Cultivation of *Nannochloropsis salina* and *Dunaliella tertiolecta* Using Shale Gas Flowback Water and Anaerobic Digestion Effluent as Cultivation Medium

THESIS

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By

Ratanachat Racharaks

Graduate Program in Food, Agricultural and Biological Engineering

The Ohio State University

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Master's Examination Committee:

Dr. Yebo Li, Advisor

Dr. Peter Ling

Dr. Jiyoung Lee

Abstract

Microalgae have been extensively studied for their capabilities in producing more lipids per acre than agricultural crops. Because of high costs, this technology has yet been proven economical on a large scale. Currently unreported is the use of flowback water from shale gas exploration as a way to reduce costs by meeting the water demand of marine microalgae cultivation. Additionally, while anaerobic digestion (AD) effluent has been shown as a promising alternative nutrient source for microalgae cultivation, unreported is the use of AD effluent as a nutrient supplement for the cultivation with flowback water.

This study hypothesized that the combination of the flowback water, as the source of water, and the AD effluent, as the source of nutrients, may act as a growth medium for marine microalgae cultivation. To validate this hypothesis, three marine microalgal strains were evaluated on the ability to adapt to different concentrations of the AD effluent in the flowback water. Among the microalgal candidates, it was determined that *Nannochloropsis salina* and *Dunaliella tertiolecta,* microalgal strains previously considered as candidates for biofuels and pigment production, were the most tolerant to the inhibitors found in the flowback water and the AD effluent. These microalgal strains were found to achieve the highest average biomass productivity in the medium composed of the flowback water supplemented with the AD effluent loading of 6%. Algal growth in this medium was then compared to growth in a

medium composed of commercial nutrients and salts at similar initial inorganic nitrogen (~132 mg L^{-1} N), salinity (~42 g L^{-1}), and pH (~7.97) levels. Comparable growth in both media was found with the specific growth rates of 0.293 and 0.348 day^{-1} and the average biomass productivities of 225 and 275 mg L^{-1} $day^{-1}$for *N. salina* and *D. tertiolecta*, respectively.

Analysis of the lipid content and profile of both strains cultivated in the medium composed of the flowback water supplemented with the 6% AD effluent further indicated that the medium had little effect on the lipid productivity of both microalgal strains. A parameter evaluated was the cetane number of the total microalgal fatty acids. The cetane number is an indicator of the potential fuel quality of the microalgal-derived biodiesel as a higher cetane number implies easier ignition and lower nitrous oxide emissions. While a minor decrease of 1.84% was observed for the cetane number of *N. salina*, no significant differences were observed for *D. tertiolecta* when compared to the values obtained in a medium composed of commercial nutrients and salts. These results suggested that comparable lipids with similar profiles could be produced by using the medium composed of the flowback water and the AD effluent.

Another objective of this research was to evaluate the effects of chemicals and microbial communities in the AD effluent and the flowback water on algal growth and sedimentation. By autoclaving the algal growth media, that contained different combinations of the nutrient and salinity sources, it was determined that the ability to examine the influence of the microbial activity was not feasible due to the significant

losses of ammonium concentrations from the autoclaving process. A greater difference in the specific growth rate of *D. tertiolecta* was found after cultivation in the autoclaved and non-autoclaved media containing commercial salts than the difference found after cultivation in the autoclaved and non-autoclaved media containing the flowback water. The specific growth rate of *D. tertiolecta* in the autoclaved media containing commercial salts and the autoclaved media containing the flowback water, however, were not significantly different. As *D. tertiolecta* is a known microalga sensitive to concentrations of free-ammonia, it was hypothesized that the higher ionic strength of the flowback water may minimize ammonia, derived from the loading of the AD effluent, from dissociating into free-ammonia in the growth media. Further research is still necessary to reveal the mechanisms involved as the autoclaving process may have also effected concentrations of unknown growth inhibitors.

This research found that cultivation of marine microalgae in shale gas flowback water is feasible and, when coupled with AD effluent, may be able to result in comparable growth and lipid productivities to values achieved by using commercial nutrients and salts. The ability to use shale gas flowback water and AD effluent to cultivate marine microalgae may reduce production costs and improve the economic viability of the algae biofuels industry.

Dedication

This document is dedicated to my family.

Acknowledgments

I would like to thank my advisor, Dr. Yebo Li, for his support and guidance over the past 6 years I have known him. Before I had background in any field of research, Dr. Li was willing to take a chance by hiring me as an intern during the first year of my education at Ohio State University. As a result, I had an invaluable experience learning from personal like Caixia Wan whose talent and dedication formed the basis of who I am as a researcher today. During this time, I also had the opportunity to meet Ting Cai, Stephen Park, and Johnathon Sheets whose prior research inspired my interest in microalgae and later formed the entire focus of the thesis presented in this paper.

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Vita

1989.....Born, Akron, Ohio

2012 .....B.S. Food, Agricultural and Biological Engineering, The Ohio State University

2012-Present .....Graduate Research Fellow, Food, Agricultural and Biological Engineering, The Ohio State University

Publications

B.J. Allred and R. Racharaks. Laboratory Comparison of Four Iron-Based Filter Materials for Drainage Water Phosphate Treatment. *Water Environment Research*. (submitted)

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Fields of Study

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Chapter 1: Introduction

1.1. Background

U.S energy consumption is estimated to grow at an average annual rate of 0.3 % from 2010 through 2035 (EIA, 2012). The continued growth in the demand for energy over the next 25 years has made microalgae an increasingly attractive option for use as a biofuel feedstock. Studied extensively by the U.S. Department of Energy, microalgae have the capability of capturing carbon dioxide, producing high-value pigments, and yielding more lipids per acre than agricultural crops (Hammouda et al., 1995; Sheehan et al., 1998). Meeting the water and nutrient requirements for algal cultivation, however, remains a significant barrier to the economic viability of algal biofuels (Jonker and Faaij, 2012).

While seawater has been proposed as an alternative water source for commercial microalgae cultivation, more inland saline water resources are required to have a significant impact on microalgae production facilities (Department Of Energy, 2009; Lansford and Solar Energy Research Institute., 1990; Olguin et al., 1997). The use of shale gas flowback water for marine microalgae cultivation has not yet been reported. Produced at a volume between 2 and 6 million gallons per well life, flowback water is an increasingly available wastewater resource that may meet the water demand of commercial marine microalgae production (M.E.Blauch et al., 2009).

Additionally, researchers have proposed a way to reduce costs associated with microalgae cultivation by using anaerobic digestion (AD) effluent to meet the nutrient requirements (Cai, 2012; Wang et al., 2010). Implemented to treat a variety of organic wastes, AD converts organic matter into biogas and produces a nutrient-rich effluent. The high levels of total phosphorus and total nitrogen, which have raised concerns regarding watershed eutrophication after land application, have also made AD effluent a likely candidate for algal cultivation (Godos et al., 2009). The use of AD effluent as a supplement for marine microalgae cultivation with flowback water, however, remains currently unreported.

1.2. Research Objectives

This study hypothesizes that the combination of the flowback water, as the source of water, and the anaerobic digestion (AD) effluent, as the source of nutrients, may act as a growth medium for marine microalgae cultivation. The following main objectives and specific aims were achieved to test the hypothesis:

Objective 1: Cultivation of marine microalgae in flowback water supplemented with AD effluent

Specific aims:

1.1. Effect of AD effluent loadings on microalgal growth in flowback water

1.2. Effect of nutrient and salinity sources on microalgal growth

1.3. Effect of nutrient and salinity sources on lipid production

Objective 2: Evaluation of the effects of chemicals and microbial communities in AD effluent and shale gas flowback water on algal growth and sedimentation

Specific aims:

2.1. Cultivation of *N. salina* and *D. tertiolecta* in autoclaved and non-autoclaved media

2.2. Sedimentation kinetics of *N. salina* and *D. tertiolecta* in autoclaved and non-autoclaved media

Chapter 2: Cultivation of Marine Microalgae in Flowback Water Supplemented with Anaerobic Digestion Effluent

Abstract

Shale gas flowback water and AD effluent were evaluated as potential water and nutrient sources for the cultivation of marine microalgae. *N. salina* and *D. tertiolecta* grew well in the flowback water with the AD effluent loadings of 2% to 10%. The highest average biomass productivities were observed at the 6% AD effluent loading in the flowback water. Subsequent experiments involved microalgae cultivation in commercial nutrients and salts at similar initial inorganic nitrogen (132 NH_4-N mg L^{-1}), pH (7.97), and salinity (42 g L^{-1}) levels. These results suggested that both microalgal strains could achieve comparable specific growth rates and average biomass productivities in the media composed solely of the wastewater resources. Additionally, the lipid content and profile obtained by using the flowback water and AD effluent medium were found to be mostly comparable to values obtained in the medium composed of the commercial nutrients and salts. These results suggested that comparable growth and lipids with similar profiles could be achieved by using the combination of the flowback water and the AD effluent as a culture medium for marine microalgae.

2.1. Introduction

Ever since the U.S. Department of Energy's Aquatic Species Program in the 1970's-1980's, a significant interest has been given to microalgae-based biofuels as they offer a number of advantages over crop-based biofuels. Microalgae have the capability of yielding more lipids per acre than agricultural crops, producing high-value pigments, and growing on non-arable land (Hammouda et al., 1995; Pittman et al., 2011; Sheehan et al., 1998). Meeting the water and nutrient requirements for algal cultivation, however, is a significant barrier to the economic viability of algal biofuels (Department Of Energy, 2009).

To meet this water and nutrient demand, the use of seawater and various low-strength wastewaters have been proposed (Pittman et al., 2011). Life cycle analysis estimated that the use of seawater can decrease water requirement by 90% (Yang et al., 2011). More inland saline water resources, however, are required as proper positioning of microalgae production facilities can have a significant impact on economic sustainability (Department Of Energy, 2009; Lansford and Solar Energy Research Institute., 1990). The saline flowback water from shale gas exploration, an increasingly available wastewater resource, may meet the water demand of commercial marine microalgae production. Additionally, while anaerobic digestion (AD) effluent been shown as a promising alternative nutrient source for microalgae cultivation, also currently unreported is the use of AD effluent as a nutrient supplement for the cultivation with flowback water.

The objective of this chapter was to evaluate biomass and lipid production from marine microalgae cultivated in the flowback water supplemented with the AD effluent. To address this objective, three specific aims were studied: 1) effect of AD effluent loadings on microalgal growth in flowback water, 2) effect of nutrient and salinity sources on microalgal growth, and 3) effect of nutrient and salinity sources on lipid production. A batch study was performed in which three different marine microalgal strains were cultivated in media composed of multiple AD effluent loadings in the flowback water. The optimal effluent loading and the most robust strains were further evaluated against a modified commercial medium at similar inorganic nitrogen, salinity, and pH levels. The performance of the microalgal strains was evaluated by comparing the growth and lipid productivities observed in both media.

2.2. Literature Review

2.2.1. Wastewater as a medium for microalgae cultivation

The water and nutrient requirements for microalgal cultivation is significant concern for feasible commercial production. A 1 hectare, 20 cm deep open pond has been estimated to require 530,000 gallons of water for algal cultivation (Department Of Energy, 2009). Additionally, nitrogen, phosphorous, and iron supplements can account for the cost of 6-8 cents per gallon of algal fuel in 1987 U.S. dollars (Benemann and Oswald, 1996). Such costs have prohibited the use of reagent- or agricultural-grade nutrients and have made water and nutrient sources from municipal, agricultural, or industrial waste streams increasingly attractive alternatives (Department Of Energy, 2009).

Microalgae have been shown to be adaptable to various chemical compounds and have been successfully cultured in wide variety of wastewaters. For example, microalgae have been found tolerant to wastewaters polluted with xenobiotic pollutants such as phenol, phenanthrene, naphthalene, and crude oil (Cerniglia et al., 1980; Narro et al., 1992; Pinto et al., 2002; Raghukumar et al., 2001; Semple et al., 1999). Additionally, microalgae cultivation in municipal and agricultural wastewaters with significant nitrogen, phosphorous, and heavy metals concentrations has been extensively studied (Cai et al., 2013b; Pittman et al., 2011). An advantage of cultivating microalgae in wastewaters is that significant quantities of lipids can also be produced. Untreated carpet mill wastewater, when coupled with microalgae cultivation, has been estimated to potentially yield 3260 to 3830 L ha^{-1} $year^{-1}$ of lipids (Chinnasamy et al., 2010). Similarly,

Wang et al. (2010) assessed batch culture-grown *Chlorella* sp. in diluted anaerobically digested dairy manure and estimated a maximal lipid productivity of ~11 mg L^{-1} day^{-1}.

2.2.2. The potential of shale gas flowback water as a water source for microalgae cultivation

Shale formations extend throughout the continental United States with an estimated 63 % (472 trillion cubic feet) of the estimated 750 trillion cubic feet of technically recoverable shale gas located in the Northeast region (EIA, 2011). Hydrocarbons, such as natural gas, are formed by either biogenic or thermogenic processes and are subsequently trapped in natural fractures of solid aggregates such as shale (Jenkins and Boyer, 2008; Soeder and Kappel, 2009). To overcome the low permeability of the shale and to make gas production economically viable, an aqueous fluid is injected in the formation at high pressures to create fissures and interconnected cracks in a process called hydraulic fracturing (Gregory et al., 2011; Wiseman, 2008). The aqueous fluid that returns to the surface after hydraulic pressure is relieved, called "flowback water", is the primary wastewater associated with shale gas production (Gregory et al., 2011). The Pennsylvania Department of Environmental Protection estimated that the gas industry alone produced 235 million gallons of wastewater in the second half of 2010 (Abdalla et al., 2011). Because of high handling costs and the scarcity of brine disposal facilities, flowback water is an increasingly available wastewater resource that may meet the water demand of commercial microalgae production (M.E.Blauch et al., 2009).

Depending on the time period of contact with the shale formation, flowback water can contain various concentrations of dissolved minerals and organic compounds (Gregory et al., 2011; GWPC et al., 2009). The created brine solution may be composed of salts, metals, oils, greases, soluble organic compounds, and radioactivity from radioactive underground sources (Abdalla et al., 2011; Gregory et al., 2011). The total dissolved solids (TDS), primarily from soluble chloride salts, has been reported to range from 66,000 mg L^{-1} to 261,000 mg L^{-1} from wells located in western Pennsylvania (Gregory et al., 2011).

While cultivation of microalgae in flowback water has not yet been reported, a potential analogue is coal seam gas (CSG) water. Similar to shale gas, CSG is mostly methane that is absorbed into a solid matrix. The pores and fractures in the coal are often saturated with water which is released when pressure in the formation is reduced during gas extraction. Also like flowback water, CSG water normally contains elevated levels of dissolved salts creating concerns regarding treatment and disposal (Hamawand et al., 2013). While research utilizing CSG water for algae cultivation is currently limited, research by Buchanan (2012) suggested that algal growth in CSG water was possibly impeded by elevated salinity levels as a lag phase between 7 and 9 times longer than other waters was observed. As the salinity of the flowback water may effect algal growth in a similar manner, the salinity of the medium with the optimum AD effluent loading in flowback water was recorded and used as a controlled factor in future experiments.

2.2.3. The potential of anaerobic digestion effluent as a source of nutrients for microalgae cultivation

Anaerobic digestion (AD) is a process in which microorganisms break down complex organic materials into simpler monomers, resulting in the production of biogas (CH_4 and CO_2) and residual effluent (Khanal, 2010). Because of the high levels of total phosphorus and total nitrogen, AD effluent has been considered a potential agricultural fertilizer to conserve and recycle valuable nutrients (Salminen et al., 2001). Over applying effluent to agricultural fields, however, can result in environmental hazards such as watershed eutrophication (Godos et al., 2009). These concerns have made application of AD effluent to algal cultivation an increasingly attractive alternative (Salminen et al., 2001).

Successful cultivation of microalgae in AD effluent has been previously reported for a variety of microalgal strains under different cultivation conditions (Cai et al., 2013a; Cai et al., 2013c; Park et al., 2010; Sheets et al., 2014; Wang et al., 2010). For example, cultures of *Synechocystis* sp. were found to grow 83% and 20% higher than *Nannochloropsis salina* at 3% and 6% AD effluent loadings, respectively (Cai et al., 2013a). Reported growth inhibition by strain specific responses as well as higher effluent concentrations is believed to be dependent on a combination of abiotic and biotic factors of the effluent. Abiotic factors may include levels of inhibitors, such as ammonia and heavy metals, or the degree of turbidity which might interfere with light penetration of the medium (Cai et al., 2013c; Pittman et al., 2011; Sheets et al., 2014). Biotic factors

may include the presence of pathogenic bacteria, predatory zooplankton, or competitive microorganisms (Pittman et al., 2011).

2.2.4. Characteristics of marine microalgae

Various marine microalgal strains have been previously studied based on their ability to adapt to different salinities, produce large quantities of intracellular lipids, and produce high-value pigments. For example, *Nannochloropsis salina* is a known marine microalga with a high lipid content (up to 50%) (Emdadi and Berland, 1989), high biomass productivity (up to 24.5 g m^{-2} day^{-1}) (Boussiba et al., 1987), and high contents of the nutritionally-valuable eicosapentaenoic acid (Krienitz and Wirth, 2006). Additionally, *N. salina* is known to produce commercially-valuable pigments such as zeaxanthin, canthaxanthin, and astaxanthin (Lubián et al., 2000).

The *Dunaliella* genus of microalgae has also been extensively studied and is unique in its robust tolerance to varying salinity levels. For example, *Dunaliella tertiolecta* is known to tolerate high salinity levels (up to ~1.0 M NaCl) as well as accumulate high levels of intracellular lipids (~67%) (Takagi et al., 2006). Similarly, *Dunaliella salina* is known to tolerate hypersaline conditions (up to ~5.5 M NaCl) as well as accumulate large amounts of the commercially-valuable pigment, β-carotene (Chen et al., 2011b).

While some microalgal strains, such as *N. salina*, have been successfully cultured in media composed of AD effluent (Cai et al., 2013c; Sheets et al., 2014), currently no studies have been reported on the culturing of *N. salina*, *D. tertiolecta*, or *D. salina* in media composed of flowback water.

2.3. Materials and Methods

2.3.1. Microalgal strains and seed cultures

Marine microalgae *Nannochloropsis salina* (849/6), *Dunaliella tertiolecta* (19/27), *Dunaliella salina* (19/18), and *Tetraselmis suecica* (66/38) were obtained from Culture Collection of Algae and Protozoa (CCAP, Oban, Scotland). Seed cultures of these strains were maintained in 2-L reactors (1-L working volume) at 25°C under constant illumination (200 μmol m^{-2} s^{-1}) using 32-watt florescent lamps (GE Lighting, Ravenna, OH, USA). The photosynthetic photon flux of the light was measured by a quantum meter (BQM, Apogee Instruments, Logan, UT, USA). Reactors were placed in a white coated chamber and each reactor was equipped with a rubber stopper and a 4.76 mm diameter stainless steel tube for air inlet/outlet. Air sparging, by the use of filter-sterilized (0.2 μm Whatman PTFE Puradisc, GE Healthcare, Maidstone, UK) ambient air (0.039% CO_2) at an airflow rate of 650 mL min^{-1}, was used to provide mixing and CO_2 for algal growth. The salinity of the culture media, described below, was maintained with 40 g L^{-1} of commercial sea salt (Instant Ocean® sea salt, Spectrum Brands, Madison, WI, USA).

N. salina and *T. suecica* cultures were cultivated in commercial f/2 media (Proline f/2 algae feed, Pentair Aquatic Eco-Systems, FL, USA) containing the following ingredients: 0.075 g L^{-1} $NaNO_3$, 0.00565 g L^{-1} $NaH_2PO_4 \cdot 2H_2O$, 1 mL L^{-1} trace elements stock solution, and 1 mL L^{-1} vitamin mix stock solution. The minor ingredients in the trace element stock solution included Na_2EDTA, $FeCl_3 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$,

$ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $Na_2MoO_4 \cdot 2H_2O$, and biotin. The vitamin stock solution contained cyanocobalamin, and thiamine HCl.

D. tertiolecta was cultured in artificial seawater media (ASW) formulated by McLachlan (1964) containing the following ingredients: 3.75 mL L^{-1} extra salts stock solution, 2.50 mL L^{-1} vitamin stock solution, 25 mL L^{-1} soil extract, and 0.50 g L^{-1} tricine. The extra salts stock solution contained 30.0 g L^{-1} $NaNO_3$, 1.20 g Na_2HPO_4, and 1.0 g L^{-1} K_2HPO_4. The vitamin stock solution contained biotin, calcium pantothenate, cyanocabalamin, folic acid, inositol, nicotinic acid, thiamine HCL, and thymine. The media were further enriched with commercial f/2 media using 1 mL L^{-1} trace elements stock solution and 1 mL L^{-1} vitamin mix stock solution. Soil extract was prepared by sieving 500-mL of air-dried deciduous woodland soil (Akron, OH, USA) though a fine mesh (2-4 mm). Deionized water (DI) at a volume of 1000-mL was then added and the resulting solution was autoclaving at 15 psi for 2 hours. The supernatant was then filtered to remove remaining particulates using filter paper (Whatman No 1 filter paper, GE Healthcare, Maidstone, UK) and then refrigerated at 4°C until use. *D. salina* was cultured in ASW media enriched with commercial f/2 media, as described above, with the addition of 35 g L^{-1} NaCl.

2.3.2. Shale gas flowback water and anaerobic digestion effluent

Shale gas flowback water was provided by a commercial oil and natural gas company (Weatherford International LTD, Geneva, Switzerland) and stored at 4°C. Prior to use, the flowback water was centrifuged for 15 min at 8,000 rpm using a tabletop

centrifuge (Sorvall RC 6+ centrifuge, Thermo Scientific, Waltham, MA, USA) to reduce the presence of suspended particulate matter.

Municipal wastewater AD effluent was collected from a commercial-scale liquid anaerobic digestion (KB Compost Services, Akron, OH, USA). The effluent was centrifuged at 3200 rpm by a continuous solid bowl decanter centrifuge (D5LL, ANDRITZ AG, Graz, Austria). The centrate/permeate was collected and stored at 4°C prior to use.

2.3.3. Cultivation of microalgae in flowback water with different anaerobic digestion effluent loadings

N. salina, D. tertiolecta, and *D. salina* were cultured in 250-mL Erlenmeyer flasks (100-mL working volume) filled with the AD effluent and the flowback water according to the loading ratios described in Table 1. All treatments were conducted in triplicates for a total of 36 reactors. Algal seed inoculum was added to each reactor at a fixed volume (10% v/v). The flasks were kept in an incubator (Innova® 43/43R incubator shaker, Eppendorf, Enfield, CT, USA) at a constant 22°C, at a rotational speed of 150 rpm. Because light intensity varied between 29 and 55 μmol m^{-2} s^{-1} inside the incubator, the positions of the reactors were randomized daily.

Before sampling, DI water was added to adjust the volume of each reactor to the initial volume recorded on Day 0, therefore compensating for both water evaporation and sampling losses. A volume of 2-mL algae suspension was collected daily. The optical density (OD) of the sample was measured at 680 nm by transferring 200-μL aliquots, in triplicates, to a microplate (Costar®, Corning, Corning, NY) which was measured by a

microplate reader (Eon® microplate spectrophotometer , Biotek, Winooski, VT, USA). The remaining sample was then centrifuged at 10,000 rpm for 15 min (Centrifuge 5810 R, Eppendorf, Enfield, CT, USA). To minimize the influence of other media constituents on OD measurements, a blank of the media was created by removing 200-µL aliquots, in triplicates, from the supernatant. The OD of the supernatant aliquots was measured and then subtracted from the OD of the corresponding algal sample. Reactors were cultured for a period of 19 days to insure that stationary phase was reached by all treatment levels.

Table 1: Experimental design for evaluating microalgal growth in the flowback water supplemented with different loadings of the anaerobic digestion effluent.

Treatment	AD effluent loading (% v/v)	*NH_4-N (mg L^{-1})
1	2	75
2	4	103
3	6	132
4	10	190

* total of both the AD effluent and the flowback water

2.3.4. Cultivation of N. salina and D. tertiolecta using different nutrient and salinity sources

The most robust strains from the screening experiment, *N. salina* and *D. tertiolecta*, were further evaluated in 2-L glass bottles (1-L working volume) with media composed of the flowback water supplemented with the 6% AD effluent. Commercials nutrients (Proline f/2 algae feed, Pentair Aquatic Eco-Systems, FL, USA) and commercial salts (Instant Ocean® sea salt, Spectrum Brands, Madison, WI, USA) were used to create modified commercial media as experimental controls. All treatments were

adjusted on Day 0 to similar inorganic nitrogen (~132 mg L^{-1} N), salinity (~42 g L^{-1}), and pH (~7.97) levels. Concentrated HCl or 10 M NaOH was used to adjust the pH of the controls to appropriate levels. Algal seed inoculum was added to each reactor at a fixed volume (10% v/v). Each treatment was performed in triplicates for a total of 12 reactors. A culture period of 14 days was chosen to insure growth transition of each culture into the stationary phase.

Each reactor was equipped with a rubber stopper and a 4.76 mm diameter stainless steel tube for air inlet/outlet. Reactors were placed in a white coated chamber in which constant illumination (200 µmol m^{-2} s^{-1}) was provided by 32-watt florescent lamps (GE Lighting, Ravenna, OH, USA). Air sparging, by the use of filter-sterilized (0.2 µm Whatman PTFE Puradisc, GE Healthcare, Maidstone, UK) ambient air (0.039% CO_2) at an airflow rate of 650 mL min^{-1}, was used to continuously mix the cultures and provide CO_2.

Before sampling, DI water was used to compensate for evaporation and sampling losses as mentioned previously. The reactors were sampled every other day by removing a sample volume of 50-mL. Aliquots of 10-mL, in triplicates, were then used to determine the dry biomass content of the algal samples in terms of ash-free dry weight.

2.3.5. Microalgal lipid analysis

Samples for lipid analysis were collected by combining individual 1-L reactors from the end of the experiment described in Section 2.3.4 into 3-L volumes. Dewatering of *N. salina* was achieved by centrifugation (Sorvall RC 6+ centrifuge, Thermo Scientific, Waltham, MA, USA) at 10,000 rpm for 15 min. Because of the susceptibility

of *D. tertiolecta* cells to break, flocculation was first performed by raising the pH of the media to 10.5 using additions of 10 M NaOH (Horiuchi et al., 2003). After flocculation, samples were decanted and centrifuged at 3,000 rpm for 15 min. Lipid content of the microalgal biomass was then determined by a modified version of Folch's method (Folch et al., 1957). Approximately 7-mL of methanol was added to 1.0 g of the thickened biomass slurry. The cell suspension was then disrupted for 30 s with an ultrasonic processor (UP400S, Hielscher Ultrasonics, Teltow, Germany) and a titanium sonotrode with a 22 mm tip. The frequency was set at 24 KHz at a power output of 100 W. After an addition of 14-mL of chloroform, sonication was repeated. The final solvent ratio of 8:4:3 chloroform:methanol:water was achieved by adding 12-mL of chloroform-methanol (2:1, v/v) followed by 0.88% KCl solution. The mixture was then shaken for 10 min in a glass centrifuge tube and then centrifuged for 5 min at 1,000 g (Sorvall Legend T+ centrifuge, Thermo Scientific, Waltham, MA, USA). The aqueous phase of the resulting biphasic solution was then removed with a pipette. The remaining organic phase was then filtered into a pre-weighed 100-mL round bottom flask using filter paper (Whatman No. 1filter paper, GE Healthcare, Maidstone, UK). The filter and centrifuge tube were then washed with 20-mL of chloroform to remove residual lipids. The chloroform was then removed with a rotary evaporator (Laborota 4001, Heidolph Instruments, Schwabach, Germany) to recover the remaining crude lipids. The weight of the crude lipids was then recorded.

Transesterification of the crude lipids into FAMEs was conducted according to Li and Watkins (2001). Crude lipids were saponified with 0.5 N NaOH in methanol at 100°C for 5 min. The resulting liberated fatty acids (FAs) were methylated in 14% boron

trifluoride in methanol at 100°C for 5 min. The resulting FAMEs were extracted with hexane and quantified by a gas chromatograph-mass spectrometer (GC-MS) (GCMS-QP2010 SE unit, Shimadzu, Columbia, MD, USA), equipped with a polar capillary column (Zebron^{TM} ZB-FFAP, Phenomenex, Torrence, CA, USA). Helium was used as the mobile phase at a flow rate of 1 mL min^{-1}. The oven temperature was raised from 50°C to 190°C at a rate of 25°C min^{-1}, then to 220°C at a rate of 3°C min^{-1} and held for 18 min. The injector, interface, and ion source temperatures were set at 250°C, 250°C, and 200°C, respectively. FAME samples of 1-µL each were injected with a split ratio of 50:1. The compounds were identified in the National Institute of Standards and Technology mass spectral database and quantified by comparing the peak area with 100 µg mL^{-1} methyl heptadecanoate as an internal standard. The cetane number of total FAs was calculated using a multiple regression model developed by Piloto-Rodriquez et al (2013).

2.3.6. Chemical composition analysis of growth media

Macronutrients, such as inorganic nitrogen and inorganic phosphorus, and other ion constituents were analyzed using an ion chromatography (IC) (ICS-5000, Dionex®, Thermo Scientific, Waltham, MA, USA). All samples were filtered to remove particulates using a 0.2 µm syringe filter and diluted with DI water to appropriate detection limits. Additionally, the concentration of 11 elements (Al, Fe, Mn, Ni, Zn, Cu, As, Mo, Pb, Ni, and Cd) in the flowback water were measured using an inductively coupled plasma-mass spectrometry (ICP-MS) (7500cx, Agilent Technologies, Santa Clara, CA, USA) following US EPA method 6062A (USEPA, 1986). Prior to ICP-MS

analysis, the samples were digested in a microwave accelerated reaction system (CEM Corporations, Matthews, NC, USA) at 190°C for 10 minutes. The digested sample was diluted 50-fold with DI water and analyzed via ICP-MS. For concentrations of barium (Ba) and strontium (Sr), the flowback water samples were sent to Service Testing and Research Lab (STAR) (OSU/OARDC, Wooster, OH). These samples were analyzed by ICP-OES after nitric acid microwave digestion following Section 3120B (Eaton, 2000). Total dissolved solids (TDS) and pH were measured by a conductivity meter (EC300, YSI, Yellow Springs, OH, USA) and a pH meter (pH 11, Oakton Instruments, Vernon Hills, IL, USA), respectively.

2.3.7. Development of calibration curves for determining algal biomass concentration

A wavelength of 680 nm was chosen to minimize the influence of non-photosynthetic solids found in the media on OD. The relationship between OD at 680 nm and algal dry biomass (g L^{-1} ash-free dry weight (AFDW)) was established for all three algal strains, and the derived correlation equations for use in the remainder of the study are shown below.

$$N.salina\ \ (g\ L^{-1}AFDW) = 0.3987 * OD_{680}$$

$$D.tertiolecta\ \ (g\ L^{-1}AFDW) = 0.9235 * OD_{680}$$

$$D.salina\ \ (g\ L^{-1}AFDW) = 0.9044 * OD_{680}$$

The ash-free dry weight (AFDW) of the algal samples was determined and reported according to Zhu and Lee (1997). Aliquots of 10-mL, in triplicates, were filtered

in a cylindrical flask through a pre-combusted and pre-weighed filter using a vacuum that was dependent on the microalgal strain. For *N. salina*, 1.6 µm glass fiber filters with 45 mm diameter (Fisherbrand G6, Thermo Fisher Scientific, Madison, WI, USA) were used under a vacuum of 20 inHg. Because *D. tertiolecta* lack cell walls and are susceptible to cell breakage during filtering (Goldman and Dennett, 1985), 0.7 µm glass fiber filters with 45 mm diameter (Whatman GF/F, GE Healthcare, Maidstone, UK) were used under a vacuum of 5 inHg. After filtration of the algal sample, filters were washed with 20-mL of 0.5 M NH_4HCO_3 solution to remove residual salt and then dried in aluminum dishes in an oven (Thelco®, Thermo Scientific, Waltham, MA, USA) for a minimum of 24 hours at 105°C. Samples were placed in a vacuum dessicator until cooled and then weighed for total suspended solids (TSS). The samples were then combusted at 550°C for 4 hours in a muffle furnace (Isotemp, Fisher Scientific, Dubuque, IA, USA) and then cooled in a vacuum desiccator and weighed. The AFDW was determined as the difference between TSS and the ash weight. See Appendix A for more details regarding the derivation of these equations as well as the selection process that resulted in the use of 680 nm.

2.3.8. Nonlinear regression and statistical analysis

Nonlinear regression analysis of the experimental data was performed to reduce human error involved in estimating the algal specific growth rate using conventional methods (Paine et al., 2012; Rees et al., 2010). A three-parameter logistic model was chosen as it provided adequate fit for most of the experimental data (R^2=0.97-0.99). Specifically, the Fit Curve personality was used with the three-parameter logistic model

(Logistic 3P) in JMP 10.0.2 (SAS Institute Inc., Cary, NC, USA), defined in the equation below.

$$Biomass\ concentration\ (g\ L^{-1}) = m(t) = \frac{c}{1 + e^{-k*(t-b)}}$$

Where:

k = specific growth rate (day^{-1})

b = inflection point (days)

c = asymptote (g L^{-1})

t = time (day)

The asymptote of the logistic function corresponds to the maximum theoretical biomass in the above equation. The inflection point corresponds to the time (day) when growth rate is maximized. The average biomass productivity was calculated by multiplying the maximum observed biomass concentration by the estimated specific growth rate.

Model appropriateness was determined by residual analysis. Analysis of significance was determined by analysis of variance (ANOVA) and Tukey HSD tests performed at a significance level of 0.05 using JMP 10.0.2 (SAS Institute Inc., Cary, NC, USA).

2.4. Results and Discussion

2.4.1. Chemical composition of shale gas flowback water and anaerobic digestion effluent

The results from the chemical composition analysis of the shale gas flowback water are shown in Table 2. Concentrations of chloride, total dissolved solids (TDS), and bromide were found to be comparable to Marcellus shale flowback water concentrations reported by Abdalla et al. (2011). Additionally, the sodium, calcium, magnesium, barium, iron, and manganese concentrations were within the range of randomly sampled Marcellus shale flowback water samples reported by Paugh (2008). These comparisons to literature values indicated that the flowback water used in this study was a realistic representation of flowback water commonly produced by shale formations such as the Marcellus.

When compared to commercial sea salt (Table 3), the flowback water had higher concentrations of the many ions in common, with the exception of sulfate. If the flowback water was hypothetically used as a growth media for marine microalgae, many of the micronutrients, such as potassium, magnesium, calcium, and sodium, required to sustain growth would be met in excess (Becker, 1994). The salinity of the flowback water (~45 g L^{-1}), while slightly higher than standard preparations of commercial sea salt (~32 g L^{-1}), has been shown not to substantially impede marine microalgal growth and may positively impact lipid yields (Hu and Gao, 2006; Takagi et al., 2006). However, this flowback water would not be able to sustain significant microalgal growth alone as key

macronutrients, such as nitrogen and phosphorus, were at insignificant levels (Pittman et al., 2011).

The results from the chemical composition analysis of the AD effluent, the envisioned source of nutrients when used in conjunction with flowback water, is shown in Table 4. As noted by previous research, the major form of nitrogen in the AD effluent was ammonium (Cai et al., 2013c; Sheets et al., 2014). Because ammonium bypasses the redox reactions required to assimilate nitrate and nitrite by microalgae, ammonium is believed to be the preferred source of nitrogen for rapid growth (Cai et al., 2013b). Excluding the presence of organic forms of nitrogen and phosphorus, the nitrogen to phosphorus ratio (N/P) was calculated to be ~14:1. While the optimum universal N/P ratio is considered to be 16:1 (Redfield, 1934), it has been shown that the optimal N/P ratio and can vary from 8.2 to 45, depending on ecological conditions (Klausmeier et al., 2004). Many popular growth media have N/P ratios greater than 16 (Andersen, 2005),

Table 2: Chemical composition of the shale gas flowback water.

Parameter	ppm
Cl	23787.18 ± 1021
Na	11455.30 ± 213
Mg	3574.96 ± 386
K	634.99 ± 12
Ca	471.77 ± 12
Br	191.65 ± 15
SO4	159.89 ± 3
NH4-N	**46.58 ± 0.66**
Li	20.90 ± 0.01
^{27}Al	17.58 ± 5
^{56}Fe	9.57 ± 0.05
^{55}Mn	1.39 ± 0.14
PO4, NO2, NO3, F	< 10
Parameter	**ppb**
^{60}Ni	102.38 ± 2
^{66}Zn	84.92 ± 94
^{63}Cu	83.45 ± 4
^{75}As	71.29 ± 45
Ba	71.09
^{97}Mo	50.23±49
^{206}Pb	44.46 ± 61
^{58}Ni	34.57 ± 3
Sr	21.03
^{111}Cd	18.36 ± 25
Property	
Salinity (g L^{-1})	45.4 ± 0.28
Total dissolved solids (mg L^{-1})	41714.2 ± 1734
pH	6.56 ± 0.22

*Bolded constituents represent macronutrients critical for microalgal growth.

Table 3: Chemical composition of commercial sea salt.

Parameter	ppm
Cl	10731.10 ± 200
Na	6234.76 ± 323
SO4	1977.98 ± 116
K	1552.38 ± 43
Mg	308.27 ± 34
Ca	145.26 ± 19
Br	116.45 ± 84
Li, NH4, NO3 PO4, NO2, F	< 10
Property	
Salinity (g L^{-1})	32

Instant Ocean™ sea salt (40 g L^{-1})

Table 4: Chemical composition of the anaerobic digestion effluent.

Parameter	ppm
NH4-N	**1447.34 ± 46**
Cl	336.88 ± 49
Na	129.95 ± 1
PO4-P	**105.77 ± 1**
Ca	83.33 ± 2
SO4	55.03 ± 3
Mg	19.54 ± 4
F, NO2, Br, NO3, Li, K	< 10
Property	
Salinity (g L^{-1})	5.25 ± 0.07
TDS (mg L^{-1})	9290.66 ± 54
pH	8.6 ± 0.01

* Bolded constituents represent macronutrients critical for microalgal growth.

2.4.2. Effect of anaerobic digestion effluent loadings on microalgal growth in flowback water

2.4.2.1. Microalgal growth curves

Growth curves of *D. salina*, *N. salina*, and *D. tertiolecta*, generated from the cultivation in the flowback water supplemented with the AD effluent loadings of 2% to 10%, are presented in Figures 1 and 2. Overall, *D. salina* (Figure 1) experienced poor growth at all loadings of the AD effluent in the flowback water. A 10-day lag phase was observed at the 4% AD effluent loading with no significant growth observed for either the AD effluent loadings of 6% or 10%. The highest biomass concentration observed was ~0.37 g L^{-1} at the 4% AD effluent loading. Both *D. tertiolecta* and *N. salina* performed substantially better than *D. salina*. For *D. tertiolecta* (Figure 2a), a 2-day lag phase was observed at the 2% AD effluent loading while a 4-day lag phase was observed at the AD effluent loadings of 4% to 10%. No lag phases were observed for *N. salina* at all studied effluent loadings. The highest biomass concentration observed for *D. tertiolecta* and *N. salina* was 1.03 g L^{-1} and 0.76 g L^{-1}, respectively, at the 10% AD effluent loading.

The poor performance of *D. salina* and the lag phases observed for *D. tertiolecta* may be attributed to changes in salinity and the presence of growth inhibitors, such as free-ammonia, in the AD effluent and flowback water media. While the ammonium (NH_4^+) in the AD effluent is generally considered nontoxic, the disassociation of the ammonium into free ammonia (NH_3) may inhibit microalgal growth (Azov and Goldman, 1982). Additionally, while *D. salina* has been reported to survive a wide range of salinity levels, the transfer of *D. salina* from a hypersaline seed culture medium to the flowback

water media may have contributed to the overall stress of the culture (Chen et al., 2011b; Chen and Jiang, 2009). Because of the poor growth of *D. salina* relative to the other microalgal strains used in this study, *D. salina* was not evaluated in further experiments.

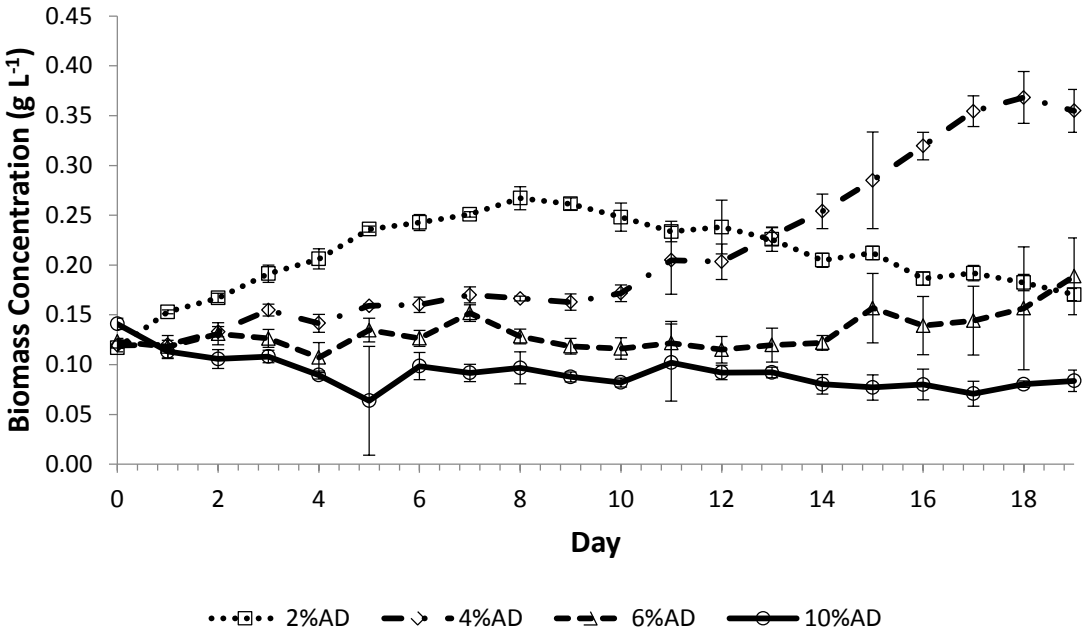

Figure 1: Effect of anaerobic digestion effluent loadings on growth of *D. salina* in the flowback water.

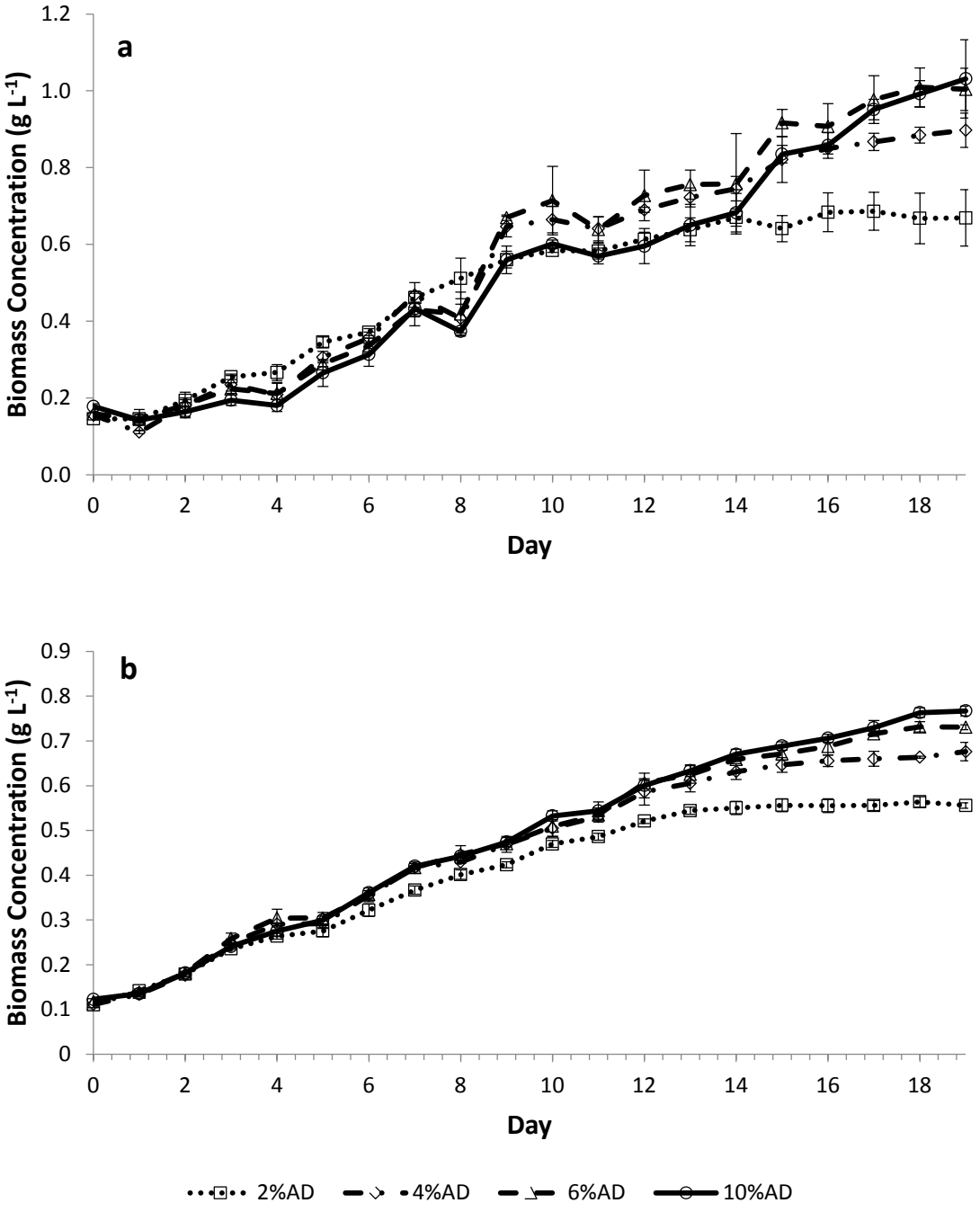

Figure 2: Effect of anaerobic digestion effluent loadings on growth of (a) *D. tertiolecta* and (b) *N. salina* in the flowback water.

2.4.2.2. Effect of AD effluent loadings on microalgal specific growth rate and average biomass productivity

The specific growth rates (μ, day^{-1}), and the average biomass productivities (mg $L^{-1}day^{-1}$) for both strains at different AD effluent loadings in the flowback water are shown in Figure 3. The impact of the AD effluent loadings on both the specific growth rate and the average biomass productivity was found to be strain specific ($p<0.05$). Both *N. salina* and *D. tertiolecta* had the highest specific growth rate at the 2% AD effluent loading (0.29 and 0.28 day^{-1}, respectively) when compared to the AD effluent loadings of 6% and 10%. The specific growth rate of *D. tertiolecta* was observed to decline as the AD effluent loadings increased and the lowest specific growth rate (0.16 day^{-1}) was obtained at the 10% AD effluent loading. While the decline in the specific growth rate was observable for *N. salina* for the AD effluent loadings of 4% to 10%, the differences are not statistically significant ($p>0.05$). For *D. tertiolecta*, the highest and the lowest average biomass productivity (239 and 175 mg L^{-1} day^{-1}, respectively) occurred at the AD effluent loadings of 6% and 10%, respectively. The AD effluent loadings of 2% to 10% were found not to significantly affect the average biomass productivity of *N. salina* ($p>0.05$).

These results suggest that *N. salina* is less sensitive than *D. tertiolecta* to increases in the AD effluent loading on the two estimated parameters. This may indicate a different degree of tolerance to inhibitors, such as free-ammonia, that may be present in the AD effluent (Pittman et al., 2011).

The observation that both *N. salina* and *D. tertiolecta* attained the highest specific growth rate (0.29 and 0.28 day^{-1}, respectively) at the 2% AD effluent loading in the flowback water (~74.6 mg L^{-1} NH_4-N) is in contrast to previously published results for microalgae cultivation using AD effluent as a nutrient source. Cai (2013c) found that *N. salina* achieved the highest specific growth rate (0.645 day^{-1}) at a 6% municipal wastewater AD effluent loading, corresponding to 160 mg L^{-1} of total nitrogen. Similarly, Sheets et al. (2014) found that *N. salina* achieved the highest specific growth rate (0.327 day^{-1}) at a 7% municipal wastewater AD effluent loading (200 mg L^{-1} of total nitrogen). These different conclusions from increased AD effluent loadings may arise from the different methods used to calculate specific growth rate. Both previously mentioned literature estimates were calculated based on the ratio of biomass concentrations at the beginning and end of exponential phase. As the specific growth rate estimates in this study were calculated based on nonlinear regression analysis of the entire span of the experimental data, the estimates in this study may be more sensitive to factors effecting algal growth.

While factors, such as differences in cultivation conditions and the age of the algal seed inoculum (Lu et al., 2012; Lu et al., 2013), may also influence these observed differences to literature values, the use of the flowback water as the basis of the growth media may also be a factor. Pittman (2011) suggested that algal growth is likely lower in industrial wastewaters as high toxin concentrations may inhibit growth. For example, Buchanan (2012) recorded a lag phase between 7 and 9 times longer when algae was grown in coal seam gas brine in oppose to seawater. As algae are sensitive to toxic

pollutants, a combination of free-ammonia, organic pollutants, and unreported heavy metals in the AD effluent and flowback water media may contribute to the observed decline in specific growth rate for both algal species (Munoz and Guieysse, 2006).

Because of the need of algal biomass to perform lipid quantification experiments, the medium composed of the 6% AD effluent loading in the flowback water was chosen for further analysis of both strains.

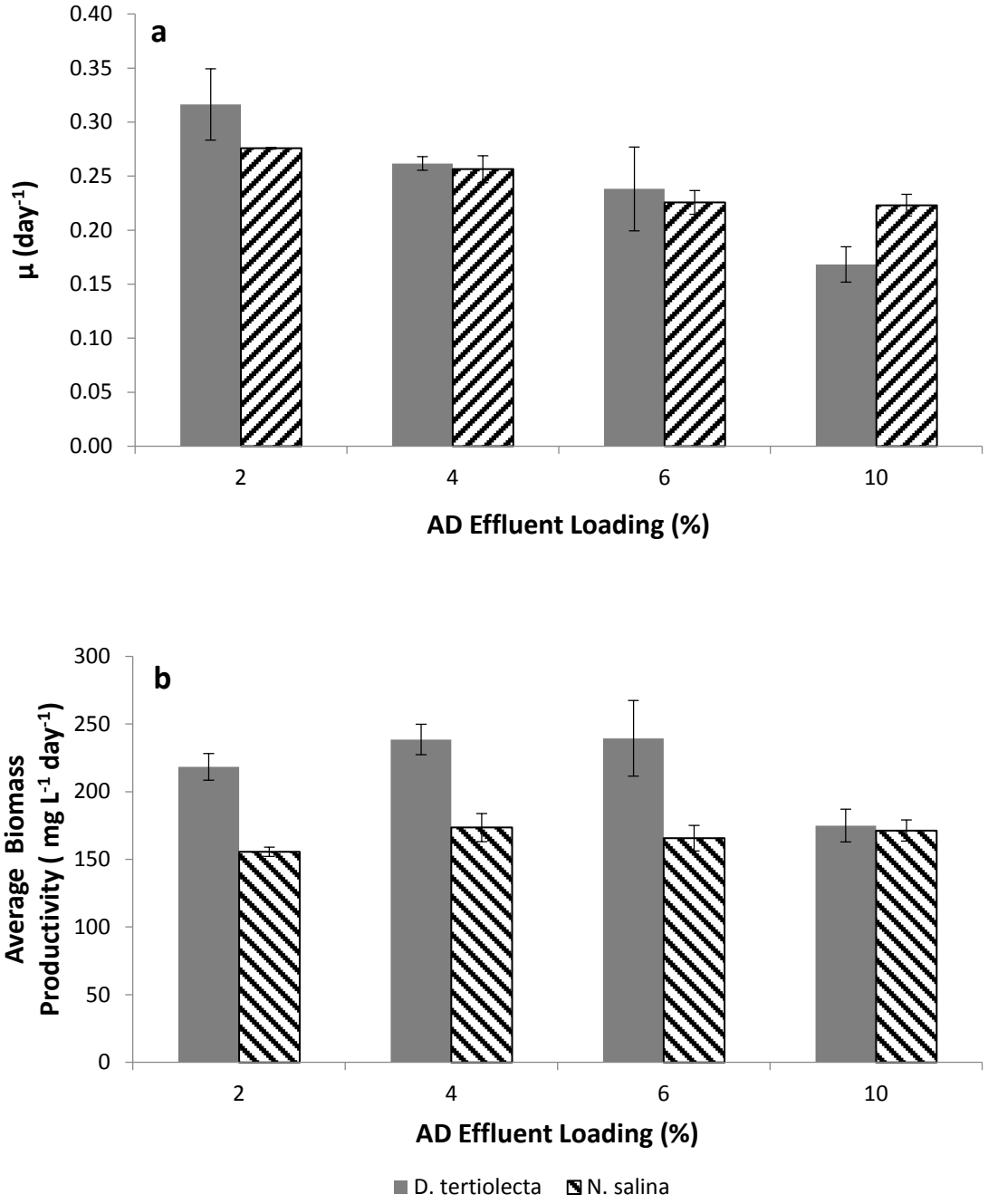

Figure 3: Effect of anaerobic digestion effluent loadings on (a) specific growth rate and (b) average biomass productivity for *N. salina* and *D. tertiolecta* cultured in the flowback water.

2.4.3. Effect of nutrient and salinity sources on microalgal growth

The specific growth rates (μ, day^{-1}) and the average biomass productivities (mg L^{-1} day^{-1}) for *D. tertiolecta* and *N. salina* grown in the media composed of the commercial nutrients and salts (control) or the 6% AD effluent loading in the flowback water can be seen in Figure 4. The results indicated that the media and the microalgal strain did not have a significant impact on the two analyzed parameters ($p>0.05$). As the main form of nitrogen in the commercial nutrients is nitrate, these results also suggested that both strains did not show observable preferences for either nitrate or ammonium at comparable inorganic nitrogen concentrations. While Chen (2011) observed a preference of nitrate over ammonium for cultures of *D. tertiolecta*, differences in the cultivation conditions may be responsible as concentrations of inhibitors, such as free-ammonia, are known to be effected by environmental parameters (Azov and Goldman, 1982).

In summary, *N. salina* and *D. tertiolecta* achieved the specific growth rates of 0.293 and 0.348 day^{-1}, respectively, in the medium composed of the flowback water supplemented with the 6% AD effluent. The average biomass productivities observed in this media were 225 and 275 mg L^{-1} day^{-1} for *N. salina* and *D. tertiolecta*, respectively. The specific growth rate and average biomass productivity for *N. salina* are comparable to the values reported by Sheets (2014) for *N. salina* grown in media composed of 7% municipal wastewater AD effluent. Specifically, the specific growth rate and the average biomass productivity were reported at 0.327 day^{-1} and 204 mg L^{-1} day^{-1}, respectively. The similar growth of each strain in both media suggests that inhibition caused by constituents in the AD effluent or the flowback water was not observed and that it is

feasible for the AD effluent and the flowback water to replace the commercial nutrients and salts at comparable inorganic nitrogen, salinity, and pH levels.

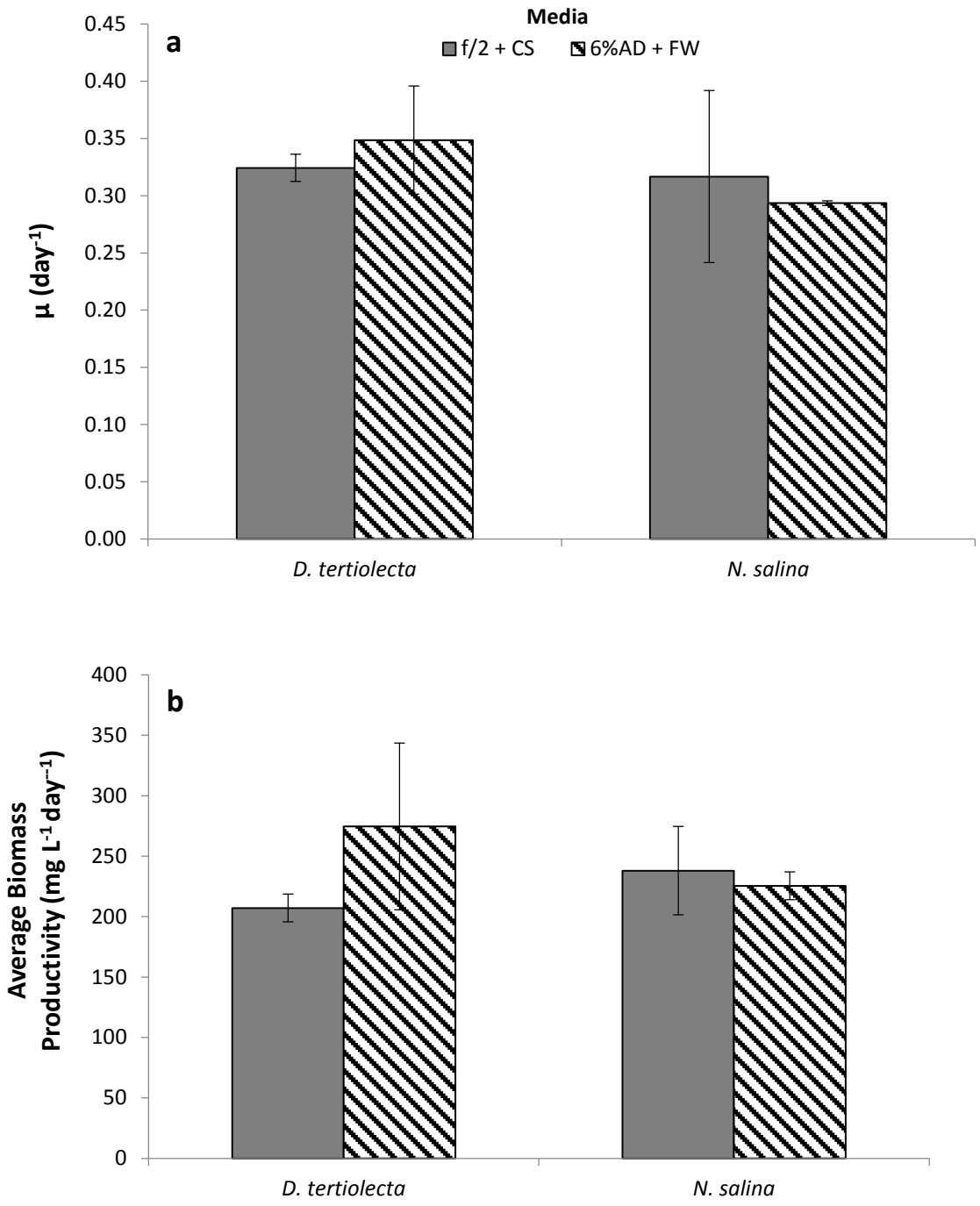

Figure 4: Effect of nutrient and salinity sources on microalgal (a) specific growth rate and (b) average biomass productivity. Media evaluated included commercial nutrients and salts (f/2+CS) and 6% anaerobic digestion effluent loading in the flowback water (6%AD+FW).

2.4.4. Effect of nutrient and salinity sources on microalgal lipid production

A summary of the results from the lipid content and profile analysis of *N. salina* and *D. tertiolecta* grown in the media composed of either the flowback water supplemented with the 6% AD effluent or the commercial nutrients and salts (control) is shown in Table 5. The lipid content and total fatty acids of *N. salina* grown in the medium composed of the flowback water supplemented with the 6% AD effluent were comparable to the control ($p>0.05$). Additionally, the amount of saturated fatty acids decreased by ~12.6% while the amount of unsaturated fatty acids increased by ~8.8% when compared to the control. In particular, the saturated fatty acid C16:0 decreased by 17.5% while unsaturated fatty acids like C16:1, C18.1 and C20:5 increased by 10.1%, 16.2%, and 34.3%, respectively. Overall, the changes in fatty acids were minor as the cetane number (CN) for *N. salina* grown in the flowback water supplemented with the 6% AD effluent decreased by only 1.84% when compared to the control. The CN is an indicator of the potential fuel quality of the microalgal-derived biodiesel as a higher CN implies easier ignition and lower nitrous oxide emissions (Cai et al., 2013a).

Eicosapentaenoic acid (EPA, C20:5) is a particularly valuable fatty acid often used as a human health supplement (Camacho-Rodríguez et al., 2014). While the observed increase in EPA is significant, the total amount (~16.9% of TFA) is comparable to the value reported by Cai et al.(2013c) for *N. salina* grown in municipal AD effluent (15.7% of TFA) and less than the value reported by Sheets et al. (2014) for *N. salina* grown in municipal AD effluent under high illumination (25.7% of TFA).

While the minor increase of 13.4% in the lipid content was observed for *D. tertiolecta* cultivated in the medium composed of the flowback water supplemented with the 6% AD effluent, no other parameter in regards to the lipid profile was found to be significantly different from the control ($p>0.05$). As the reported lipid contents of both *N. salina* and *D. tertiolecta* were obtained near the end of exponential phase, there is a potential that the lipid content can be increased by nutrient starvation, salt stress, or other environmental parameters (Chen, 2011; Xin, 2010).

Table 5: Effect of nutrient and salinity sources on microalgal lipid content and profile.

	N. salina		*D. tertiolecta*	
	f/2+CS	**6% AD+FW**	**f/2+CS**	**6% AD+FW**
Lipid Content ($g\ g^{-1}$ TVS)	0.302± .029	0.308 ± 0.022	0.120 ± 0.002	0.135± 0.003
Total Fatty Acids (TFAs) ($g\ g^{-1}$ lipids)	0.899 ± 0.030	0.767 ± 0.114	0.470 ± 0.020	0.532 ± 0.029
Total Saturated FAs ($g\ g^{-1}$ TFAs)	0.414 ± 0.005	0.362 ± 0.004	0.330 ± 0.014	0.341 ± 0.023
Total Unsaturated FA's ($g\ g^{-1}$ TFAs)	0.586 ± 0.005	0.638 ± 0.004	0.670 ± 0.014	0.659 ± 0.023
FAME Constituent (% of TFAs)				
C 14:0	4.81 ± 0.24	6.34 ± 0.21	2.35 ± 0.39	2.91 ± 1.05
C 14:1			0.43 ± 0.04	0.22 ± 0.38
C 16:0	33.42 ± 0.25	27.55 ± 0.31	26.31 ± 0.07	27.21 ± 0.20
C 16:1	23.78 ± 0.89	26.17 ± 0.96	2.39 ± 0.18	2.90 ± 0.63
C 18:0	3.17 ± 0.50	2.25 ± 0.46	4.29 ± 0.94	4.02 ± 1.10
C 18:1	13.14 ± 0.26	15.27 ± 0.20	9.62 ± 0.63	7.92 ± 1.59
C 18:1t	2.01 ± 0.23	1.55 ± 0.24	4.69 ± 0.21	6.37 ± 0.25
C 18:2	2.45 ± 0.10	1.34 ± 0.05	8.33 ± 0.35	9.23 ± 0.64
C 18:3			41.60 ± 2.11	39.21 ± 4.17
C 20:0	0.15 ± 0.27			
C 20:2				
C 20:4	4.36 ± 0.12	2.65 ± 0.12		
C 20:3				
C 20:5 EPA	12.55 ± 0.12	16.87 ± 0.03		
Cetane Number	58.39 ± 0.13	57.32 ± 0.08	45.93 ± 0.93	46.50 ± 1.68

f/2+CS = commercial nutrients and salts, 6%AD+FW = 6% anaerobic digestion effluent loading in the flowback water

2.5. Conclusions

The use of the shale gas flowback water, as the source of water, and the AD effluent, as the source of nutrients, for the cultivation of marine microalgae is feasible for certain microalgal strains. While *D. salina* experienced poor growth, *N. salina* and *D. tertiolecta* both achieved growth comparable to cultivation in commercial nutrients and salts when the starting nitrogen, salinity, and pH levels were controlled. The lipid content and profile of both strains when grown in the medium composed of the flowback water and AD effluent were also comparable to yields obtained with commercial media and salts. These results suggested that comparable growth and lipids with similar profiles could be achieved by replacing the modified commercial medium with the flowback water and AD effluent medium for marine microalgae cultivation.

Chapter 3: Effects of Chemicals and Microbial Communities in Anaerobic Digestion Effluent and Shale Gas Flowback Water on Algal Growth and Sedimentation

Abstract

The ability to examine the influence of the microbial community of the medium composed of the flowback water and AD effluent was complicated by observed losses in ammonia after autoclaving. It was speculated that the volatilized ammonia was in the form of free-ammonia and that the differences in ionic strength between the evaluated media resulted in a different degree of free-ammonia toxicity experienced by both strains. In the case of *D. tertiolecta*, a strain of microalgae that is relatively sensitive to free-ammonia, higher biomass productivities were achieved in the flowback water compared to commercial salts. As this was not observed previously, differences in the cultivation conditions may also affect the severity of ammonia-toxicity. Further research is suggested to explore the impact of the ionic strength of the flowback water on algal growth inhibition.

3.1. Introduction

Microalgae-bacteria associations have commonly been found in ecosystems consisting of high microalgal production (Cole, 1982; Fouilland, 2012). Symbiotic relationships have been observed to occur when heterotrophic bacteria support the growth of algae by providing CO_2, vitamins, re-mineralized nutrients, and other growth dependent compounds (Gonzalez and Bashan, 2000; Grossart and Simon, 2007). Microalgae, in return, have been observed to increase the growth of bacteria by providing O_2 and dissolved organic carbon (Cole, 1982; Fouilland, 2012; Grossart and Simon, 2007). Despite the association of bacteria with algae in nature, applications of algal-bacterial relationships to biotechnology are relatively rare.

Algal-bacterial relationships may be able to improve the overall economics of microalgal biofuels. While current methods, such as nutrient depletion, attempt to increase microalgal lipid accumulation through stress, overall increases in lipid yields are minimal as algal growth is concurrently reduced (Mata et al., 2010; Yang et al., 2011). Certain bacterial strains, however, have been observed to increase both the growth and lipid content of microalgae simultaneously (de-Bashan et al., 2002). Additionally, bacteria have been observed to produce extracellular polymeric substances that promote flocculation of microalgae (Lee et al., 2009). The flocculation of microalgae by bacteria may reduce substantial costs involved in the harvesting and dewatering of microalgae after cultivation (Uduman et al., 2010).

Microbial communities have been found in both shale gas flowback water and anaerobic digestion (AD) effluent (Chouari et al., 2005; Struchtemeyer and Elshahed,

2012). Because of the diverse populations of microbes found in both wastewaters, it is currently uncertain the degree to which algal-bacterial interactions are occurring. While there are many potential benefits to an algal-bacterial consortium, negative interactions cannot be overlooked. Bacteria may compete for limited nutrients and produce algicidal extracellular metabolites (Doucette, 1995; Fukami et al., 1997; Grossart and Simon, 2007). In this case, the chemicals and salts present in the shale gas flowback water may act as natural buffers and prevent biological contamination. The use of seawater has been similarly used in the past to provide an osmotic barrier to invasive competitors such as herbivorous zooplankton, fungi, and lytic bacteria (Benemann and Oswald, 1996; Vasseur et al., 2012).

The objective of this chapter was to evaluate the effects of chemicals and microbial communities present in both the AD effluent and the shale gas flowback water on algal growth and sedimentation. To address this objective, two specific aims were studied: 1) cultivation of *N. salina* and *D. tertiolecta* in autoclaved and non-autoclaved media and 2) sedimentation kinetics analysis of *N. salina* and *D. tertiolecta* in autoclaved and non-autoclaved media. Autoclaving was chosen as the method to sterilize and thus minimize the influence of viable microbes found in the media. It was anticipated that a comparison to non-autoclaved media, as a result, would allow observations to be made on the impact of viable microbes on microalgae growth and sedimentation. A batch study was performed in which *N. salina* and *D. tertiolecta* were grown in autoclaved and non-autoclaved media composed of either wastewater-derived or commercially-derived nitrogen and salinity sources. A novel method to measure sedimentation kinetics using

the kinetic analysis feature of a microplate reader was also proposed and applied in the hopes of determining the existence of bioflocculant-producing bacteria which may increase the sedimentation rate of microalgae cultures.

3.2. Literature Review

3.2.1. Growth-promoting bacteria

A bacterium previously studied for its ability to promote microalgal growth and lipid production is *Azospirillum brasilense*. Also known as the plant-growth-promoting bacteria (PGPB), *A. brasilense* has been observed to produce plant hormones which effect plant growth by interfering with the hormonal metabolism of the host plant (Gonzalez and Bashan, 2000; Patten and Glick, 1996). The first study of the effects of *A. brasilense* on microalgae was conducted by Gonzalez and Bashan (2000) who co-immobilized *Chlorella vulgaris* and *A. brasilense* in alginate beads. A significant promotion of *C. vulgaris* growth was observed within 1 day of inoculation which continued for 6 days. Both the total number of algal cells and the pigment concentrations were observed to significantly increase. Gonzalez and Bashan (2000) concluded that the mechanism behind PGPB's effect on terrestrial plants may be similar to its effects on microalgae such as *C. vulgari*. Particularly, the production of phytohormones by PGPB may potentially stimulate microalgal growth (Bajguz and Czerpak, 1996; Gonzalez and Bashan, 2000).

Additionally, de-Bashan et al. (2002) analyzed the lipid and pigment content of *C. vulgaris* and *C. sorokiniana* co-immobilized with *A. brasilense*. After a 10 day study, Bashan et al. (2002) reported significant increases in microalgal cell sizes. Lipid content in all algal strains were also reported to increase with the number of different fatty acids increasing from four to eight. The accumulation of lipids, however, was determined by Bashan et al. (2002) not to be stress-induced as microalgal populations were also

observed to increase. The ability to increase both algal biomass and lipid content simultaneously is significant as previous attempts at lipid accumulation have focused on nutrient depletion, which happens when growth rate is reduced and microalgal stress is increased (Mata et al., 2010; Yang et al., 2011). As a result, finding and utilizing growth-promoting bacteria, such as *A. brasilense*, may be critical for the economics of commercial microalgae cultivation as the current trade-offs between microalgal growth rate and lipid content can be avoided. By comparing microalgal growth in autoclaved and non-autoclaved media, an additional aim of this chapter was to determine the possible presence and influence of growth-promoting bacteria in either the AD effluent or the flowback water.

3.2.2. Bioflocculant-producing microbes

The dilute nature of microalgal cultures makes the harvesting and dewatering step operationally costly as substantial energy inputs are required to concentrate microalgal biomass. Major techniques currently employed include centrifugation, flocculation, filtration and screening, gravity sedimentation, flotation, and electrophoresis techniques (Chen et al., 2011a; Uduman et al., 2010). Despite the large number of techniques, most are considered too costly and energy intensive for the primary harvesting of microalgae. Flocculation is considered a superior method because it can treat large quantities of microalgal cultures and can be applied to a wide range of species (Uduman et al., 2010). Inorganic chemical flocculants, such as alum and ferric chloride, however, are considered too expensive for large scale operations (Schenk et al., 2008).

Technologies based on microbial flocculants may circumnavigate these current problems. Oh et al. (2001) conducted a study in which flocculent-producing bacteria were isolated from soil samples. Out of the 100 bacterial strains isolated, strain AM49 (*Paenibacillus* spp.) was determined to have the highest flocculation activity when co-cultured with the *C. vulgaris*. The flocculent efficiency of 83% was observed which was higher than the flocculent efficiencies of either aluminum sulfate (72%) or polyacrylamide (78%). Similar promising results were also obtained from Lee et al. (2009). Lee et al. (2009) conducted a study in which atmospheric bacteria (*Pseudomonas stutzeri* and *Bacillus cereus*) were allowed to naturally inoculate 1-L culture flasks where *Pleurochrysis carterae* was the only form of organic carbon present. By doing so, Lee et al. (2009) was able to select microbes which were able to coexist with the alga *P. cartera.* Results from this experiment showed that stress, as a result of nutrient depletion, caused the microbes to produce extracellular polymeric substances (EPS) that promoted flocculation of the algae. The average recovery efficiency of the microalgae was over 90%. Furthermore, microalgal cells were not damaged, retained their integrity, and followed predicable flocculation/settling behavior.

These promising results indicate that microbial bioflocculation of microalgae may prove to be an energy efficient, low cost, and safe commercial harvesting method. By comparing microalgal sedimentation kinetics in autoclaved and non-autoclaved media, an additional aim of this chapter was to determine the possible presence and influence of bioflocculant-producing microbes in either the AD effluent or the flowback water.

3.2.3. Microbial communities in shale gas flowback water and anaerobic digestion effluent

Bacteria are known to survive not only the thermodynamically harsh conditions during hydraulic fracturing injection, but also survive the chemically complex, saline fracturing wastewater. Bacterial growth and, as a result, microbiologically influenced corrosion has become such a significant problem to shale gas exploration that biocides have been added to fracturing fluids (Fichter et al., 2008; Struchtemeyer et al., 2012). Even with the addition of biocides, however, bacteria have been known to persist. Struchtemeyer and Elshahed (2012) reported that sequences affiliated with the phylum *Firmicutes* dominated fracturing wastewater samples from two wells located in the Barnett Shale formation in North Central Texas (USA). While many of the reported families, such as *Bacillaceae* and *Clostridiaceae*, contained spore-forming genera that have known resistance to biocides, sulfide-producing bacteria, halophilic bacteria, and non-spore forming thermophiles were also reported. Davis et al. (2012) similarly analyzed two wells located in the Barnett Shale formation in North Central Texas (USA) and found the predominance of halophilic bacteria. The observed lineages contained a variety of metabolic and physiological capabilities which included chemolithoautotrophs, aerobic heterotrophs, anaerobic fermenters, and anaerobic respiratory sulfate reducers. Completely distinct microbial communities were reported in both studies when compared to the source water used for hydraulic fracturing.

Anaerobic digestion sludge and effluent is even more so characterized by having a diverse and complex microbial community. 16S rRNA gene clones have been retrieved

from various prokaryotic taxa such as Proteobacteria, *Chloroflexi*, *Firmicutes*, *Spirochaetes*, and *Bacteroidetes* (Narihiro and Sekiguchi, 2007). Additionally, uncultured bacterial phyla (WWE1) in abundance of 12% of the total bacteria on the basis of rRNA have also been reported in digestion sludge of a municipal wastewater treatment plant (Chouari et al., 2005) . The first obligate syntrophic propionate-oxidizing anaerobe was also purported to be cultured from an aerobic sludge bed reactor treating sugar beet wastewater (de Bok et al., 2005). With the existence of such a large and diverse microbial community, some researchers involved in the use of AD effluent as a nutrient source for algal cultivation have pretreated the effluent using methods such as filtration and autoclaving to reduce the potential for contamination (Park et al., 2010; Wang et al., 2010). In this study, autoclaving was chosen as the method to evaluate the influence of microbial contaminates on microalgae growth.

3.2.4. Current methods to measure microalgal sedimentation kinetics

Because of the possibility of observing microalgae flocculation as a result of microbial or chemical interactions, a method to measure sedimentation kinetics was pursued in this study. Conventional methods to measure algae sedimentation involve the use of jars (Vandamme et al., 2010) or cylindrical glass tubes (Papazi et al., 2010). Algal suspensions are placed in these containers and measurements using OD are used to assess the microalgal removal percentage using the below equation (Salim et al., 2011):

$$recovery\ (\%) = \frac{OD_{t_0} - OD_t}{OD_{t_0}} * 100$$

Where:

OD_{t_0} = OD of algal suspension at time zero

OD_t = OD at half the height of the clarified layer at time t

Similarly to how Salim et al. (2011) adapted this method for use with cuvettes, it was postulated in this study that the method could be applied to 96-well microplates. Microplate readers with kinetic analysis have been used previously in endotoxin quantification assays, biological activity studies, and molecular biology assays for the quick and reliable analysis of a large number of samples (Lourenco, 2011). Because software is used to control when measurements are taken, using a microplate reader also has the benefit of removing the need to monitor the sample.

3.3. Materials and Methods

3.3.1. Cultivation of N. salina and D. tertiolecta in autoclaved and non-autoclaved media

N. salina and *D. tertiolecta* were cultured in 250-mL Erlenmeyer flasks (100-mL working volume) filled with media according loading ratios described in Table 6. All treatments were conducted in triplicates for a total of 36 reactors. Algal seed inoculum was added to each reactor at a fixed volume (10% v/v). Cultivation conditions were similarly described in Section 2.3.3. The reactors were sampled every other day for a period of 18 days using methods described previously in Section 2.3.3. The OD of the samples were measured with a microplate reader at 680 nm by transferring 200-µL aliquots, in triplicates, to a microplate.

To test the influence of nutrient and salinity source, commercials nutrients (Proline f/2 algae feed, Pentair Aquatic Eco-Systems, FL, USA) and commercial salts (Instant Ocean® sea salt, Spectrum Brands, Madison, WI, USA) were used as experimental controls. Because the factor of interest with using commercial salts was to test the influence of the salt/chemical constituents in the flowback water, the ammonium content of the flowback water (~47 mg L^{-1} NH_4) was accounted for by adding NH_4Cl to commercial salts at equivalent amounts. Because a significant amount of ammonium was volatilized and lost during autoclaving of the AD effluent, NH_4Cl was added to the autoclaved AD effluent to match the non-autoclaved samples. In effect, the total inorganic nitrogen of the AD effluent and the flowback water was matched separately

using commercial nutrients and commercial salts, supplementing with NH_4Cl as appropriate.

Autoclaving at 121°C for 15 min was chosen as the method to sterilize and thus minimize the influence of viable microbes found in the media. It was anticipated that a comparison to the non-autoclaved media, as a result, would allow observations to be made on the impact of viable microbes on microalgal growth and sedimentation. To avoid the formation of precipitates while autoclaving, samples of the AD effluent and the flowback water were autoclaved separately and then combined afterwards. The pH of the media after autoclaving was adjusted to 7.97, as described in Section 2.3.4, using concentrated HCl or 0.2 μm filtered 10 M NaOH to minimize possible microbial contamination. All media preparations and inoculations were performed in a biosafety cabinet. In summary, all media preparations were adjusted on Day 0 to similar inorganic nitrogen (~132 mg L^{-1} N), salinity (~42 g L^{-1}), and pH (~7.97) levels.

Table 6: Experimental design for evaluating the effects of chemicals and microbial communities in the anaerobic digestion effluent and the shale gas flowback water on algal growth and sedimentation.

Treatment	Nutrient Source	Salinity Source	Sterilization Factor	NH4-N (mg L^{-1})	NO3-N (mg L^{-1})	Total N (mg L^{-1})
1	6% AD	FW	Autoclaved	132		132
2	6% AD	FW	Non-autoclaved	132		132
3	6% AD	CS	Autoclaved	132		132
4	6% AD	CS	Non-autoclaved	132		132
5	f/2	FW	Autoclaved	47	85	132
6	f/2	FW	Non-autoclaved	47	85	132

CS = commercial salts, FW = flowback water, 6% AD = 6% anaerobic digestion effluent, f/2 = commercial nutrients

3.3.2. Evaluation of a microplate-based method to measure sedimentation kinetics

To test the capability of a microplate reader to analyze the sedimentation kinetics of microalgae, microalgal seed cultures of *N. salina*, *D. tertiolecta*, *D. salina*, and *T. suecica* in exponential phase were transferred to a 96-well microplate in 200 μL aliquots, in triplicates. *T. suecica* was particularly chosen for this analysis as it is a known microalga capable of auto-flocculating (Salim et al., 2011). A 2% (v/v) sodium azide solution at different loadings (2, 5, 10, and 20 μL) was used as a positive control for sedimentation as sodium azide is a known toxin for microalgae (Keilin, 1936). The 2% sodium azide solutions were directly added to the well with a pipette. The sedimentation kinetics of microalgae samples without sodium azide additions were also analyzed. Using the kinetic analysis function of the microplate reader (Eon® microplate spectrophotometer, Biotek, Winooski, VT, USA), wells were monitored at 680 nm every 30 min for a total of 12 hrs. Percent change in OD at 680 nm was calculated based on the following equation:

$$\%Change\ in\ OD_{680\ nm} = \frac{OD_t - OD_{t_0}}{OD_{t_0}} * 100$$

Where:

OD_t = OD at time t

OD_{t_0} = initial OD

3.3.3. Analysis of sedimentation kinetics

Daily samples from the experiment described in Section 3.3.1 were analyzed by transferring 200 μL aliquots, in triplicates, to a 96-well microplate. The % change in OD at 680 nm was analyzed using methods described in Section 3.3.2. As a control, the % change in OD was compared against microalgal cultures grown, in triplicates, in commercial nutrients and commercial salts (f/2+CS) at similar initial inorganic nitrogen (~132 mg L^{-1} N) ,salinity (~42 g L^{-1}), and pH (~7.97) levels to the treatments mentioned in Table 7. Cultivation conditions and sampling procedures for the controls were similarly described in Section 3.3.1.

3.4. Results and Discussion

3.4.1. Cultivation of N. salina and D. tertiolecta in autoclaved and non-autoclaved media

3.4.1.1. Specific growth rate

A comparison of the specific growth rates for *N. salina* and *D. tertiolecta* grown in the autoclaved and non-autoclaved media are shown in Figure 5. Autoclaving was found to be a significant factor ($p<0.05$) and resulted in a 14.9% and a 68.8% increase in the specific growth rate for *N. salina* and *D. tertiolecta*, respectively. The nutrient and salinity source also had a significant interaction with autoclaving with the autoclaved 6% AD effluent loading in commercial salts and the autoclaved 6% AD effluent loading in flowback water having 58.9% and 18.9% higher specific growth rates than the non-autoclaved counterparts ($p<0.05$). No significant differences were observed in the specific growth rates attained in the autoclaved 6% AD effluent loading in commercial salts or the autoclaved 6% AD effluent loading in flowback water ($p>0.05$).

The differences between the autoclaved and non-autoclaved media for both strains may be a result of free-ammonia inhibition and not because of anticipated differences in microbial activity. A key observation that suggested a possible effect of ammonia is that a significant amount of ammonium was lost (~39%) after autoclaving the AD effluent. Park et al. (2010) also autoclaved AD effluent and observed very little changes in the ammonium concentrations and speculated that significant volatilization of the ammonia would occur if the primary form of ammonia was free-ammonia. Because the ammonia

losses were minimal, Park et al. (2010) hypothesized that the ammonia content was primarily in the form of ammonium ions stabilized by constituents in the effluent.

Based on these observations, this may suggest that the main form of ammonia in the non-autoclaved AD effluent was free-ammonia, which was primarily lost after autoclaving. This is consistent with both the results in Section 2.4.2 in which a significant decrease in the specific growth rate was observed for *D. tertiolecta* at higher loadings of the AD effluent and previous research which suggests that *D. tertiolecta* is sensitive to the concentration of free-ammonia (Azov and Goldman, 1982; Chen, 2011). Because the ammonium lost was replaced with NH_4Cl in this study, an ion form that would have been stabilized by constituents in the medium, free-ammonia toxicity would not have been as apparent in the media with the autoclaved AD effluent. This is consistent with the observed difference in the specific growth rate for *D. tertiolecta* between the autoclaved and non-autoclaved media containing the AD effluent (Figure 5b). Because a smaller difference in the specific growth rate was observed for *N. salina* between the autoclaved and non-autoclaved media containing the AD effluent, these results also suggest that *N. salina* is less affected by the free-ammonia concentration in the medium. Again, this is also supported in Section 2.4.2 as only a gradual decline in the specific growth rate for *N. salina* was observed at higher AD effluent loadings.

The magnitude of the difference between the autoclaved and non-autoclaved 6% AD effluent loading in commercial salts and the autoclaved and non-autoclaved 6% AD effluent loading in flowback water further suggests that the ability to ionize free-ammonia into its stable form, ammonium, is also dependent on the constituents in the

medium. This is supported by literature which indicates that saline waters reduce the apparent toxicity of free-ammonia as ion concentrations increase the electrostatic forces among ions and molecules to an extent that free-ammonia ionizes into ammonium. Additionally, the extent of the electrostatic forces is affected by both the concentration and charge of ions in solution (Soderberg and Meade, 1991).

As can be seen in Table 2 and 3, the flowback water contained higher concentrations of ions, such as Na^{+}, and multivalent ions, such as Mg^{2+}, when compared to standard preparations of commercial salts. As mentioned above, the differences in the specific growth rates between the autoclaved and non-autoclaved 6% AD effluent loading in commercial salts is higher than the differences obtained from the autoclaved and non-autoclaved 6% AD effluent loading in flowback water. This is consistent with the belief that the lower ionic strength of commercial salts is resulting in a higher concentration of free-ammonia in the non-autoclaved 6% AD effluent loading in commercial salts than the non-autoclaved 6% AD effluent loading in flowback water. As *D. tertiolecta* is more sensitive to free-ammonia, this may explain the pronounced difference between the specific growth rates observed for both strains from cultivation in the autoclaved and non-autoclaved 6% AD effluent loading in commercial salts.

While an explanation based on free-ammonia is feasible considering previous experimental results, changes in microbial activity after autoclaving may be also be having an effect. Previous research has noted that the significant population of bacteria in AD effluent may cause contamination to algae production systems if left untreated (Cai et al., 2013b; Wang et al., 2010). Bacteria may compete for limited nutrients or produce

algicidal extracellular metabolites (Doucette, 1995; Fukami et al., 1997; Grossart and Simon, 2007). As the increase in specific growth rate for *N. salina* in the autoclaved media was to a lesser extent than *D. tertiolecta*, this would indicate that an explanation based on bacterial-interactions is strain specific, complicating the viability of this explanation.

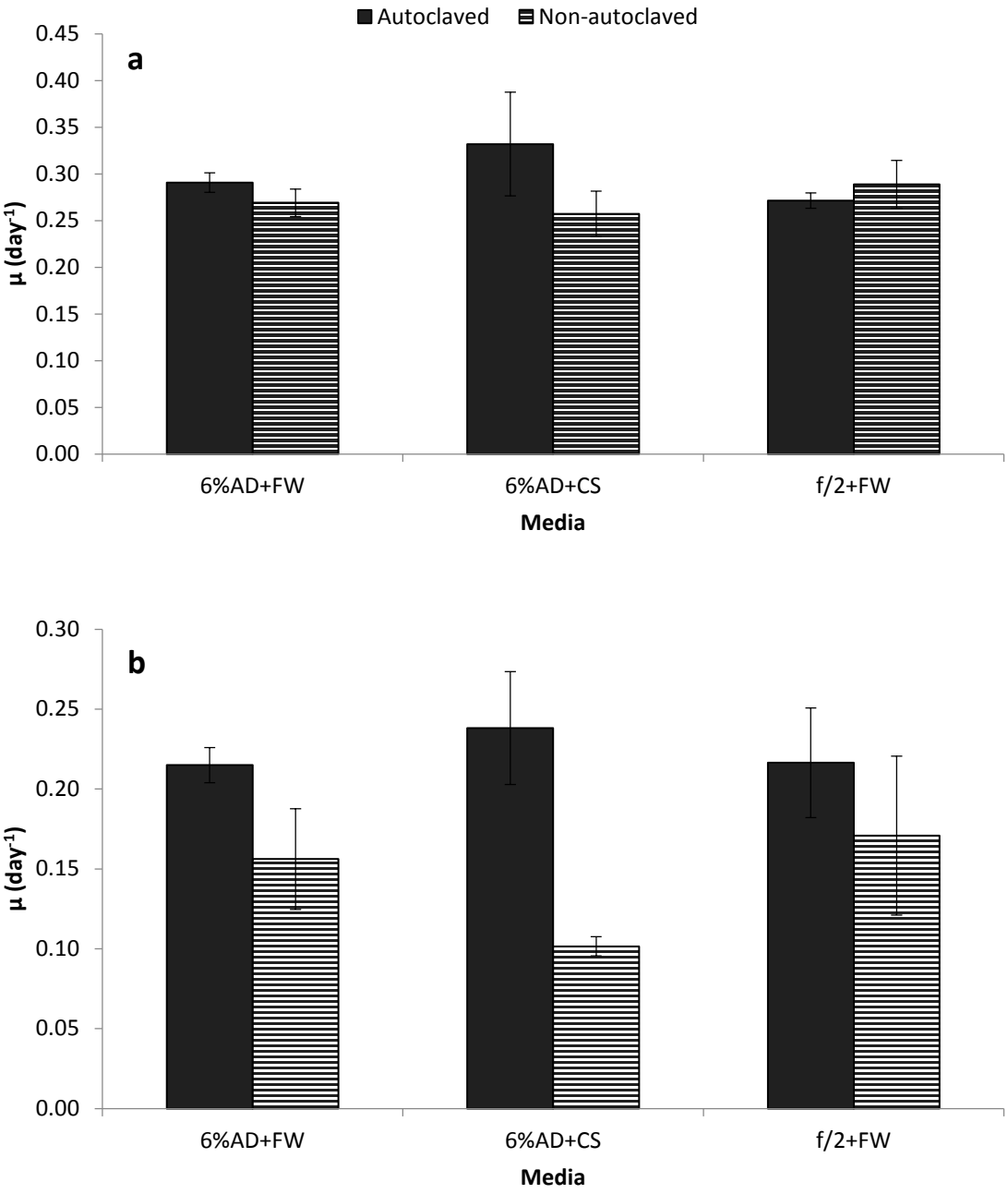

Figure 5: Effect of autoclaving media on specific growth rate of (a) *N. salina* and (b) *D. tertiolecta*. Media evaluated included 6% anaerobic digestion effluent loading in the flowback water (6% AD+FW), 6% anaerobic digestion effluent loading in commercial salts (6% AD+CS), and commercial nutrients in the flowback water (f/2+FW).

3.4.1.2. Average biomass productivity

The average biomass productivities for both strains cultivated in the various media are summarized in Figure 6. The effect of autoclaving was found to be significant and an increase of 64% was observed in the average biomass productivity for *D. tertiolecta* in the autoclaved media ($p<0.05$). In contrast, autoclaving did not have a significant effect on the average biomass productivity for *N. salina* ($p>0.05$). Additionally for *D. tertiolecta*, the autoclaving media with the 6% AD effluent loading resulted in a 39.1% increase in the average biomass productivity compared to the non-autoclaved 6% AD effluent loading and the autoclaved and non-autoclaved commercial nutrients. In regards to the salinity source, *D. tertiolecta* achieved an average biomass productivity 40.3% higher in the flowback water when compared to commercial salts. The salinity source had no significant impact on *N. salina* in regards to biomass productivity ($p>0.05$).

These differences, as mentioned previously in Section 3.4.1.1, may be explained by the removal of free-ammonia by autoclaving and the replacement of free-ammonia with NH_4Cl. The preference of *D. tertiolecta* for the flowback water may also indicate that high ion concentrations in the flowback water may be stabilizing the ammonium ions, preventing free-ammonia from accumulating to inhibitory levels (Soderberg and Meade, 1991). A possible explanation why this preference for the flowback water over commercial salts was not observed in Section 2.4.3 for *D. tertiolecta* is the difference in cultivation conditions. In this experiment, the algae were rotated on a shaker at 150 rpms, while the experiment in Section 2.4.3 was air sparged. Because air sparging results in a

significant formation of aerosols, it is possible that the free-ammonia in the 1-L cultures was volatilized through ammonia stripping (Liao et al., 1995). This explanation is also supported by the screening experiment conducted in Section 2.4.2 in which rotation at 150 rpms was also utilized and the appearance of an initial lag phase for *D. tertiolecta* in the AD effluent and flowback water media was observed, indicating possible free-ammonia inhibition. This may suggest that the flowback water with comparable loadings of ammonium to commercial salts may be able to improve the growth performance of microalgae sensitive to free-ammonia because of the inherently higher ionic strength.

Overall, the highest average biomass productivities observed were 162 and 203 mg L^{-1} day^{-1} for *N. salina* and *D. tertiolecta*, respectively, in the autoclaved 6% AD effluent loading in flowback water. In contrast, the highest specific growth rates observed were 0.332 and 0.238 day^{-1} for *N. salina* and *D. tertiolecta*, respectively, in the autoclaved 6% AD effluent loading in commercial salts. Because of the significant interference of autoclaving on the ammonium concentrations, the degree to which the inherent microbial activity of the flowback water and the AD effluent is effecting the microalgal growth remains uncertain.

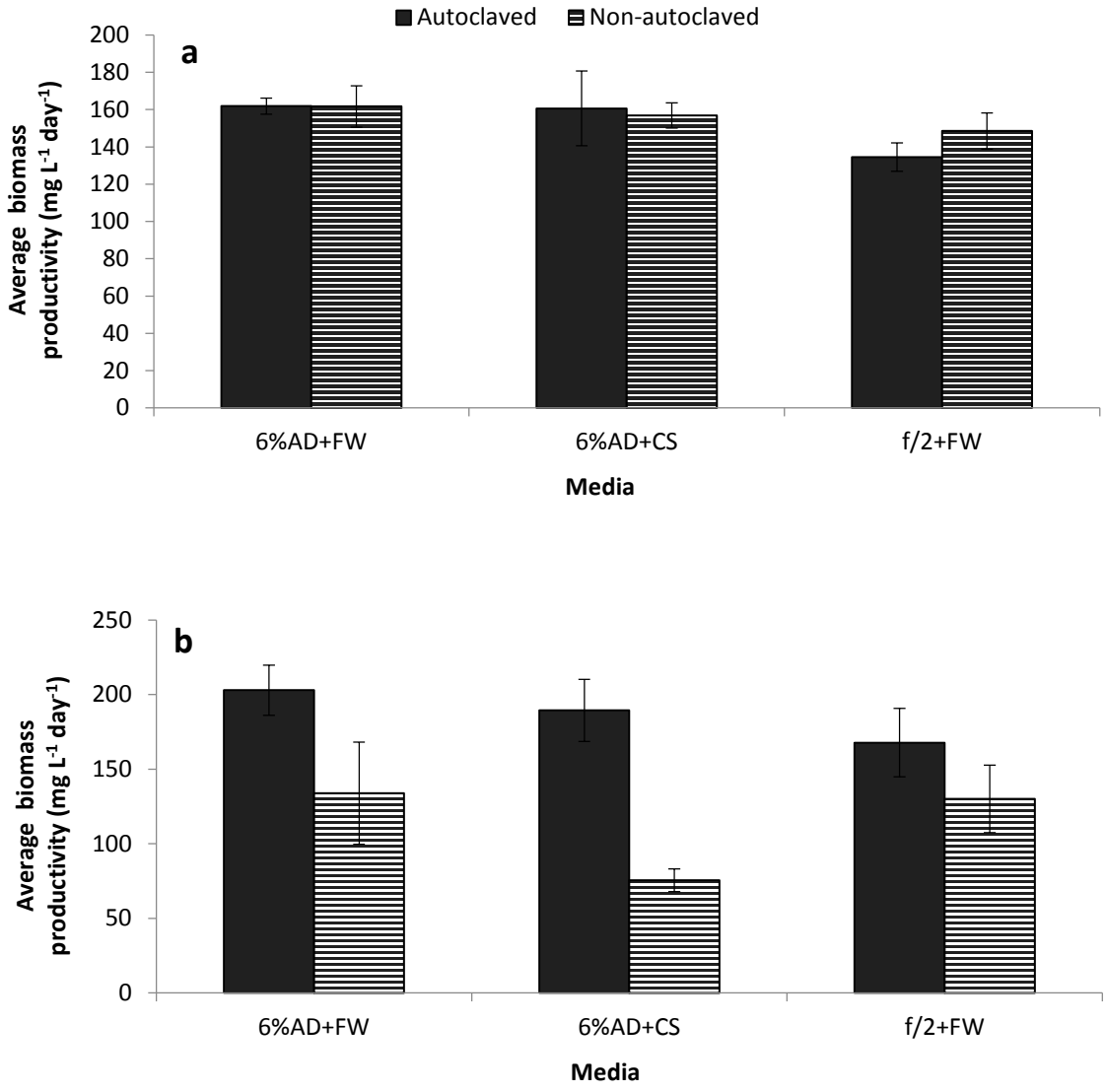

Figure 6: Effect of autoclaving media on average biomass productivity of (a) *N. salina* and (b) *D. tertiolecta*. Media evaluated included 6% anaerobic digestion effluent loading in the flowback water (6% AD+FW), 6% anaerobic digestion effluent loading in commercial salts (6% AD+CS), and commercial nutrients in the flowback water (f/2+FW).

3.4.2. Sedimentation kinetics of N. salina and D. tertiolecta in autoclaved and non-autoclaved media

3.4.2.1. Evaluation of a microplate-based method to measure sedimentation kinetics

Results from the preliminary sedimentation experiment are shown in Figure 7. Each strain without the addition of sodium azide was observed to have a unique curve that developed as a result of a change in OD at 680 nm over time. While *T. suecica* is a known microalga capable of auto-flocculating (Salim et al., 2011), the % change in OD at 680 nm did not suggest that flocculating or settling was occurring under the given conditions. The appearance of a logistic curve for *N. salina*, however, suggested that settling was occurring naturally. A possible explanation for this settling is the inherent lack of motility for *N. salina* cells compared to the other tested algal strains. Previous research has shown that actively photosynthesizing microalgal cells with high metabolism rates and unicellular mobility are naturally resistant to settling. Called the net electronegative zeta shielding effect, active algal cells are stabilized by the presence of charged carboxylic and amine groups on the cell surface (Danquah et al., 2009; Vandamme et al., 2013). However when microalgal cells have a low metabolic rate, such as when the cells are at a period of low growth or are exposed to darkness, the net electronegative zeta shielding effect is reduced, causing cells to agglomerate and settle (Danquah et al., 2009). The lack of natural motility for *N. salina* combined with the exposure to dark conditions inside the microplate reader may have reduced the net electronegative zeta shielding effect to a point that settling was observed.

Upon addition of 2% sodium azide, a known algal toxin, the % change in OD increased for all microalgal strains except *N. salina*. The addition of sodium azide for the motile strains is most likely resulting in the death of the microalgal cells, causing charge-stabilizing metabolic processes to cease and for the cells to begin settling. The observable difference between the % change in OD after sodium azide addition suggested that settling of microalgal cells may be observable by a microplate reader under kinetic analysis.

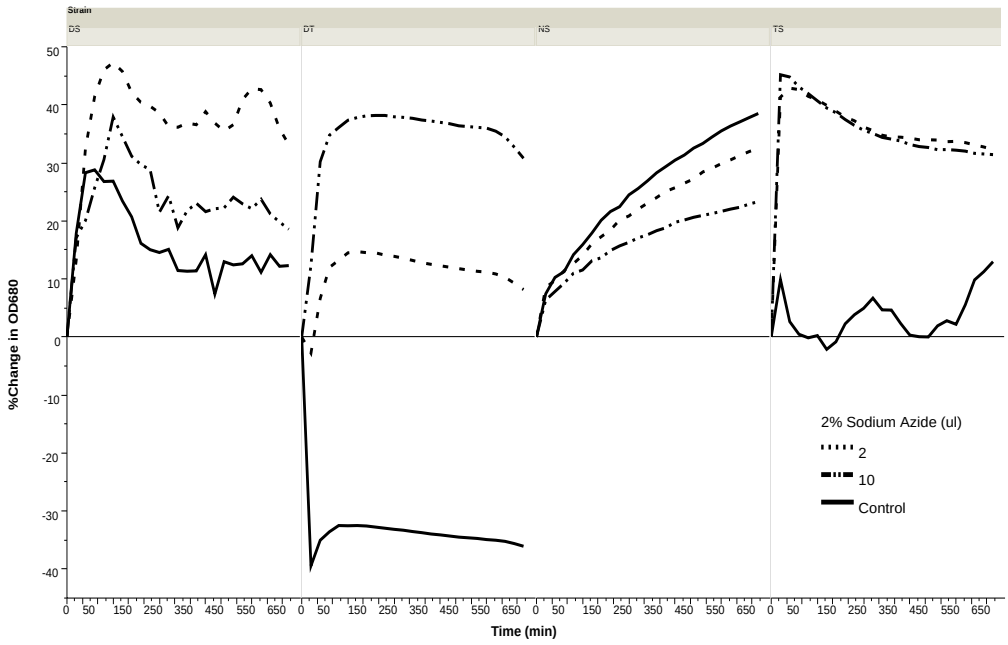

Figure 7: Microplate-based sedimentation kinetics of marine microalgae under different loadings of 2% sodium azide. Microalgal strains evaluated included *D. salina* (DS), *D. tertiolecta* (DT), *N. salina* (NS), and *T. suecica* (TS).

3.4.2.2. Sedimentation kinetics of *N. salina* and *D. tertiolecta*

Experimental results from the sedimentation kinetics of *N. salina* and *D. tertiolecta* grown in the autoclaved and non-autoclaved media are shown in Figure 8 and 9. For *N. salina* (Figure 8), the % change in OD was apparent for all media on Day 0. Additionally, the % change in OD was significantly affected by the day of analysis ($p<0.05$). This is supported by previous research which observed that the settling characteristics of microalgae are dependent on the current growth phase (Salim et al., 2013). For example, Konno (1993) and Lavoie and de la (1987) observed that the settling velocity of *N. linearis* was faster in both the stationary and declining phase compared to the exponential phase. The largest % change in OD for *N. salina* observed in this study, however, occurred on Day 4 in the medium composed of the autoclaved 6% AD effluent loading in commercial salts. Additionally, the highest % change in OD observed for the control occurred on Day 6-8. As researchers like Lavoie and de la Noue (1987) measured sedimentation based on the decreased transmittance of algal cells in a cuvette, it is currently uncertain if this indicates method, algal strain, or condition specific results.

Furthermore, the results indicated that the salinity and nutrient source were significant factors ($p<0.05$) and that *N. salina* had the highest and lowest % change in OD with the 6% AD effluent loading in commercial salts and the commercial nutrients in flowback water, respectively. Because of the high amount of total dissolved solids in the flowback water, it is possible that the increased concentration of ions is aiding in the stabilization of the electrical charge of the algal cells (Vandamme et al., 2013).

For *D. tertiolecta* (Figure 9), substantial % changes in OD were observed on Day 2 and Day 6. These peaks occurred for the autoclaved media such as the 6% AD effluent loading in flowback water, the 6% AD effluent loading in commercial salts, and the commercial nutrients in flowback water. Additionally, peaks were also observed for the non-autoclaved media such as the 6% AD effluent loading in flowback water and the commercial nutrients in flowback water. The media with the flowback water as the salinity source or the 6% AD effluent as the nutrient source were significantly higher than the commercial salts or the commercial nutrients ($p<0.05$). Autoclaving was also found to be significant on both nutrient and salinity sources for *D. tertiolecta*, with the autoclaved 6% AD effluent and the autoclaved flowback water having a significantly higher effect on % change in OD. The media that produced the highest means in regards to % change in OD were the autoclaved commercial nutrients in flowback water (32.9%), the non-autoclaved 6% AD effluent loading in flowback water (25.5%), and the autoclaved 6% AD effluent loading in commercial salts (23.1%), which were 290%, 247%, and 233% higher than the control, respectively.

A possible explanation for the observed increase in sedimentation with the flowback water for *D. tertiolecta* is the high concentration of Mg^{2+} and Ca^{2+} ions found in the flowback water. Previous research indicated that a pH increase above 10.2 caused Mg^{2+} and Ca^{2+} ions to form hydroxide precipitates that caused algal cells to settle and flocculate (Folkman and Wachs, 1973; Smith and Davis, 2012). As shown in Table 7, none of the various media had a final pH > 9. Because pH was not monitored throughout the cultivation period, it is uncertain the degree to which pH is affecting sedimentation.

This explanation is also complicated by the fact that similar changes in OD were not observed for *N. salina* in the flowback water.

In support of these experimental results for *D. tertiolecta*, sedimentation was observed in Eppendorf centrifuge tubes during sampling for certain media, such as the autoclaved 6% AD effluent loading in commercial salts and the autoclaved 6% AD effluent loading in flowback water. Significant sedimentation was not observed, however, for non-autoclaved counterparts. Sedimentation was also observed for *D. tertiolecta* in the previous media at the end of the culture period, contrasting the trend of decreasing % change in OD shown in Figure 9. It is uncertain whether the mechanics of settling for *D. tertiolecta* have changed so that sedimentation measurements via increasing changes in OD are no longer valid. This may indicate a limitation to using kinetic measurements in a microplate reader as a method to measure sedimentation via % change in OD as the sedimentation mechanics must result in increased light attenuation. Increased light attenuation might not occur if sedimentation is caused by the formation of loose flocs which might allow light to bypass the settled cells. The formation of loose flocs has been known to occur if flocculation is induced by extracellular polymeric substances, such as in the case of microbial-induced flocculation (Lee et al., 2009).

A significant finding was that the pH for *D. tertiolecta* in the autoclaved 6% AD effluent loading in flowback water and the autoclaved 6% AD effluent loading in commercial salts significantly decreased to a pH of 4.02 and 4.46 on Day 18, respectively. Cultures of *N. salina* in the autoclaved 6% AD in commercial salts were also observed to have a low pH of 5.21 on Day 18. Under normal cultivation conditions

of marine microalgae, the pH of the media rises as dissolved CO_2 is removed by photosynthesis (Chen, 1994). As can be seen in Table 7, most of the cultures of *N. salina* and *D. tertiolecta* had final pH values near or higher than the starting pH. It is currently uncertain what may be causing the acidification of the culture media. As flocculation of *D. tertiolecta* in the previously mentioned media was still observed first-hand in Eppendorf centrifuge tubes on Day 18, this observation is in oppose to previous research which indicates that a pH rise is necessary to flocculate marine microalgae (Besson and Guiraud, 2013; Horiuchi et al., 2003; Wu et al., 2012). Low pH has been observed to flocculate freshwater microalgae by causing organic matter adhering to microalgal cells to accept protons, causing instability and subsequent flocculation (Liu et al., 2013). Because this mechanism is independent of Mg^{2+} and Ca^{2+} ions and more dependent on the presence of suspended organic matter, this might explain why the changes in OD for *D. tertiolecta* were observed highest in media composed of the AD effluent or the flowback water. Again, as this conflicts with the observations for *N. salina*, it is uncertain of the exact sedimentation mechanisms effecting *N. salina* and *D. tertiolecta* related to differences in autoclaving, nutrient source, and salinity source. Further research is necessary to determine the reproducibility and mechanics behind these unusual phenomenon and whether the observed increases in sedimentation can be observed on a larger scale.

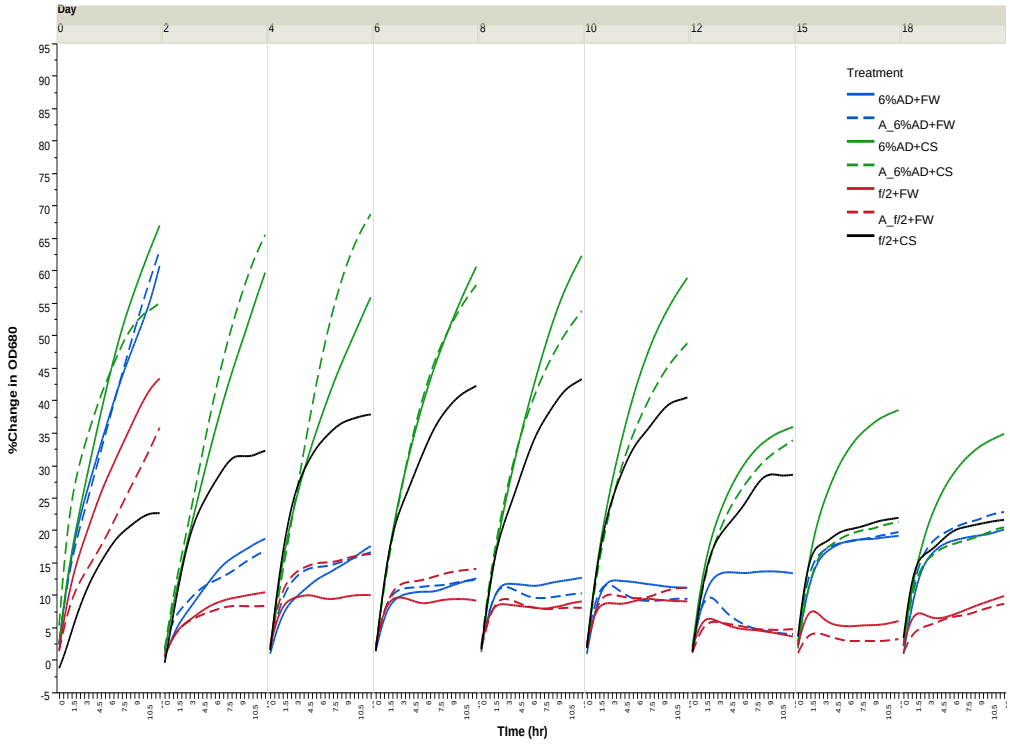

Figure 8: Effect of autoclaving media on microplate-based sedimentation kinetics of *N. salina*. Media evaluated included 6% anaerobic digestion effluent loading in the flowback water (6% AD+FW), 6% anaerobic digestion effluent loading in commercial salts (6% AD+CS), and commercial nutrients in the flowback water (f/2+FW). A medium composed of commercial nutrients and salts (f/2+CS) was used as a control. Autoclaved media are denoted by *A*_.

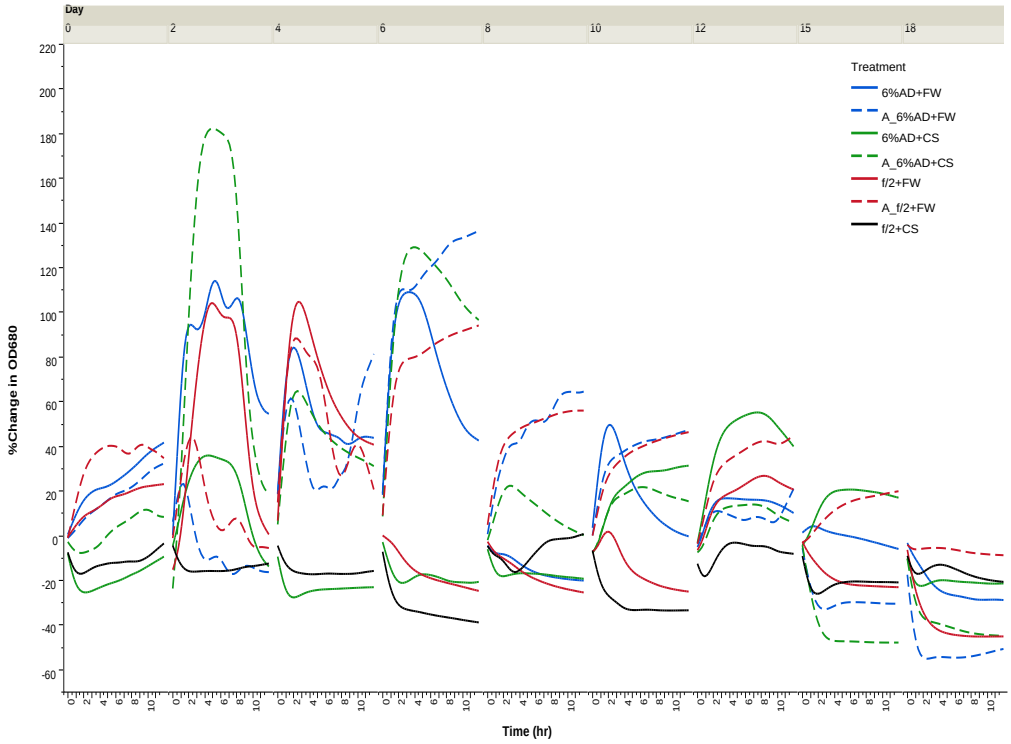

Figure 9: Effect of autoclaving media on microplate-based sedimentation kinetics of *D. tertiolecta*. Media evaluated included 6% anaerobic digestion effluent loading in the flowback water (6% AD+FW), 6% anaerobic digestion effluent loading in commercial salts (6% AD+CS), and commercial nutrients in the flowback water (f/2+FW). A medium composed of commercial nutrients and salts (f/2+CS) was used as a control. Autoclaved media are denoted by *A*_.

Table 7: The pH of autoclaved and non-autoclaved media before and after algal cultivation.

Strain	Media	Day 0	Day 18
N. salina	6% AD+FW	7.65	8.25 ± 0.04
	A_6% AD+FW	7.62	7.10 ± 0.40
	6% AD+CS	7.61	6.60 ± 1.73
	A_6% AD+CS	7.63	**5.21 ± 0.02**
	f/2+FW	7.62	9.00 ± 0.08
	A_f/2+FW	7.63	9.00 ± 0.12
	f/2+CS	7.62	8.94 ± 0.21
D. tertiolecta	6% AD+FW	7.65	7.27 ± 0.06
	A_6% AD+FW	7.62	**4.02 ± 0.05**
	6% AD+CS	7.61	6.71 ± 0.28
	A_6% AD+CS	7.63	**4.46 ± 0.26**
	f/2+FW	7.62	7.74 ± 0.07
	A_f/2+FW	7.63	7.76 ±0.12
	f/2+CS	7.62	8.45 ± 0.06

Media evaluated included 6% anaerobic digestion effluent loading in the flowback water (6% AD+FW), 6% anaerobic digestion effluent loading in commercial salts (6% AD+CS), and commercial nutrients in the flowback water (f/2+FW). A medium composed of commercial nutrients and salts (f/2+CS) was used as a control. Autoclaved media are denoted by *A*_.

3.5. Conclusion

The impact of autoclaving various media was found to be strain specific. While these parameters did not have a major effect on *N. salina* in regards to the specific growth rate or the average biomass productivity, dramatic changes occurred with *D. tertiolecta* cultures. For example, autoclaving resulted in a 56.2% increase in the specific growth rate for *D. tertiolecta*. A most likely explanation for these differences is based on three main assumptions: 1) tolerance to free-ammonia toxicity is strain dependent, 2) autoclaving resulted in the loss of free-ammonia, 3) the higher ionic strength of the flowback water prevented ammonia dissociation into free-ammonia. If these assumptions prove to be true, it might indicate that the flowback water may be able to improve the growth of microalgae sensitive to free-ammonia concentrations compared to commercial salts at the same salinity. Future research is necessary to explore impact of the ionic strength of the flowback water on free-ammonia toxicity and possible microbial interactions with algal growth. Because of the inconsistencies in the results of the sedimentation kinetics between the two strains, is it difficult to conclude the extent of sedimentation and the mechanisms responsible. As a result, future research is also suggested to determine the reproducibility and mechanics behind the unusual sedimentation kinetics observed.

Chapter 4: Conclusions and Recommendations

Marine microalgae cultivation utilizing shale gas flowback water and AD effluent as sources of water and nutrients has yet been reported. The results from the initial evaluation of microalgae in various loadings of the AD effluent loading in the flowback water suggested that successful cultivation is highly dependent on the microalgal strain's ability to tolerate inhibitors found in the flowback water and the AD effluent. While *D. salina* experienced poor growth overall, *N. salina* and *D. tertiolecta* were able to tolerate the AD effluent loadings of 2% to 10% in the flowback water. The highest average biomass productivities for *D. tertiolecta* and *N. salina* were observed at the 6% AD effluent loading in the flowback water (132 NH_4-N mg L^{-1}).

N. salina and *D. tertiolecta* were then cultivated in the medium composed of the 6% AD effluent loading in the flowback water and compared to cultivation in a medium composed of commercial nutrients and salts at similar initial inorganic nitrogen (~132 mg L^{-1} N), salinity (~42 g L^{-1}), and pH (~7.97) levels. It was determined that there was no significant difference in the specific growth rates or the average biomass productivities obtained by cultivation in the different media. Further analysis of the lipid content and profile of both strains suggested that the medium had little effect on lipid productivity. The cetane number of the fatty acid methyl esters from *D. tertiolecta* was similar in both media while only a minor decrease of 1.84% was observed for *N. salina* grown in the flowback water supplemented with the 6% AD effluent. These results suggested that

comparable lipids with similar profiles could be achieved by using the AD effluent and the flowback water.

An evaluation of the effects of chemicals and microbial communities in the AD effluent and the shale gas flowback water on algal growth and sedimentation was also performed. Because of significant losses in the ammonium concentrations after autoclaving, the ability to examine the influence of the microbial community of the medium composed of the AD effluent and the flowback water was complicated. It was observed, however, that the ionic strength of the flowback water may have a significant impact on the ionization of ammonia. As commercial salts have a less concentration of ions, the ionic strength is lower and thus free-ammonia toxicity was believed to be more likely. In the case of *D. tertiolecta*, a strain of microalgae that is relatively sensitive to the concentration of free-ammonia, a higher biomass productivity was observed in the flowback water compared to the commercial salts. Because of inconsistencies in the observations regarding the sedimentation kinetics of *N. salina* and *D. tertiolecta*, it is currently unclear to what degree the flowback water and the AD effluent is affecting microalgae sedimentation and whether the developed microplate-based method has validity as an experimental tool.

Overall, the research conducted in this thesis suggests that cultivation of marine microalgae in shale gas flowback water is feasible and, when coupled with municipal anaerobic digestion effluent, may be able to result in comparable growth and lipid productivities to commercial nutrients and salts at similar initial nitrogen, salinity, and pH levels. The ability to use shale gas flowback water and AD effluent to cultivate marine

microalgae may remove a significant economic barrier to the algae biofuels industry in regards to water and nutrients required for cultivation. Further research is required in determining if similar growth rates and biomass productivities can be achieved with flowback waters from other shale gas exploration sites. Additionally, research in the continuous culture of microalgae in AD effluent and flowback water medium is also needed as it is currently uncertain if constituents like toxic metals will accumulate over time to levels inhibitory to microalgal growth. As the flowback water will eventually require disposal after microalgal cultivation, further research is still required in wastewater treatment technologies, such as desalinization.

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Appendix A: Wavelength Selection for Algal Biomass Estimations Based on Optical Density

A.1. Introduction

The assessment of microalgae cultivated in wastewater is potentially problematic because of the possible presence of non-algal solids and particles. For example, shale gas flowback water has been reported to contain elevated concentrations of total dissolved solid, minerals, hydrocarbons, metals, oils, greases, and soluble organic compounds (Abdalla et al., 2011; Gregory et al., 2011). Conventional methods to assess microalgal growth, such as dry weight, are inaccurate for cells grown in medium containing non-cellular solids (Shuler, 2002). Similarly, optical density (OD), the most common measurement in microbiology laboratories to assess microbial growth, is also complicated by the presence of non-photosynthetic biomass (Griffiths et al., 2011; Myers et al., 2013). As such, a study was conducted with the following aims: 1) evaluate the relationship between wavelength, OD, and algal dry biomass and 2) select the wavelength which minimized interference of non-algal particulates on biomass approximations for future research involving the flowback water. Chosen wavelengths were analyzed concurrently with the microalgal experiments involving growth in the AD effluent and the flowback water. Accuracy of the chosen wavelengths was then assessed by comparing the algal dry biomass measurements to the biomass concentrations predicted by OD during microalgal cultivation experiments used for lipid analysis.

A.2. Literature Review

Optical density (OD) is often used as a rapid measurement for suspended biomass concentrations and is the most common measurement in microbiology laboratories to assess microbial growth (Myers et al., 2013). The premise behind most laboratory studies involving OD is the use of the Beer-Lambert Law (Figure 12).

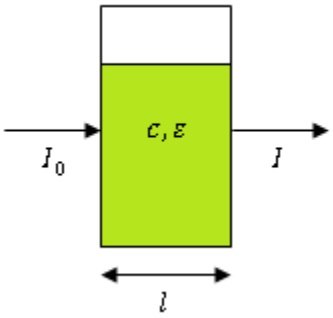

Figure 10: Typical laboratory cuvette used with optical density measurements.

$$OD = -log\left[\frac{I}{I_0}\right] = \varepsilon * c * l$$

Where:

I_0 = incident intensity of light

I = measured intensity of light

ε = attenuation coefficient

c = sample concentration

l = path length

As shown in this equation, the optical density of a sample is directly related to the cell concentration in the sample, as well as the path length and the attenuation coefficient. The attenuation coefficient characterizes the degree which a species or suspension attenuates light. The path length defines the distance between the incident intensity of light and the measured intensity of light. To use the Beer-Lambert law, both the attenuation coefficient and the path length (standardized at 1 cm for most spectrophotometers) are assumed constant. Specifically, it is assumed that the attenuation coefficient is derived from light absorption that occurs in a single-phase, homogenous solution. With these assumptions, optical density is linearly correlated in the following equation with the measured species, such as biomass (Griffiths et al., 2011; Hsiu et al., 2010; Myers et al., 2013).

$$[X] = m * OD$$

Where:

$[X]$ = concentration of species (biomass)

Difficulty in appropriately applying this equation to microalgal biomass measurements arises as microalgal suspensions are typically two-phase solutions composed of a solid (microalgal biomass) and a liquid (growth media) phase. The inherent refractive index of each phase and the resulting mismatch of the refractive index of the suspension results in an attenuation coefficient being composed of both absorption and scattering components. Wavelengths which correspond to an absorbance maximum by chlorophyll *a* in algae (e.g. 680 nm) maximize the absorption component while

wavelengths which fall outside the absorbance maximum (e.g. 550) maximize the scattering component. Because scattering may redirect light away from the photosensor or cause light to be reemitted at a lower frequency, the linear relationship between OD and sample concentration is no longer completely valid (Griffiths et al., 2011; Hsiu et al., 2010; Myers et al., 2013).

Despite these difficulties, researchers have used a variety of wavelengths without a consensus on whether wavelength choice is dependent on culturing conditions. Table 8 shows the wide range of wavelengths previously considered for different strains and media. Research conducted by Myers et al. (2013) and Griffiths et al. (2011) suggested that wavelengths associated with scattering (e.g. 550 nm) may prolong the linear correlation between OD and sample concentration as they are not dependent on cell pigment. However, it was also suggested that such wavelengths may be inappropriate in medium containing components which may interfere with cell scattering, such as the presence of non-photosynthetic material. Because of the lack of research regarding appropriate wavelength choice for media such as wastewater, this study aimed to select the wavelength that was least effected by non-algal particulates and thus most appropriate for future research involving flowback water.

Table 8: Previously used wavelengths to approximate microalgal biomass.

Microalgae Strain	Wavelength (nm)	Media	Reference
Dunaliella tertiolecta	405	Erdschreiber's medium	(Chen, 2011)
Nannochloropsis salina	440	Diluted municipal AD effluent	(Cai et al., 2013c)
Chlorella vulgaris	515	MBL Medium Woods-Hole	(Al-lwayzy et al., 2014)
Chlorella vulgaris *Dunaliella tertiolecta*	540	Various artificial media	(Gouveia and Oliveira, 2009)
Botryoccus braunii	650	Kessler's medium	(Krzeminska et al., 2014)
Chlorella sp.	680	Diluted manure AD effluent	(Wang et al., 2010)
Aphanizomenon flos-aquae	750	ASN-1-TR medium	(Schanz and Zahler, 1981)

A.3. Materials and Methods

A.3.1. The relationship between wavelength, optical density, and algal dry biomass

N. salina, *D. tertiolecta*, *D. salina*, and *T. suecica* were cultivated in 2-L reactors (1-L working volume) at 25°C under constant illumination (200 µmol m^{-2} s^{-1}) using 32-watt florescent lamps (GE Lighting, Ravenna, OH, USA). Reactors were placed in a white coated chamber and each reactor was equipped with rubber stoppers and 4.76 mm diameter stainless steel tubing for air inlet/outlet. Air sparging, by the use of filter-sterilized (0.2 µm Whatman PTFE Puradisc, GE Healthcare, Maidstone, UK) ambient air (0.039% CO_2) at an airflow rate of 650 mL min^{-1}, was used to continuously mix the cultures. Reactors were filled to 1-L with appropriate seed culture media, described in Section 2.3.1, and seed inoculum for an initial optical density of 0.1 at 680 nm. The cultivation period was 18 days.

Before sampling, evaporation and previous sampling losses were accounted for by additions of DI water, as described previously. A sample volume of 50-mL was removed daily from each reactor. Optical density (OD) at wavelengths between 300-800 nm in increments of 10 nm was measured with a microplate reader (Eon® microplate spectrophotometer, Biotek, Winooski, VT, USA) using 96-well, flat bottom microplates (Costar®, Corning, Corning, NY). All microplate measurements were performed using 200-µL aliquots, in triplicates.

The dry biomass content of the algal samples was determined and reported in terms of ash-free dry weight (AFDW) as previously described. To minimize the influence of algal growth media on OD measurements, a blank of the media was created by

removing 200-µL aliquots, in triplicates, from the collected filtrate before additions of the NH_4HCO_3 wash. The OD of the aliquots was measured using the method mentioned previously and then subtracted from the OD of the corresponding algal sample.

A.3.2. Impact of wavelength on microalgal growth estimations

The experiment involving the evaluation of marine microalgae in AD effluent and flowback water was previously described in Section 2.3.3. In addition to the previously mentioned experimental design, the OD of the daily algal samples was also measured at 800 nm, a wavelength determined in Appendix A.4.1 to result in the highest correlation coefficient between OD and algal dry biomass. As previously described in Section 2.3.3, a blank of the media was created by removing 200-µL aliquots, in triplicates, from the supernatant after centrifugation at 10,000 rpm for 15 min (Centrifuge 5810 R, Eppendorf, Enfield, CT, USA). The OD of the supernatant aliquots was measured and then subtracted from the OD of the corresponding algal sample.

A.3.3. Evaluation of optical density as a method to approximate algal biomass

Evaluation of OD as a proxy for algal biomass estimations at 680 nm and 800 nm was performed concurrently with the experiment described in Section 2.3.4. In addition to the ash-free dry weight (AFDW) of the 50-mL algal samples, OD was also measured at 680 nm and 800 nm by measuring 200-µL aliquots, in triplicates, with a microplate reader. To minimize the influence of algal growth media on OD measurements, a blank of the media was created by removing 200-µL aliquots, in triplicates, from the collected filtrate before additions of the NH_4HCO_3 wash. The OD of the aliquots was measured

using the method mentioned previously and then subtracted from the OD of the corresponding algal sample.

A.4. Results and Discussion

A.4.1. The relationship between wavelength, optical density, and algal dry biomass

In order to identify optimal wavelengths for the measurements of algal biomass, correlation curves between algal dry biomass concentration (g L^{-1}AFDW) and OD were obtained. The results of 51 independent correlations are summarized in Figures 11 and 12 as plots between the correlation coefficient (R^2) and wavelength. For *N. salina*, *D. tertiolecta*, and *T. suecica* (Figures 11a, 11b, and 12b respectively), the maximum R^2 was obtained at a wavelength of 800 nm (R^2 =0.93, 0.92, and 0.97 respectively). In contrast, the widely used wavelength of 680 nm produced the lowest R^2 for *N. salina*, *D. salina,* and *T. suecica* (R^2=0.81, 0.86, and 0.87 respectively). These results are supported by previous research which suggested that wavelengths outside the maximal absorbance range of chlorophyll-*a* (400-460 nm and 680 nm) minimize correlation discrepancies between OD and biomass. In particular, Griffiths et al. (2011) found that a wavelength of 750 nm was less affected by the pigment content of *Chlorella vulgaris* grown under high and low nitrogen conditions. Similarly, Myers et al. (2013) observed almost identical correlations between dry weight and OD for 550 nm yet a difference of 34% for 680 nm for *Nannochloropsis* sp. under varying nitrogen conditions. The increase in robustness in OD measurements observed for these wavelengths is attributed to the independence of light scattering on pigment levels for light attenuation (Myers et al., 2013).

The influence of algal pigments on the linear correlation between OD and biomass is further illustrated by observing the trends of both R^2 vs. wavelength and OD vs. wavelength in Figures 11 and 12. For example, *N. salina* (Figure 11a) is an algal

strain devoid of photosynthetic pigments other than chlorophyll-*a* and resultantly showed clear absorbance peaks at 440 nm, 490 nm, 580 nm, 630 nm, and 680 nm. The peaks near 490 nm result from absorption by carotenoids while the remaining peaks are attributed to absorption by chlorophyll-*a* (Gitelson et al., 2000). As can be seen in Figure 11a, these peaks corresponded to local minimums in the plot of R^2 vs. wavelength. This inverse relationship between absorbance peaks and R^2 local minimums was not as apparent for *D. tertiolecta*, *D. salina*, and *T. suecica* and may be complicated by the presence of chlorophyll-*b* in addition to chlorophyll-*a* (Bustillos-Guzmán et al., 2001; Masuda et al., 2002; Stramski et al., 1993).

It is worth noting that *D. salina* was found to be unique in having a maximum R^2 at a wavelength of 300 nm (R^2=0.97). As this wavelength is well outside the visible spectrum and within ultraviolet range, it is seldom considered a candidate for algal biomass correlations. Interestingly, *D. tertiolecta* showed a similar response at 300 nm with a relatively high R^2 (R^2=0.91). Since both *N. salina* and *T. suecica* show a relatively poor correlation at 300 nm (R^2=0.86 and 0.90), it can be hypothesized that a high correlation at 300 nm does not necessitate the presence or absence of chlorophyll-*b*. It remains uncertain whether this characteristic is unique to *Dunaliella* sp. or if other unknown factors are attributing to this unusual correlation.

Wavelengths of 680 nm and 800 nm were chosen for further analysis as they represent wavelengths that either produced an absorbance maxima or the maximum R^2 for the selected algal strains. A detailed representation of the linear regression between biomass concentration (g L^{-1} AFDW) and OD for all four algal strains can be seen in

Figures 13 and 14. The calculated root-mean-square error (RMSE) from linear regression analysis is summarized in Table 9. A common phenomenon of the linear regression models observed for the entire 18 day growth period is the tendency to under predict biomass concentrations at low and high ODs and the tendency to over predict at mid-ranged ODs (Figures 13 and 14). *N. salina* (Figure 13a), for example, showed considerable fluctuation over the range of ODs. While the wavelength of 800 nm reduced the magnitude of this error for all strains when compared to 680 nm (Table 9), this fluctuation between OD and biomass was not eliminated. Even the highly correlated wavelength of 300 nm for *D. salina* showed observable fluctuations and areas of over and under prediction. This persistent error was also observed by Griffiths (2011) who attributed this error to changing pigment, age, and condition of the cells under culture.

Based on these results, correlation equations for use in the remainder of the study were established between biomass concentration (g L^{-1} AFDW) and OD for 680 nm and 800 nm (below). Equations were calculated based upon the region of the correlation curve which produced normally distributed residuals to minimize prediction errors during exponential growth. It is accepted that such a method, as noted above, will under predict biomass concentrations at the beginning and end of the growth period. *T. suecica* was omitted from future experimentation as a result of equipment limitations.

$$N.salina\ \ (g\ L^{-1}AFDW) = 0.3987 * OD_{680}$$

$$N.salina\ \ (g\ L^{-1}AFDW) = 0.8034 * OD_{800}$$

$$D.tertiolecta\ \ (g\ L^{-1}AFDW) = 0.9235 * OD_{680}$$

$$D.tertiolecta\ \ (g\ L^{-1}AFDW) = 1.5418 * OD_{800}$$

$$D.salina\ \ (g\ L^{-1}AFDW) = 0.9044 * OD_{680}$$

$$D.salina\ \ (g\ L^{-1}AFDW) = 1.6982 * OD_{800}$$

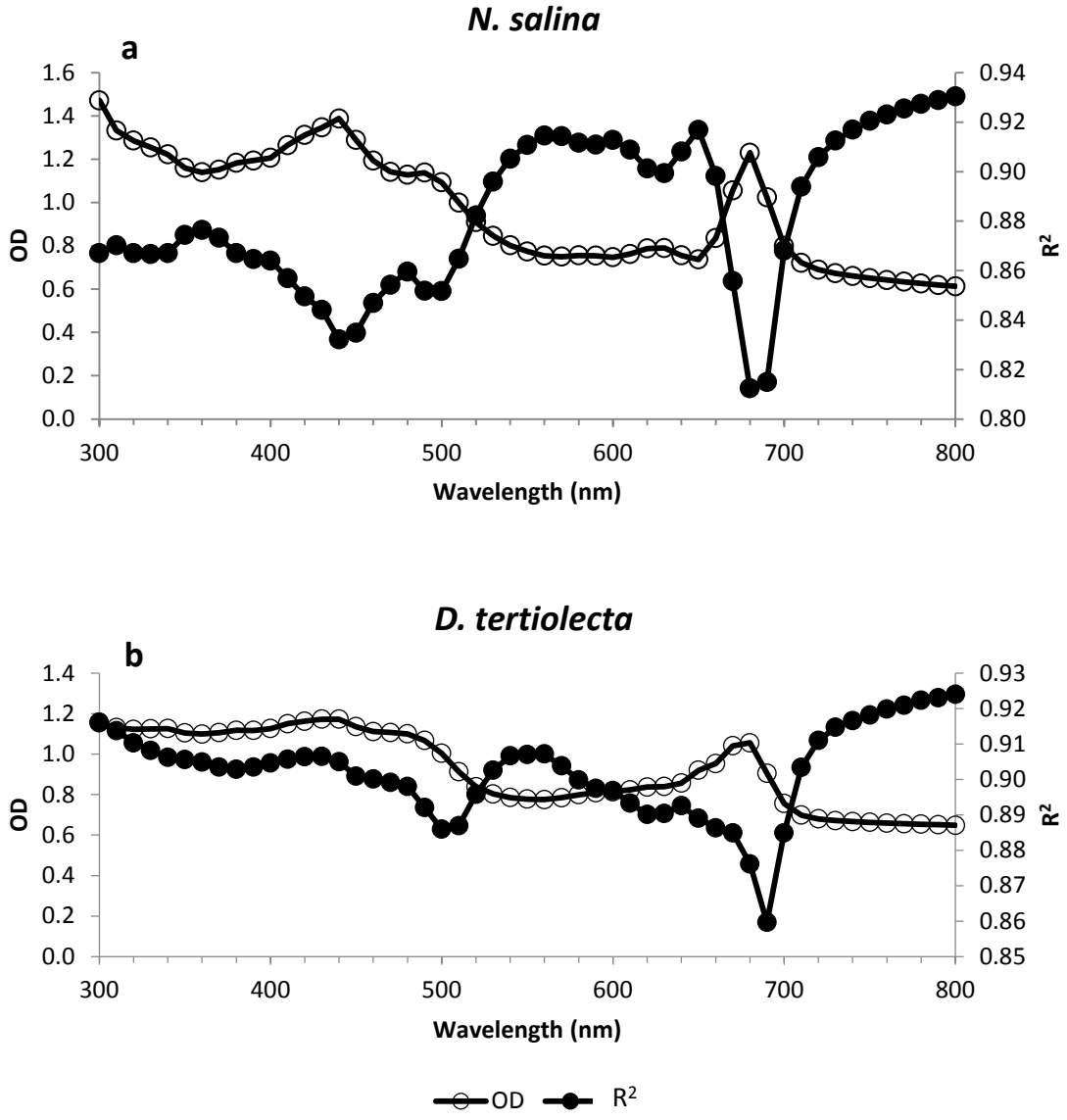

Figure 11: Effect of wavelength selection on the correlation coefficient (R^2) between algal dry biomass and optical density for (a) *N. salina* and (b) *D. tertiolecta*. Optical density (OD) spectra of the microalgae were arbitrarily chosen to display significant absorbance peaks.

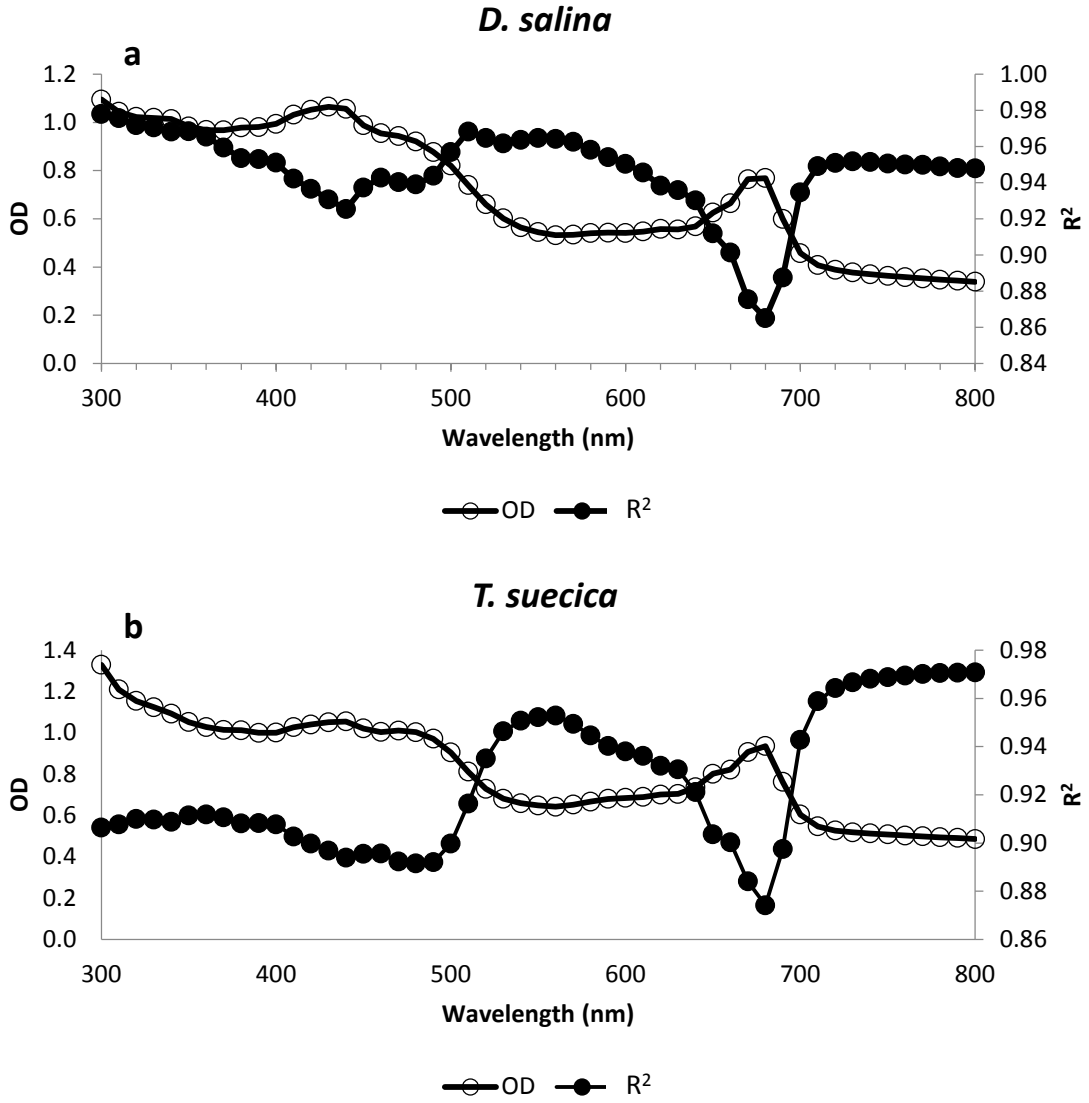

Figure 12: Effect of wavelength selection on the correlation coefficient (R^2) between algal dry biomass and optical density for (a) *D. salina* and (b) *T. suecica*. Optical density (OD) spectra of the microalgae were arbitrarily chosen to display significant absorbance peaks.

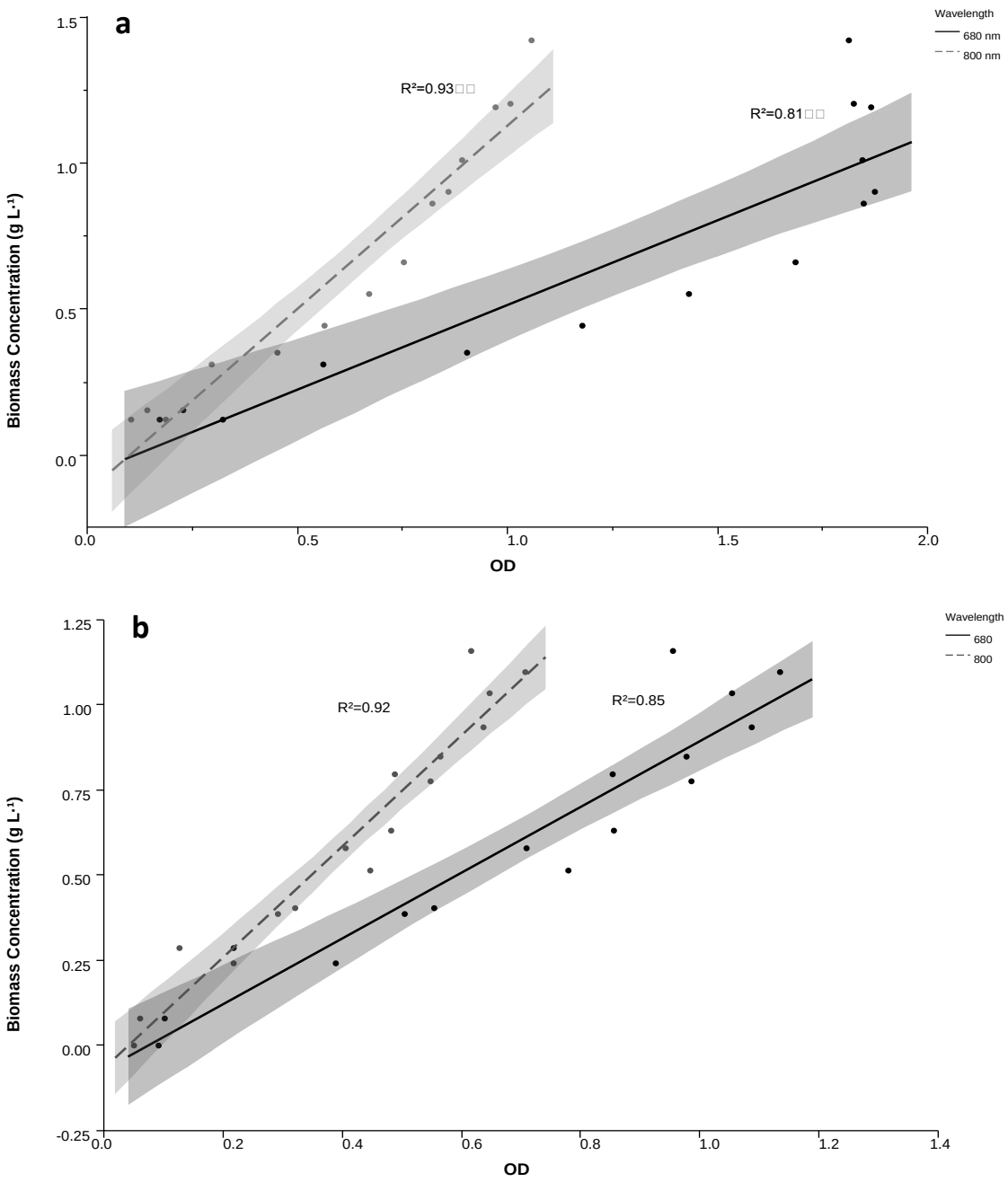

Figure 13: Effect of wavelength selection on the performance of using optical density for biomass concentration approximations of (a) *N. salina* and (b) *D. tertiolecta*. Shaded regions represent 95% confidence intervals.

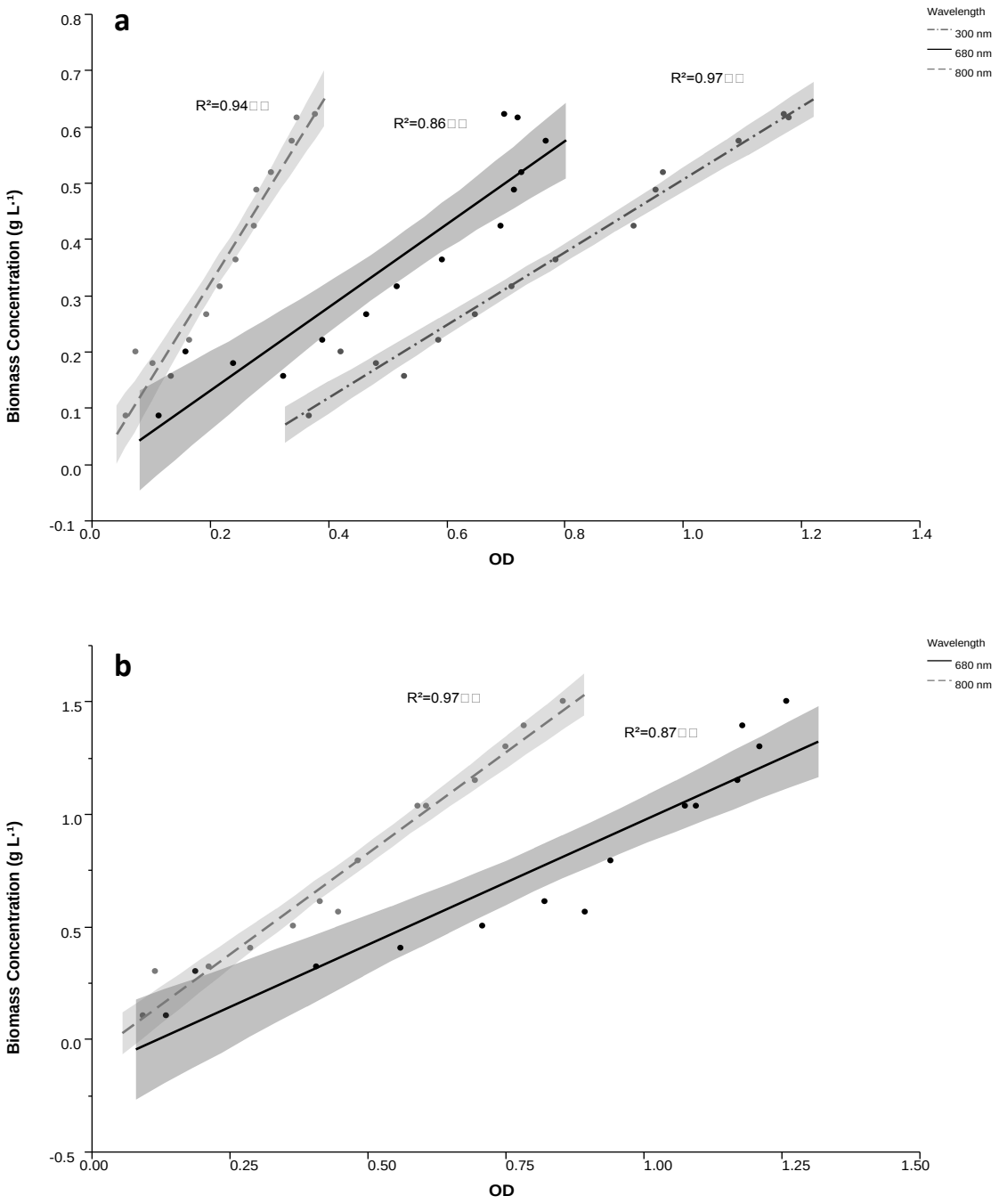

Figure 14 Effect of wavelength selection on the performance of using optical density for biomass concentration approximations of (a) *D. salina* and (b) *T. suecica*. Shaded regions represent 95% confidence intervals.

Table 9: Effect of wavelength selection on the root-mean-square error (RMSE) of the linear regression between optical density and algal biomass concentration.

	RMSE (%)		
Strain	**300 nm**	**680 nm**	**800 nm**
N. salina		19.7	12.0
D. tertiolecta		9.1	7.5
D. salina	2.8	7.0	4.3
T. suecica		16.6	8.0

A.4.2. Impact of wavelength on microalgal growth estimations

The specific growth rates, μ (day^{-1}), estimated by the fitted model for both strains at different loadings of AD effluent loading in flowback water, are shown in Figure 15a. The estimated specific growth rate was found to be dependent on both wavelength and algal strain ($p<0.05$). For example, the wavelength of 800 nm systematically underestimated the specific growth rate when compared to 680 nm for *D. tertiolecta*. Estimates for *N. salina*, however, were not significantly altered by wavelength choice ($p>0.05$).

Average biomass productivities (mg $L^{-1}day^{-1}$) are summarized in Figure 15b. ANOVA analysis indicated that the average biomass productivity was dependent on both the wavelength and the AD effluent loading ($p<0.05$). For example, while *D. tertiolecta* was observed to obtain the highest average biomass productivity in the 6% AD effluent loading in the flowback water as estimated by the wavelength of 680 nm, the wavelength of 800 nm estimated that the highest average biomass productivity was observed in the 2%AD effluent loading in the flowback water.

The cause of the different growth parameter estimates based on both wavelength and algal strain is currently uncertain. As indicated by previous research, factors such as changes in cell size, aggregation, cell structure, or in the prevalence of suspended solids in the media may contribute to changes in OD (Griffiths et al., 2011; Myers et al., 2013).

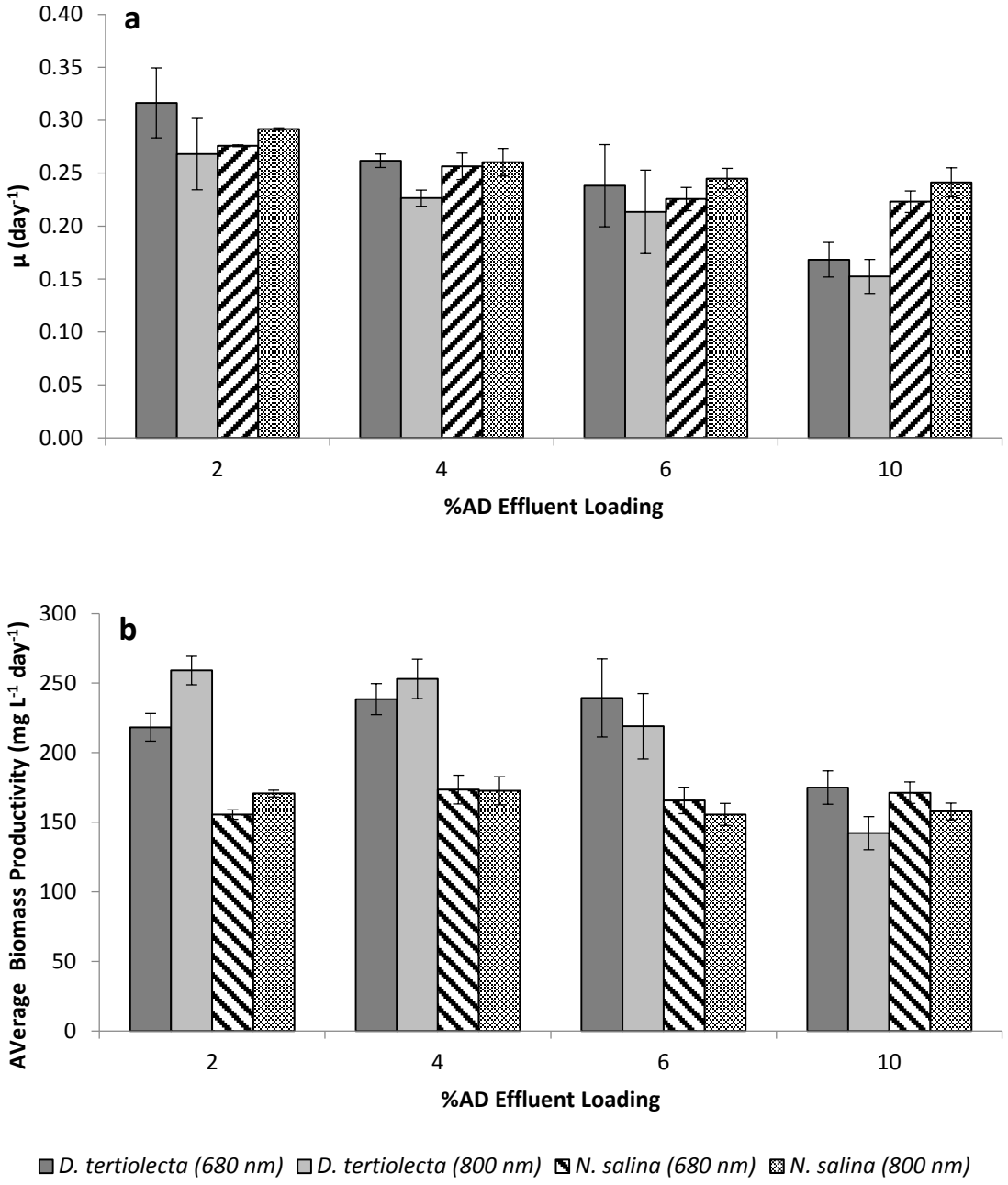

Figure 15: Effect of wavelength selection on the estimated (a) specific growth rate and (b) average biomass productivity for *N. salina* and *D. tertiolecta* cultured in multiple anaerobic digestion effluent loadings in the flowback water. Estimates were calculated based on optical density at either 680 nm or 800 nm.

A.4.3. Evaluation of optical density as a method to approximate algal biomass

A.4.3.1. Algal growth curves

Algal growth curves created from 680 nm, 800 nm, and AFDW are shown in Figures 16 and 17 for *N. salina* and *D. tertiolecta*, respectively. For both strains, biomass concentrations derived from AFDW for the 6% AD effluent and flowback water media are significantly higher ($p<0.05$) than the media based on the commercial nutrients and salts. Wavelength was also determined to be a significant factor in regards to media type and that the wavelength of 800 nm estimated a significantly lower biomass concentration with the 6% AD effluent loading in flowback water media compared to the media based on the commercial nutrients and salts ($p<0.05$). Biomass concentrations estimated by 680 nm for both media, however, showed no significant difference ($p>0.05$).

The similar biomass concentrations estimated at 680 nm for both media and for both strains suggested that not only was algal growth similar in both media, but also that the differences in biomass concentrations derived from 800 nm and AFDW may be caused by the presence of suspended solids in the 6% AD effluent loading in flowback water media. Since inoculation size was controlled, the calculated difference of ~0.07 ± 0.02 g L^{-1} AFDW between the two media on Day 0 further suggested the presence of suspended solids in the 6% AD effluent loading in flowback water media. It was initially believed that a wavelength of 800 nm would overestimate algal biomass correlations in such a media as light attenuation is based primarily on light scattering by suspended particles. The statistically lower biomass concentration with the 6% AD effluent loading in flowback water media by 800 nm seemed to suggest that the opposite is true; that the

presence of suspended solids lowered the estimated biomass concentration at 800 nm. A possible explanation is that the presence of additional particles in the media may be redirecting initially scattered light back towards the photo sensor, lowering the estimated OD (Myers et al., 2013; Wind and Szymanski, 2002). These results suggested that a wavelength of 680 nm may be less effected by the presence of non-algal solids in the media and therefore more appropriate for calculating algal biomass concentrations based on OD.

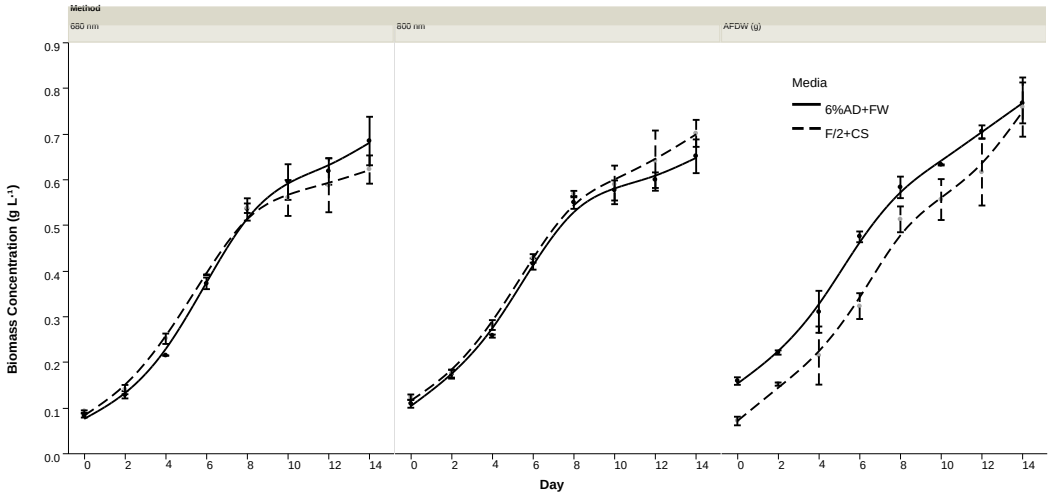

Figure 16: Effect of biomass quantification method on the estimated growth of *N. salina* in media composed of different nutrient and salinity sources. Growth curves were calculated from approximations based on ash-free dry weight (AFDW) or optical density at 680 nm or 800 nm. Media evaluated included commercial nutrients and salts (F/2+CS) and 6% anaerobic digestion effluent loading in the flowback water (6%AD+FW).

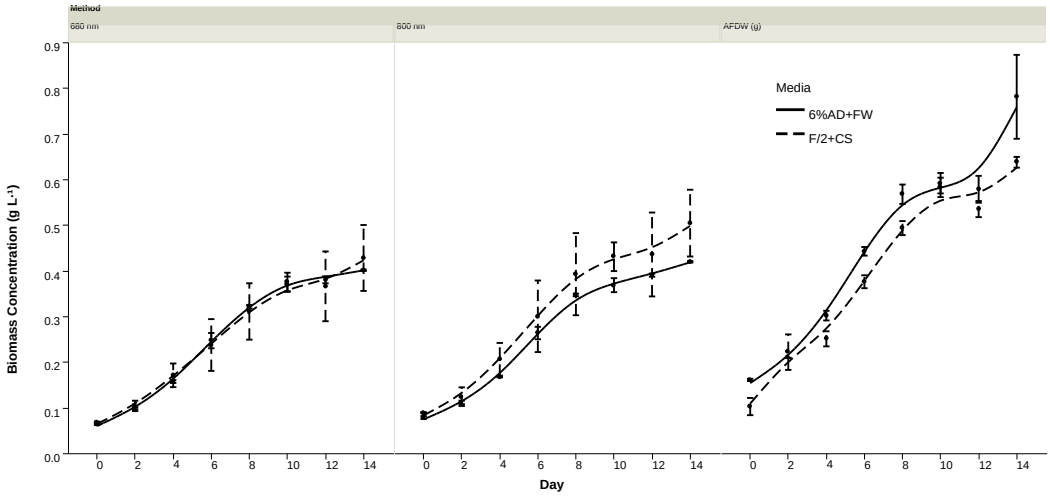

Figure 17: Effect of biomass quantification method on the estimated growth of *D. tertiolecta* in media composed of different nutrient and salinity sources. Growth curves were calculated from approximations based on ash-free dry weight (AFDW) or optical density at 680 nm or 800 nm. Media evaluated included commercial nutrients and salts (F/2+CS) and 6% anaerobic digestion effluent loading in the flowback water (6%AD+FW).

A.4.3.2. Logistic growth parameters

The effect of biomass approximations by OD and AFDW on the on the estimated specific growth rates (μ, day^{-1}) and the estimated average biomass productivities (mg $L^{-1}day^{-1}$) for *N. salina* and *D. tertiolecta* cultivated in the two different media can be seen in Figure 18. In regards to the specific growth rate (Figure 18a), the estimates based on 680 nm for *N. salina* were significantly higher than the estimates derived from AFDW ($p<0.05$). Additionally, the estimates for the average biomass productivity (Figure 18b) based on AFDW were significantly higher than both methods based on OD ($p<0.05$). Significant differences between the different media from using estimates based on OD or AFDW were not observed ($p>0.05$).

The significant difference of the estimates for the average biomass productivity when AFDW is used compared to both OD methods may suggest a limitation and weakness to using OD as a proxy for biomass. As discussed in Appendix A.4.1, deviations from linearity between OD and biomass cannot be avoided because of inherent changes in pigment, age, and condition of the cells under culture (Griffiths et al., 2011). Because these changes become more apparent with prolonged cultivation, estimates near the end of the growth curve may contain inherently more deviations and thus may be inherently more inaccurate.

The theory behind using a wavelength based on scattering, such as 800 nm, is that the inherent deviations in OD from changes in cell pigments are minimized, as discussed in Appendix A.4.1. As seen in Figure 18b, the average biomass productivity estimates based on 800 nm are ~5% closer to the estimates based on AFDW than the

estimates by 680 nm in the control media. The estimates based on the wavelength of 800 nm, however, are not significantly different from the values estimated by 680 nm ($p>0.05$). Wavelengths based on scattering are also further complicated by the presence of suspended particles which may interfere with OD, as mentioned in Appendix A.4.3.1. As seen in Figure 18b, the larger estimates for average biomass productivity when 800 nm is used is no longer apparent when compared to 680 nm in the media composed of the flowback water supplemented with the 6% AD effluent.

In general, wavelength choice had little impact on the logistic model parameters based on OD. This is likely because parameters such as growth rate are determined by the changes and trends with OD over time and not point value estimates. Additionally, the presence of suspended solids in the 6% AD effluent loading in flowback water media, mentioned in Appendix A.4.3.1, were not found to significantly affect the estimated trajectory of the growth curves as significant differences between the two media were no observed for either the estimated specific growth rate or the estimated average biomass productivity. The wavelength of 680 nm was chosen for future experiments with the media composed of the flowback water and the AD effluent to avoid potential complications with suspended particulates, as previously discussed in Appendix A.4.3.1. Because of the inherent inaccuracies with calculations based on OD (see Appendix A.4.3.2), the specific growth rates and the average biomass productivities in Section 2.4.4 were reported based on estimates derived from AFDW.

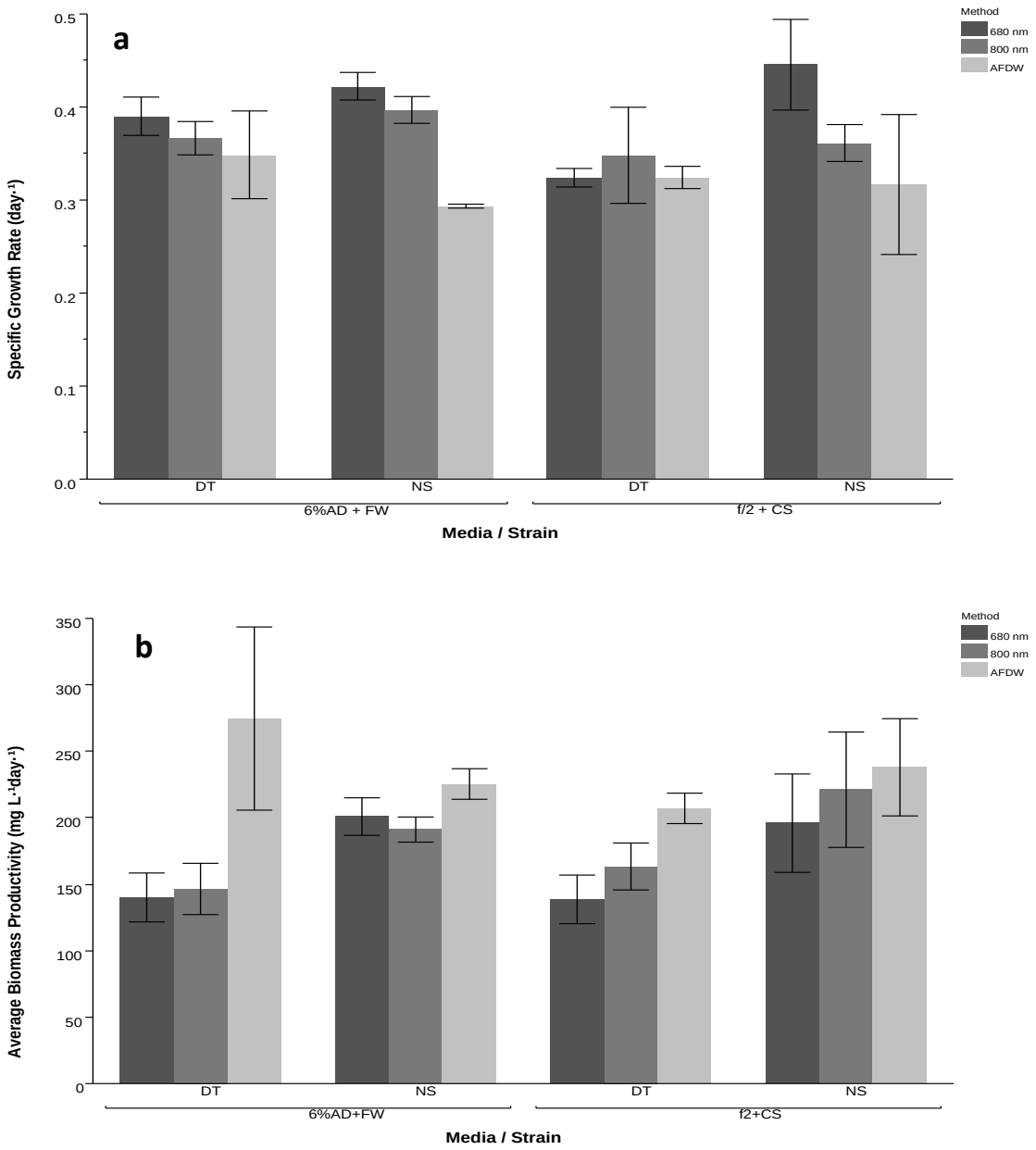

Figure 18: Effect of biomass quantification method on the estimated (a) specific growth rate and (b) average biomass productivity for *N. salina* and *D. tertiolecta* cultivated in media composed of different nutrient and salinity sources. Estimates were calculated from approximations based on ash-free dry weight (AFDW) or optical density at 680 nm or 800 nm. Media evaluated included commercial nutrients and salts (F/2+CS) and 6% anaerobic digestion effluent loading in the flowback water (6%AD+FW). Microalgal strains evaluated included *D. tertiolecta* (DT) and *N. salina* (NS).

A.5. Conclusions

Because of the possible interference of wastewater particulate matter, the relationship between algal dry biomass and OD at wavelengths between 300 to 800 nm for various algal strains was first studied. The majority of strains exhibited a correlation coefficient (R^2) minimum at 680 nm, which corresponded to a chlorophyll-*a* absorption maxima. This indicated that the pigment content varied with the different stages of algal growth. In contrast, the wavelengths outside the absorption range of chlorophyll-*a* for all strains corresponded to increases in R^2. For example, the wavelength of 800 nm resulted in the highest R^2 for the majority of the microalgae studied. The wavelengths of 680 nm and 800 nm were further studied and the results of the microalgal screening experiment and the algal dry biomass estimates suggested that 680 nm was the most appropriate wavelength for future experiments involving the flowback water and the AD effluent as it was found least effected by suspended solids.