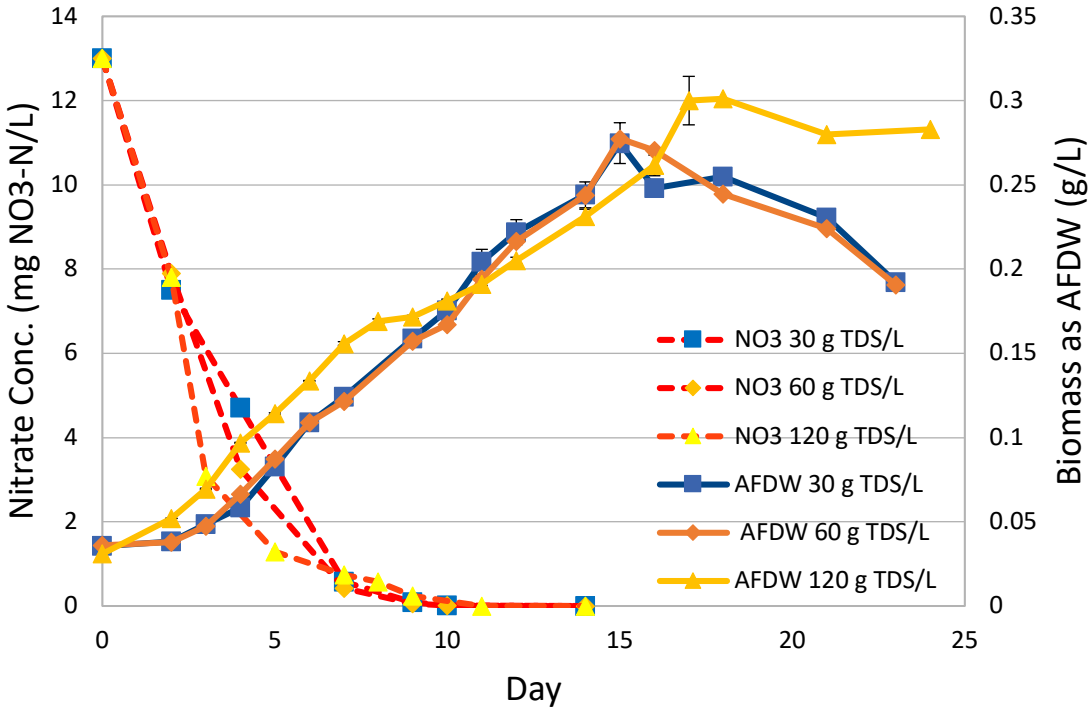

Figure 3.4. 8: *D. tertiolecta* flask experiment PW media nitrate and AFDW concentrations for the salinity conditions 30, 60, and 120 g TDS/L (Exp. D1).

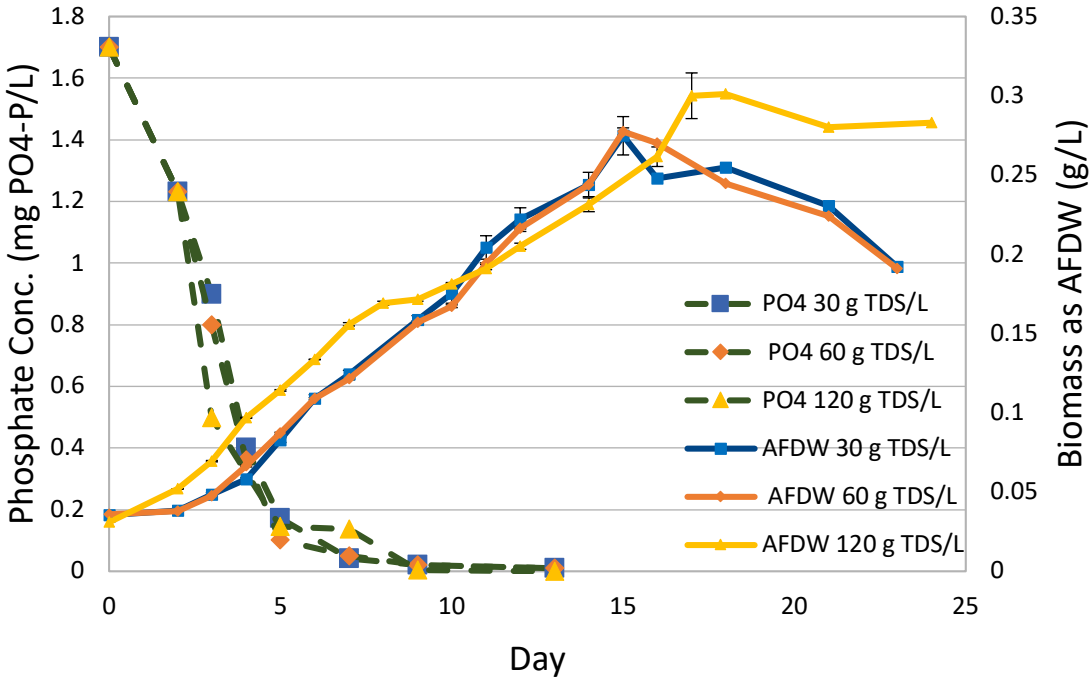

Figure 3.4. 9: *D. tertiolecta* flask experiment PW media phosphate and AFDW concentrations the salinity conditions 30, 60, and 120 g TDS/L (Exp. D1).

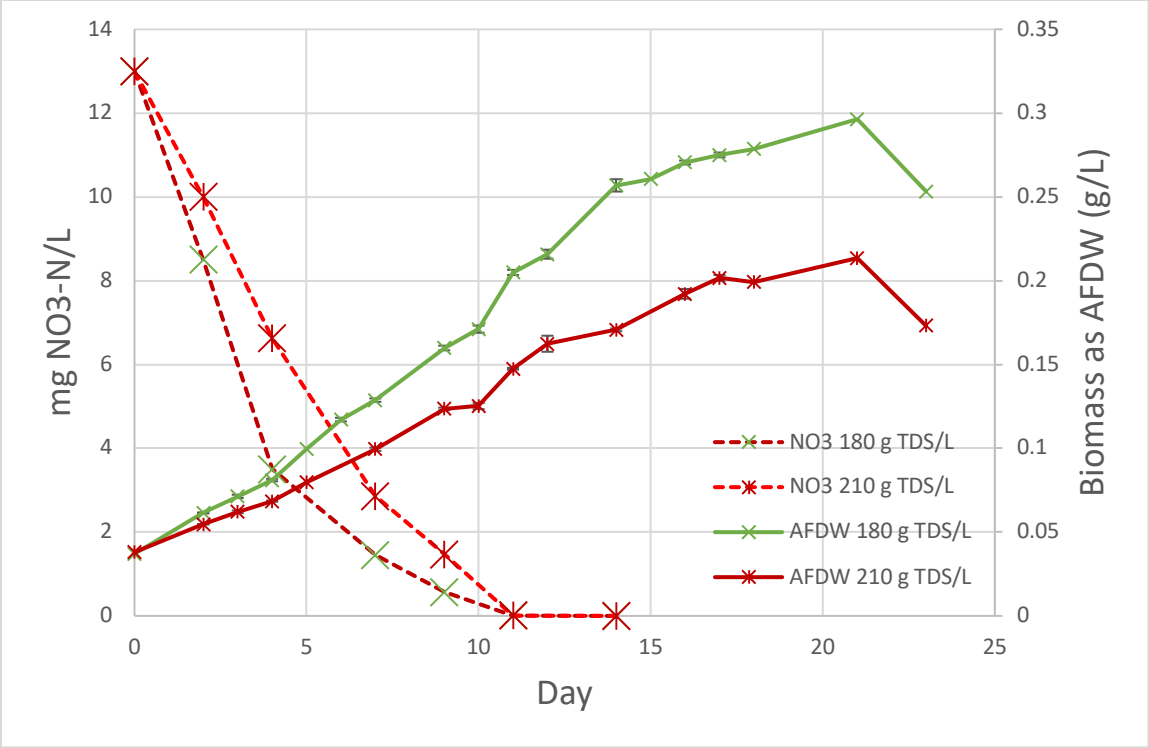

Figure 3.4. 10: *D. tertiolecta* flask experiment PW media nitrate and AFDW concentrations for salinity conditions 180 and 210 g TDS/L (Exp. D1).

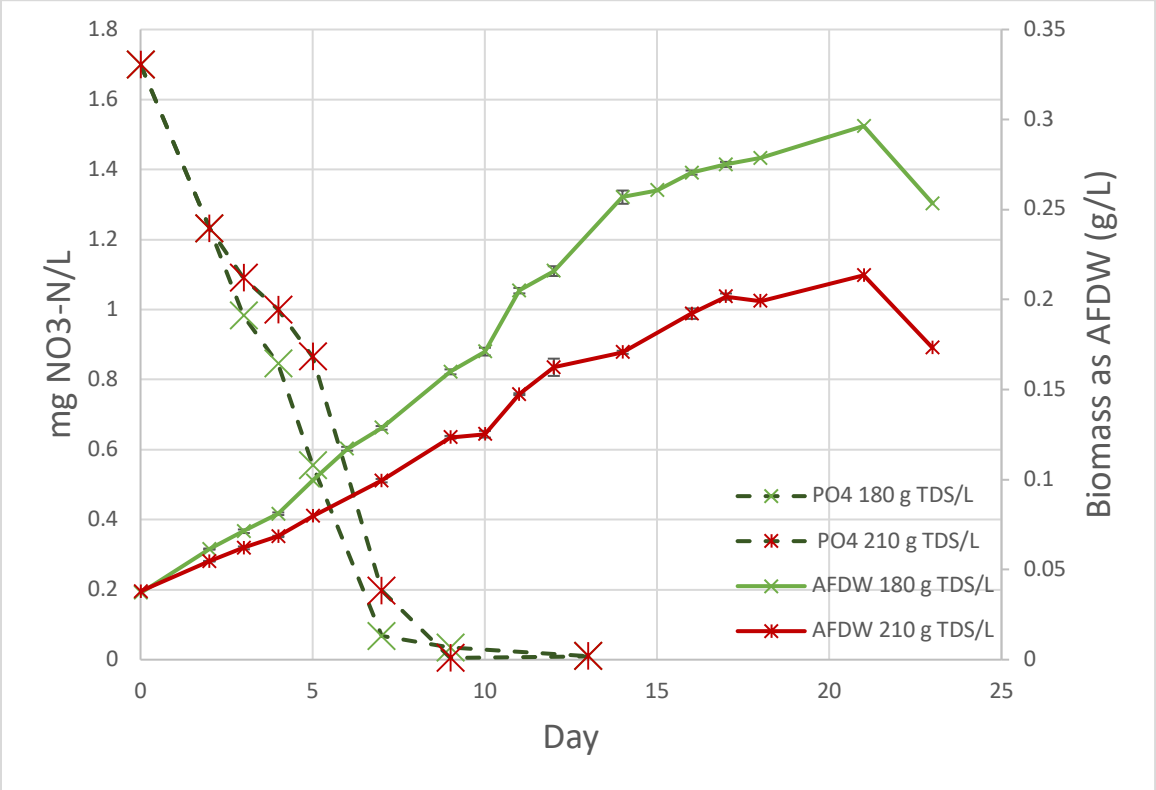

Figure 3.4. 11: *D. tertiolecta* flask experiment PW media phosphate and AFDW concentrations for salinity conditions 180 and 210 g TDS/L (Exp. D1).

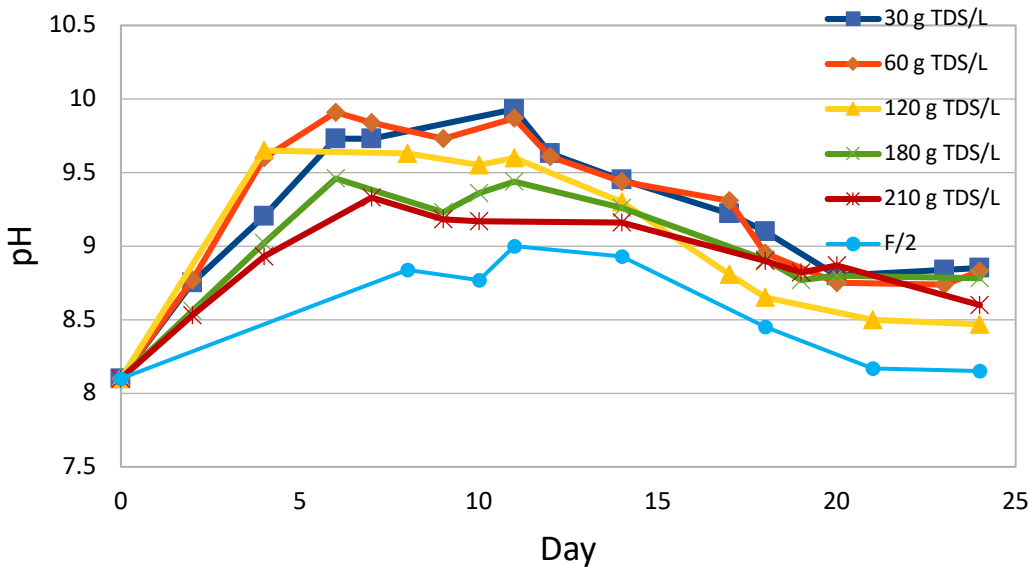

Figure 3.4. 12: *D. tertiolecta* PW media with nitrate flask experiment pH measurements at a range of salinities (Exp. D1).

3.4.2: D. tertiolecta lipid Productivity and character at varying salinities in PW (Exp. D1)

D. tertiolecta's lipid productivity varied with salinity in the PW media (Figure 3.4. 13 & Figure 3.4. 14). The 30 and 60 g TDS/L flasks followed a different pattern than those of the 120, 180, and 210 TDS with higher initial rates. In the lower salinity conditions (30 and 60 TDS), lipid concentration reached a peak at Day 8 of close to 0.06 g/L, while lipid content peaked at 40% on day 5. From day 8 to 15 lipid concentration and its percentage of biomass then decreased to a low value of around 0.045 g/L. after day 15, coinciding with the culture reaching stationary phase, lipid concentration increased until the end of the experiment reaching the max value of 0.070 g/L (Figure 3.4. 15 & Figure 3.4. 16). The 120, 180, and 210 g TDS/L conditions did not exhibit this early bump in lipid content (Figure 3.4. 17 & Figure 3.4. 18). For these flasks lipid

concentration and content increased at a somewhat constant rate until the culture entered stationary phase on day 17. For the 120 and 180 g TDS/L conditions there was a decrease in lipids by day 21. Over the course of the experiment the lipid content varied between 20 and 35% for the mid and high range salinities tested. Overall, the lipid content of biomass was within the range of many reports in the literature for *D.* tertiolecta, for example 22% in Aravinthan & Harrington, (2013), 36-42% in Tsukahara & Sawayama (2005), 20-23% in Tang et al., (2011), and 37% in Tafreshi & Shariati (2008). Values approaching 70% published in Takagi et al. (2006) through salt stress was not observed in this study.

Nitrogen starvation of an algal culture is an often-cited method for inducing lipid production, and there have been conflicting claims whether lipid productivity can be increased for *D. tertiolecta* under nitrogen stressed conditions (Huesemann and Benemann, 2009). The author has not found any published results testing the effect on lipids due to nitrogen starvation at salinities higher than seawater (About 35 g TDS/L). Chen et al. (2011) reported a time dependent lipid content increase in a nitrate deplete media for *D. tertiolecta* on day 3 that disappeared by day 7. In that study, overall lipid production was highest in a standard nitrate medium after 7 days due to higher growth rate despite a reduction in lipid content in the nitrate depleted condition from day 3 to 7. Huesemann and Benemann (2009) state that nitrate limitation has been shown to slightly decrease lipid content in *D. tertiolecta* in some studies. In the closely related *Dunaliella salina*, Weldy and Huesemann (2007) demonstrated that nitrogen limitation only slightly boosted lipid content (from 38% to 44%) with a one third reduction in lipid

productivity due to decreased biomass growth. Overall, growth under nitrogen deficient conditions has not been shown to increase lipid productivity which would improve the economics of biofuel conversion.

In this experiment lipid content decreased shortly after nitrate depletion in the 30 and 60 g TDS/L conditions (Figure 3.4. 19 & Figure 3.4. 20). However, after day 15 the lipid content began to increase again, with a maximum concentration on day 21. The peak in lipid concentration and content at the point of nitrate depletion might explain the time dependent increase in lipid content reported in Chen et al. (2011). The later stationary phase lipid enrichment after nitrate depletion would not increase the overall lipid productivity at these lower salinity ranges. For peak lipid productivities maintaining nitrogen in the media would be required at these lower salinity levels. Another important conclusion is there is large variation in lipid content over the course of the experiment which might have ramifications for biomass conversion to fuel.

The early lipid peak observed in the 30 and 60 g TDS/L conditions coincided with that of pH. Figure 3.4. 21 & Figure 3.4. 22 show how peak lipid content and pH both occur on day 8 of the experiment. Since carbon uptake dominates changes to pH this can be assumed to the time of maximum sugar synthesis for algae cells. A portion of this surplus fixed carbon appears to be being converted in lipid molecules. This would be consistent with algae cells storing carbon as energy for later use ("fattening up"). It seems that nutrient depletion in the media (C, N, and/or P) reduces carbon fixation after day 8. As a result, algae cells tap into their fat reserves to maintain biomass growth.

Maintaining culture conditions during this peak might be a key to optimizing lipid productivity.

In the 120, 180, and 210 g TDS/L salinities the lipid content of biomass does increase after nitrate depletion but does not seem to greatly enhance productivity (Figure 3.4. 23 & Figure 3.4. 24). The lipid content of the biomass only increased modestly from 25-35% of biomass after day 11. Upon reaching the maximum biomass concentration around day 16, lipid content either decreased (120 and 180 g TDS/L flasks) or leveled off (210 g TDS/L). Maintaining nitrate concentrations in the media may yield higher lipid productivity by maximizing biomass growth. There was no clear advantage to harvesting the algae biomass after growth in nitrate depleted conditions at or above 120 g TDS/L. No early peak was seen as compared to the lower salinity conditions of 30 and 60 g TDS/L. It is possible that glycerol production to maintain osmotic balance directed fixed carbon away from lipid synthesis. It is also possible that there is a decreased energy surplus of the cells at higher salinity that can be stored as triglycerides.

The variation in time dependence of lipid productivity for the low (30 and 60 g TDS/L) and high (120, 180, and 210 g TDS/L) has implications for when to harvest the biomass for peak lipid content. For the lower salinity conditions of 30 and 60 g TDS/L the *D. tertiolecta* biomass should be removed at or just after nitrate depletion in the media after maximizing the growth rate with sufficient nutrients (Figure 3.4. 25). This would maximize the lipid productivity at a value of 6.7 mg/L/Day and 6.3 mg/L/Day (Figure 3.4. 26). At salinities at or above 120 g TDS/L biomass would reach peak lipid

content and concentration once the culture reaches a stationary phase. Once biomass growth ceases harvesting would be warranted before decay in the lipid fraction of biomass.

The types of fatty acid chains in triglycerides converted to methyl esters (FAME) indicated that the D. tertiolecta biomass grown in PW was suitable for biodiesel and was consistent in character. The FAME chromatograms displayed distinct peaks for palmitic (C16:0), oleic (C18:1), linoleic (C18:2), and α - γ linolenic acid (C18:3) (Figure 3.4. 27). Palmitic acid dominated the percentage of total FAME for each salinity over the duration of the experiment (analytical problems arose for the 210 g TDS/L lipid samples and only day 6 displayed distinct peaks). The percentage of palmitic acid varied from 45-75% in the analyses (Figure 3.4. 28, Figure 3.4. 29, Figure 3.4. 30, & Figure 3.4. 31). Peak palmitic acid between day 12-15 is consistent for the range of salinities. The remaining fraction of FAME is split between the carbon chain lengths C18:1, C18:2, and C18:3 which vary from 5-30%. The polyunsaturated C18:3 is usually the second most dominant with no clear pattern over time or with ionic strength, except that it varies inversely with C16:0. The character of FAME is important in determining the ability of a fuel to auto ignite and is represented by the cetane number (Fahkry and Maghraby, 2013). The dominance of palmitic acid would improve the quality of biodiesel derived from the *D. tertiolecta* biomass yielding better performance for a cold start, lower nitrous oxide emissions, and smoother engine performance. The lowest cetane value calculated was 59.3 with a high of 74, which are all significantly higher than the minimum biofuel standard of 47 (Racharaks et al., 2015). Larger amounts of the

unsaturated C16:0 would also improve the oxidative stability of the derived biodiesel compared to unsaturated fatty acids. Importantly the FAME profile at different salinities and over time remains fairly consistent and favorable for conversion to biodiesel.

The FAME profiles of *D. tertiolecta* measured here are more conducive to biodiesel quality that those measured in other studies. A number of published results found linolenic acid (C18:3) as the dominant FAME for *D. tertiolecta* (Racharaks et al., 2015), (Tang et al., 2011), Chen et al., 2011). Figure 3.4. 32 displays the fatty acid profiles typical of these studies with linolenic acid representing from 32 to 45% with a corresponding decrease in fraction represented by palmitic acid. A higher linolenic acid component would decrease the cetane number and oxidative stability of the derived biodiesel. The reason for the difference in the fatty acid profile found in this study is unclear. It could represent a difference in the *D. tertiolecta* strain. Both Chen et al., (2011) and Tang et l. (2011) were most likely using the same strain since studies were conducted in the same lab (University of Texas, UTEX LB999). The strain used in Racharaks et al. (2015) was obtained from Scotland. The strain used in this study was originally obtained from UTEX, but it is possible genetic differences exist.

In summary, the results show that *D. tertiolecta* can be cultivated in a PW medium and produce biomass of sufficient lipid content and character for biodiesel production. The highest lipid content achieved was over 40% and would make a practical feedstock for hydrothermal liquefaction or transesterification to biodiesel. Cultivation strategies would best focus on maintaining high growth rates rather that

limiting nutrients for lipid enrichment. The FAME profile is consistent over a wide range in salinity (30 – 180 g TDS/L) and would create a diesel fuel with a high cetane number and oxidative stability. Further research could focus on using salinity stress to increase lipid content in a dense biomass as was reported in Takagi et al. (2006).

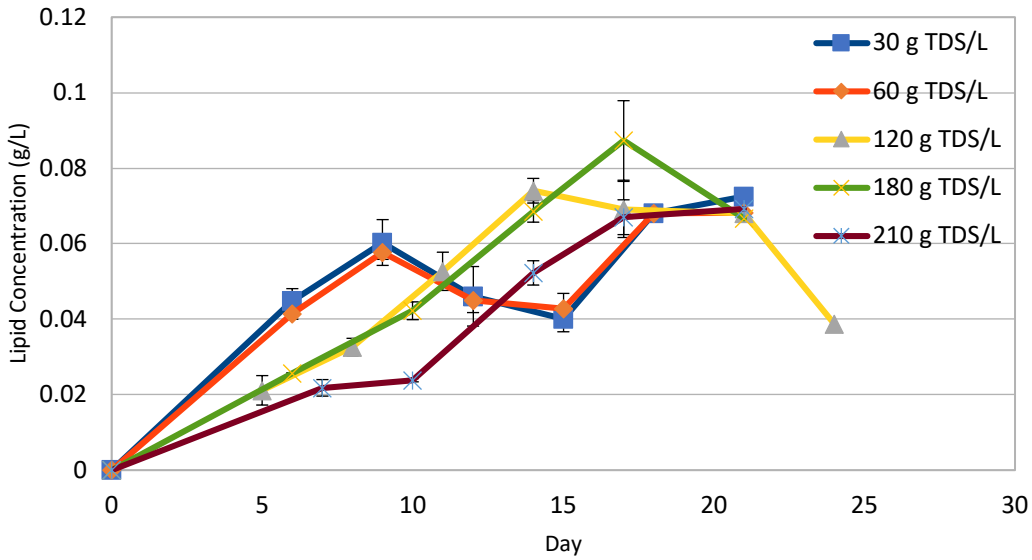

Figure 3.4. 13: *D. tertiolecta* PW flask experiment lipid concentration measurements (Exp. D1)

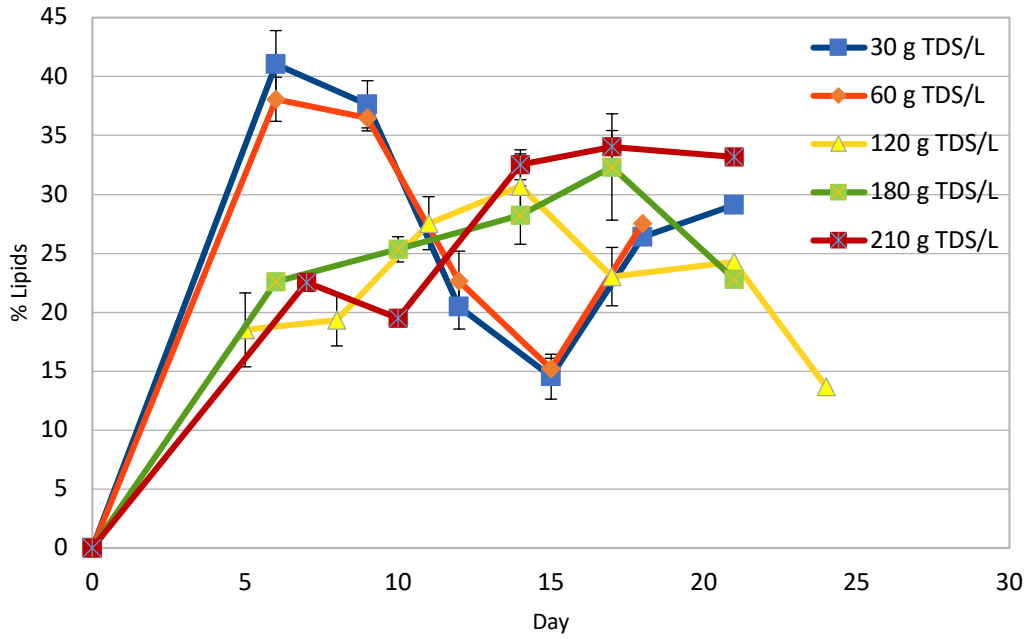

Figure 3.4. 14: *D. tertiolecta* PW flask experiment (Exp. D1) lipid content of biomass using nitrate as a nitrogen source (initial 13 mg NO_3-N/L).

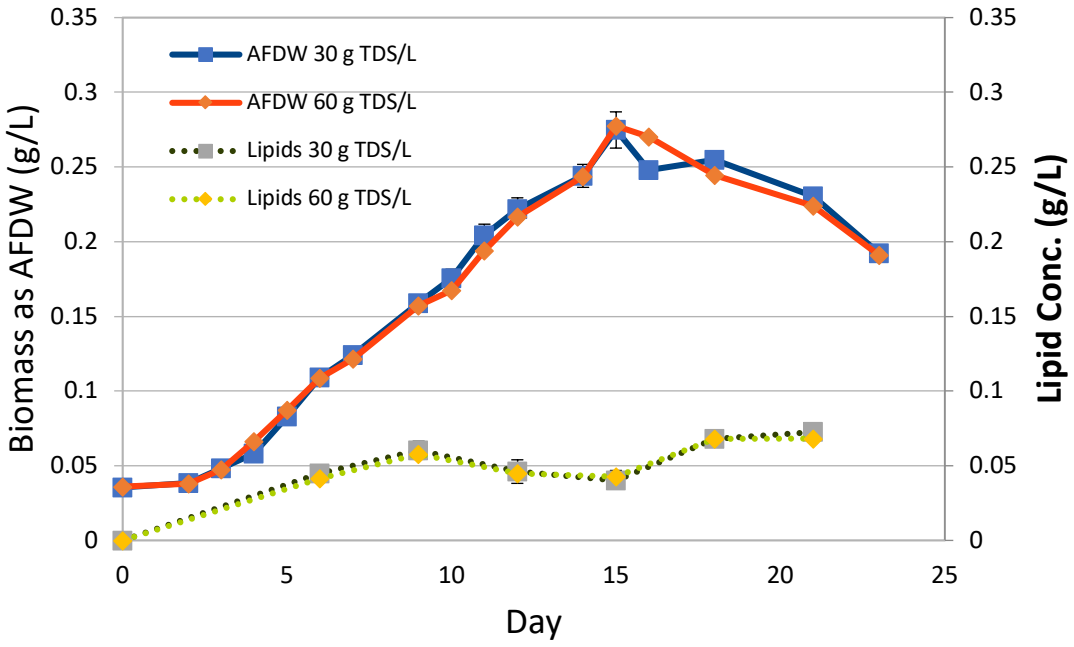

Figure 3.4. 15: *D. tertiolecta* PW flask experiment (Exp. D1) biomass and lipid concentration.

Nitrate was used as a nitrogen source (initial 13 mg NO_3-N/L) for 30 and 60 g TDS/L conditions.

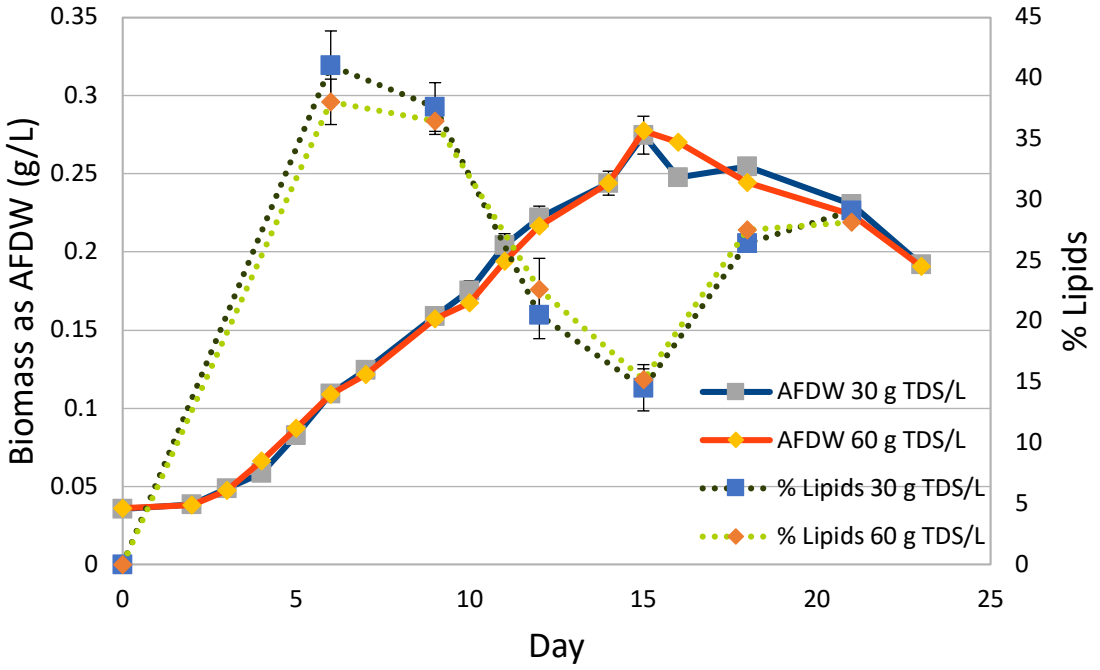

Figure 3.4. 16: *D. tertiolecta* PW flask experiment (Exp. D1) biomass concentration and lipid content for the 30 and 60 g TDS/L conditions.

Nitrate was used as a nitrogen source (13 mg NO_3-N/L).

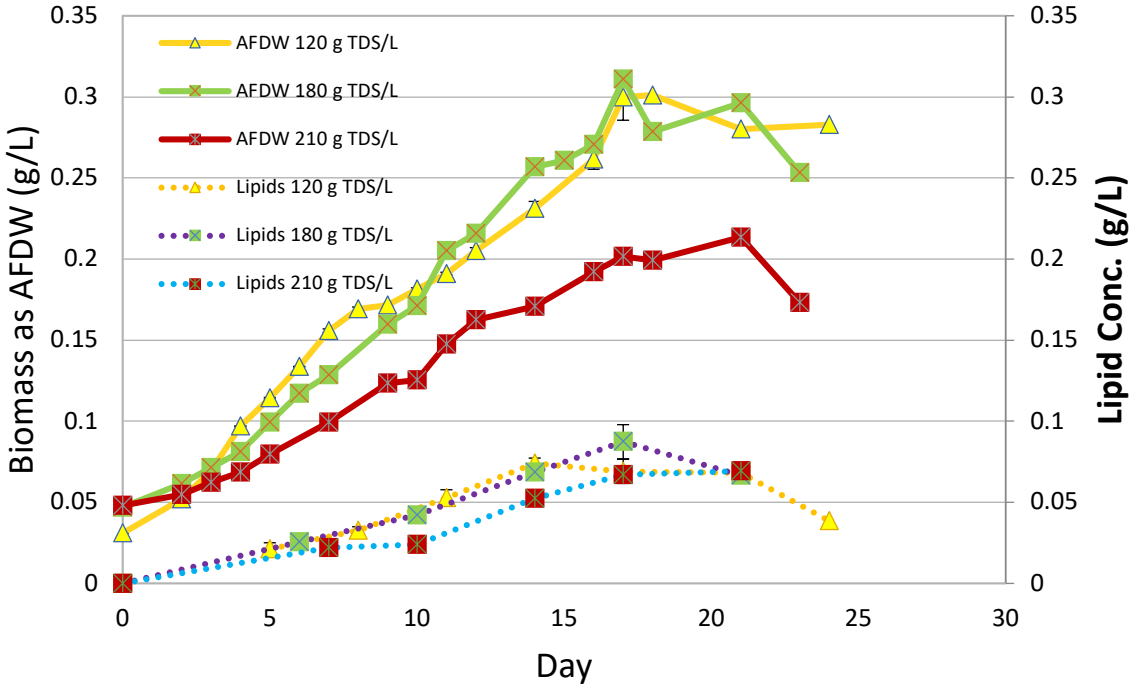

Figure 3.4. 17: *D. tertiolecta* PW flask experiment (Exp. D1) biomass and lipid concentration for 120, 180, and 210 g TDS/L conditions.

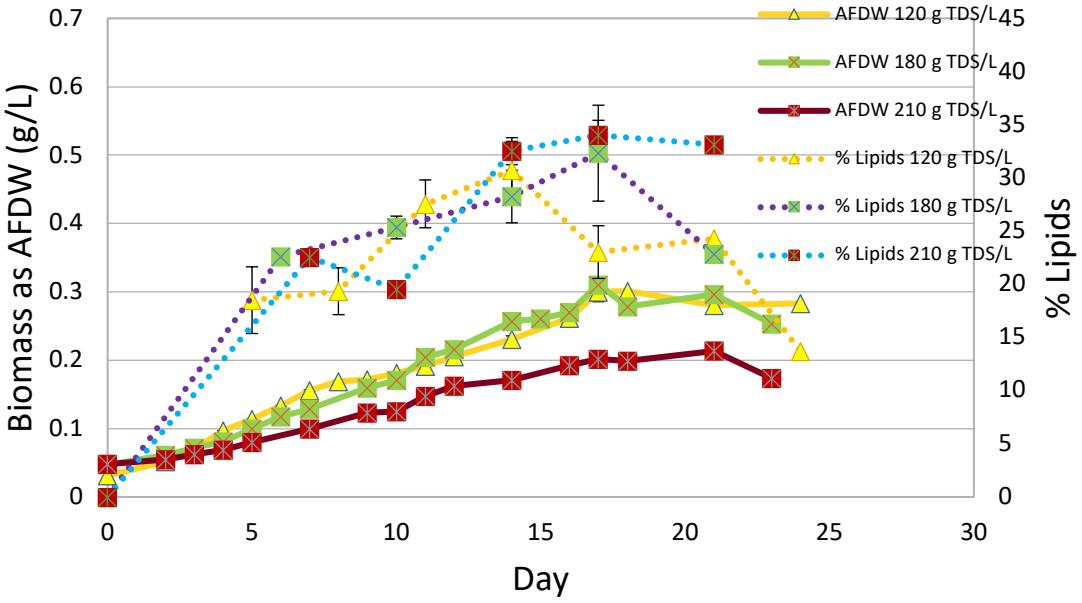

Figure 3.4. 18: *D. tertiolecta* PW flask experiment (Exp. D1) biomass concentration and lipid content for 120, 180, and 210 g TDS/L conditions.

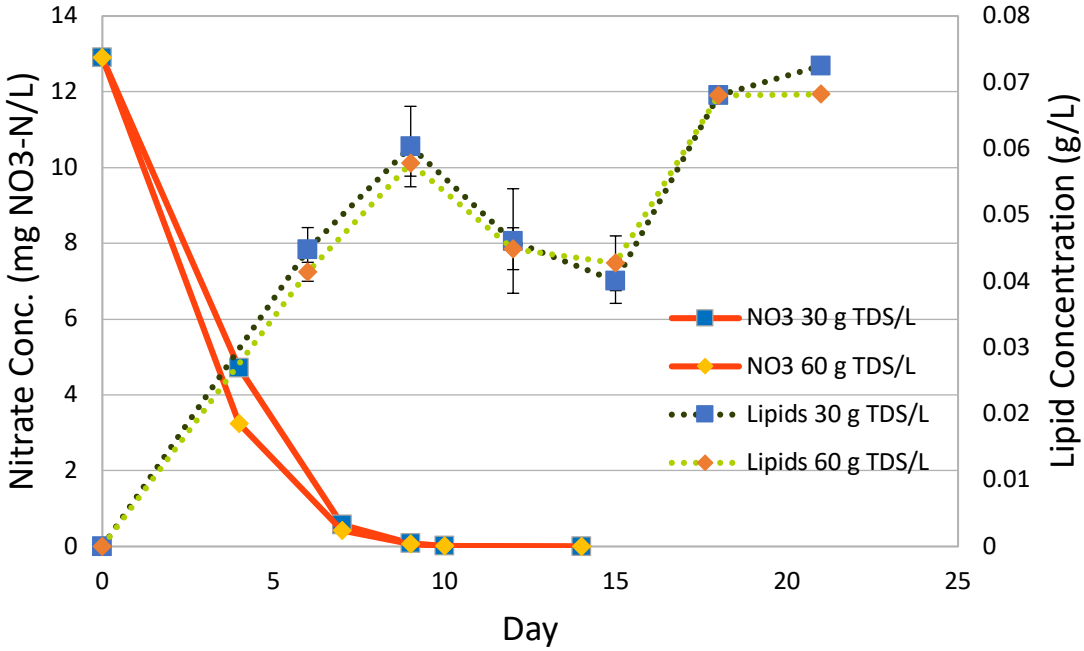

Figure 3.4. 19: *D. tertiolecta* PW flask experiment (Exp. D1) lipid and nitrate concentrations for the 30 and 60 g TDS/L conditions.

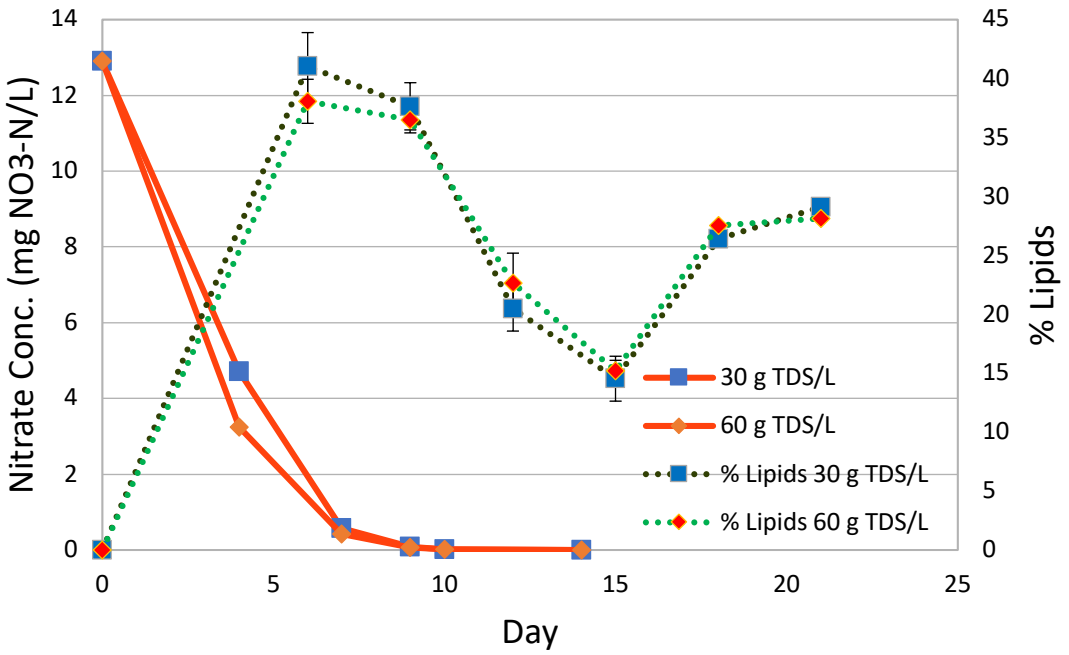

Figure 3.4. 20: *D. tertiolecta* nitrate PW flask experiment (Exp. D1) lipid content and nitrate concentration for the 30 and 60 g TDS/L conditions.

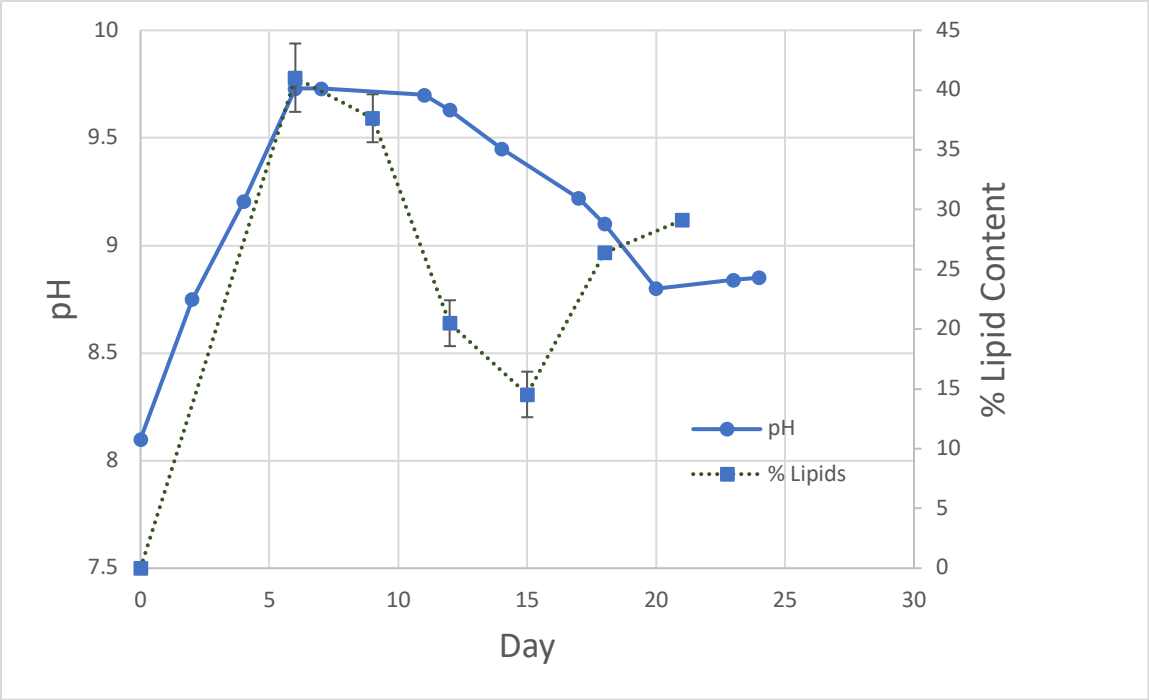

Figure 3.4. 21: *D. tertiolecta* pH and lipid content measurements in 30 g TDS/L PW media.

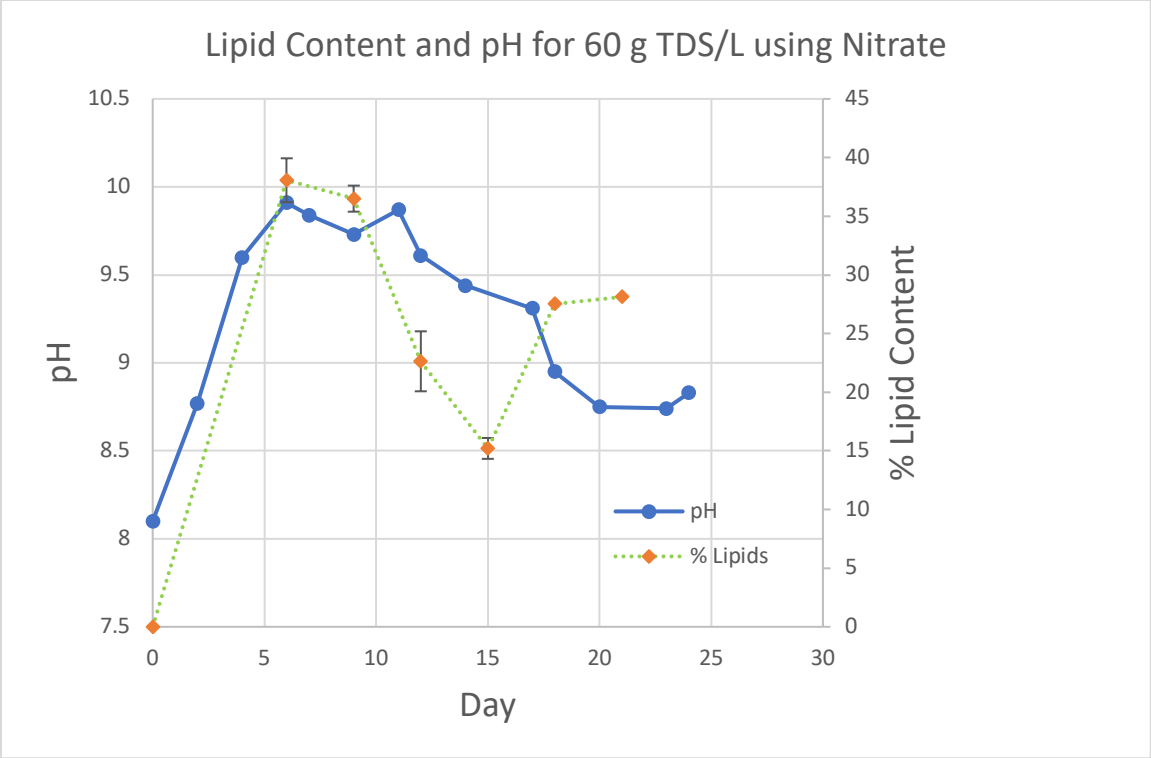

Figure 3.4. 22: *D. tertiolecta* pH and lipid content measurements in 60 g TDS/L PW media.

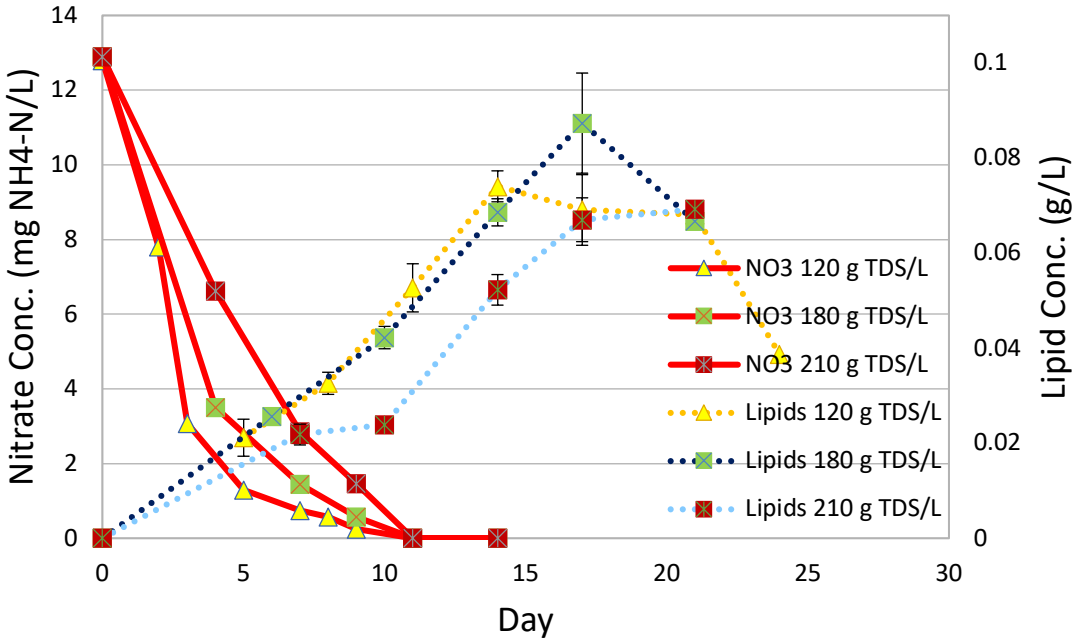

Figure 3.4. 23: *D. tertiolecta* PW flask experiment (Exp. D1) nitrate and lipid concentration for 120, 180, and 210 g TDS/L conditions.

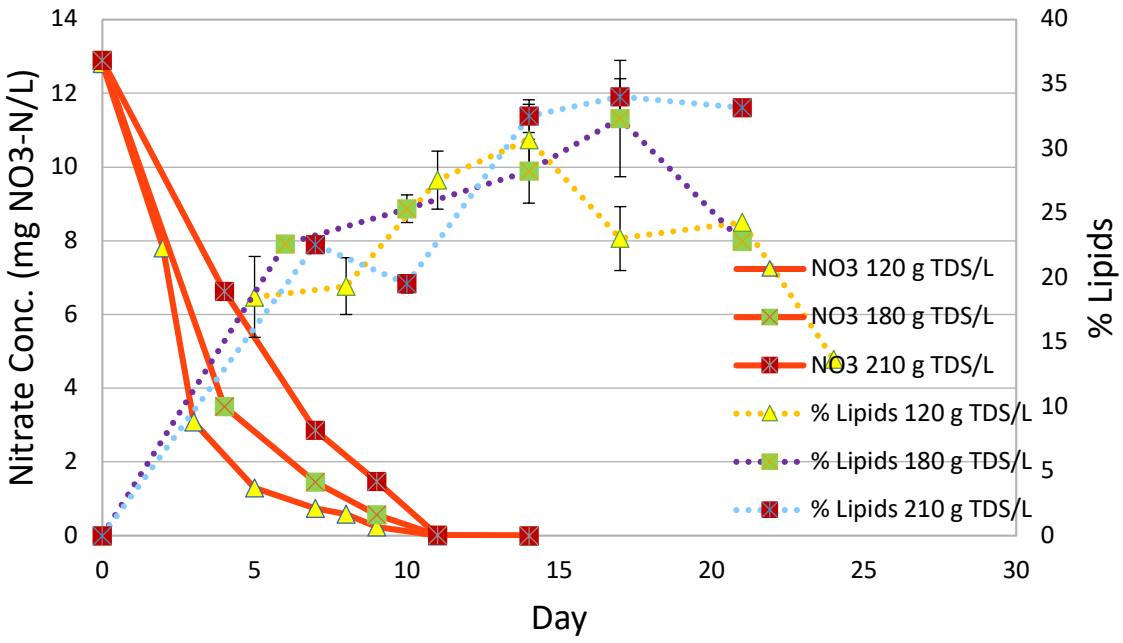

Figure 3.4. 24: *D. tertiolecta* PW (Exp. D1) nitrate concentration and lipid content for 120, 180, and 210 g TDS/L conditions.

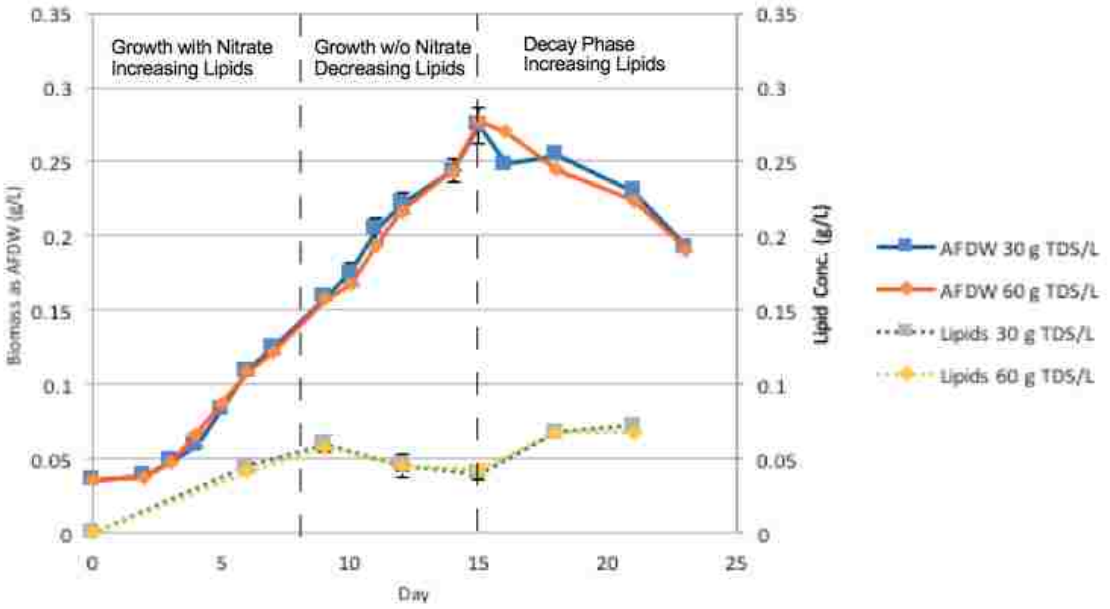

Figure 3.4. 25: *D. tertiolecta* PW (Exp. D1) biomass and lipid concentration for 30 and 60 g TDS/L conditions.

Three phases of culture growth and decay are defined.

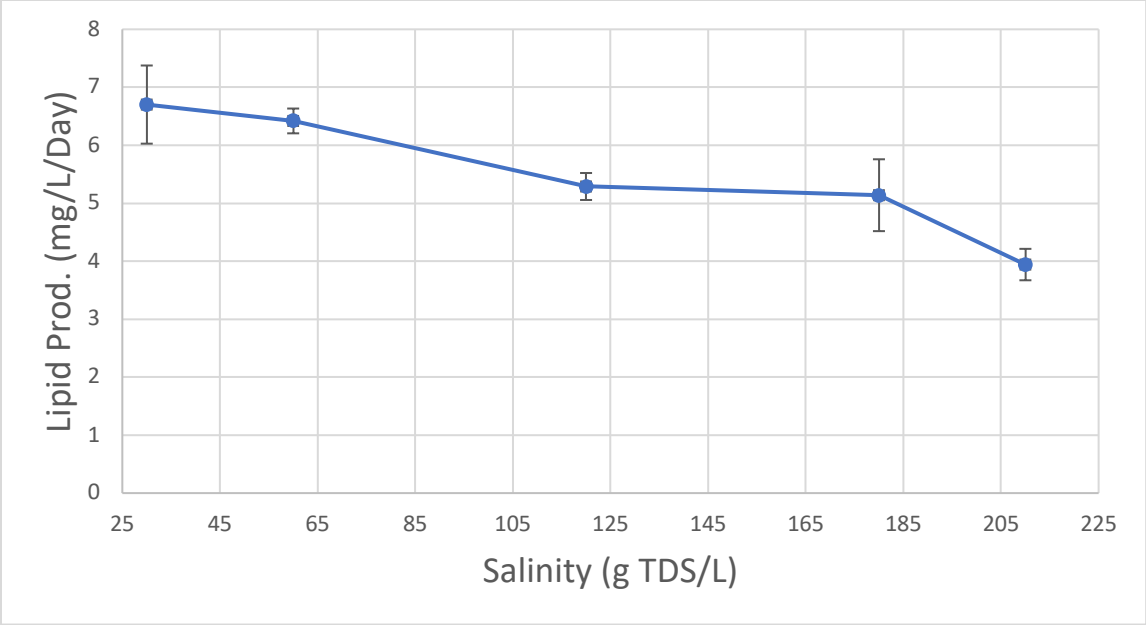

Figure 3.4. 26: *D. tertiolecta* PW (Exp. D1) lipid productivity by salinity.

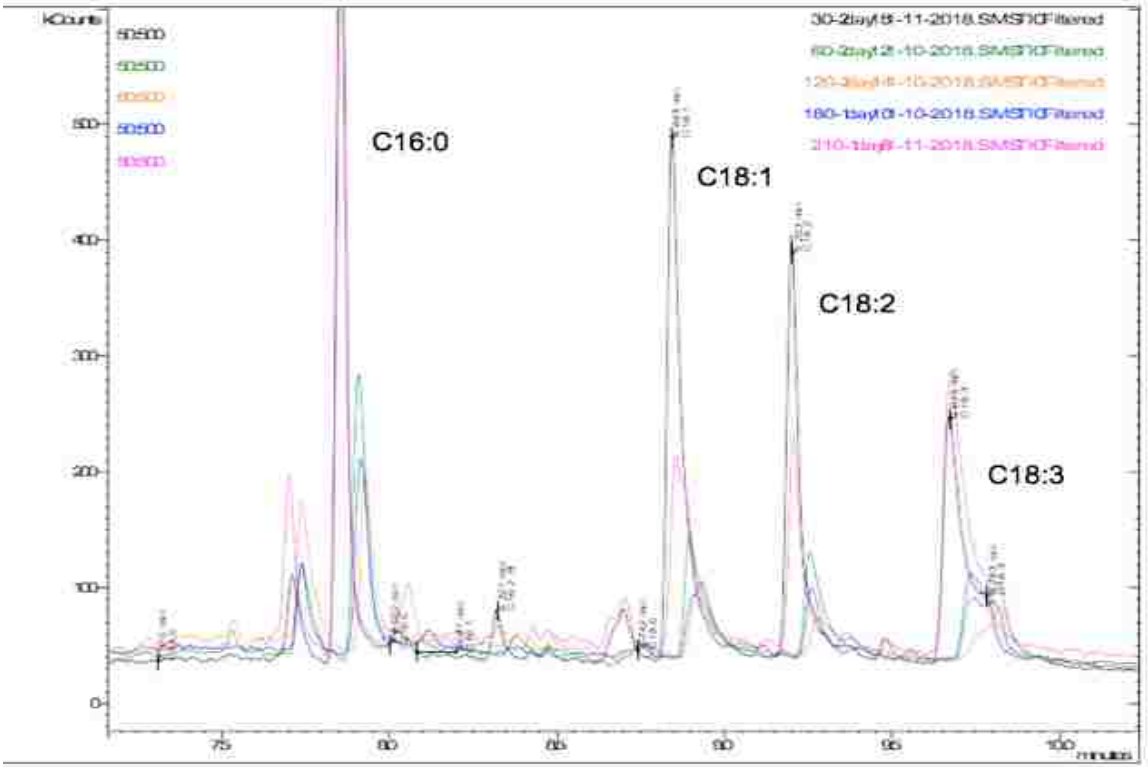

Figure 3.4. 27: Composite Chromatogram of *D. tertiolecta* FAMEs at all salinities tested.

The first number in the legend is the TDS concentration. The distinct peaks are palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and α and γ linolenic acid (C18:3).

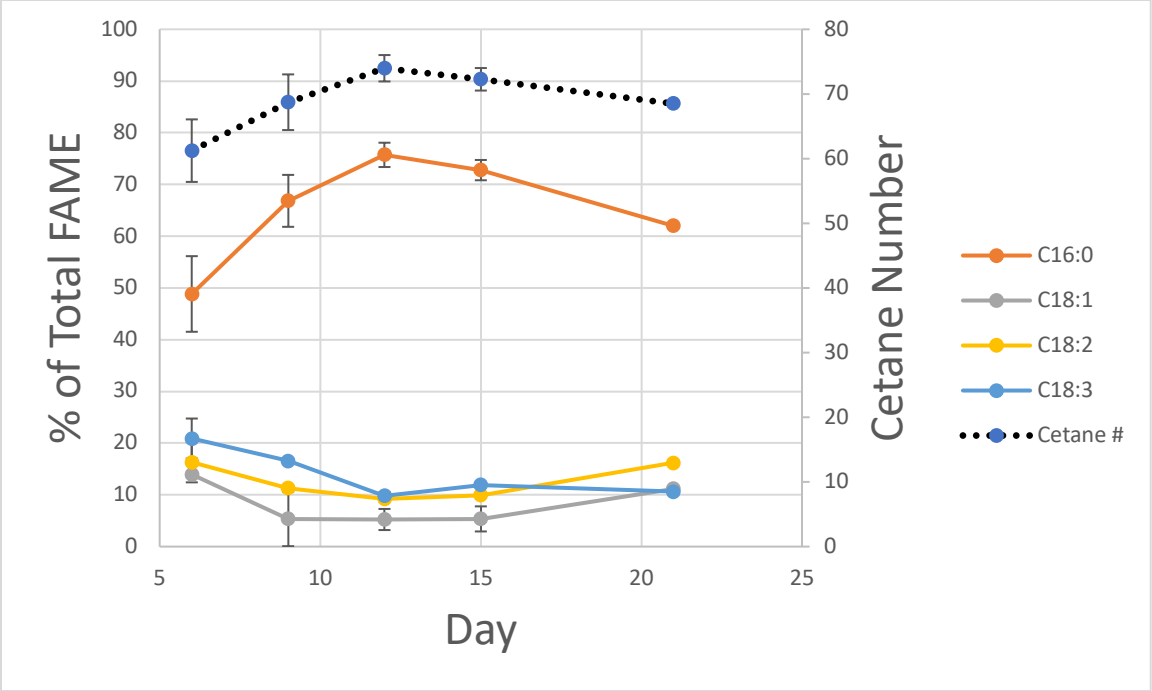

Figure 3.4. 28: *D. tertiolecta* PW flask experiment % FAME and cetane number for the 30 g TDS/L condition using nitrate.

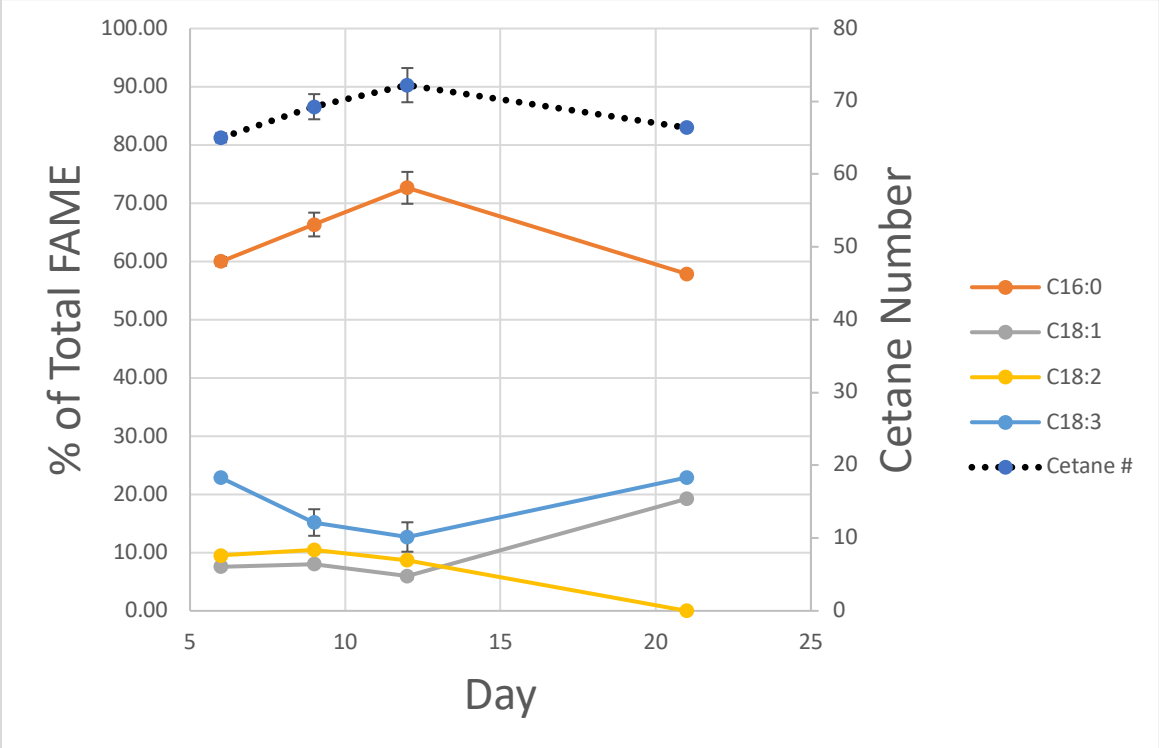

Figure 3.4. 29: *D. tertiolecta* PW flask experiment % FAME and cetane number for the 60 g TDS/L condition using nitrate.

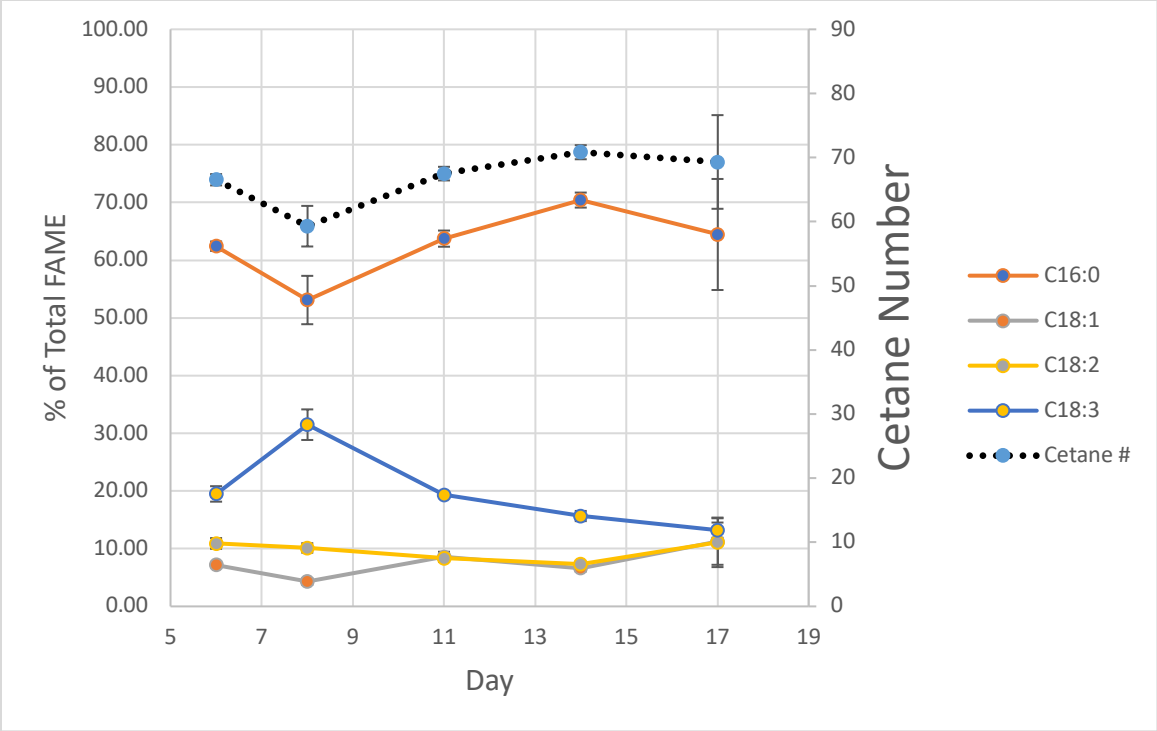

Figure 3.4. 30: *D. tertiolecta* PW flask experiment % FAME and cetane number for the 120 g TDS/L condition using nitrate.

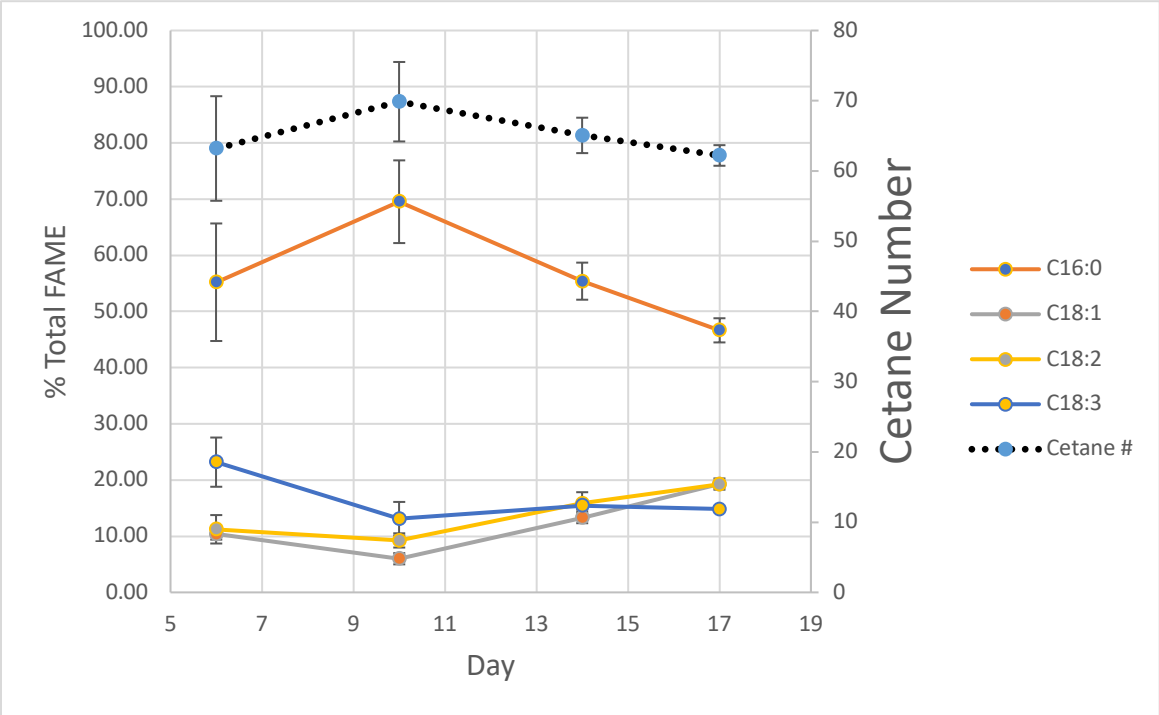

Figure 3.4. 31: *D. tertiolecta* PW flask experiment % FAME and cetane number for the 180 g TDS/L conditions using nitrate.

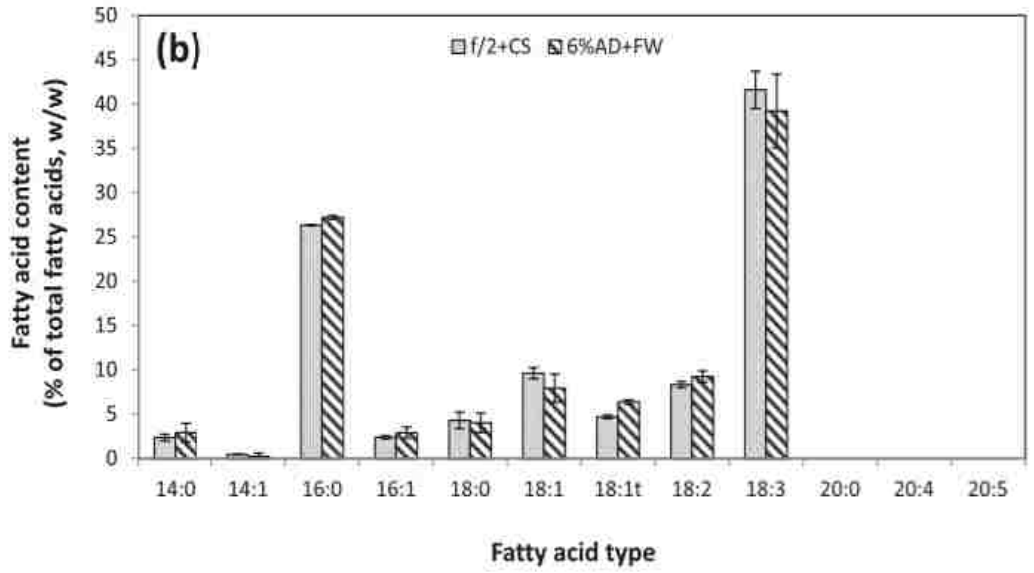

Figure 3.4. 32: *D. tertiolecta* lipid profile from Racharaks et al., (2015). The media was a mixture of shale gas flowback water and anaerobic digester effluent.

3.4.3: *D. tertiolecta* growth in PW using ammonium and higher phosphate concentrations (Exp. D2 & D3)

The objective of these experiments was to evaluate growth and lipid productivity of *D. tertiolecta* in PW using ammonium as a nitrogen source at different initial concentrations. Ammonium produced strong growth at the lowest initial concentration tested, with suggested ammonia inhibition at higher (Figure 3.4. 33). A final biomass density of around 0.37 g AFDW/L was reached with a growth rate of 21.8 mg/L/D between day 2 and 10. Doubling the initial ammonium concentration to 26 mg NH_4-N/L produced much lower growth and biomass concentration of 0.08 g AFDW/L and 5.6 mg/L/D. At the starting concentration of 39 mg NH_4-N/L, initially no growth occurred until around day 14. At this point, the culture shifted to rapid growth. Biomass was added at a rate of approximately 26.8 mg/L/Day reaching a concentration of 0.31 g AFDW/L. Growth did not occur in the highest initial ammonium concentrations tested

of 52 and 70 mg NH4-N/L. Early in the experiment algae cells were inactive (not motile). By around Day 7, cell mortality was high (as evidence of lysised cells), and green algae "pellets" were no longer observed in centrifuged samples. The results highlighted the opposite effects of ammonium which can both stimulate and inhibit growth depending on the concentration.

As for nitrate algae growth was associated with initial pH increases. Figure 3.4. 34 displays pH measurements for the course of the experiment. The 13 mg NH4-N/L condition had the highest pH at 9.8 on day 8. In the higher ammonium concentrations of 39, 52, and 70 mg NH4-N/L pH quickly increased to around 8.65, where it remained roughly constant. Upon entering growth phase, the pH increased for the 39 mg NH4-N/L condition highlighting the link between growth and more alkaline conditions. Although ammonium uptake releases protons in the media (Goldman et al.,1980), no effect is seen in the pH measurements indicating that the uptake of inorganic carbon (bicarbonate) is more dominant. It is possible that in the 13 mg NH4-N/L flasks that H+ release from ammonium uptake is slowing the progression to more a more alkaline pH until ammonium was depleted from the media. The later pH decrease seen cannot be due to ammonium uptake for it was depleted much earlier in the experiment on day 4.

Nutrient removal was correlated with increases in algae biomass, and as for nitrate suggests that *D. tertiolecta* can store nitrogen and phosphorus for later growth. Figure 3.4. 35 & Figure 3.4. 36 display total ammonium and phosphate measurements for the experiment. The 13 mg NH_4-N/L condition rapidly removed both ammonium and phosphate from the media both being absent by day 4. Growth continued for another

two weeks before reaching a decay phase (Figure 3.4. 37). This would be consistent with *D. tertiolecta* storing phosphorus as polyphosphate and ammonium in acid vacuoles as was proposed for *D. salina* (Pick and Weiss, 1991). Nutrient removal was less or non-existent at higher ammonium conditions. All conditions exhibited a loss of ammonium probably due to ammonia volatilization. The no growth conditions of 70 and 52 mg NH_4-N/L were measured to be losing total ammonium at the rate of 1.7 and 1.2 mg NH_4-N/L/day respectively. In flasks with an initial concentration of 39 mg NH_4-N/L total ammonium decreased gradually until the growth phase was entered, upon which it was removed in a few days. It is hypothesized that ammonia volatilization from the media was responsible for the vast majority of ammonium removal in the 70 and 52 mg NH_4-N/L conditions, and in the 39 mg NH_4-N/L before it entered a growth phase. With phosphate, no growth conditions (52 & 70 g NH_4-N/L) displayed an initial phosphate concentration drop followed by an increase. This could be attributed to cell death/lysis releasing stored phosphate back into the media.

Ammonia is known to limit algae division in a variety of phyla (Collos and Harrison, 2014) (Eustance et al., 2013). There is evidence it interferes with photosynthetic electron transport, directly affecting the cells ability to obtain energy (Markou et al., 2016). The 39 mg NH_4-N/L condition displayed bimodal behavior with an extended lag period until day 14, when rapid growth commenced. During the lag phase total ammonium was gradually decreasing at a rate close to 1.4 mg NH_4-N/L (Figure 3.4. 38). It is hypothesized that at around 21 mg total NH_4-N/L the free ammonia fell below levels inhibitory to *D. tertiolecta* cells and/or they became acclimatized. The result was

rapid growth that utilized an efficient nitrogen source and reduced free ammonia further inducing a positive feedback loop. Any inhibitory effects of ammonia should be avoided for algal biofuels cultivation, and with that in mind, the concentration of total ammonium near 13 mg NH_4-N/L would produce optimal growth. The pH increase was more pronounced in media were *D. tertiolecta* was utilizing bicarbonate, and the resulting alkaline conditions would exacerbate ammonia inhibition/toxicity. While the inorganic carbon and nitrogen already in PW can enhance algae productivity, care must be taken to avoid potential inhibitory or toxic effects of free ammonia.

At low concentration (13 mg N/L) ammonium appears to be a better nitrogen source than nitrate in hypersaline PW media. Figure 3.4. 39 shows biomass measurements on nitrate and ammonium at the same concentration in 120 g TDS/L strength PW. Growth with ammonium had both a higher rate (21.8 mg/L/D vs. 16.2 mg/L/D) and peak biomass concentration (0.37 vs. 0.30 g AFDW/L). Ammonium was removed from the media twice as fast as nitrate (2.6 mg NH_4-N vs. 1.3 mg NO_3-N/L) (Figure 3.4. 6). *D. tertiolecta* like all algae must reduce nitrate to ammonium to utilize it in cellular metabolism (Payne, 1973). This requires an energy expenditure that can decrease growth. The *Dunaliella* genus preferentially uptakes ammonium over nitrate (Beardall and Giordano, 2009). Chen et al., (2011) suggests a similar effect by reporting around equal growth on 1 mM NH_4 and 2.3 mM NO_3. *D. tertiolecta* growing on ammonium has been stated to double its chlorophyll content (suggesting enhanced photosynthetic activity) along with a 20% increase in cell size (Giordano and Bowes, 1997). It seems probable that *D. tertiolecta* (and many species in the genus) have

adapted to environments where free ammonia concentrations are below toxic levels. The lowest initial ammonium concentrations tested in this experiment might be ideal to produce robust growth for biofuel production.

Interestingly, *D. tertiolecta's* growth on the same initial concentration of ammonium (13 mg NH_4-N/L) and phosphate (1.7 mg PO_4-P/L) in PW under hypersaline and saline conditions differed. Figure 3.4. 40 displays biomass measurements for the experiment at the salinities of 120 g TDS/L and 30 g TDS/L (close to sea water). In the hypersaline media *D. tertiolecta* exhibited little to no lag phase before growth, while the saline did not display growth for approximately 10 days. Upon entering the growth phase, the flasks at 30 g TDS/L exhibited a similar growth rate to those at 120 g TDS/L (19 mg/L/Day vs. 21.8 mg/L/Day). During the lag phase total ammonium concentrations were decreasing at a rate of about 0.56 mg/L/Day (Figure 3.4. 41). It is proposed that in the 30 g TDs/L condition ammonia inhibition was initially preventing growth. Once free ammonia concentrations were reduced by the gradual degassing, *D. tertiolecta* was able to enter a rapid growth phase that removed ammonia from solution. This depleted free ammonia concentrations eliminating any inhibitory effect.

The results indicate ammonia toxicity is reduced at higher salinity. This could be due to the lower fraction of free ammonia that occurs with increasing ionic strength. Harkness et al., (2015) refers to decreasing free ammonia in hypersaline PW. Racharaks et al. (2015) comes to a similar conclusion cultivating *D.* tertiolecta in hypersaline PW and anaerobic digester effluent (with a high total ammonium content). Maeda et al., (1990) attempted to measure and predict the pKa of the NH_4^+/NH_3 system with sodium

(and lithium chloride). They modeled the system with the specific interaction model (SIM) which is based on the Pitzer equations. Their predicted and measured results show that the pKa increases from the standard condition value of 9.25 to above 9.6 in a 2M NaCl solution. This would greatly reduce the amount of free ammonia at pH values near the pKa with increasing salinity. Decreased free ammonia would help *D. tertiolecta* or other algae species survive in ammonium rich hypersaline PW media and utilize it as a nitrogen source. Harkness et al. (2015) reported a direct correlation between salinity and ammonium concentration of PW. This would work to the benefit of algae cultivation in that the higher total ammonium concentration would be less toxic at high ionic strength.

A higher initial concentration of phosphate in the PW medium produced slightly lower growth. Figure 3.4. 42 shows the biomass production of *D. tertiolecta* in a 120 g TDS/L and 13 mg NH_4-N/L PW media at a low (1.7 mg PO_4-P/L) and high (8 mg PO_4-P/L) beginning phosphate concentration. Growth rates and peak biomass concentration were similar but slightly lower for the high phosphate condition (18.9 vs. 21.8 mg/L/Day and 0.34 vs. 0.37 g AFDW/L). Figure 3.4. 43 displays biomass and nutrient levels for the high phosphate condition. Phosphate was continuously removed until the decay phase at a rate of approximately 0.44 mg PO_4-P/L/Day. Figure 3.4. 44 plots the phosphorus content of the biomass based on phosphate removal and biomass concentration. The % P in the high PO_4 condition ranges 2.1-4.5%, while the low PO_4 between 0.5-2.1%. *D. tertiolecta* has been shown to store phosphate in other experiments (Rao, 2009). Rao (2009) states that higher phosphate is associated with increased growth, but that was

not observed in the study. It seems at the low initial phosphate concentration that the lack of the nutrient phosphorus is not the limiting factor. This bodes well for cultivation in PW which likely would have phosphate levels of at least 1.7 mg PO_4-P/L.

The lower levels of ammonium detected in hydraulically fractured shale derived PW by Harkness et al., (2015) might by ideal for cultivation with higher levels potentially toxic. Likely *D. tertiolecta* can tolerate higher level of total ammonia at higher salinity due to the decreased fraction of free ammonia (Racharaks et al., 2015). This could be used to enhance growth rates in a hypersaline PW medium. Nitrate could also be added to a media as a back-up nitrogen source. It is unclear from this study at exactly what level phosphate would be growth limiting due to *D. tertiolecta's* ability to store polyphosphate. It appears that the low levels detected in many PW samples would be sufficient for growth thus reducing costs. Further work could explore optimum nutrient concentrations in saline and hypersaline PW.

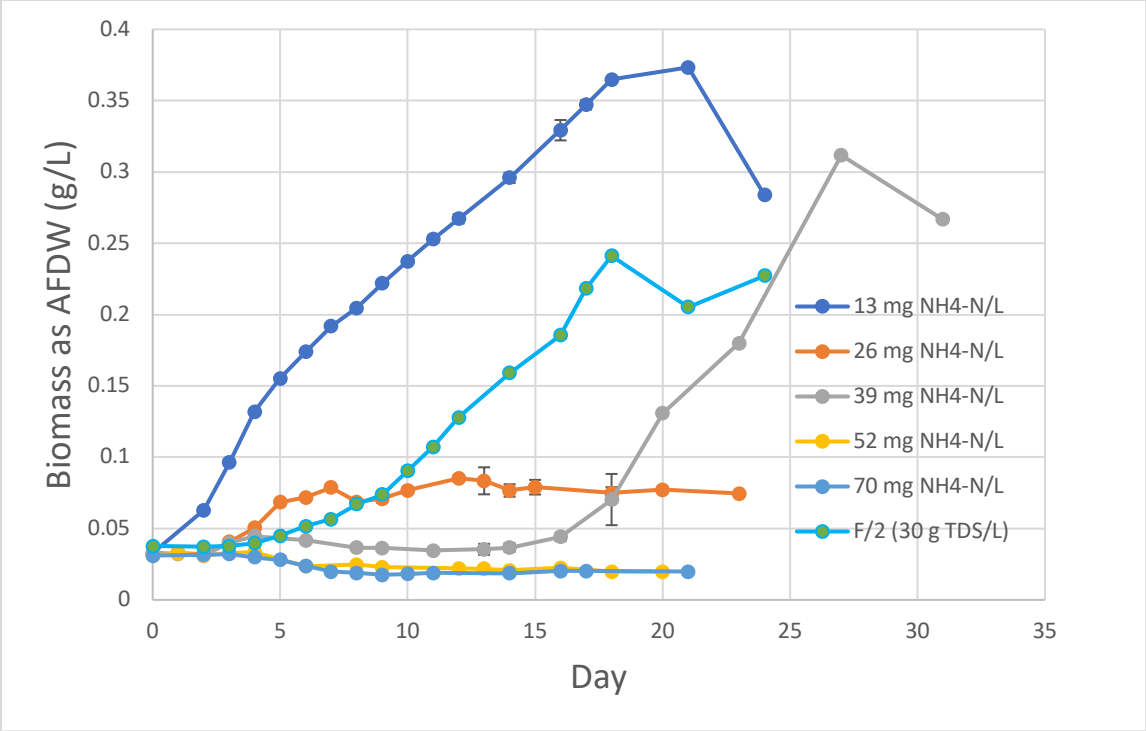

Figure 3.4. 33: *D. tertiolecta* biomass measurements in a 120 g TDS/L PW media (Exp. D2 & D3) at different initial ammonium concentrations.

The commercial F/2 media is shown for comparison.

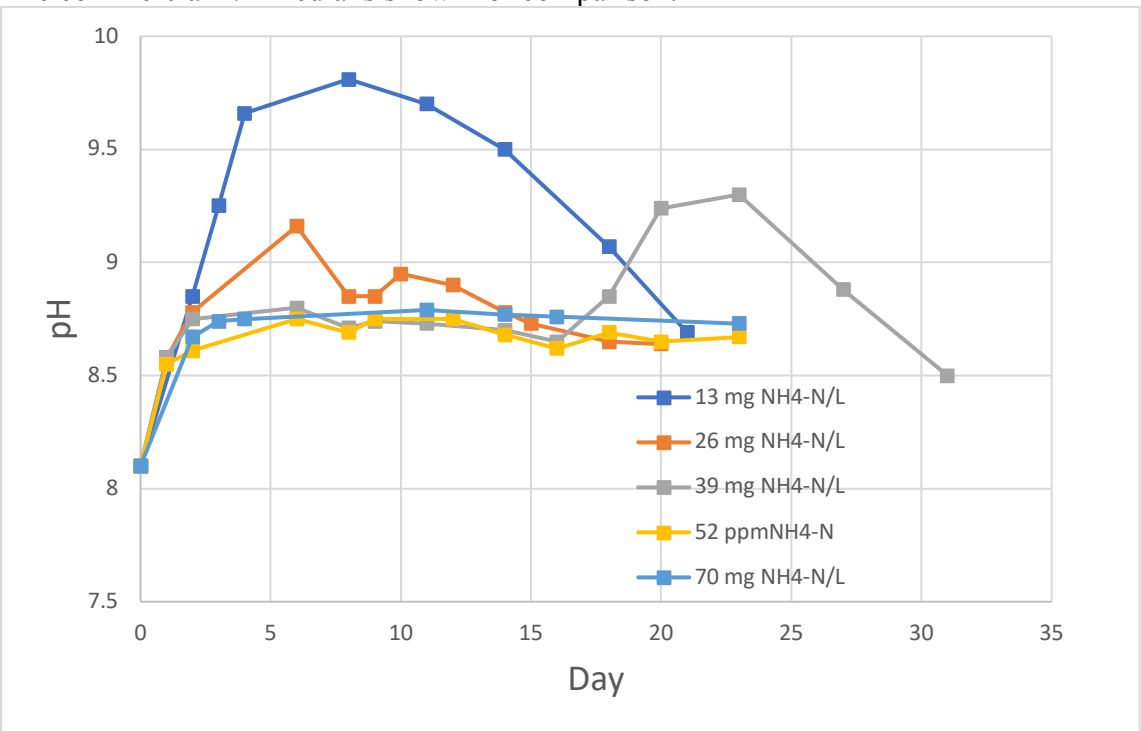

Figure 3.4. 34: *D. tertiolecta* pH measurements in a 120 g TDS/L PW media at different initial ammonium concentrations.

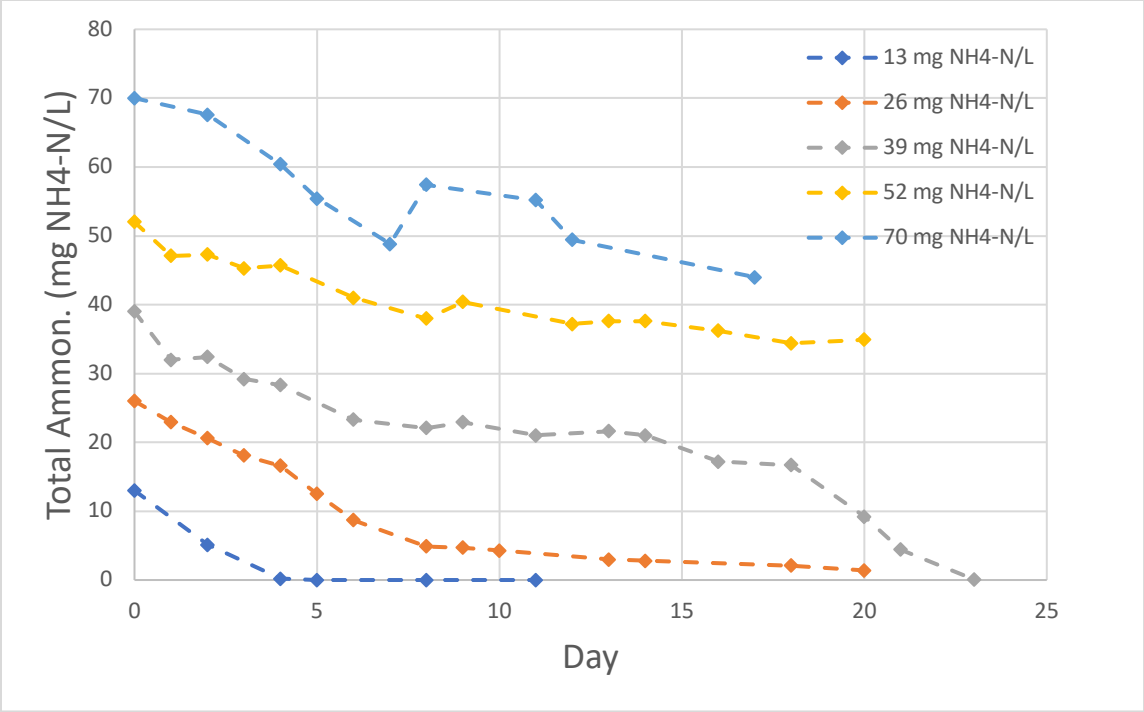

Figure 3.4. 35: *D. tertiolecta* ammonium measurements using 120 g TDS/L PW media at different initial concentrations.

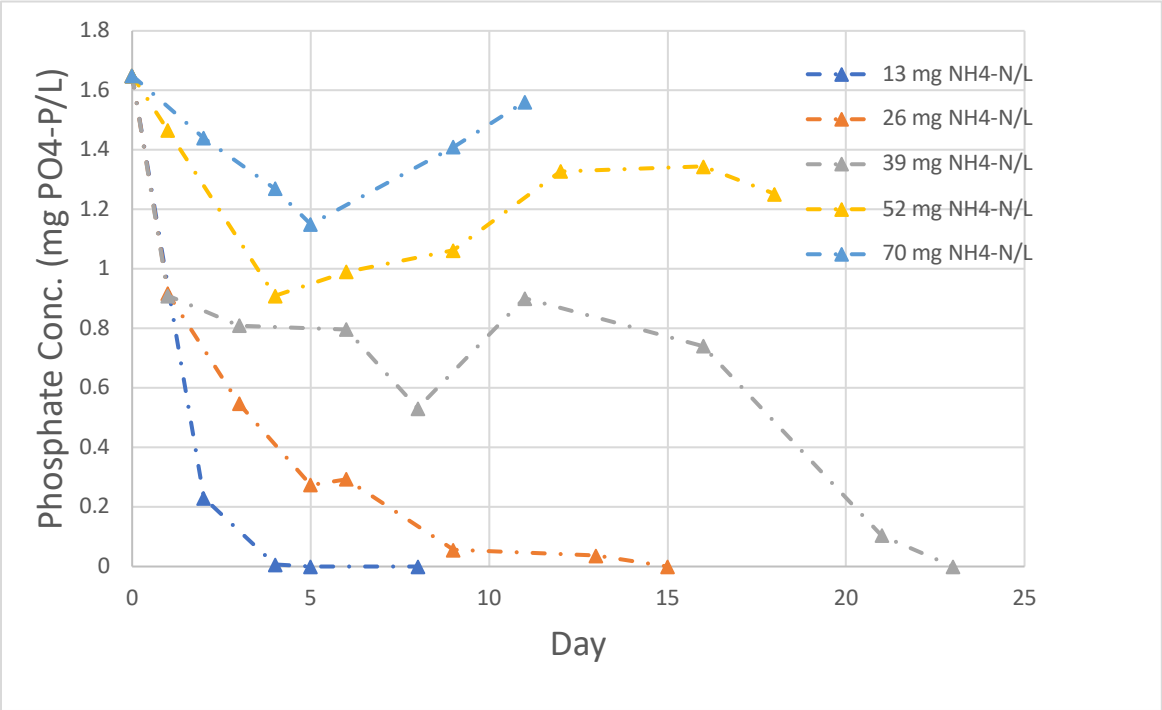

Figure 3.4. 36: *D. tertiolecta* phosphate measurements using 120 g TDS/L PW media at different initial concentrations.

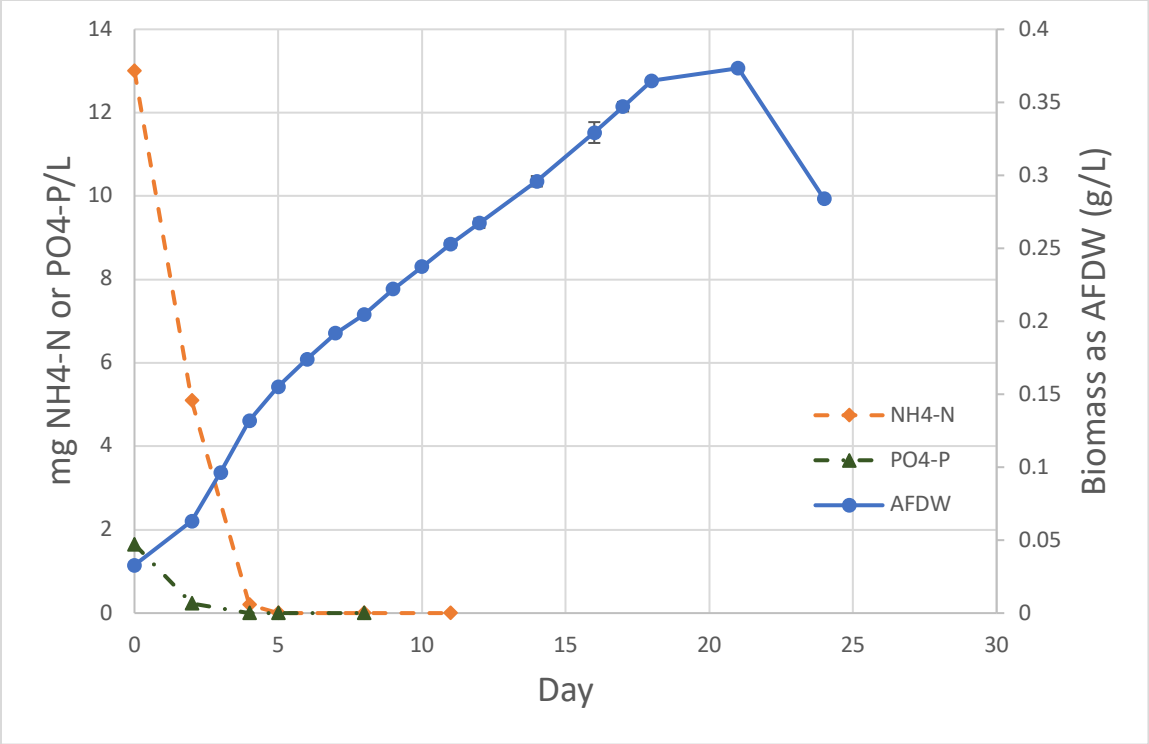

Figure 3.4. 37: *D. tertiolecta* biomass, ammonium, and phosphate measurements in a 120 g TDS/L PW media (Initial Concentrations 13 mg NH4-N/L and 1.7 PO4-P/L).

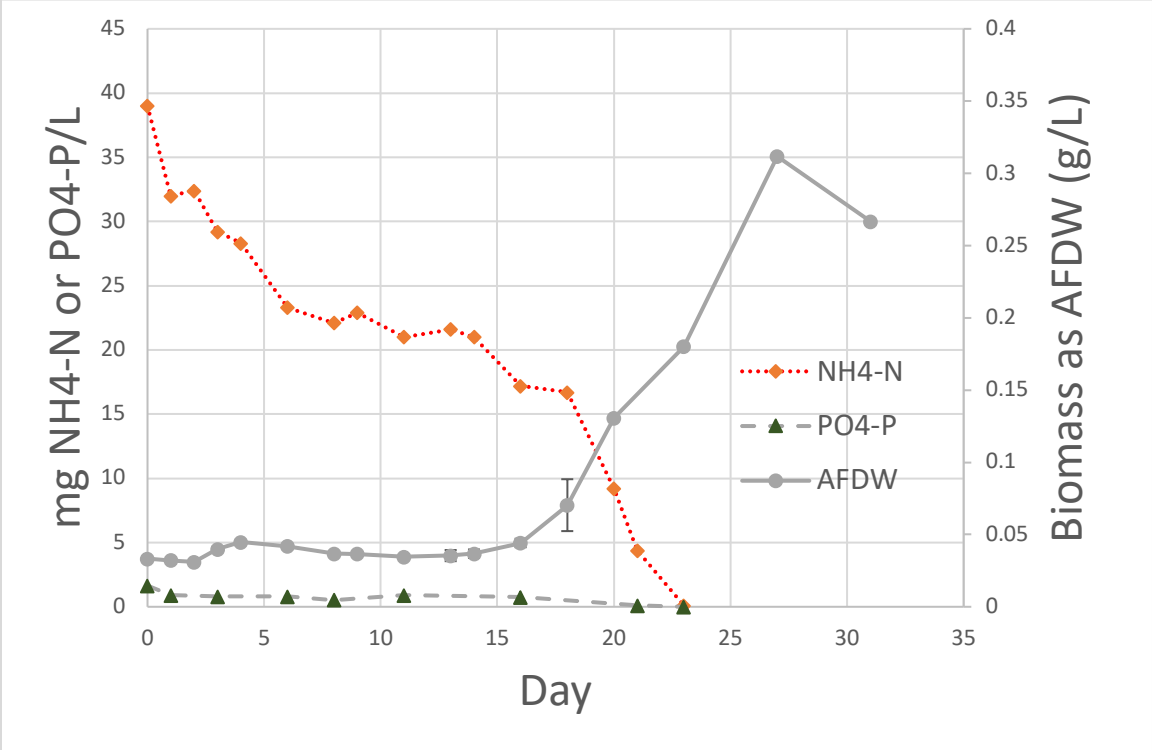

Figure 3.4. 38: *D. tertiolecta* biomass, ammonium, and phosphate measurements in a 120 g TDS/L PW media (Initial Concentrations 39 mg NH4-N/L and 1.7 PO4-P/L).

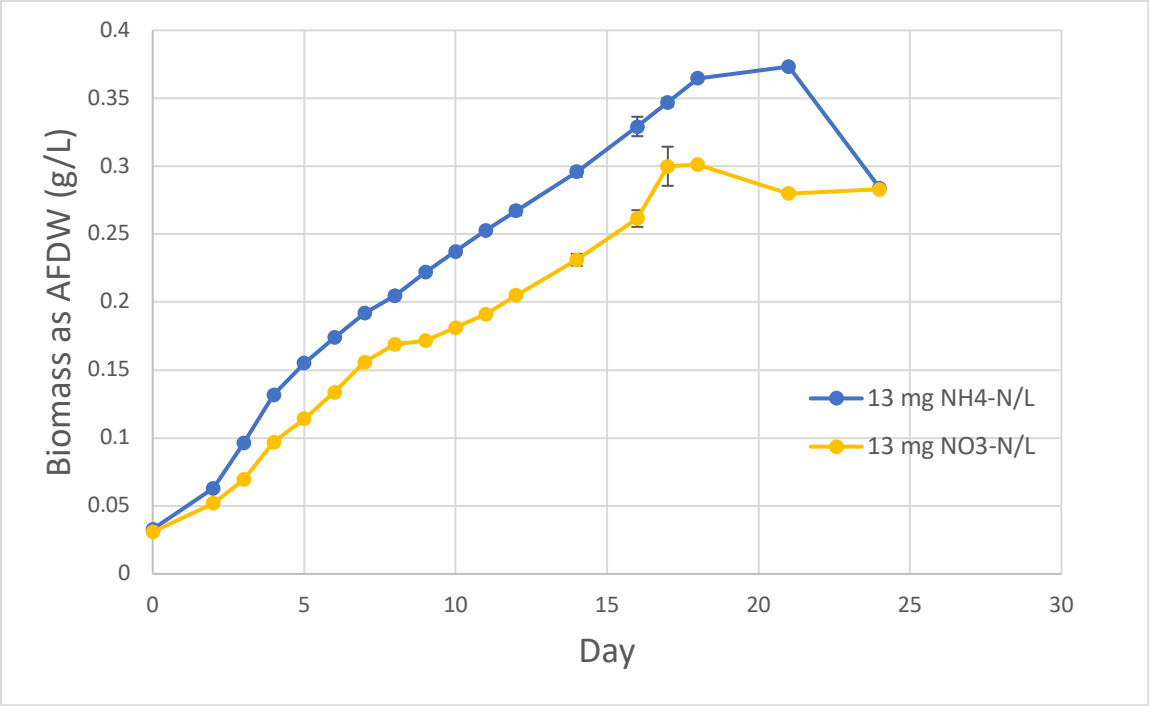

Figure 3.4. 39: *D. tertiolecta* biomass measurements in a 120 g TDS/L PW media at the same nitrogen concentration with ammonium and nitrate.

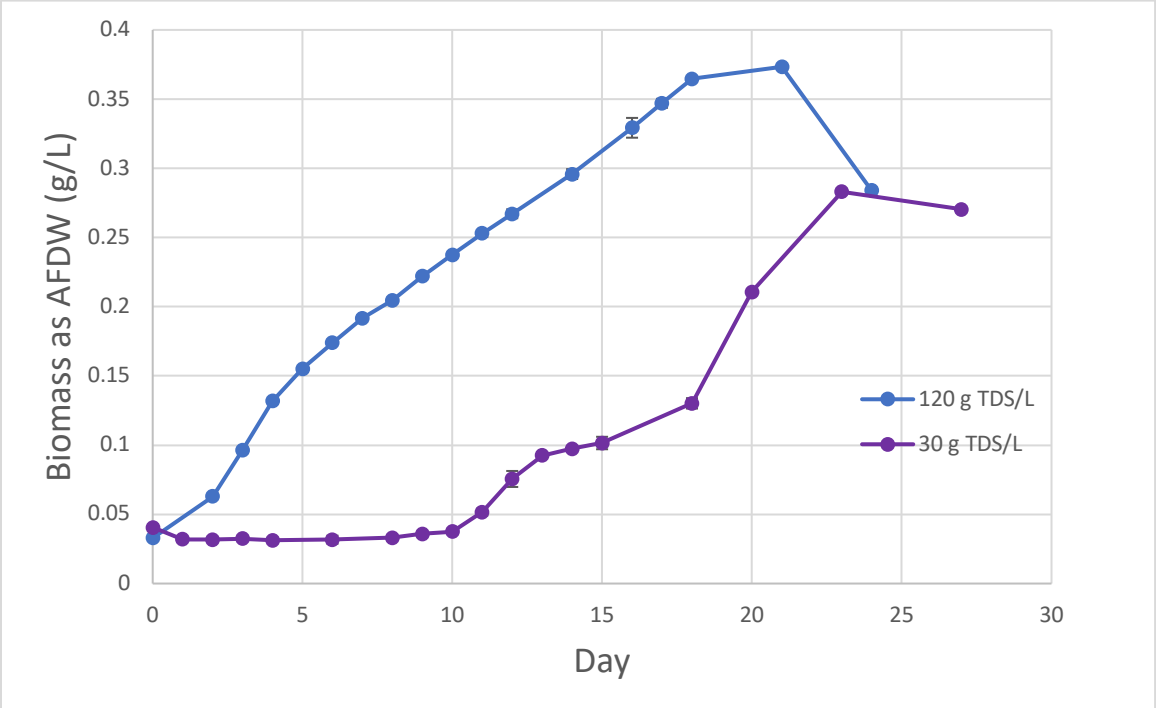

Figure 3.4. 40: *D. tertiolecta* biomass measurements in 120 g TDS/L and 30 g TDS/L PW media at the same initial ammonium (13 mg NH_4-N/L) and phosphate (1.7 mg PO_4-P/L) concentrations.

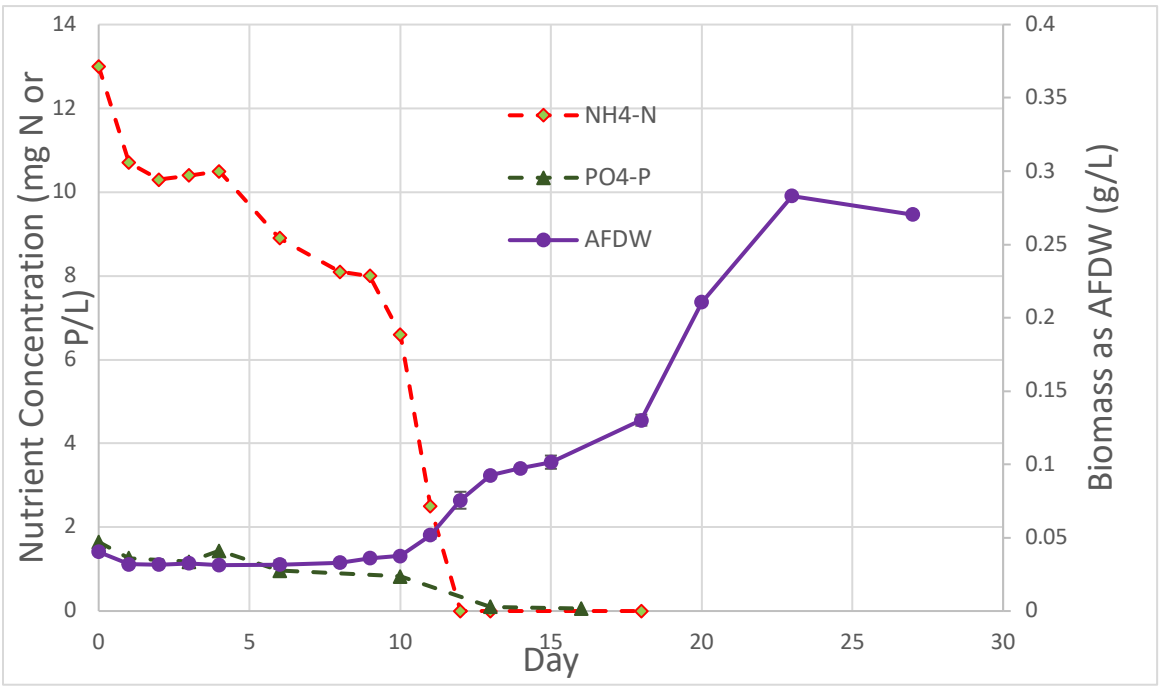

Figure 3.4. 41: *D. tertiolecta* biomass, ammonium, and phosphate measurements in a 30 g TDS/L PW media (initial Concentrations 13 mg NH4-N/L and 1.7 PO4-P/L).

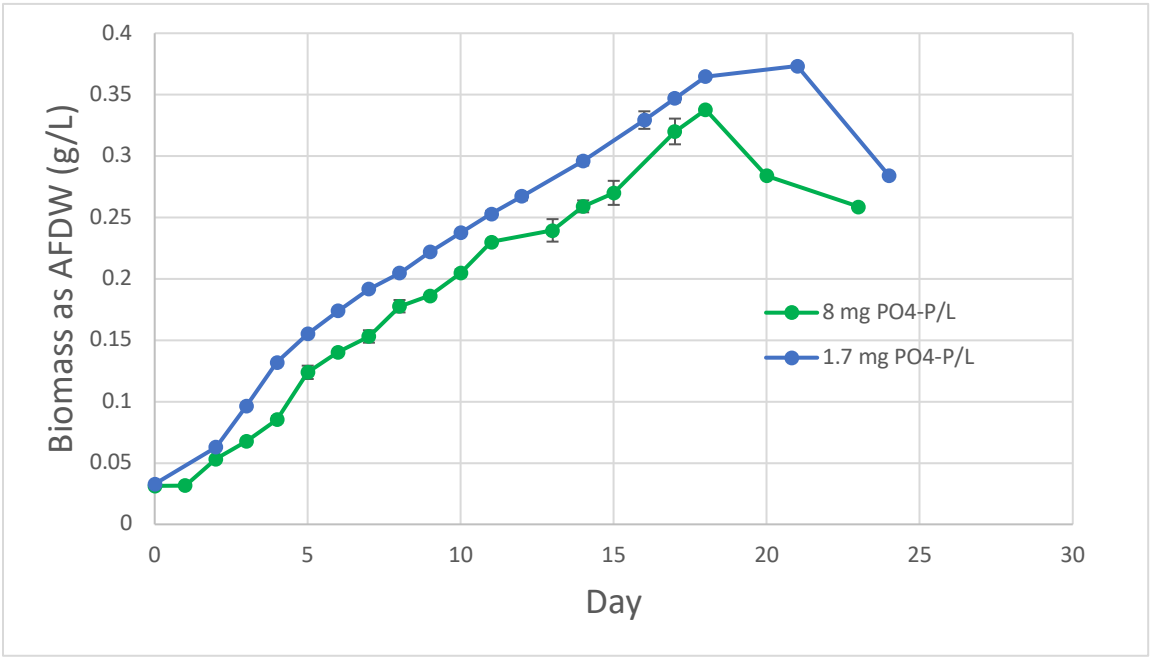

Figure 3.4. 42: *D. tertiolecta* biomass measurements in a 120 g TDS/L PW media at an initial high (8 mg PO_4-P/L) and low (1.7 mg PO_4-P/L) phosphate concentration with 13 mg NH_4-N/L.

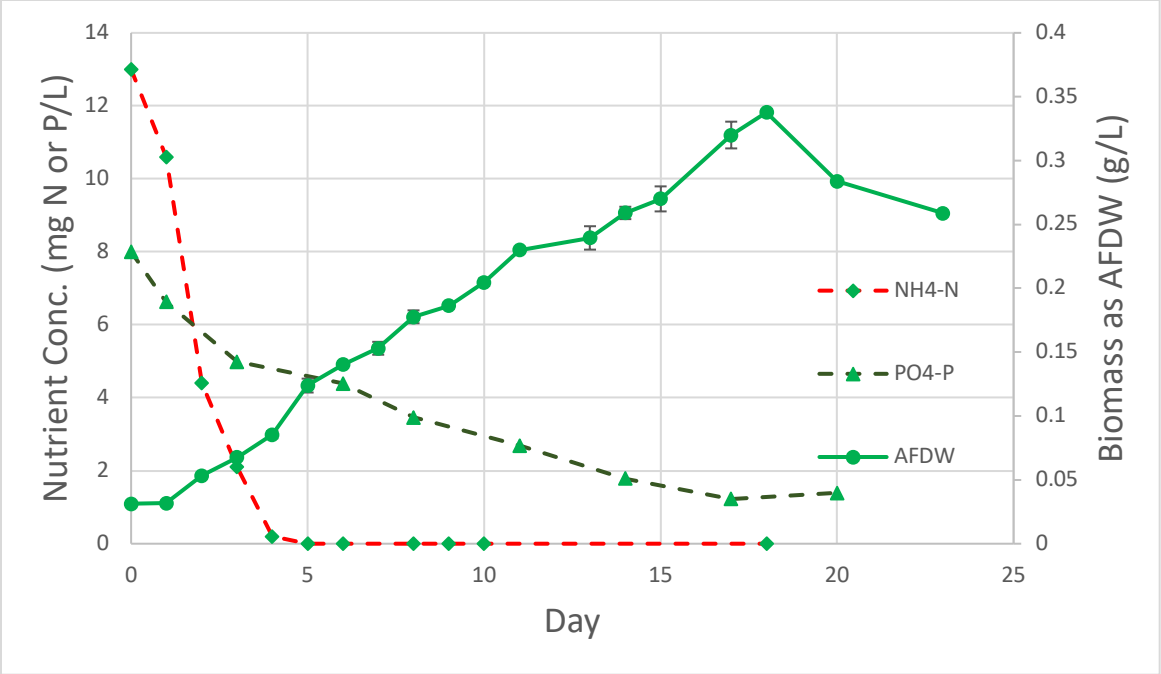

Figure 3.4. 43: *D. tertiolecta* biomass, ammonium, and phosphate measurements in a 120 g TDS/L PW media (Initial Concentrations 13 mg NH4-N/L and 8 PO4-P/L).

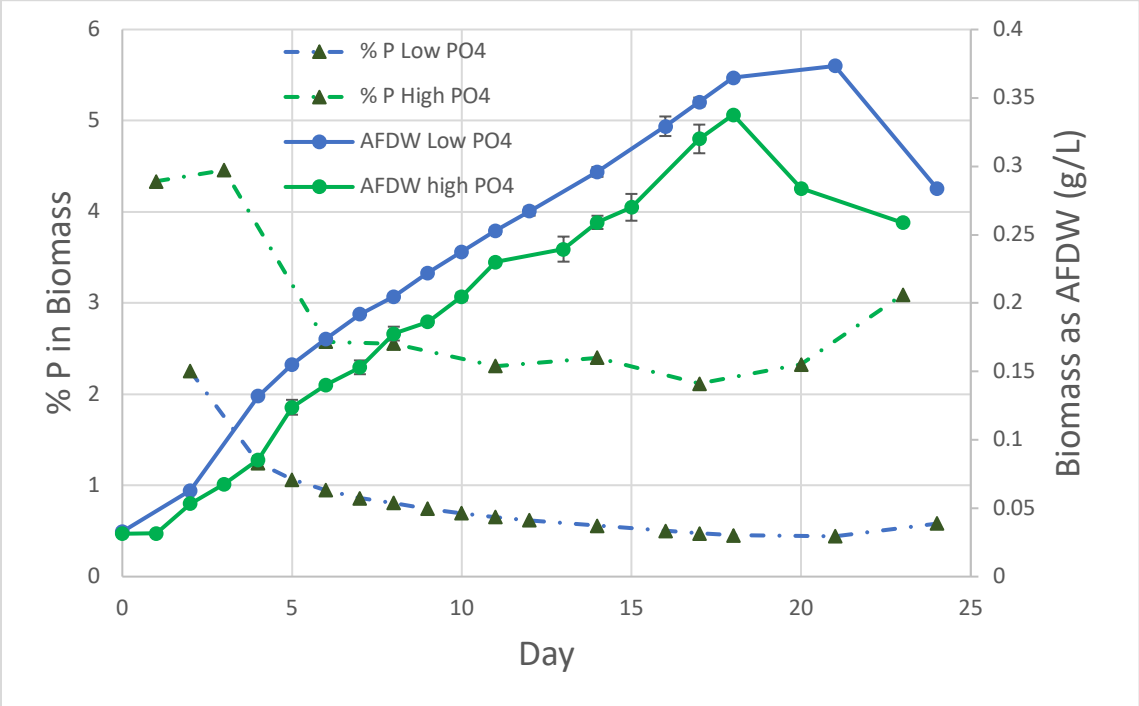

Figure 3.4. 44: *D. tertiolecta* biomass measurements and % phosphorus in a 120 g TDS/L PW and 13 mg NH_4-N/L media at low (1.7 mg PO_4-P/L) and high (8 mg PO_4-P/L) initial concentrations.

3.4.4: *D. tertiolecta* lipid productivity and character using ammonium in PW (Exp.: D2 & D3)

D. tertiolecta's lipid production was enhanced using ammonium over nitrate in the hypersaline PW. At 120 g TDS/L lipid content and concentration peaked earlier for flasks growing on ammonium rather than nitrate (Figure 3.4. 45). For the initial concentrations of 13 mg NH_4/L and 1.7 mg PO_4-P *D. tertiolecta's* oil content reached a maximum on day 8 with values of 0.90 g lipids/L and a 44% fraction. This compares to a peak on day 14 for nitrate with 0.074 g lipids/L and a 30.7% fraction. This results in over a doubling in lipid productivity for ammonium rates of 11.2 mg/L/Day vs. 5.3 mg/L/Day. Flasks with a higher initial concentration of phosphate reached a peak oil content on day 17 with a density of 0.11 g lipids/L and a 34.6% fraction. While the lipid concentration is higher than the low phosphate condition, lipid productivity is lower (6.5 mg/L/Day) due to the delayed peak. In terms of the producing algae oil, *D. tertiolecta* performed best on the low initial ammonium and phosphate concentrations tested.

Harvesting for maximum biomass and oil content would need to be done at different times in a low ammonium and phosphate medium. Figure 3.4. 46 shows that peak oil content clearly occurs on day 8 well before peak biomass on 21. Biomass growth continues for long after day 8 as the lipid content and concentration decrease. It seems that accumulated fat and nutrient reserves are being used for biomass accumulation before reaching a maximum on day 21. For processes that require an oleaginous biomass such as transesterification optimal harvest would at or near Day 8. If overall biomass is the chief priority, harvest should wait for approximately two more weeks until peak biomass. To the best of the author's knowledge this result has not

been shown in the algae literature for *D. tertiolecta*. Early lipid enrichment is not seen in the high P condition. Maximum lipid content and concentration essentially occur near the time of peak biomass (Figure 3.4. 47). There would be no advantage to harvesting before stationary growth phase in this condition. The precise time frame would differ in outdoor raceway cultivation, but the relative timing of oil and biomass peak concentrations would likely apply.

In this study nitrate depletion did not lead to clear lipid enrichment, but there is evidence of a time dependent effect with ammonium. Figure 3.4. 48 & Figure 3.4. 49 display nutrient, lipid concentration, and lipid content for the low ammonium/phosphate condition. Nitrogen and phosphate are depleted from the medium by day 4. Lipid enrichment continues under nutrient starvation conditions until day 8, after which there is a decrease in lipid content of the biomass and a smaller proportional decrease in concentration. This seems similar to the time dependent enrichment effect reported by Chen et al., (2011). While lipid content and concentration did increase with nitrate in PW, it appears to be more of a continuation of the pre-nitrate depletion trend. The final content and concentration are lower than for ammonium. Figure 3.4. 50 proposes growth phases for the low ammonium/P flasks. Since ammonium is linked to a higher chlorophyll content it seems likely that each cell is absorbing a higher proportion of the incoming light energy. This might make a surplus that can be stored as lipids during the short period of growth/enrichment just after ammonium depletion. *D. tertiolecta* cells likely have an ammonium reserve stored in acid vacuoles during this time (Pick and Weiss, 1991). After Day 8 photosynthetic

activity decreases probably due to nutrient starvation, and cells tap into their fat energy reserves to maintain biomass growth. This is supported by pH measurements which reported a peak pH on day 8 of 9.8 with a following gradual decrease (Figure 3.4. 51). This would indicate decreased uptake of inorganic carbon after day 8 that could be allocated for energy storage as lipids. The nutrient starved lipid depleting phase of growth lasted for almost two weeks and resulted in almost a doubling of the biomass.

In the high initial phosphorus flasks lipid content and concentration also reached a maximum well after ammonium depletion from the media, while phosphate was still present (Figure 3.4. 52 & Figure 3.4. 53). This seems part of a clear trend that extends from Day 5 until Day 17. As stated previously, there is not a distinct peak a few days after ammonium depletion as for the low P condition. As a result, *D. tertiolecta* looks to be progressing through a different set of phases that yield a lower lipid production rate (Figure 3.4. 54). It seems apparent that the continued presence of phosphate in the media induces a different metabolic pattern. As mentioned for the Polyculture this may be due to algae cells diverting energy from growth to phosphate storage. While both the low P and High P display lipid enrichment after nitrogen stress, simultaneous phosphorus stress would improve lipid yield for biodiesel applications. The lipid productivity of *D. tertiolecta* in hypersaline PW might be optimized using low concentrations of ammonium already present in PW to yield high biomass growth, then followed by a few days of nutrient starvation (absence of N and P) for enriched oil content.

The character of fatty acid methyl esters (FAME) derived from *D. tertiolecta* extracted lipids using ammonium also appear sufficient for quality biodiesel. Figure 3.4. 55 displays GC/MS chromatograms from *D. tertiolecta* lipids grown in hypersaline PW at low NH_4 and Low P conditions described previously. They are taken from samples ranging over Day 8 to 24 of the experiment. As for the nitrate grown *D. tertiolecta*, C16:0, C18:3, C18:2, and C18:1 are dominant. A significant lesser peak is found at C18:0. FAME profiles look similar over time, indicating that only overall lipid content could be considered for harvesting for biodiesel applications (Figure 3.4. 56). C16:0 consistently composed the majority of FAME in nitrate GC/MS analyses. The ammonia analyses state, that while dominant, it composes 41-45% of the total. The overall Cetane number and derived biofuel should be similar to that indicated by the profiles of nitrate grown *D. tertiolecta*. Stearic acid would increase the cetane number and decrease viscosity of any derived biodiesel.

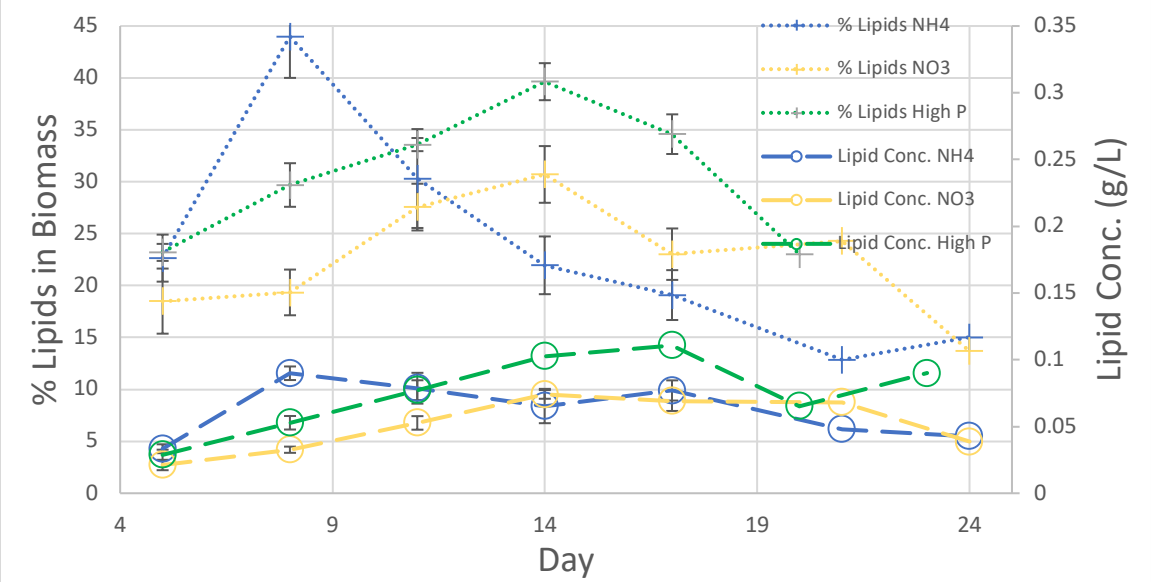

Figure 3.4. 45: *D. tertiolecta* lipid content and concentration in 120 g TDS/L PW media.

Initial concentrations of 13 mg NH_4-N/L & 1.7 mg PO_4-P/L (NH4), 13 mg NO_3-N/L & 1.7 mg PO_4-P/L (NO_3), and 13 mg NH_4-N/L & 1.7 mg PO_4-P/L (High P).

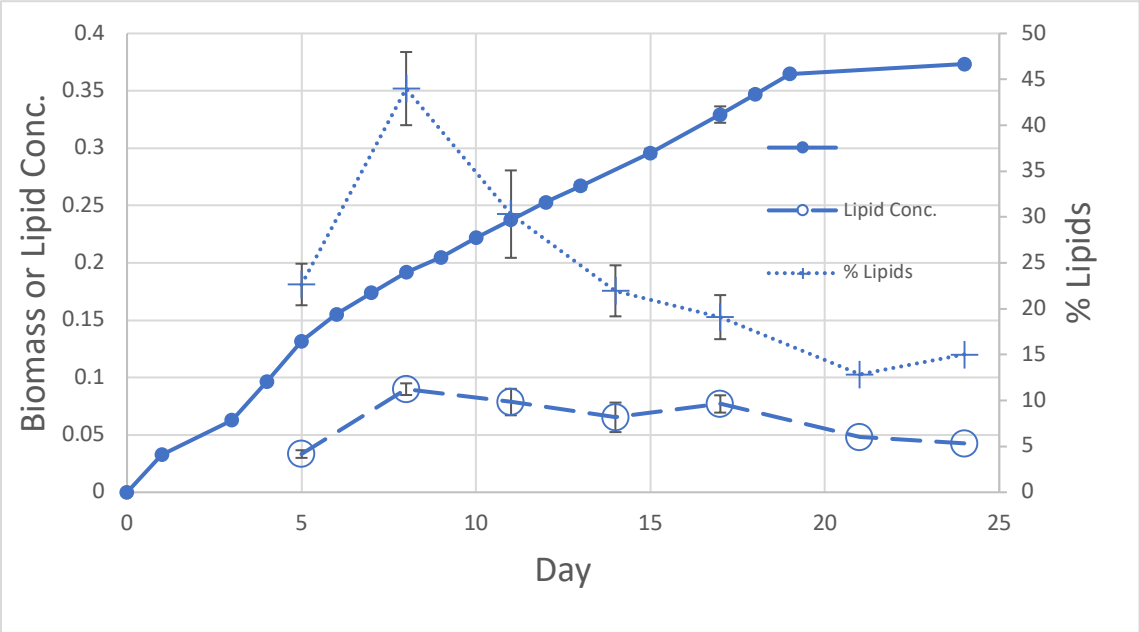

Figure 3.4. 46: *D. tertiolecta* lipid content, biomass, and lipid concentration in 120 g TDS/L PW media at initial concentrations of 13 mg NH_4-N/L and 1.7 mg PO_4-P/L.

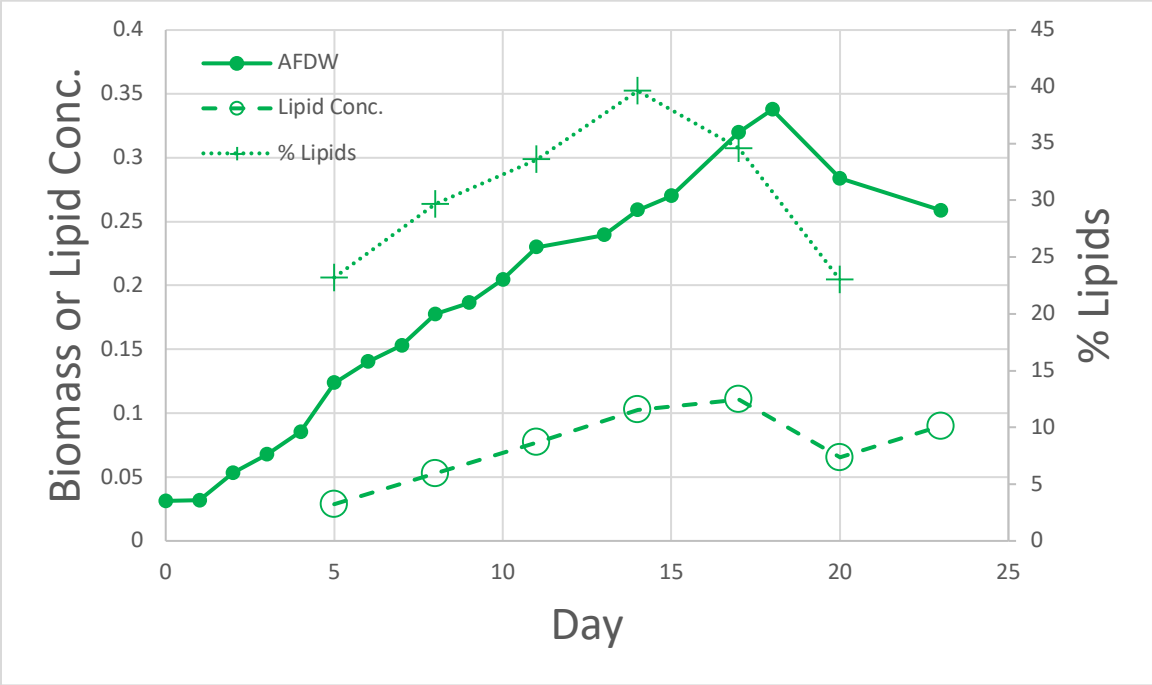

Figure 3.4. 47: *D. tertiolecta* lipid content, biomass, and lipid concentration in 120 g TDS/L PW media at initial concentrations of 13 mg NH_4-N/L and 8 mg PO_4-P/L.

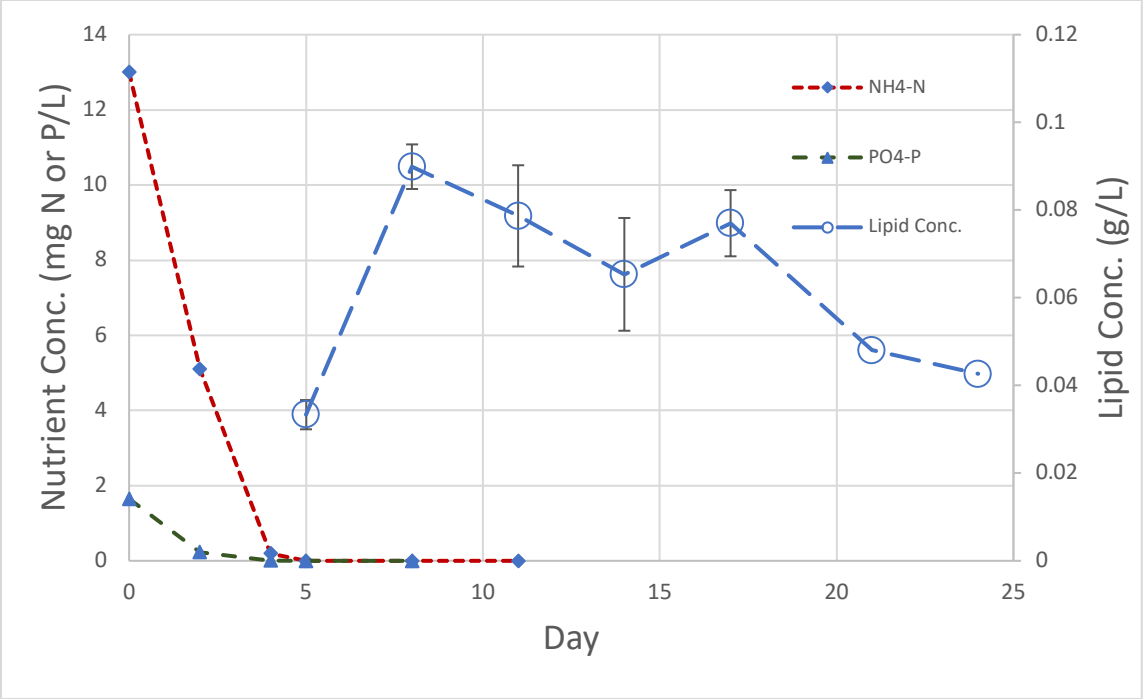

Figure 3.4. 48: *D. tertiolecta* ammonium, phosphate, and lipid concentration in 120 g TDS/L PW media at initial concentrations of 13 mg NH_4-N/L and 1.7 mg PO_4-P/L.

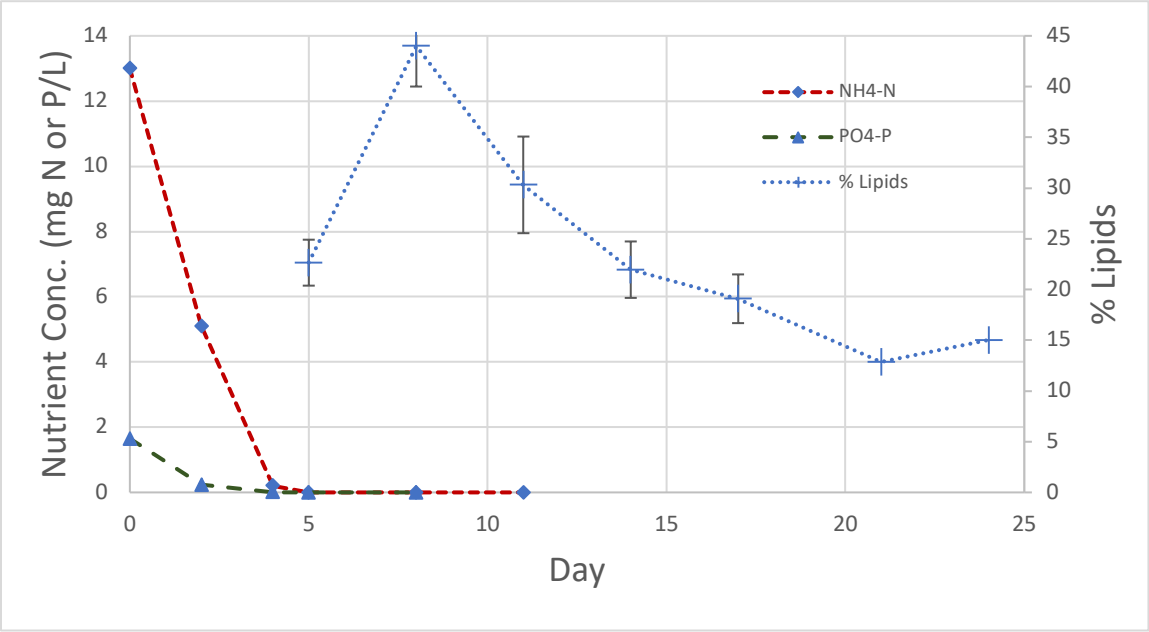

Figure 3.4. 49: *D. tertiolecta* ammonium, phosphate, and lipid content in 120 g TDS/L PW media at initial concentrations of 13 mg NH_4-N/L and 1.7 mg PO_4-P/L.

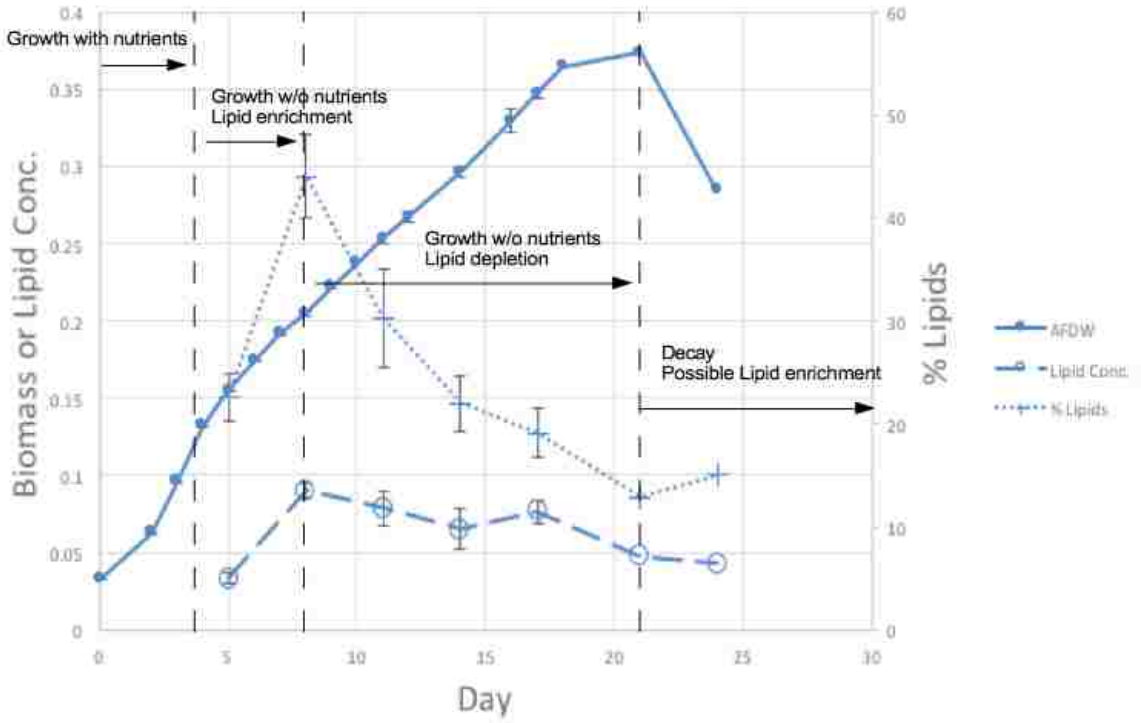

Figure 3.4. 50: Proposed growth phases for *D. tertiolecta* grown in a 120 g TDS/L PW media at initial concentrations of 13 mg NH_4-N and 1.7 mg PO_4-P/L.

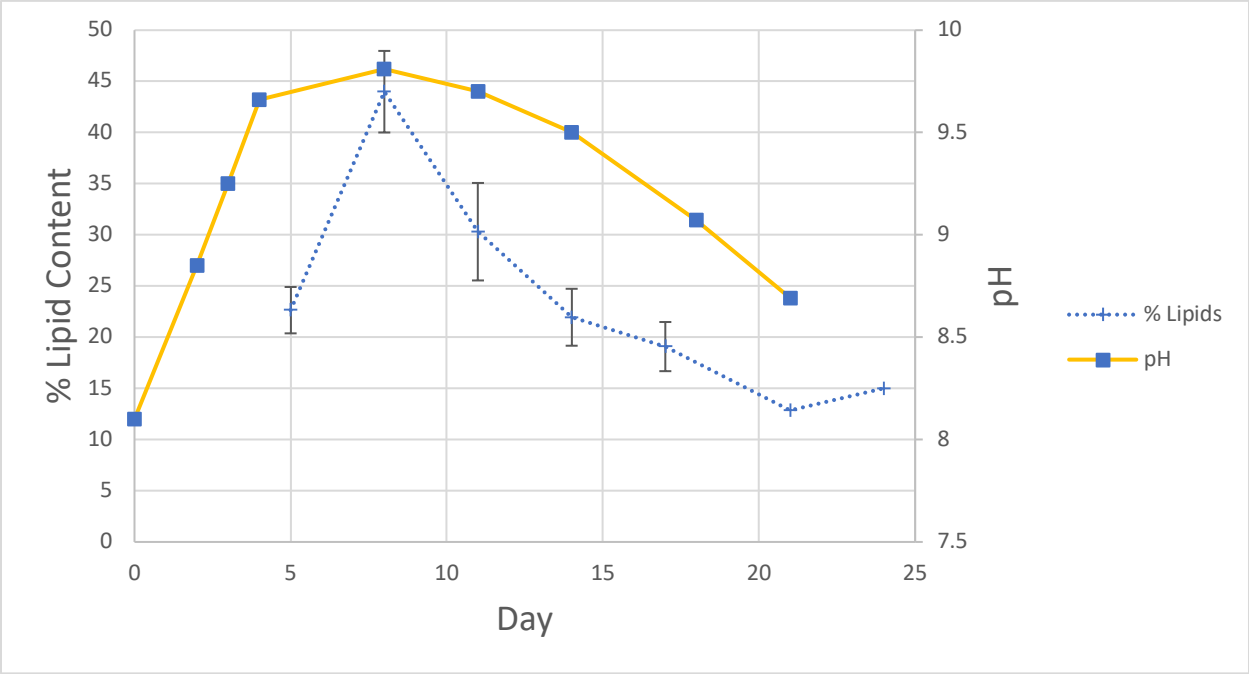

Figure 3.4. 51: Lipid fraction and pH measurements for *D. tertiolecta* in 120 g TDS/L PW media at initial concentrations of 13 mg NH_4-N and 1.7 mg PO_4-P/L.

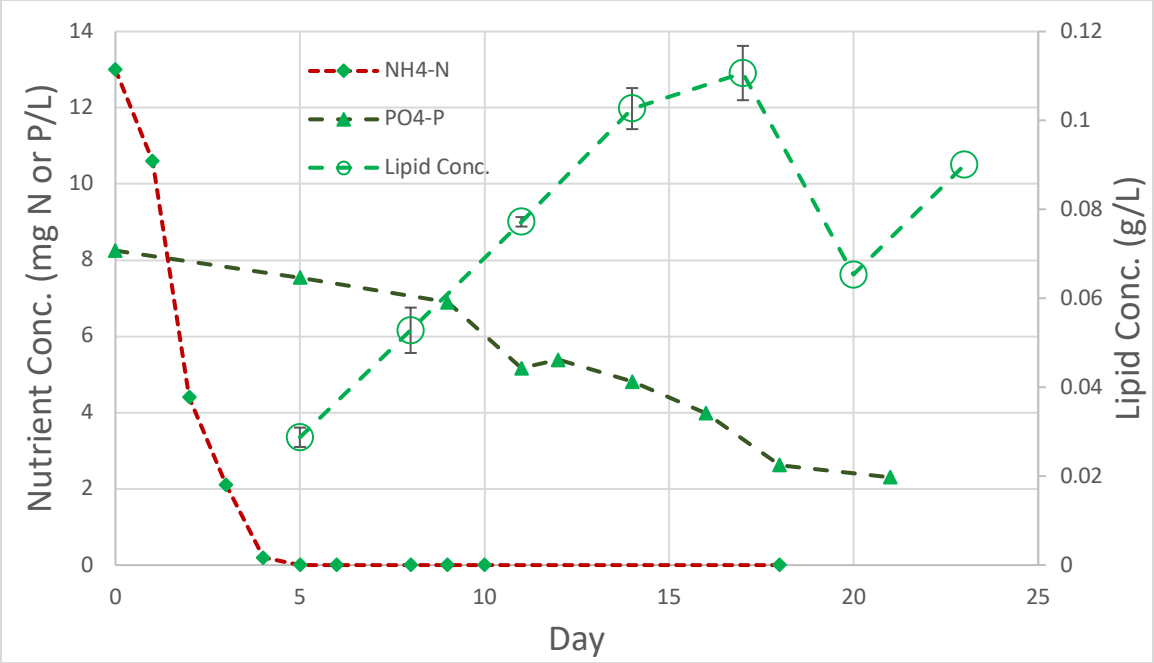

Figure 3.4. 52: *D. tertiolecta* ammonium, phosphate, and lipid concentration in 120 g TDS/L PW media at initial concentrations of 13 mg NH_4-N/L and 8 mg PO_4-P/L.

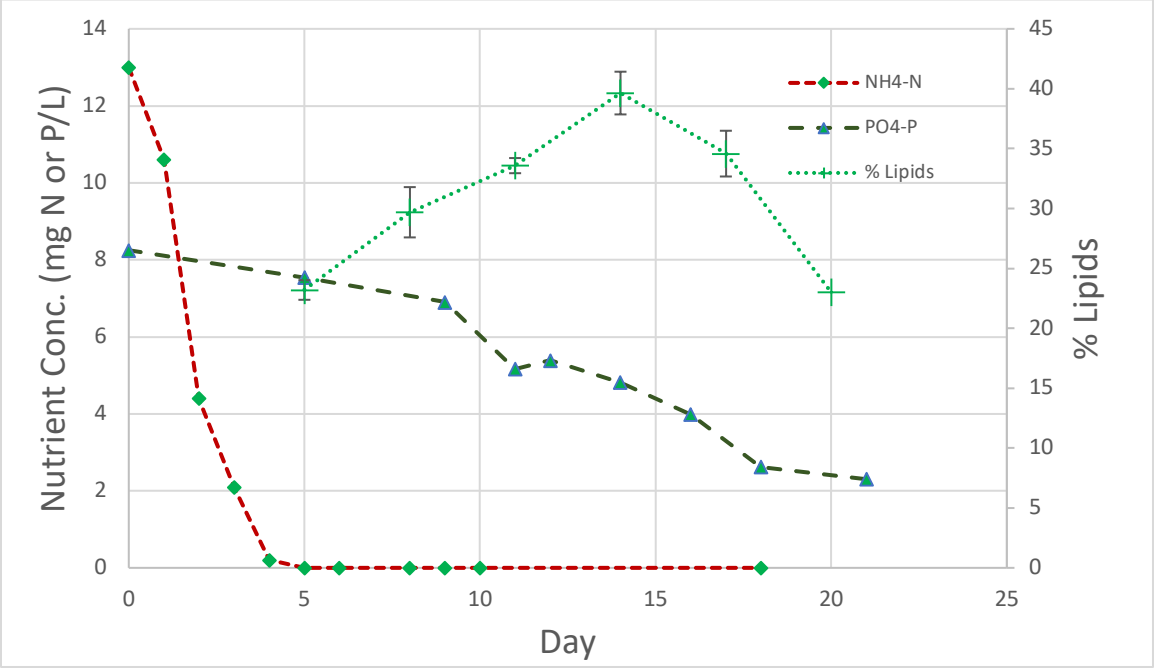

Figure 3.4. 53: *D. tertiolecta* ammonium, phosphate, and lipid content in 120 g TDS/L PW media at initial concentrations of 13 mg NH_4-N/L and 8 mg PO_4-P/L.

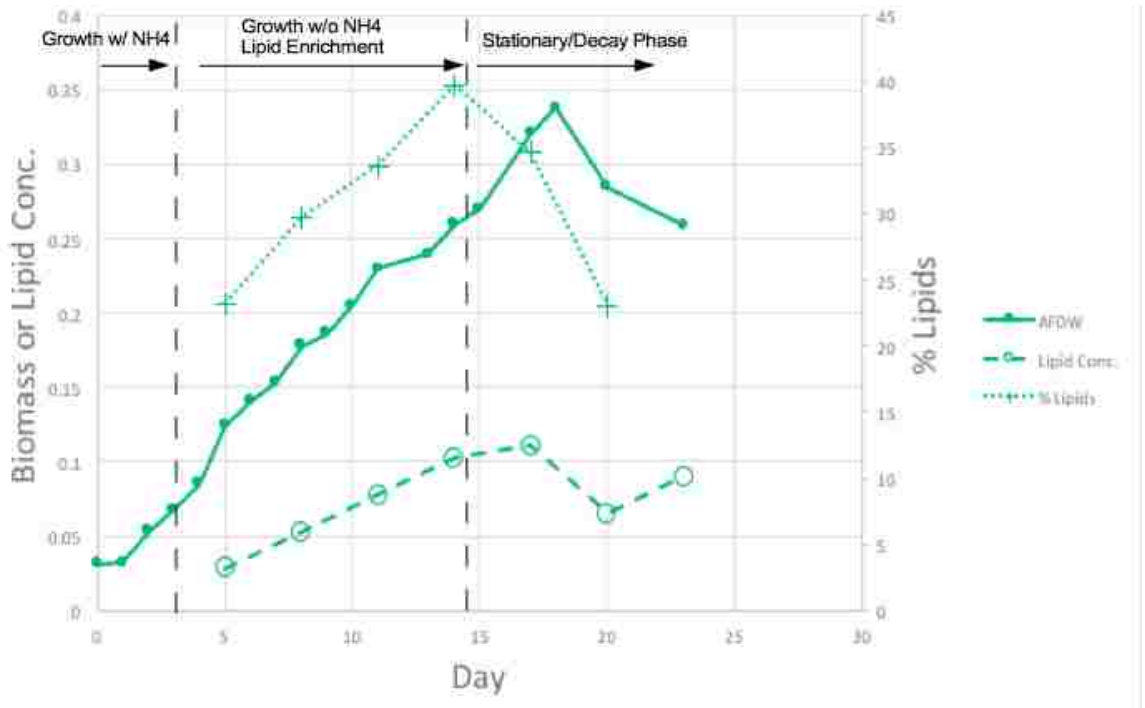

Figure 3.4. 54: Proposed growth phases for *D. tertiolecta* grown in a 120 g TDS/L PW media at initial concentrations of 13 mg NH_4-N and 1.7 mg PO_4-P/L.

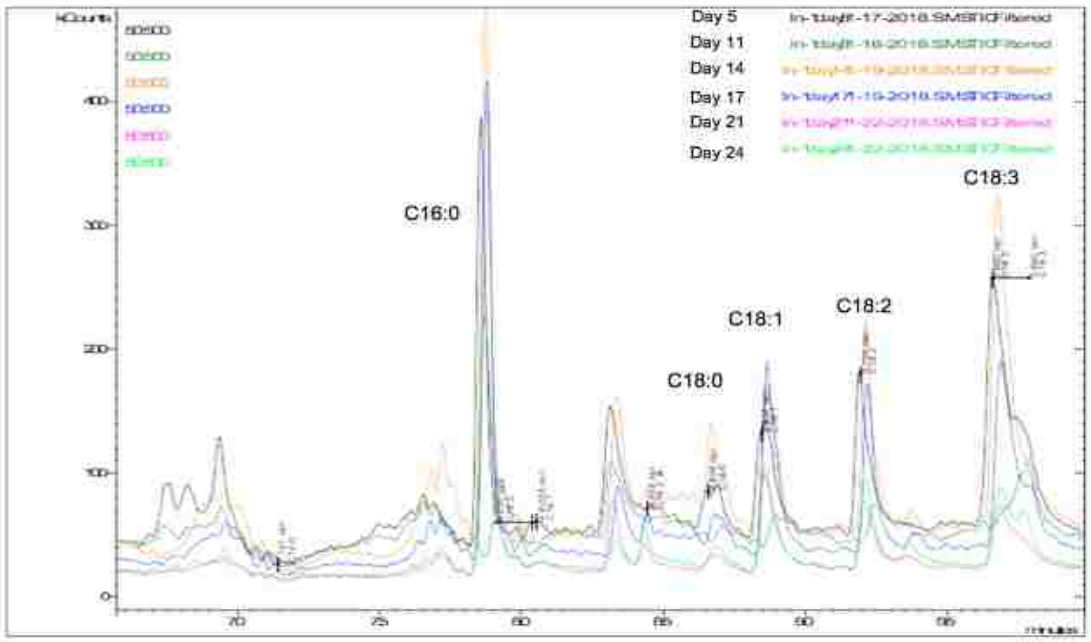

Figure 3.4. 55: Composite Chromatogram of *D. tertiolecta* FAMEs grown in a 120 g TDS/L PW media at initial concentrations of 13 mg NH_4-N and 1.7 mg PO_4-P/L .

The distinct peaks are palmitic acid (C16:0), C16:2, stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and α and γ linolenic acid (C18:3).

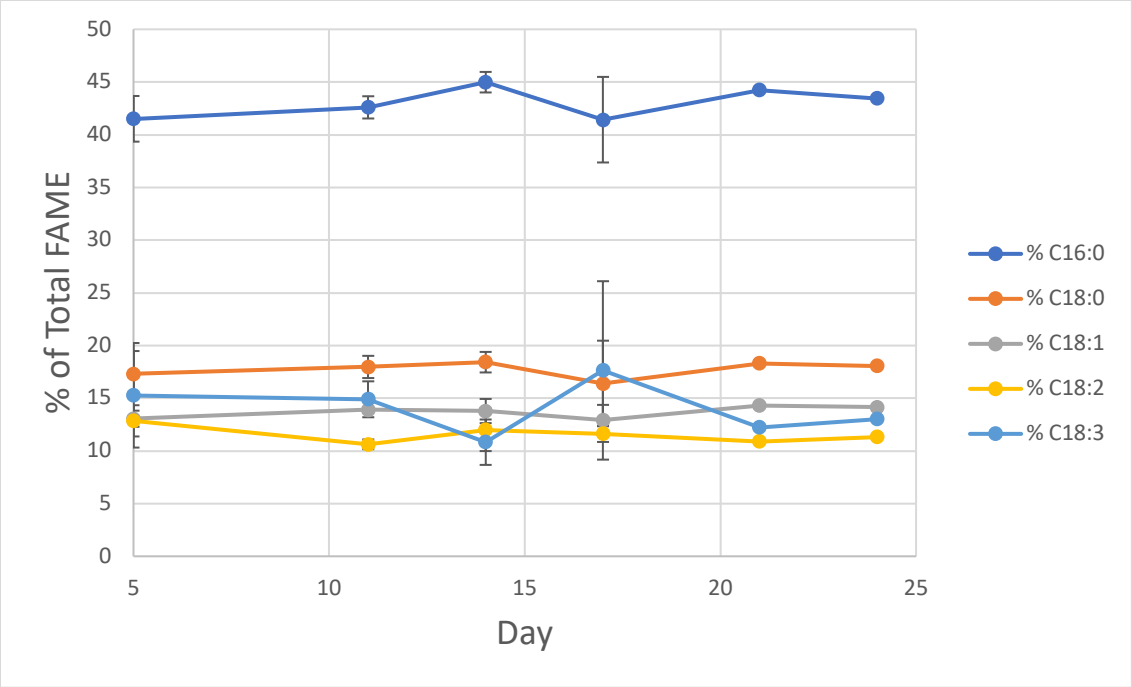

Figure 3.4. 56: FAME profile of *D. tertiolecta* grown in 120 g TDS/L PW media at initial concentrations of 13 mg NH_4-N and 1.7 mg PO_4-P/L.

3.5: Modeling the effect of free ammonia levels on growth in the PW media

In this study, using ammonium/ammonia as a nitrogen source for *D. tertiolecta* produced wildly varying growth effects for *D. tertiolecta* depending on the concentration, pH, and salinity. Not only did algae cells fail to grow at higher concentrations (≥ 52 mg NH_4-N/L), but high cell mortality was observed in microscopic view (Figure 3.5. 1). Most likely this is a result of the of free ammonia that more easily diffuses across membranes than the protonated ammonium and interferes with photosynthetic systems (Gutierrez et al., 2016). It is not possible to measure free ammonia levels easily in the media, but they can be calculated from pH and total ammonium values using the pKa for the ammonium/ammonia system. Due to the high ionic strength of the PW, the standard condition pKa value of 9.25 cannot be assumed

to be accurate. Benjamin (2002) states that the specific interaction model (SIM) can accurately predict activity coefficients in solutions with ionic strength in excess of 1 M by adding extra terms. Maeda et al. (1990) used the specific interaction model (also called the Pitzer Equations) to predict the pKa values of the ammonium/ammonia system in sodium/lithium chloride solutions of high ionic strength. Predictions closely matched observations. The pKa increased with ionic strength falling near 9.7 at 3M. Depending on pH, this would reduce considerably the amount of free ammonia at higher salinity. This effect would be more pronounced for pH values near the pKa, which is where the measurements in this study mostly fall. The pKa values, adjusted for ionic strength using SIM, can be used to estimate the level of free ammonia in hypersaline PW media.

In this study pKa values for NH_4/NH_3 species were estimated using equations from Maeda et al. (1990). Sodium and chloride are the most abundant ions in the PW media, and modeled pKa values assumed these electrolytes determined the activity coefficients of the acid base system. The true ionic strength is likely higher due to the presence of divalent ions such as Ca, Mg, SO_4, and CO_3, but this method likely yields an effective lower range increase for the pKa. Plotting pKa vs. sodium chloride concentration yields a linear relationship that increases with salinity (Figure 3.5. 2). The pKa at 120 g NaCl/L would be 9.62 and 9.35 in a 30 g NaCl/L solution. Figure 3.5. 3 shows that over the range in pH measured in the flask experiments the free ammonia species is significantly reduced from standard condition at 30 g TDS/L with a greater

effect at 120. The pKa values determined by the salinity can be used with the equation below to find the ammonia concentration.

$$[NH_3] = 10^{-pKa}/(10^{-pH} + 10^{-pKa}) * \text{Total } NH_4$$

Predicted pKa values, measured pH, and total ammonium were used to estimate the concentration of free ammonia in the *D. tertiolecta* media over time at different initial total ammonium concentrations (Figure 3.5. 4). The results predictably show the highest free ammonia levels in the initial conditions of 70 and 52 mg NH_4-N/L, where *D. tertiolecta* failed to grow and ultimately suffered high cell mortality (cell lysis observed microscopically). NH_3 concentrations are below 2 mg /L at the beginning but rise quickly with the increasing pH caused by photosynthetic activity to around 8.6-7. This increases free ammonia above (and in the case highest initial concentration well above) 4 mg /L. *D. tertiolecta* cells do not die immediately but are mostly dead by day 14 as evidenced in flask photos by the loss of pigmentation (Figure 3.5. 5). On the other extreme in the 13 mg NH_4-N/L estimated free ammonia never rose above 1 mg/L and strong growth was observed.

Initial conditions in between resulted in inhibition and growth responses. The 26 mg NH_4-N/L flasks exhibited far lower growth rates and ultimate culture density than at half the concentration (Figure 3.5. 6). It was exposed to free ammonia levels of around 2.5 mg/L. Cells did not appear to recover to this exposure even after concentrations fell. The 39 mg NH_4-N/L condition displayed a different pattern of initial inhibition following

by robust growth (when compared to the lowest total ammonium concentration) (Figure 3.5. 7). The concentration of free ammonia quickly spiked to almost 4 mg/L on day 2. It slowly fell until day 14 with a slight decrease in biomass density. During this time, there is some evidence of cell mortality by a small increase in media phosphate possibly caused by cell lysis. On day 14 the culture shifted into a growth phase as free ammonia levels reached around 2.4 mg NH_3-N/L. While this increased ammonia levels in the short term, the growing cells quickly absorbed ammonia nitrogen at a growth rate comparable to the lowest initial concentration. It is hypothesized that the 36 mg NH_4-N/L flasks were able to grow after free ammonia level fell in the range of 2-2.5 mg/L with possible cell acclimatization.

The different growth patterns displayed by the salinity conditions of 30 and 120 g TDS/L can be explained by higher free ammonia levels at lower salinity. At a pH of 8.8 which approximates those found in the cultures on day 2, free ammonia should be around 39% less in the hypersaline vs. the saline media based on the different pKa values. Ammonia species concentrations are far higher on day 2 in 30 g TDS/L media because of the lower pKa, ranging just above 2.5 mg/L (Figure 3.5. 8). Levels remain here until the culture enters a growth phase on day 10, and never enter the higher ranges experienced by cultures with high mortality. Interestingly, pH is increasing from day 2-10 before the growth phase indicating perhaps increased photosynthetic activity. It is hypothesized that early free ammonia levels of 2.5-2.6 mg NH_3-N/L caused growth inhibition. Cells where able to gradually acclimatize to the toxicity as their photosynthetic energy production increased. Eventually cells reached a level of cellular

metabolism required for rapid growth which quickly depleted ammonia nitrogen from the media eliminating any inhibitory effect. Racharaks et al. (2015) also noted increased ammonia inhibition for *D. tertiolecta* in lower strength media consisting of PW and anaerobic digester effluent. They made a similar hypothesis that ions in solution shield NH_4^+ species from deprotonation. As a general rule, it is proposed that ammonia toxicity will be reduced at higher ionic strength for *D. tertiolecta* and probably other species. In addition, higher total ammonium concentrations might be a way of maintaining growth rates in hyper-saline conditions, where nutrient uptake can be less efficient.

The measured and estimated results suggest *D. tertiolecta* experiences ammonia toxicity at levels between 2-2.5 mg NH_3-N/L. The 26 mg NH_4-N/L condition entered this range early on and experienced a significant growth reduction. Both the 39 mg NH_4-N/L and 30 g TDS/L conditions were able to enter growth phase after free ammonia levels fell to this value and/or after adaptation to it. At higher levels cells are stressed to maintain metabolism, perhaps due to interference with their photosynthetic activity by NH_3 diffusion across their membranes. This agrees with Chen et al. (2011) which reported strong growth at 1 mM of NH_4 (very close to the lowest ammonium concentration in this study), but none at higher concentrations of 10, 20, and 50 mM. Using a pKa of 9.35 and a buffered reported pH of 7.8 this would yield ammonia species levels of 3.8, 7.6, and 19.1 mg NH_3-N/L respectively. These levels are all higher than what produced growth in this study with PW media.

An inhibition threshold of 2-2.6 mg NH_3-N/L also is supported, with caveats, by results from two other studies. Racharaks et al. (2015) presents findings indicating lower growth in media at an ionic strength of 0.4 M than at 0.91 M with an initial concentration of 132 mg NH_4-N/L. Using calculated pKas from this study at those ionic strengths and reported initial pH values from their work, free ammonia levels of 2.4 and 2.0 mg NH_3-N/L are estimated for the low and high ionic strengths. This falls in the range found here of ammonia inhibition, and the 20% higher concentrations at the low ionic strength could be having an effect. Gutierrez et al. (2016) concluded that *D. tertiolecta* experienced ammonia inhibition at a concentration of 1.9 mg NH_3-N/L (Figure 3.5. 9). Free ammonia levels were calculated using equilibrium constants not specified, which affects comparison with this study. While growth was reduced above 1.9 mg NH_3-N/L, it did not cease until levels much higher than what was found here (13.2 mg NH_3-N/L). Cultures were reported to have a constant ammonia level under aeration. This may have stripped free ammonia gas reducing toxicity. Also, variation in sensitivity among *D. tertiolecta* strains is possible along with better acclimatization in Gutierrez et al. (2016). In summary, ammonia concentrations at or higher than 2-2.5 mg/L do not increase growth and have potentially inhibitory and lethal effects on a culture. For the purposes of cultivating *D. tertiolecta* in PW, lower concentrations would be preferred, and higher levels found in some sources might be inhibit or prevent growth.

Similar modeling of the free ammonia concentrations in Polyculture PW flasks experiments suggest it can tolerate and thrive on much higher levels. In the 60 g TDS/L media the pKa would be 9.42. In the 13 mg NH_4-N condition free ammonia spiked at 3.3

mg N/L while displaying strong growth (Figure 3.5. 10). *D. tertiolecta* did not exhibit growth at this level. The Polyculture was exposed to even higher levels in the greater initial ammonium conditions. Media pH values above 10 contributed to the high free ammonia concentrations. Free ammonia level topped 30 NH_3-N/L in the 70 mg NH_4-L condition. *D. tertiolecta* would likely experience high cell mortality under similar concentrations. The initial biomass growth rates in Figure 3.3. 33 suggest a small inhibitory effect in higher ammonia concentrations. Peak free ammonia values did not seem to affect growth curves of the Polyculture. *P. kessleri* composed the majority of the algae fraction at higher ammonium concentrations. The results suggest this species strain has higher affinity and tolerance to free ammonia.

The ability of species closely related to *Parachlorella* to tolerate and thrive on ammonia has been shown in other studies. Gutierrez et al. (2016) reported that *Chlorella sorokiniana* growth was unaffected by at a wide range of free ammonia concentrations. There was no reduction in cell division between 0 -12 mg NH_3/L. Tam & Wong (1996) found that *Chlorella vulgaris* (96% homology to *P. kessleri*) exhibited no difference in growth rates or maximum biomass density concentrations between 20 – 250 mg NH_3-N/L. Lu et al. (2018) set a concentration of 476 mg NH_3-N/L as the toxicity threshold for *Chlorella sp.*, which would exceed any concentrations published for PW samples. If free ammonia inhibits photosynthetic systems in algae, then the genus *Chlorella* and *Parachlorella* must possess a protection mechanism. The author was unable to find any published works on *C.* aponinum with ammonium present in the media. The reduced abundance of the cyanobacteria at higher concentrations suggest

its ability to cope with free ammonia is less robust. For practical purposes, *P. kessleri* seems to be able to tolerate and thrive on ammonium nitrogen already present in much of the PW.

If ammonium rich PW is to be used as a growth medium for algae cultivation ammonia toxicity should be studied for candidate species in a variety of conditions. Responses seem to vary by species in this study which is supported in the literature [Collos and Harrison, (2014) & Gutierrez et al. (2016)]. Different algae cultures and PW chemistries might have unique interactions. Algae growth in high alkalinity PW might exacerbate an algae species susceptibility to free ammonia. Maintaining pH at or below 8 (Which is commonly done through CO_2 addition.) and lower total ammonium concentrations might be prudent standard operating procedure for a variety of algae strains.

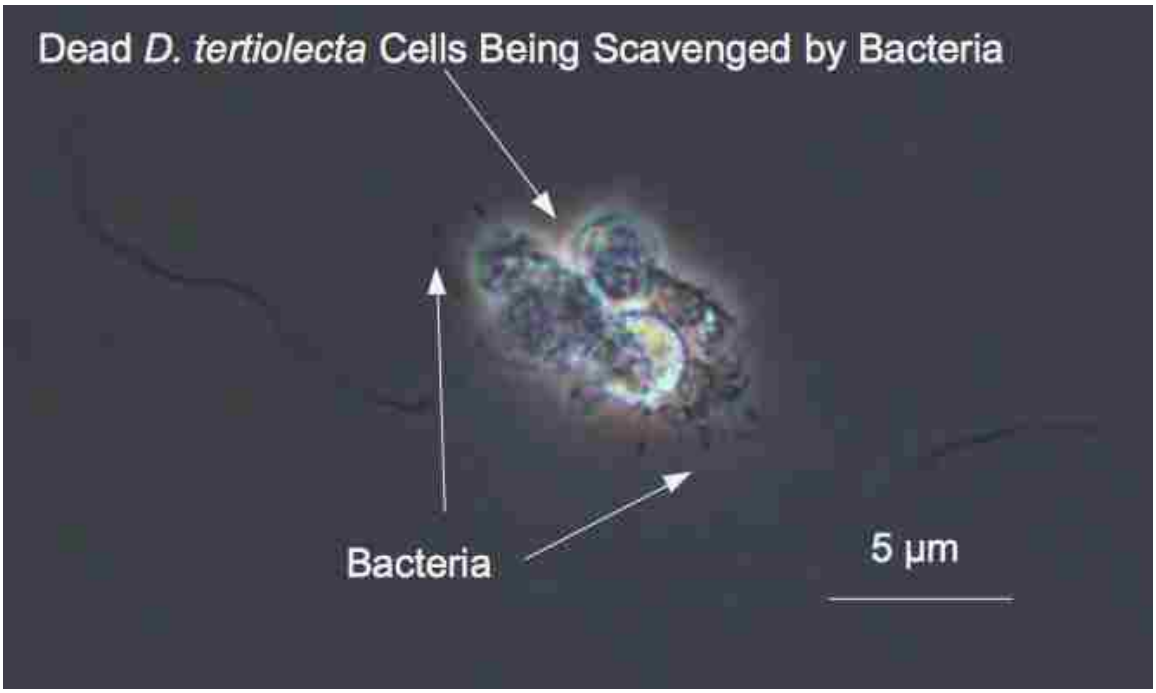

Figure 3.5. 1: Microscopic image of dead *D. tertiolecta* cells being scavenged by bacteria under phase contrast (800X).

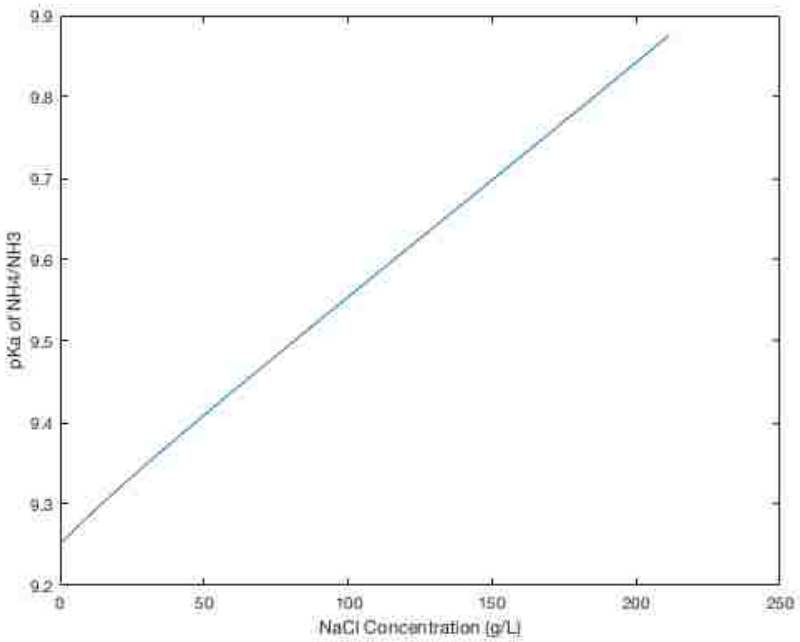

Figure 3.5. 2: The pKa values for ammonia/ammonium species as a function of salinity using equations from Maeda et al. (1990) based on the specific interaction model.

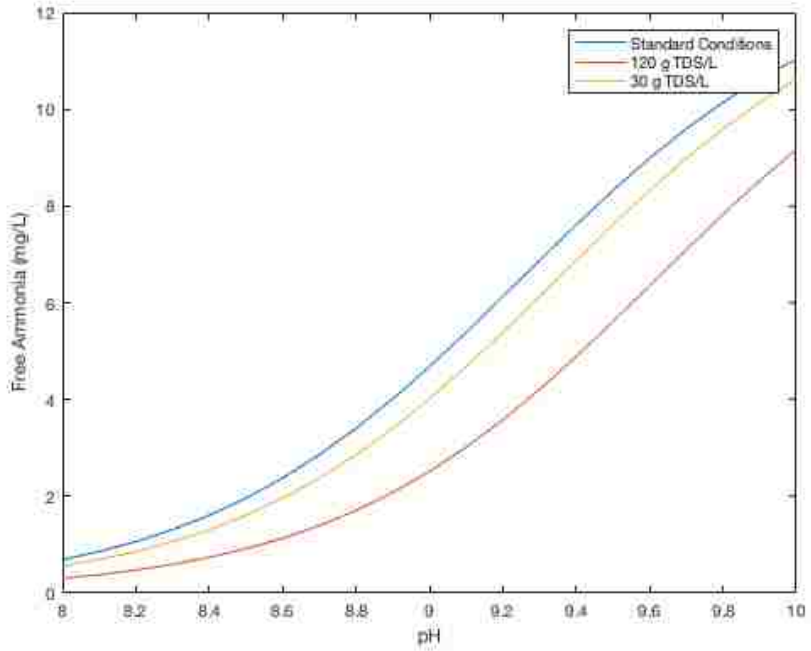

Figure 3.5. 3: Free ammonia levels in different solutions over a pH range of 8-10 assuming a total ammonium concentration of 13 mg NH_4-N/L.

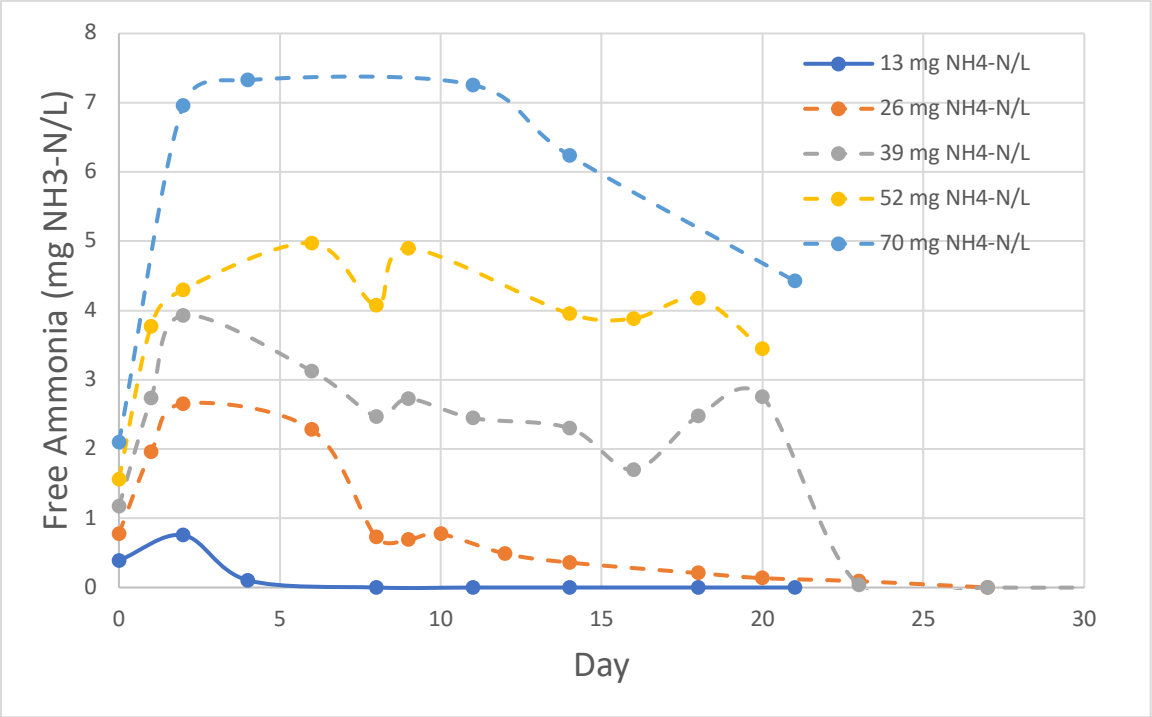

Figure 3.5. 4: Estimated free ammonia in the 120 g TDS/L PW media at a range of different initial total ammonium concentrations. Dashed lines displayed growth inhibition.

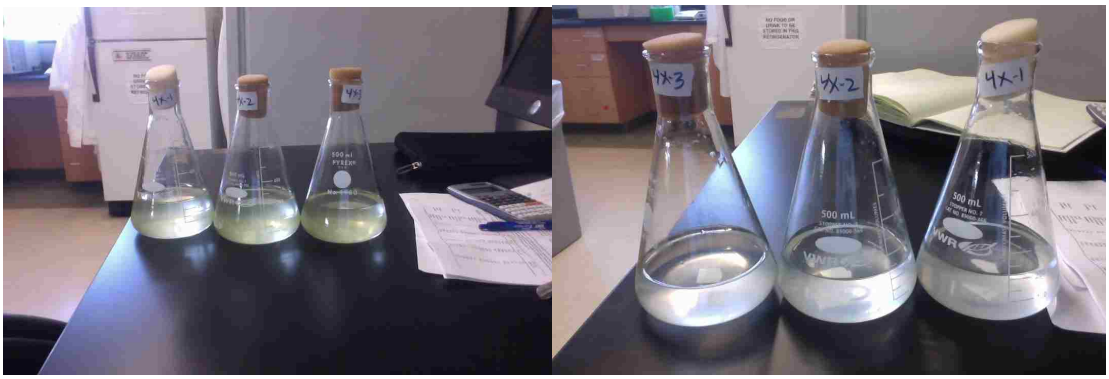

Figure 3.5. 5: Flask photos of *D. tertiolecta* growing in PW at an initial concentration of 52 mg NH_4-N/L on Day 2 (left) and Day 14 (right).

Note the presence of a yellow color on Day 2 and an absence on Day 14.

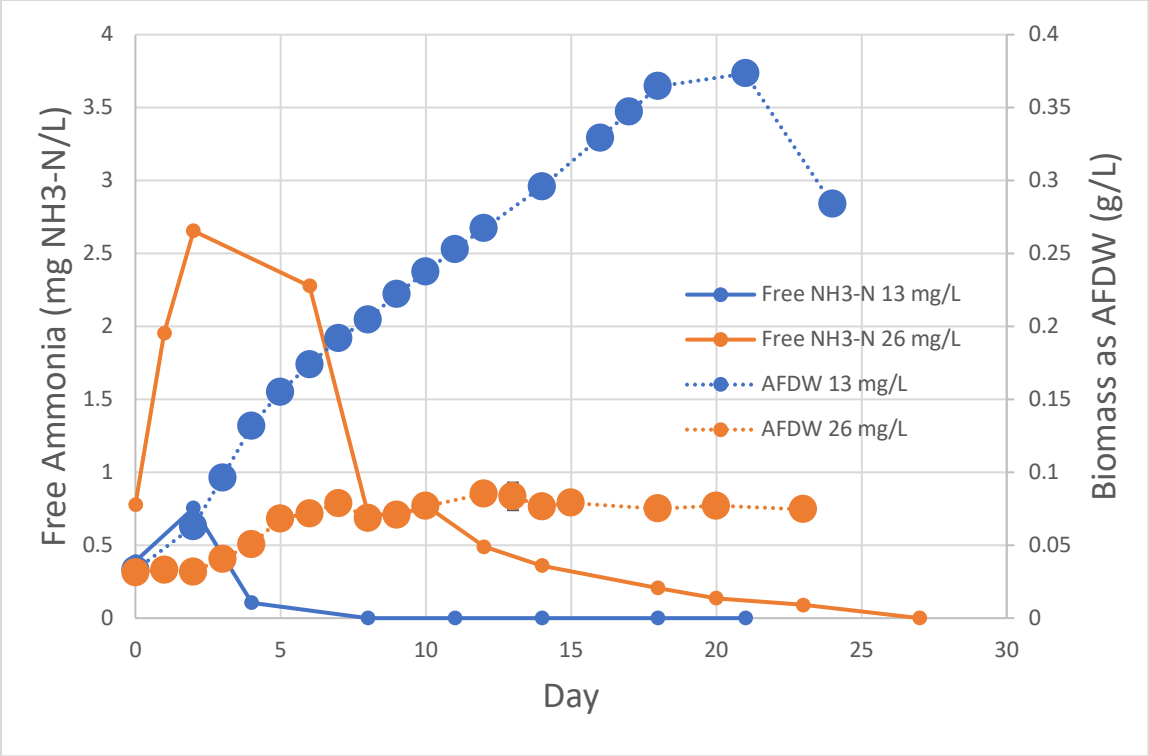

Figure 3.5. 6: Estimated free ammonia and measured biomass densities for *D. tertiolecta* in 120 g TDS/L PW media at initial concentrations of 13 and 26 mg NH_4-N/L.

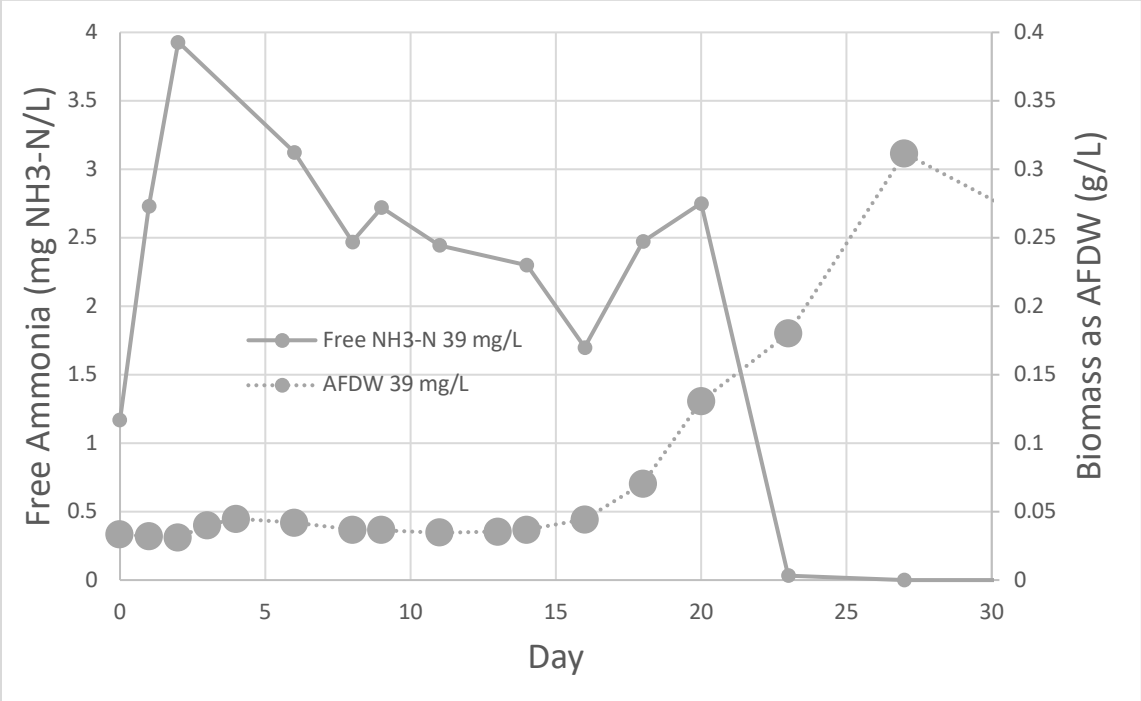

Figure 3.5. 7: Estimated free ammonia and measured biomass densities for *D. tertiolecta* in 120 g TDS/L PW media at an initial concentration of 39 mg NH_4-N/L.

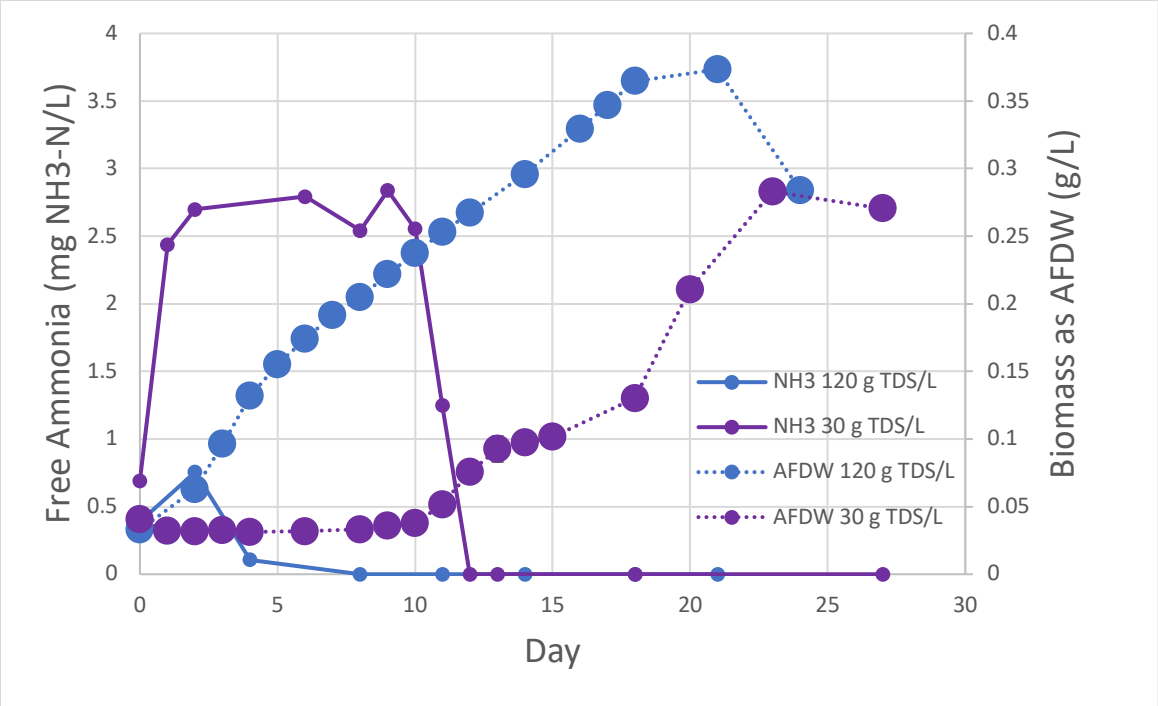

Figure 3.5. 8: Estimated free ammonia and measured biomass densities for *D. tertiolecta.*

In 30 and 120 g TDS/L PW media at an initial concentration of 13 mg NH_4-N/L. Growth inhibition and growth are labeled for the 30 g TDS/L salinity.

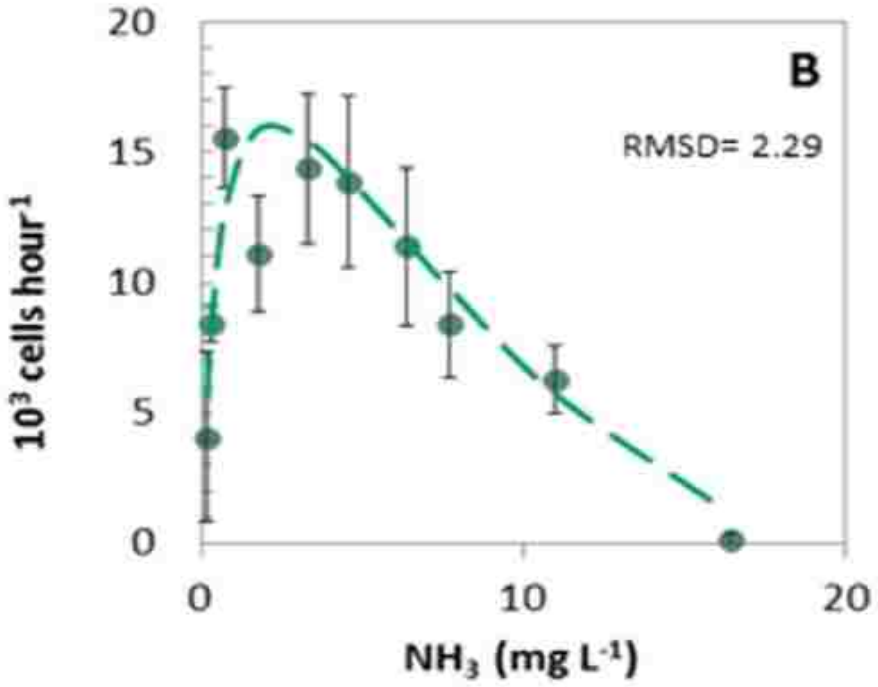

Figure 3.5. 9: Growth rate vs. ammonia concentration for *D. tertiolecta* taken from Gutierrez et al. (2016).

The dashed green line in the modeled growth kinetics.

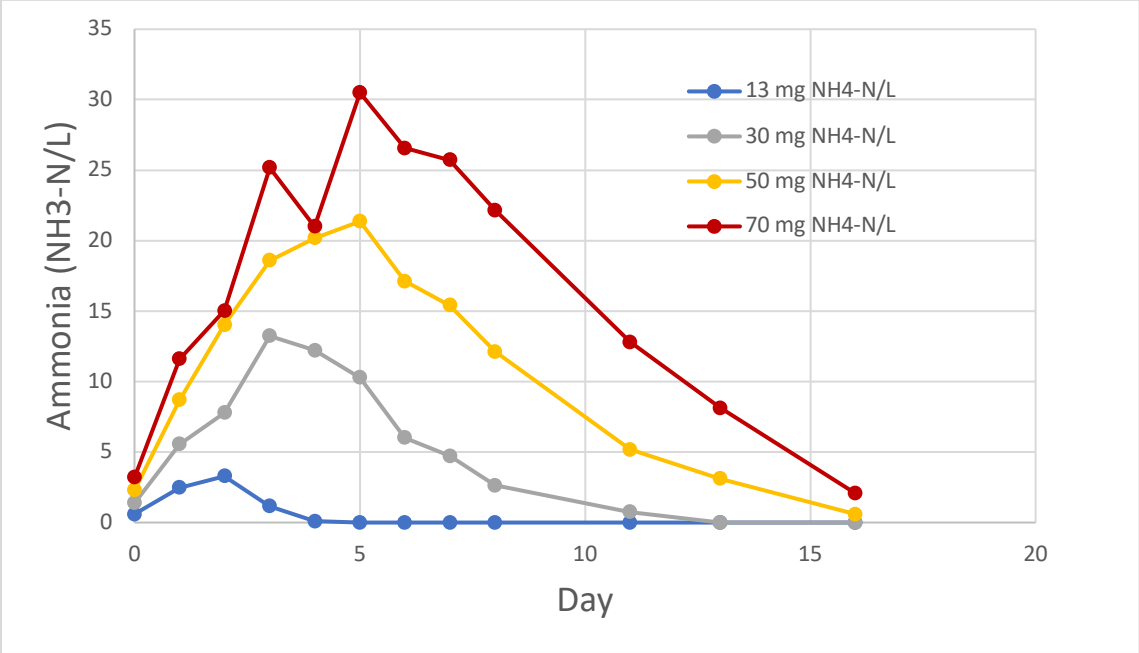

Figure 3.5. 10: Polyculture free ammonia levels in 60 g TDS/L PW media at a range of initial ammonium concentrations.

Chapter 4: Summary and Conclusion

4.1: Summary of the Polyculture's suitability for cultivation in PW

Based on the results of this study, the Polyculture (and its two-dominant algae species) are potential candidates for biofuel production in PW. Growth remained consistent and strong (45-50 mg/L/Day) over a wide range of salinity from 15-60 g TDS/L using nitrate (Figure 4.1. 1). A higher biomass productivity was achieved at the 60 g TDS/L salinity using ammonium, which is likely already present in PW, can be added with wastewater, and/or can be recycled from biofuel production such as anaerobic digestion. Compared to the commercial F/2 media, growth in PW was greater most likely because the Polyculture can utilize the higher concentration of bicarbonate. Growth could be boosted further with increased inorganic carbon (CO_2 aeration or

bicarbonate addition), nutrients, and/or illumination. High inorganic carbon concentration, ammonium, and low phosphate produced a sustained growth rate of 97.5 mg/L/Day in tubular photobioreactors. The results from the flask tests suggest that concentrations of around 13 mg NH_4-N and 1.7 mg PO_4-P produce the highest biomass productivity. These nutrient levels could be maintained with a continuous feed of enriched media. Many sources of PW have nutrient concentrations at or above these values. Experiments in outdoor raceway ponds could define the optimized illumination, nutrient, and pH levels.

Growth compares favorably with other species grown in PW and outdoor ponds in New Mexico. Racharaks et al. (2015) cultivated *D. tertiolecta* at around 42.1 mg/L/D and *Nannochloropsis salina* at around 41.7 mg/L/D under similar flask growth conditions. Higher growth rates were claimed in Racharaks et al., (2015), but they were achieved in a closed photoreactor system that would not be scalable for biofuel production. Aravinthan and Harrington (2013) claimed a growth rate of 49.7 mg suspended solids/L/Day using *D. tertiolecta* in brackish conditions (using suspended solids often yields a higher biomass than AFDW). At higher nutrient levels (10X those tested in flask tests here) and constant aeration, *N. salina* was reported to create biomass at a rate of 140 mg/L/Day (Graham et al., 2017), but once again these were not outdoor open pond conditions. Hodgkiss et al., 2016 reported a similar growth rate of around 120 mg/L/Day in coal bed methane waste water with a wild algae strain in the family *Chlamydomonadaceae*. The waste water in their study was brackish with a higher alkalinity of 23 mM. *Chlorella sp.* was cultivated with a biomass production rate

of 93 mg/L/D in high rate algal ponds in New Mexico (Weissmen and Goebel, 1988). Under optimized conditions it is possible the Polyculture could match or exceed these growth rates with a higher salinity tolerance (60 g TDS/L) than all except the *Dunaliella* genus. The commonly studied species *N. salina* has a much lower salinity threshold for optimal growth of around 40 g NaCl/L, and thus the Polyculture could extend cultivation to higher salinities (Bartley et al., 2013). Once again, the true potential of any species for biofuel production must be tested in realistic industrial scale cultivation systems.

The lipid content and productivity of the Polyculture are suitable for conversion to fuel. The maximum lipid productivity found in this study of 12 mg/L/Day shows promise and could be improved. Higher biomass growth at a salinity of 60 g TDS/L followed by a few days in a nitrogen or phosphorus deficient media could increase this value. Prior to harvest salinity stress could be used to increase lipid content and has been shown to increase lipid yield by 21% for *Chlorella* (Sibi et al., 2016). Also, exposure to increased concentrations of metal ions such as iron and copper have been claimed to increase lipid content in *Chlorella* (Li et al., 2013). Nutrient starvation would reduce protein content which improves the efficiency of anaerobic digestion and quality of fuel from hydrothermal liquefaction. The lipid derived FAME content is capable of producing suitable biodiesel due to the high percentage of saturated C16 and C18 content. Any issues involving viscosity could be handled by blending with unsaturated FAMEs from another source. Further testing is needed to explore how this unique Polyculture would perform in outdoor raceway ponds under different conditions.

The mixotrophic capabilities of *P. kessleri* could improve productivity of its cultivation in PW. Wang et al., 2012 documented a biomass density of over 6 g cell dry/L and productivity of around 280 mg/L/D growing the closely related *Chlorella kessleri* mixotrophically on 200mM of glycerol. In addition, the algae cells were able to thrive on sugars and ethanol. Piasecka et al. (2017) states that *P. kessleri* has the same potential for photoheterotrophic growth. Thus, biomass productivity can be greatly increased by using waste byproducts from biofuel production or other industrial processes. Glycerol is the chief byproduct of the transesterification of triglycerides to FAMEs. Also, processed algae biomass from biodiesel conversion commonly contains residual carbohydrates including sugar that could be fed back to the algae ponds. Sugar rich byproducts such as beet molasses can be added to media to increase growth (Piasecka et al., 2017). It is also possible, that the closely related *C. vulgaris* can metabolize petroleum compounds that might already be present in a PW medium (Kauss and Hutchison, 1970). This could serve a dual function of remediation of this challenging wastewater. Carbon dioxide is less available at higher salinity and adding an already reduced carbon source could significantly increase growth (Tafreshi & Shariati, 2007). Further experimentation with mixotrophic growth in a PW medium could fully explore its potential.

The Polyculture species were able to adapt to a variety of environmental stresses. Growth occurred over a pH range of around 4 – 10.6. The pH in outdoor ponds can exceed 11 under high photosynthetic conditions, which the Polyculture can tolerate (Park et al., 2011). Purposely allowing pH to increase to these levels can kill of

algae predators (Benemann et al., 1978). Acidification can occur in low alkalinity media when using ammonium (Eustance et al., 2013). The culture would likely survive sudden extreme swings in acidity or alkalinity whatever the cause. It was observed to grow in PW with a strong odor of hydrogen sulfide gas. The Polyculture tolerated metal concentrations in the PW media of 2.42 mg Cu/L and 0.386 mg As/L that inhibit some algae species. While algae growth was affected at higher salinities, it could survive levels as high as 120 g TDS/L for three weeks. During cultivation, this could be a useful characteristic to survive temporary spikes in salinity. Adjusting the salinity of the media might also be a way to manage algae predators by temporarily spiking to kill such organisms as rotifers and amoebas. Additional testing could better define the limits of the Polyculture.

4.2: Summary of the *D. tertiolecta's* suitability for cultivation in PW

D. tertiolecta is a strong candidate for biofuel production in PW media at salinities above 60 and below 210 g TDS/L. Growth rates while lower than the Polyculture below 60 g TDS/L are consistent from 30 – 120 g TDS/L at around 16-17 mg/L/D in PW using nutrient levels equal to the F/2 media. For cultivation, growth rates might be acceptable from 120 to above 200 g TDS/L. At a salinity of 120 g TDS/L ammonium levels of 13 mg NH_4-N/L boosted growth by around 28%. A continuous nutrient feed might be one technique improving productivity in hypersaline PW media and avoiding free ammonia inhibition. Growth rates were 240% higher in the high alkalinity PW as opposed to the F/2 commercial media. *Dunaliella's* increased growth on bicarbonate has been described previously, and its impact may be enhanced in

hypersaline media [Srinivasan et al., (2015) & Beardal and Giordano, (2009)]. Many sources of PW have alkalinity values up to 77 mM (Graham et al., 2017). Dissolved inorganic carbon already present or added should raise growth rates above those measured in this study to values as high as 60 mg/L/Day for *Dunaliella* HRAP cultivation (Huesemann & Benemann (2009) & Tafreshi and Shariati (2008)). Other algae species, such as those in the Polyculture, have a higher biomass productivity below 60 g TDS/L. Despite *D. tertiolecta's* slightly higher growth at lower salinities near seawater, its halotolerance lends it to cultivation at higher ionic strength. The number of algae species that can grow at 120 g TDS/L is small and the amount of PW with salinities in this range large. As always evaporation in outdoor conditions will only increase the TDS of the media. If this challenging media is to be used for large scale cultivation production, further study is warranted for this species as a candidate.

Lipid measurements of *D. tertiolecta* show its potential as an oleaginous biofuel feedstock. Peak oil content of 40-45% was achieved in some of the conditions (Figure 4.2. 1). Like biomass, lipid production was consistent over a broad salinity (Figure 3.4. 26). Maximum oil concentration did not necessarily match biomass in some conditions, and this would be an important factor in determining when to harvest. The highest lipid productivity of 11.2 mg/L/Day was obtained using low initial concentrations of ammonium and phosphate (13 mg NH_4-N and 1.7 PO_4-P/L) under alkaline conditions (pH 9.8). A time dependent lipid enrichment peak occurred a few days after ammonium depletion in the media. This peak probably corresponds with peak inorganic carbon uptake as determined by pH. In media with nitrate, N stress did not seem to clearly

enrich fat content of the biomass to the point it improved lipid productivity. The lipid derived FAME profiles would produce a high cetane biodiesel. Proportionally less C18:3 was found than reported in other studies (Racharaks et al., 2015 & Chen et al., 2011) improving the quality (in terms of cetane number and oxidative stability) of derived biodiesel.

Ammonia toxicity is a concern for *D. tertiolecta* cultivation utilizing produced water. Free ammonia concentrations of around 2-2.5 mg NH_3-N/L cause severe inhibition of growth. The potentially high concentrations of ammonium (over 300 mg NH_4/L) found in PW samples (with or without photosynthetic induced pH increases) could easily exceed this threshold. Algae utilization of bicarbonate clearly produces more alkaline conditions while increasing growth. Care would be needed to maintain overall concentrations in the range of optimal growth while keeping pH well below the pKa of the ammonium system. Carbon dioxide addition to the media could prevent alkaline pH values reducing the fraction of ammonia species and increasing the availability of inorganic carbon. *D. tertiolecta* can survive free ammonia concentrations in excess of 2-2.5 mg/L allowing time to restore more favorable growth conditions. Acclimatization or selection of different *D.* tertiolecta strains might reduce the susceptibility to ammonia. Interestingly, it is possible that free ammonia concentrations less than 2 mg N/L might stimulate growth. One potential advantage of ammonia toxicity is that it may be possible to reduce C18:3 content of the derived biodiesel improving oxidative stability and engine ignition (Gutierrez et al., 2016).

D. tertiolecta's high glycerol content could be used as an organic carbon source for mixotrophic growth of other algae strains or as a supplement to improve oil yields of other biomass feedstocks. Many members of the *Chlorella* genus are considered for biofuel production and have enhanced growth in mixotrophic conditions. The biofuel candidates *Nannochloropsis, Chlorella* sp., *Scenedesmus* sp., and *Haematococcus* were documented to have higher biomass and lipid productivities in mixotrophic conditions using glycerol (Andruleviciut et al., 2014). Glycerol produced as a byproduct of *D. tertiolecta's* conversion to biodiesel or other methods could be utilized by capable algae species to increase yields. In addition, glycerol remains in the growth media after *Dunaliella* harvesting (Tafreshi and Shariati, 2008). Mixotrophic algae could be relied upon to utilize the left-over glycerol and nutrients of the spent media. Cao et al. (2016) reported glycerol addition can improve the oil yield of rice straw with Hydrothermal liquefaction. A practical process for utilizing *D. tertiolecta's* glycerol to improve algae biofuel production warrants further study.

4.3: Design and operational ramifications for algae biofuel production and hypersaline wastewater remediation

Algae biofuel production utilizing the challenging PW media is clearly feasible. The saline and hypersaline conditions of many PW water sources are not a barrier to algae growth. Both algal cultures in this study exhibited adequate growth for cultivation in hypersaline PW that spans the range of many measured samples. Ishika et al. (2017) proposed cultivation over a range of salinities to efficiently recover nutrients from spent saline media, and the algae cultures in this study would be potential candidates. Other

species in the *Dunaliella* genus could extend the salinity range and improve productivity. *D. salina* has an even higher salt tolerance up to the saturation point of sodium chloride (Oren, 2005). This could extend cultivation salinities to over 300 g TDS/L. Another member of the genus, *D. viridis,* has been reported to possess higher growth rates between 100 and 200 g TDs/L (Cifuentes et al., 2001). Results from study and others indicate that algae species could be cultivated in the full range of PW salinities measured.

Hypersaline cultivation of algae even has advantages over lower ionic strength. The number of algae herbivores that can survive these conditions is much reduced (Tafreshi and Shariati, 2008). In open pond systems, high ionic strength PW could be used to kill off any algae grazers with lower halotolerance than the algae. Fungal parasitism and viral infection have the ability to also reduce biomass productivity (Park et al., 2011). Hypersaline media would likely inhibit both these classes of organisms. Invasive algae species with less optimal oil yields would have a much lower chance of establishing themselves at salinities comparable to brine lakes.

Nutrient levels already present in PW are adequate for high algae biomass productivity. Many studies have reported ammonium concentrations in excess of the 13 mg NH_3-N/L that resulted in the highest growth rates. Overall, the reduced nitrogen in ammonium seems to produce higher growth for the cultures in this study, and that might be broadly applicable to more strains and species. The low concentrations of phosphate (1.7 mg PO_4-P/L), that led to the highest growth rates in these experiments, are most likely commonly found in PW. Trace metal such as iron are also usually

present in PW samples (Graham et al., 2017). PW with high alkalinity has elevated levels of inorganic carbon that improve growth rates. Operating costs could be reduced by about a quarter by removing the need for nutrient addition. Species capable of growth in the hypersaline conditions would improve the efficiency of utilizing the nutrient levels already present (Ishika et al., 2017). Removing cost associated with nutrient addition should improve the economic outlook for algae biofuel production.

Lower phosphate concentration might improve biomass production and high phosphate lipid productivity. In this study both the species in the Polyculture and *D. tertiolecta* exhibited mildly inhibited growth at the elevated phosphate concentration of 8 vs. 1.7 mg PO_4-P/L. Species appear to be likely storing the extra phosphate as polyphosphate inclusions. Phosphate bonds in ATP require 31.8 kJ/Mol for formation and are a crucial part of how cells store and transmit energy (Madigan et al., 2015). It is likely that in this study algae cells diverted energy away from growth to store it in polyphosphate bonds. This had the effect of slightly reducing the growth rate compared to the lower phosphate condition. Maintaining low phosphate concentrations (near 1.7 mg PO_4-P/L) seems to be a viable strategy to optimize growth rates. While growth rates at the high phosphate condition were lower, lipid productivity was higher. It appears in this study that nitrogen stress without phosphate limitation results in a higher peak lipid content of the algae (47% vs. 35%). This effect might apply to other potential algae biofuel candidates.

Though this study focusing on algae cultivation for biofuel production, algae appear capable of quickly removing nutrients from hypersaline waste water. The

Polyculture was able to sustain a removal rate around 12 mg NH_4-N/L/Day in the tubular bioreactors. *D. tertiolecta* displayed a lower removal rate of around 3 mg NH_4-N/L/Day. Hypersaline oilfield disposed of in surface streams contains high levels of ammonium that could cause eutrophication and formation of disinfection byproducts in downstream water treatment plants (Harkness et al., 2015). Both cultures in this study and likely other algae could remove ammonium from discharged PW removing this risk. Both the Polyculture and *D. tertiolecta* were able to efficiently uptake phosphate. This ability could be used reduce eutrophication in surface water from discharged waste.

4.4: Future research directions and potential

The results from this study need to be tested at the pilot scale in realistic outdoor cultivation conditions. Though the species presented here appear to be strong candidates for biofuel production, results may vary in an open raceway system. A number of different environmental factors would vary possible affecting biomass and lipid productivity. Diurnal and seasonal temperature cycles, changes in light intensity, PW character variation, and many other factors would exert an influence. Many of these variables would be site specific. Pilot scale experiments exploring these parameters would be informative to determine the viability of large scale algae cultivation in PW.

The influence of potentially toxic components of PW should be more thoroughly explored. Heavy metal concentrations in this wastewater can be high and may influence biofuel production. For example, Cu, Cr, Zn, Cd and Pb were shown to inhibit growth in *Chlorella vulgaris* and *D. tertiolecta* (Ouyang et al., 2012 & Tsuji et al., 2002). Also, algae

produce biomolecules called phytochelators that sequester metals intracellularly in vacuoles (Hirato et al., 2001). This could contribute to toxicity during anaerobic digestion or nutrient recycling. Many biorefinery schemes suggest utilizing left over biomass from biofuel production for fertilizer and animal feed. Enriched heavy metal content of the biomass could be prohibitive for these uses.

Mixotrophic growth of algae cultures has the potential to increase biomass and lipid yields. Additional experiments should be performed in saline and hypersaline media using different substrates (particularly glycerol). Various industrial sources could be tested such as food waste. The mixotrophic potential of *Dunaliella* has not been fully defined yet and might boost growth in hypersaline media. Lipid productivity and character under these conditions should also be better defined.

If large scale algae biofuel production is to become reality PW might be the only viable water source in arid inland regions. Algae can be cultivated in this challenging media. The economics of the processes need to be explored further to determine whether a reasonable path exists. While low petroleum prices currently exist, this may not remain the case. The need to reduce carbon emissions will remain relevant. Algae derived biofuels may yet represent a significant source of world energy consumption.

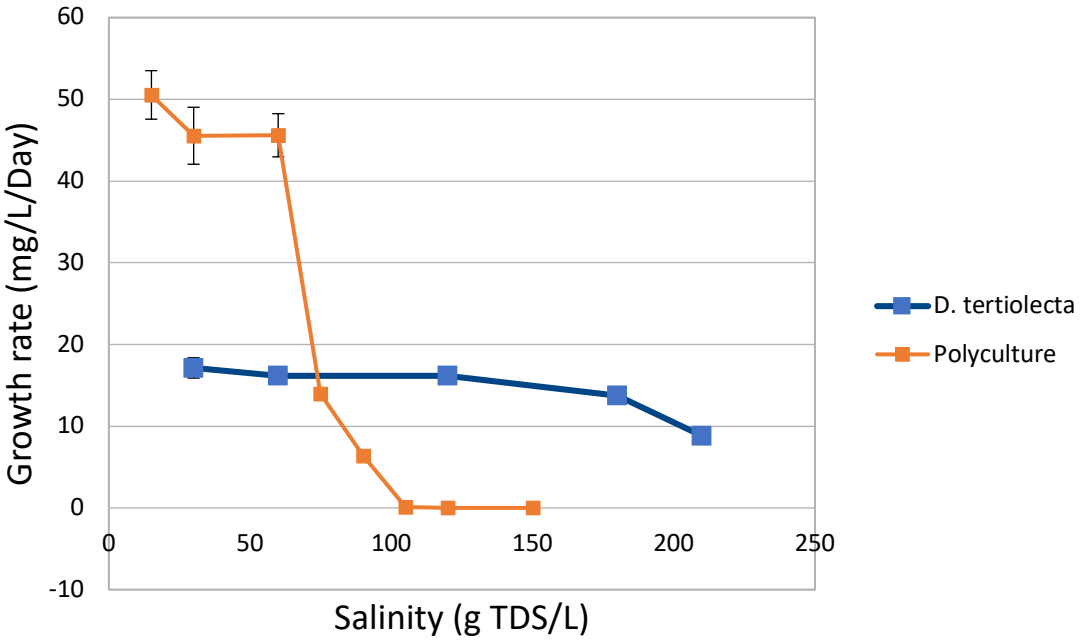

Figure 4.1. 1: Polyculture and *D. tertiolecta* growth rates in PW media at a range of salinities. Initial nitrate, phosphate, and trace metal levels were kept constant.

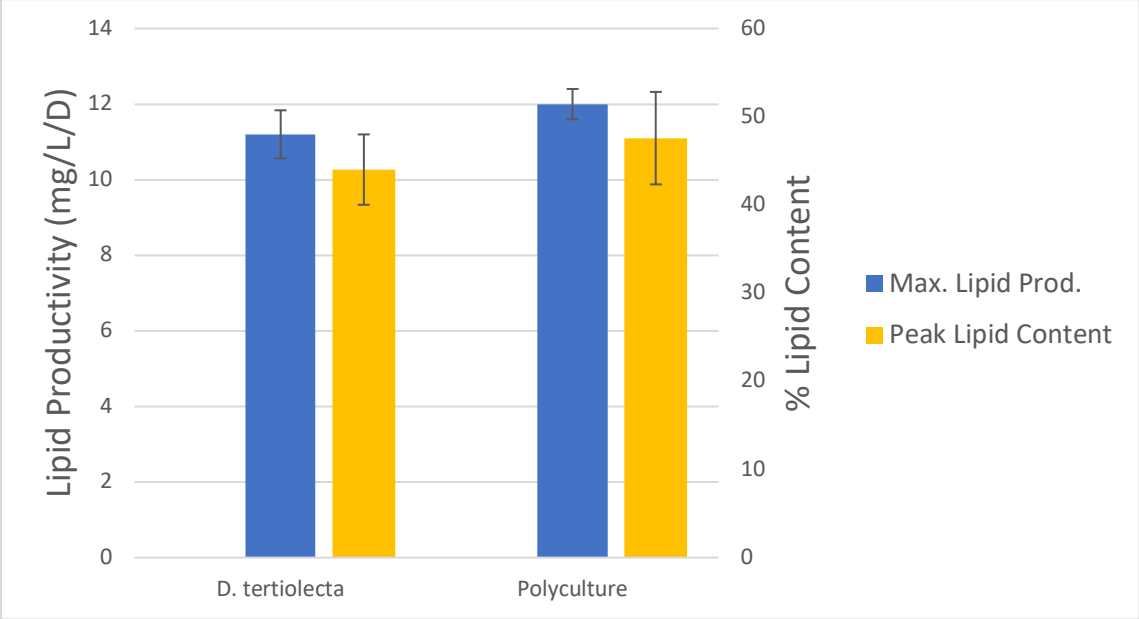

Figure 4.2. 1: Maximum measured lipid productivity and content for *D. tertiolecta* and the Polyculture in PW media.

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