Cultivation of marine microalgae using shale gas flowback water and anaerobic digestion effluent as the cultivation medium

Ratanachat Racharaks, Xumeng Ge, Yebo Li*

Department of Food, Agricultural and Biological Engineering, The Ohio State University/Ohio Agricultural Research and Development Center, 1680 Madison Ave., Wooster, OH 44691-4096, USA

*Corresponding author. Tel.: +1 330 263 3855. *E-mail address:* li.851@osu.edu (Y.li).

Abstract

The potential of shale gas flowback water and anaerobic digestion (AD) effluent to reduce the water and nutrient requirements for marine microalgae cultivation was evaluated with the following strains: *Nannochloropsis salina*, *Dunaliella tertiolecta*, and *Dunaliella salina*. *N. salina* and *D. tertiolecta* achieved the highest biomass productivity in the medium composed of flowback water and AD effluent (6% v/v). Growth in the above unsterilized medium was found to be comparable to that in sterilized commercial media with similar initial inorganic nitrogen concentrations, salinity, and pH levels. Specific growth rates of 0.293 and 0.349 day⁻¹ and average biomass productivities of 225 and 275 mg L⁻¹ day⁻¹ were obtained for *N. salina* and *D. tertiolecta*, respectively. The lipid content and fatty acid profile of both strains in the medium were also comparable to those obtained with commercial nutrients and salts.

Key words: flowback water; anaerobic digestion effluent; shale gas; algae; biofuels

1. Introduction

The continued growth in the demand for energy 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 growing on non-arable land, producing high-value byproducts, and yielding more lipids per acre than terrestrial crops (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. More abundant water resources for inland sites are required to address water conservation concerns as the proper positioning of microalgae production facilities can impact the cost, availability, and transportation of water (Ferrell and Sarisky-Reed, 2010).

Flowback water from shale gas exploration is one of the promising water resources to meet the water demand of marine microalgae production. Shale formations extend throughout the continental U.S. and contain an estimated $3.29 \times 10^{13} \text{ m}^3$ of technically recoverable shale gas (U.S. Energy Information Administration, 2013). To make shale gas production more 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. Flowback water – the aqueous fluid that returns to the surface after hydraulic pressure is relieved – is the primary wastewater associated with shale gas production (Gregory et al., 2011). Produced at a volume of 7,570–22,700 m^3 per well life (Blauch et al., 2009), flowback water can contain high concentrations of total dissolved solids (up to 261,000 mg L^{-1}), primarily soluble chloride salts (Gregory et al., 2011). Because of high handling costs and the scarcity of brine disposal facilities (Blauch et al., 2009), flowback water is an increasingly available wastewater resource that may meet the water demand of commercial marine microalgae cultivation.

1
2
3
4 1 Additionally, researchers have proposed a way to reduce costs associated with microalgae
5
6 2 cultivation by using anaerobic digestion (AD) effluent as a nutrient supplement. Used to treat a
7
8 3 variety of organic wastes, AD converts organic matter into biogas and produces a nutrient-rich
9
10 4 effluent. Because of its high levels of total phosphorus and total nitrogen, AD effluent has been
11
12 5 considered a potential agricultural fertilizer. Over-applying effluent to agricultural fields,
13
14 6 however, can result in environmental hazards such as watershed eutrophication. These concerns
15
16 7 have made AD effluent an attractive nutrient source for algal cultivation (Cai et al., 2013a;
17
18 8 Sheets et al., 2014; Wang et al., 2010). However, no report was found on the use of AD effluent
19
20 9 in conjunction with flowback water for the cultivation of marine microalgae.
21
22
23
24
25

26 10 Various marine microalgal strains have been previously studied for their ability to adapt
27
28 11 to different salinities, produce large quantities of intracellular lipids, and produce high-value
29
30 12 pigments. For example, *Nannochloropsis salina* is a marine microalga with a high lipid
31
32 13 production rate ($4.0 \text{ g m}^{-2} \text{ day}^{-1}$) (Boussiba et al., 1987) and a high content of the nutritionally-
33
34 14 valuable eicosapentaenoic acid (Krienitz and Wirth, 2006). The *Dunaliella* genus of microalgae
35
36 15 has also been extensively studied and is unique in its robust tolerance to varying salinity levels.
37
38 16 For example, *Dunaliella tertiolecta* is known to tolerate high salinity levels (up to ~1.0 M NaCl)
39
40 17 as well as accumulate high levels of intracellular lipids (~67%) (Takagi et al., 2006). Similarly,
41
42 18 *Dunaliella salina* is known to tolerate hypersaline conditions (up to ~5.5 M NaCl) as well as
43
44 19 accumulate large amounts of the commercially-valuable pigment, β -carotene (Chen et al.,
45
46 20 2011a). While some microalgal strains, such as *N. salina*, have been successfully cultured in
47
48 21 media composed of AD effluent (Cai et al., 2013a; Cai et al., 2013b; Sheets et al., 2014),
49
50 22 currently no studies have been reported on the culturing of *N. salina*, *D. tertiolecta*, or *D. salina*
51
52 23 in media composed of flowback water.
53
54
55
56
57
58
59
60
61
62
63
64
65

The purpose of this study was to evaluate biomass and lipid production from marine microalgae cultivated in the flowback water from shale gas exploration supplemented with AD effluent from a municipal wastewater treatment facility. To address this goal, three specific objectives were studied: 1) the effect of AD effluent loadings on microalgal growth in flowback water, 2) the effect of nutrient and salinity sources on microalgal growth, and 3) the effect of nutrient and salinity sources on lipid production. A batch study was performed in which three marine microalgal strains were cultivated in media composed of flowback water and AD effluent at different loadings. 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 rate, lipid content, and fatty acids observed in both media.

2. Materials and Methods

2.1. Microalgal strains and seed cultures

Marine microalgae *N. salina* (849/6), *D. tertiolecta* (19/27), and *D. salina* (19/18) were obtained from the Culture Collection of Algae and Protozoa (Oban, Scotland). Seed cultures of these strains were maintained in 2-L reactors (1-L working volume) at 25°C under constant illumination ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$) using 32-watt fluorescent lamps (GE Lighting, Ravenna, OH, USA). The photosynthetic photon flux of the light was measured by a BQM quantum meter (Apogee Instruments, Logan, UT, USA). Reactors were placed in a white coated chamber and each reactor was equipped with a rubber stopper and two 4.76-mm diameter stainless steel tubes for the air inlet and outlet. Ambient air (0.039% CO_2) blown through a 0.2- μm Whatman PTFE

1 Puradisc filter (GE Healthcare, Maidstone, UK) at an airflow rate of 650 mL min⁻¹ was used to
2 provide mixing and CO₂ for algal growth.

3 *N. salina* cultures were cultivated in a commercial medium originally formulated by
4 Guillard and Ryther (1962), using Proline f/2 Algae Feed (Pentair Aquatic Eco-Systems, Apopka,
5 FL, USA), which contained the following ingredients: 0.075 g L⁻¹ NaNO₃, 0.00565 g L⁻¹
6 NaH₂PO₄·2H₂O, 1 mL L⁻¹ trace elements stock solution, and 1 mL L⁻¹ vitamin mix stock solution.
7 The minor ingredients in the trace element stock solution included Na₂EDTA, FeCl₃·6H₂O,
8 CuSO₄·5H₂O, ZnSO₄·7H₂O, CoCl₂·6H₂O, MnCl₂·4H₂O, Na₂MoO₄·2H₂O, and biotin. The
9 vitamin stock solution contained cyanocobalamin and thiamine HCl. The salinity of the culture
10 medium was maintained with 40 g L⁻¹ of Instant Ocean[®] sea salt (Spectrum Brands, Madison,
11 WI, USA).

12 *D. tertiolecta* was cultured in a modified artificial seawater (ASW) medium, originally
13 formulated by McLachlan (1964), that contained the following ingredients: 3.75 mL L⁻¹ extra
14 salts stock solution, 2.50 mL L⁻¹ vitamin stock solution, 25 mL L⁻¹ soil extract, and 0.50 g L⁻¹
15 tricine. The extra salts stock solution contained 30.0 g L⁻¹ NaNO₃, 1.20 g Na₂HPO₄, and 1.0 g L⁻¹
16 K₂HPO₄. The vitamin stock solution contained biotin, calcium pantothenate, cyanocobalamin,
17 folic acid, inositol, nicotinic acid, thiamine HCL, and thymine. The medium was further enriched
18 with Proline f/2 Algae Feed using the concentration mentioned previously for *N.salina*. Instant
19 Ocean[®] sea salt was added to the ASW medium as described previously. Soil extract was
20 prepared by sieving 500 mL of air-dried deciduous woodland soil (Akron, OH, USA) through a
21 2–4 mm mesh. Deionized (DI) water at a volume of 1000 mL was then added, and the resulting
22 solution was autoclaved at 121°C at 103 kPa for 2 hours. The supernatant was then filtered using
23 Whatman No. 1 filter paper (GE Healthcare, Maidstone, UK) to remove remaining particulates

1 and then refrigerated at 4°C until use. *D. salina* was cultured in an ASW medium enriched with
2 Proline f/2 Algae Feed, as described previously, with the addition of 40 g L⁻¹ of Instant Ocean®
3 sea salt and 35 g L⁻¹ NaCl.

2.2. Shale gas flowback water and anaerobic digestion effluent

Shale gas flowback water was provided by Weatherford International LTD (Houston, TX, USA), a commercial oil and natural gas company, and stored at 4°C. Prior to use, the flowback water was centrifuged for 15 min at 8,000 rpm using a Sorvall RC 6+ tabletop centrifuge (Thermo Scientific, Waltham, MA, USA) to reduce the presence of suspended solids. Municipal wastewater AD effluent was collected from a commercial-scale liquid anaerobic digester (KB Compost Services, Akron, OH, USA), centrifuged at 3200 rpm by a D5LL continuous solid bowl decanter centrifuge (Andritz AG, Graz, Austria), and stored at 4°C prior to use.

The chemical composition of the flowback water and the AD effluent are shown in Table 1. The concentrations of total dissolved solids (TDS), chloride, and bromide in the flowback water were 41714 mg L⁻¹, 23787 mg L⁻¹, and 192 mg L⁻¹, respectively, which were comparable to the concentrations reported by Hayes (2009) for flowback water samples obtained from the Marcellus shale formation. Additionally, the concentrations of sodium, magnesium, and calcium were 11455 mg L⁻¹, 3575 mg L⁻¹, and 472 mg L⁻¹, respectively, which were within the range of randomly sampled Marcellus shale flowback water samples reported by Paugh (2008). These comparisons indicated that the flowback water used in this study was a realistic representation of the flowback water commonly produced by shale gas exploration.

2.3. Experimental cultivation conditions

2.3.1. Cultivation 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 flowback water and AD effluent according to the loading ratios described in Table 2. Algal seed inoculum was added to each reactor at a fixed volume of 10% v/v. The flasks were kept in an Innova® 43/43R incubator shaker (Eppendorf, Enfield, CT, USA) at a constant 22°C and a rotational speed of 150 rpm. Because light intensity varied between 29 and 55 $\mu\text{mol m}^{-2} \text{s}^{-1}$ inside the incubator, the positions of the reactors were randomized daily. All treatments were conducted in triplicate. Optical density (OD) measurements were taken daily for a cultivation period of 19 days to insure that the stationary phase was reached by all treatment levels.

2.3.2. Cultivation using different nutrient and salinity sources

N. salina and *D. tertiolecta* were further cultivated in 250-mL Erlenmeyer flasks (100-mL working volume) with media as described in Table 3. To evaluate the influence of the AD effluent as a nutrient source and the flowback water as a salinity source, treatments with commercial algal nutrients (Proline f/2 algae feed, Pentair Aquatic Eco-Systems, FL, USA) and commercial sea salts (Instant Ocean® sea salt, Spectrum Brands, Madison, WI, USA) were used as controls. To reduce the potential for biotic contaminants in the controls, the commercial nutrients and salts were autoclaved separately at 121°C at 103 kPa for 15 min prior to addition in the media. All treatments were initially adjusted to similar inorganic nitrogen (132 mg L⁻¹ N), salinity (42 g L⁻¹), and pH (7.6) levels as the medium composed of the flowback water with 6% AD effluent loading. To account for the inherent ammonium content of the flowback water (47

1 mg L⁻¹ N), ammonium chloride was added at equivalent amounts to the media. Commercial sea salts were used as the salinity source for the controls. Concentrated HCl or 10 M NaOH was used to adjust the pH of the controls to appropriate levels. All treatments were conducted in triplicate using inoculation volumes and cultivation conditions previously described in Section 2.3.1. OD measurements were taken every other day for a period of 18 days.

2.3.3. Cultivation in 2-L reactors using different nutrient and salinity sources

To further demonstrate the capabilities of the flowback water and the AD effluent to replace commercial nutrients and salts, cultivation was scaled-up by utilizing 2-L glass bottles (1-L working volume) under constant illumination (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and airflow rate (650 mL min⁻¹) as previously described in Section 2.1. *N. salina* and *D. tertiolecta* were cultivated in media composed of either: 1) flowback water supplemented with 6% AD effluent, or 2) commercial nutrients and salts at equivalent starting inorganic nitrogen (132 mg L⁻¹ N), salinity (42 g L⁻¹), and pH (7.9) levels. Similar to the previous experimental conditions, the commercial nutrients and salts were autoclaved separately prior to addition, the inoculation volume was fixed at 10% v/v, and each treatment was performed in triplicate. Algal biomass was determined gravimetrically every other day and the averaged values of duplicate measurements derived from algal total volatile suspended solids were reported.

For the analysis of algal lipids and fatty acids, algal biomass was harvested at the beginning of the stationary phase. Harvesting of *N. salina* cultures was achieved with a Sorvall RC 6+ centrifuge (Thermo Scientific, Waltham, MA, USA) operated at 10,000 rpm for 15 min. Because *D. tertiolecta* cells were observed to rupture under high speed centrifugation, flocculation was first performed for *D. tertiolecta* cultures by raising the pH of the media to 10.5

1 using additions of 10 M NaOH (Horiuchi et al., 2003). After flocculation, samples were decanted
2 and centrifuged at 3,000 rpm for 15 min.

3 4 **2.4. Analytical methods**

5 **2.4.1. Algal growth and biomass determination**

6 Before sampling for algal growth determination, DI water was added to each reactor to
7 adjust the volume to the initial volume, therefore compensating for losses due to evaporation and
8 sampling. For OD measurements, a sample volume of 2 mL of algae suspension was collected
9 and measured at 680 nm with an Eon[®] microplate spectrophotometer (Biotek, Winooski, VT,
10 USA). Each sample was measured three times using a fixed volume of 200 µL. The remaining
11 sample was then centrifuged at 10,000 rpm for 15 min using a Centrifuge 5810 R (Eppendorf,
12 Enfield, CT, USA). To minimize the influence of media constituents, OD measurements were
13 corrected by subtracting the OD of the supernatant from the OD of the corresponding algal
14 sample. Averaged values of the three measurements were reported.

15 The linear relationship between the OD at 680 nm and algal dry biomass (total volatile
16 suspended solids, TVSS) was established for all three algal strains. The TVSS of the algal
17 samples was determined and reported according to the Standard Methods for the Examination of
18 Water and Wastewater (APHA, 2005) using a 10 mL sample, which was filtered using a pre-
19 combusted filter under vacuum. For *N. salina*, 1.6 µm Fisherbrand G6 glass fiber filters with a
20 45 mm diameter (Thermo Fisher Scientific, Madison, WI, USA) were used under a vacuum of 20
21 inHg. To minimize the potential for *D. tertiolecta* cells to rupture during filtration, 0.7 µm
22 Whatman GF/F glass fiber filters with a 45 mm diameter (GE Healthcare, Maidstone, UK) were

1 used under a vacuum of 5 inHg. After filtration of the algal sample, filters were washed with 20
2 mL of 0.5 M NH_4HCO_3 solution to remove residual salts (Zhu and Lee, 1997).

2.4.2. *Algal lipid and fatty acids analysis*

The lipid content of the microalgal biomass was 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 a UP400S ultrasonic processor (Hielscher Ultrasonics, Teltow, Germany) equipped with 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 a solution of 0.88% KCl. The mixture was then shaken for 10 min in a glass centrifuge tube and then centrifuged for 5 min at 1,000 g in a 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 with Whatman No. 1 filter paper (GE Healthcare, Maidstone, UK) and dried with a Laborota 4001 rotary evaporator (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 fatty acid methyl esters (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 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 QP-2010 SE gas chromatograph-mass spectrometer (Shimadzu,

Columbia, MD, USA) equipped with a ZebronTM ZB-FFAP polar capillary column (Phenomenex, Torrence, CA, USA). The compounds were identified using the National Institute of Standards and Technology (NIST) mass spectral database and quantified by using methyl heptadecanoate as an internal standard. The cetane number of total fatty acids was calculated using a multiple regression model developed by Piloto-Rodríguez et al. (2013). The bis-allylic position equivalent value of total fatty acids was calculated using the equation described by Bucy and Marchese (2012).

2.4.3. Chemical composition analysis of growth media

Macronutrients, such as inorganic nitrogen and inorganic phosphorus, and other ion constituents were analyzed using a Dionex[®] ICS-5000 ion chromatograph equipped with a Dionex[®] IonPac AS anion-exchange column and a Dionex[®] IonPac CS19 cation-exchange column (Thermo Scientific, Waltham, MA, USA). Prior to analysis, samples were filtered with a 0.2 µm syringe filter to remove suspended particulates and diluted with DI water to appropriate detection limits. Total dissolved solids (TDS) and salinity were measured by an EC300 conductivity meter (YSI, Yellow Springs, OH, USA) whereas pH was measured with a pH meter (Oakton Instruments, Vernon Hills, IL, USA).

2.4.4. Nonlinear regression and statistical analysis

Nonlinear regression analysis of the algal growth data was performed using the statistical software JMP 10.0.2 (SAS Institute Inc., Cary, NC, USA). A three-parameter logistic model was chosen as it provided adequate fit for most of the experimental data ($R^2=0.97-0.99$). The average biomass productivity was calculated by multiplying the maximum observed biomass

concentration by the estimated specific growth rate. Analysis of significance was determined by analysis of variance (ANOVA) and Tukey HSD tests performed at an α level of 0.05.

3. Results and Discussion

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

Four AD effluent loadings (2%, 4%, 6%, and 10%) in the flowback water were tested with corresponding ammonium nitrogen levels and nitrogen to phosphorus ratios (N/P) shown in Table 2. Ammonium was found to be the only significant source of nitrogen in either the flowback water or the AD effluent. While the N/P ratios varied between 35 to 18 for the AD effluent loadings between 2% to 10%, respectively, it has been previously reported that the optimal N/P ratio depends on the ecological condition and can vary from 8.2 to 45 (Klausmeier et al., 2004). Microalgal growth curves of *D. salina*, *D. tertiolecta*, and *N. salina* are presented in Figure 1. Overall, *D. salina* experienced poor growth at all loadings of the AD effluent in the flowback water (Figure 1a). A 10-d lag phase was observed at the 4% AD effluent loading with no significant growth observed at either the AD effluent loadings of 6% or 10%. Both *D. tertiolecta* and *N. salina* performed substantially better than *D. salina* (Figure 1b and 1c). For *D. tertiolecta* (Figure 1b), a 2-d lag phase was observed at the 2% AD effluent loading, while a 4-d lag phase was observed at the AD effluent loadings of 4% to 10%. No significant lag phases were observed for *N. salina* at all studied effluent loadings (Figure 1c). The specific growth rates and average biomass productivities for *D. tertiolecta* and *N. salina*, derived from nonlinear regression analysis, are shown in Figure 2. Both *D. tertiolecta* and *N. salina* achieved the highest specific growth rates of 0.316 and 0.276 day⁻¹, respectively, at the 2% AD effluent loading in the flowback water (Figure 2a). The specific growth rate of *D. tertiolecta* was observed to decline as

the AD effluent loadings increased. While the decline in the specific growth rate was observable for *N. salina* for the AD effluent loadings of 4% to 10%, the change in the specific growth rate was not significantly different ($p>0.05$). As shown in Fig. 2b, *N. salina* and *D. tertiolecta* achieved their highest average biomass productivity (173 and $239 \text{ mg L}^{-1} \text{ day}^{-1}$, respectively) at the AD effluent loading of 6% and 4%, respectively. However, the change in the average biomass productivity was not statistically significant between the effluent loadings of 2-10% for *N. salina*, or 2-6% for *D. tertiolecta* ($p>0.05$).

The observation of lag phases and the decline in both the microalgal specific growth rate and average biomass productivity at higher loadings of the AD effluent support previous research which suggested that high AD effluent concentrations can inhibit microalgal growth (Cai et al., 2013a; Cai et al., 2013b; Sheets et al., 2014; Wang et al., 2010). Growth inhibition from high effluent concentrations is believed to be dependent on a combination of abiotic factors, such as free-ammonia concentrations and the degree of turbidity (Eustance et al., 2013; Wang et al., 2010), as well as biotic factors, such as the presence of pathogenic or competitive bacteria (Ma et al., 2014). Additionally, these results suggest that either tolerance to these inhibitory factors or the ability to utilize effluent for cellular growth is algal strain-dependent.

Concentrations of ammonium nitrogen (75 to 190 mg L^{-1}) in the flowback water/AD effluent media are likely not high enough to impede growth for *D. salina*, because a specific growth rate of 0.5 day^{-1} was observed by Giordano (1997) for this microalga with $140 \text{ mg L}^{-1} \text{ N}$ ($10 \text{ mM NH}_4\text{Cl}$). Similarly, phosphorus concentration and N/P ratio may also play a minimal role in the observed impeded growth for *D. salina*. No growth of *D. salina* was detected in the 10% AD effluent medium which has a phosphorus concentration of 11 mg L^{-1} and N/P ratio of 18 (Table 2), while Gibor (1956) observed significant growth of *D. salina* at phosphorus

1 concentrations from 4.6 to 46 mg L⁻¹ (0.02-0.2 µg mL⁻¹ KH₂PO₄) or N/P ratios in range of 3-30.
2 However, Giordano (1997) reported that *D. salina* required approximately 4 weeks to reach the
3 maximum division rate after cells were transferred from a seed culture with nitrate to a medium
4 with ammonium, which might explain the poor growth performance of *D. salina* in this study. In
5 contrast, it was reported that *D. tertiolecta* did not show an acclimation period after transfer to a
6 different nitrogen source (Hulatt et al., 2012). Therefore, strain-dependent responses to changes
7 in the nitrogen source may be a reason for the different performance of *D. salina* and *D.*
8 *tertiolecta* grown in flowback water supplemented with AD effluent. Further research on the
9 impact of different nutrient and metal concentrations on algal growth, however, is required to
10 better understand the ability of different algal strains to tolerate and utilize these wastewater
11 resources.

12 Because of the poor growth of *D. salina* relative to the other microalgal strains used in
13 this study, *D. salina* was not evaluated in further experiments. The medium with the highest
14 average biomass productivity (i.e. 6% AD effluent loading in the flowback water) was chosen for
15 further analysis of *N. salina* and *D. tertiolecta* to maximize the algal biomass is needed to
16 perform lipid content and fatty acids analyses.

18 **3.2. Effect of nutrient and salinity sources on growth of *N. salina* and *D. tertiolecta***

19 The specific growth rates and average biomass productivities for *D. tertiolecta* and *N.*
20 *salina*, cultivated in media composed of commercial nutrients and flowback water (f/2+FW), 6%
21 AD effluent loading and flowback water (6%AD+FW), or 6% AD effluent loading and
22 commercial salts (6%AD+CS), are shown in Figure 3. No significant difference (p>0.05) in the
23 specific growth rate (Figure 3a) or the average biomass productivity (Figure 3b) was found for *N.*

1 *salina* between the three analyzed media. While no significant difference ($p>0.05$) in the specific
2 growth rate was also found for *D. tertiolecta* among the analyzed media, the 6%AD+CS medium
3 resulted in the lowest average biomass productivity ($76 \text{ mg L}^{-1} \text{ day}^{-1}$).

4 To further validate the ability of the flowback water to replace commercial sea salts and
5 the AD effluent to replace commercial nutrients, cultivation of the microalgae was scaled-up
6 using 2-L reactors and the specific growth rate and average biomass productivity are shown in
7 Fig. 4. No significant difference ($p>0.05$) in either the specific growth rate (Figure 4a) or the
8 average biomass productivity (Figure 4b) was observed for either strain using the 6%AD+FW
9 medium or the commercial nutrients and commercial salts medium (f/2+CS). Under these
10 conditions, *N. salina* and *D. tertiolecta* achieved specific growth rates of 0.293 and 0.349 day^{-1} ,
11 respectively, in the medium composed of flowback water supplemented with 6% AD effluent.
12 The average biomass productivities observed in this medium were 225 and $275 \text{ mg L}^{-1} \text{ day}^{-1}$ for
13 *N. salina* and *D. tertiolecta*, respectively. The specific growth rate and average biomass
14 productivity for *N. salina* are comparable to the values reported by Sheets et al. (2014) for *N.*
15 *salina* grown in a medium composed of 7% municipal wastewater, AD effluent, and commercial
16 salts. Specifically, the specific growth rate and the average biomass productivity were reported at
17 0.327 day^{-1} and $204 \text{ mg L}^{-1} \text{ day}^{-1}$, respectively (Sheets et al., 2014).

18 These results indicated that the use of the flowback water as a salinity source and AD
19 effluent as a nutrient source had a similar effect on the growth of *N. salina* and *D. tertiolecta*
20 when compared to media composed of commercial nutrients and salts at the same initial
21 inorganic nitrogen, salinity, and pH levels. The presence of unknown inhibitors in either the
22 flowback water or the AD effluent was not found to significantly impede algal growth.

23 Furthermore, the comparable growth of both algal strains in the unsterilized AD effluent and

1 flowback water media to the sterilized commercial media suggested that the presence of
2 pathogenic or competitive microorganisms was minimal.

3 As shown in Table 3, the main form of nitrogen in the commercial nutrients and AD
4 effluent was nitrate and ammonium, respectively. Since ammonium bypasses the redox reactions
5 required to assimilate nitrate and nitrite by microalgae, ammonium is believed to be the preferred
6 source of nitrogen for rapid algal growth (Cai et al., 2013b; Giordano, 1997). The similarity in
7 specific growth rates and average biomass productivities observed when either the AD effluent
8 or the commercial nutrients was used as a nutrient source suggested that *N. salina* did not show
9 observable preference for either nitrate or ammonium nitrogen at a loading of 132 mg L⁻¹ (9.44
10 mM). Similarly, Sheets et al. (2014) reported that the growth rate of *N. salina* in AD effluent was
11 comparable to commercial nutrients at a loading of 200 mg L⁻¹ total nitrogen. While Chen et al.
12 (2011b) also found that *D. tertiolecta* could use either ammonium or nitrate as a nitrogen source,
13 a high level of ammonium (10 mM NH₄Cl) was found to inhibit growth. While *D. tertiolecta*
14 was found in this study to have a lower average biomass productivity in the 6%AD+CS medium,
15 comparable specific growth rates and average biomass productivities between the 6%AD+FW
16 and the f/2+FW media using the 250-mL flasks (Figure 3), and between the 6%AD+FW and the
17 f2+CS media using the 2-L reactors (Figure 4), suggested that factors other than just ammonium
18 inhibition alone were involved.

19 Growth inhibition from high concentrations of ammonium can arise from the
20 disassociation of ammonium into free ammonia (NH₃), a known inhibitory compound to
21 microalgae (Eustance et al., 2013). A possible explanation for the lower average biomass
22 productivity in the 6%AD+CS medium for *D. tertiolecta* is the lower ionic strength of the
23 commercial salts compared to the flowback water. The ionic strength of the media composed of

the flowback water or the commercial salts was calculated by using the equations outlined by Soderberg and Meade (1991) for the following ions: Ca^{2+} , Mg^{2+} , Na^+ , K^+ , SO_4^{2-} , and Cl^- . The ionic strength of the medium composed of the flowback water (0.91 M) was 129 % higher than the ionic strength of the medium composed of the commercial salts (0.40 M). The higher ionic strength of the flowback water may have increased the electrostatic forces among the ions and molecules in the medium, stabilizing the ammonium ions in the AD effluent and preventing free ammonia from accumulating to inhibitory levels (Soderberg and Meade, 1991). As a decrease in the average biomass productivity was not observed for the 6%AD+CS medium with *N. salina*, this could also indicate that the tolerance to free ammonia is algal strain-specific. Further research is needed to evaluate the influence of ions in the flowback water on the dissociation of ammonium into free ammonia and the toxicity of free ammonia to microalgae.

Additionally, the overall effects of algal cultivation and the growth media on the pH of the media can be seen in Table 4. Changes in the pH of the media may be attributed to two main mechanisms: (1) alkalization of the media via the release of hydroxyl ions as a result of algal dissolved inorganic carbon (DIC) uptake and (2) acidification of the media via the release of protons as a result of algal utilization of ammonium (Eustance et al., 2013). Since the pH for all experimental conditions in this study was below the pK_a of the ammonium/ammonia system (pK_a 9.3), the dominant form of this nitrogen species was ammonium. The fluctuation in final pH between the different conditions may suggest that one of the mechanisms is dominant over the other. For example, the pH of the AD effluent and flowback water media increased by ~ 0.60 when *N. salina* was cultured in 250-mL flasks, suggesting that the effect of algal DIC uptake may be dominant under these experimental conditions (Experiment 1 and 2 in Table 4). The final pH decreased by ~1.01, however, when *N. salina* was cultured in the 6%AD+CS medium

(Experiment 2 in Table 4), suggesting that the effect of ammonium utilization was dominant when the flowback water was replaced with commercial salts. In addition, the final pH was the highest when *N. salina* was cultured in the f/2+FW medium, suggesting that the effect of algal DIC uptake was most dominant when nitrate was used as the nitrogen source. Similarly, the final pH was higher when *N. salina* was cultured in the f/2+CS medium than in the 6%AD+FW medium in 1-L reactors (Experiment 3 in Table 4). Together, these results suggested that the flowback water may have a higher buffer capacity than the commercial salts used in this study, therefore minimizing the influence of these opposing mechanisms. A high buffer capacity is desirable with growth media utilizing ammonium as the nitrogen source as a significant decrease in pH from the utilization of the ammonium by the algae can inhibit growth (Eustance et al., 2013). Further evaluation on the buffer capacity, kinetics of the nitrogen species, DIC level, and dissolved oxygen level during cultivation with flowback water is suggested to clarify the influence of the flowback water on the dominance of different nitrogen species.

A comparison of the overall results for the 6%AD+FW medium indicated that *D. tertiolecta* had a 35% decrease in the specific growth rate between experiments involving 250-mL flask reactors (Figure 2 vs. Figure 3) and a 48% increase when experiments involved 2-L reactors (Figure 2 vs. Figure 4). As the cultivation conditions for the experiments involving the 250-mL flasks were identical (i.e. 100-mL working volume, light intensity between 29 and 55 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and rotational speed of 150 rpm), this may indicate changes in the specific growth rate as a result of natural variability in the seed inoculum for this strain of microalgae. Despite attempts in this study at choosing the seed inoculum during the exponential phase of growth, minute changes in the age of the inoculum have been known to impact the rate of algal growth (Fogg and Thake, 1987). The increase in specific growth rate for *D. tertiolecta* when 2-L reactors

1 were utilized may also indicate an inherent sensitivity to changes in environmental conditions
2 (i.e. 1-L working volume, a light intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, and mixing via ambient air) for
3 this strain of microalgae. In contrast, no significant difference ($p>0.05$) was observed in the
4 specific growth rate for cultures of *N. salina* grown in the 6%AD+FW medium under the
5 different experiments conducted in this study.

3.3. Effect of nutrient and salinity source on microalgal lipid content and fatty acid profiles

6
7
8 A summary of the results from the lipid content and fatty acid profiles of *N. salina* and *D.*
9 *tertiolecta*, cultivated in either the 6%AD+FW medium or the f/2+CS medium, is shown in
10 Figure 5 and Table 5. The lipid content and total fatty acids (Table 5) of *N. salina* in the
11 6%AD+FW medium were comparable to the values obtained with the f/2+CS medium. Palmitic
12 acid (C16:0), palmitoleic acid (C16:1), and eicosapentaenoic acid (C20:5) were the major fatty
13 acids derived from the lipids of *N. salina* cultivated in either media. While the content of
14 eicosapentaenoic acid (C20:5), a particularly valuable fatty acid for freshwater aquaculture and
15 biotechnology applications (Krienitz and Wirth, 2006), was observed to increase by 34.3% in the
16 6%AD+FW medium, the total amount (16.9% of TFA) was comparable to the value reported by
17 (Cai et al., 2013b) for *N. salina* grown in municipal AD effluent and commercial salts (15.7% of
18 TFA) and less than the value reported by Sheets et al. (2014) for *N. salina* grown in municipal
19 AD effluent and commercial salts under high illumination (25.7% of TFA).

20 Additionally, the amount of saturated fatty acids (SFA) for *N. salina* decreased by about
21 12.6% and the amount of unsaturated fatty acids (UFA) increased by about 8.8% with the
22 6%AD+FW medium. Despite the changes in fatty acids, the cetane number (CN) decreased by
23 only about 1.84% between the two media. The CN is an indicator of the potential fuel quality of

1 the microalgal-derived biodiesel as a higher CN implies easier ignition and lower nitrous oxide
2 emissions. The structure and type of fatty acids influence the CN as the CN decreases with
3 increasing unsaturation and increases with increasing chain length. Standards for biodiesel can
4 vary from a minimum CN of 47 (ASTM D6751) to 51 (EN14214) (Knothe, 2005). While the
5 percentage of total polyunsaturated fatty acids (PUFA) was similar for *N. salina* cultivated in
6 either media, the types of PUFAs were different as the resulting bis-allylic position equivalent
7 (BAPE) value was about 16.2% higher in the 6%AD+FW medium. BAPE is a measure of the
8 oxidative stability of the biodiesel and is calculated based on the number of methylene groups
9 adjacent to two double bonds in the fatty acids. The oxidative stability of the biodiesel decreases
10 with increasing BAPE and BAPE values of 81 and 51 would be sufficient to pass the ASTM
11 D6751 and EN 14214 standards, respectively (Bucy and Marchese, 2012). While these results
12 may suggest that lipids derived from *N. salina* cultured in the 6%AD+FW medium may have
13 lower oxidative stability than lipids derived from commercial media, the results were not
14 statistically significant ($p>0.05$).

15 For *D. tertiolecta*, a minor increase of 13.4% in the lipid content was observed for *D.*
16 *tertiolecta* cultivated in the 6%AD+FW medium. Linolenic acid (C18:3) and palmitic acid
17 (C16:0) were the major fatty acids derived from the lipids of *D. tertiolecta* cultivated in either
18 media. No other parameter in regards to the fatty acid profile was found to be significantly
19 different from values obtained in the f/2+CS medium ($p>0.05$). While the CN of *N. salina* fatty
20 acids in both media were higher than the minimum CN of 47 required by the biodiesel fuel
21 standard ASTM D6751, the CNs for *D. tertiolecta* in the 6%AD+FW medium (45.9) and the
22 f/2+CS medium (46.5) were slightly lower than this biodiesel fuel standard. Additionally, the
23 BAPE value of *D. tertiolecta* lipids was ~25.4% higher than *N. salina* lipids. These results

1 suggest that *D. tertiolecta* lipids have significantly lower oxidative stability than the *N. salina*
2 lipids analyzed in this study ($p < 0.05$). The possible instability of the biofuel produced from *D.*
3 *tertiolecta* lipids was also noted by Tang et al. (2011) as *D. tertiolecta* was similarly observed to
4 have a high content of C18:3 unsaturated chains. As the reported lipid contents of both *N. salina*
5 and *D. tertiolecta* were obtained near the end of the exponential phase, there is a potential that
6 the microalgal lipid content can be increased by nutrient starvation or changes in other
7 environmental parameters (Chen et al., 2011b).

4. Conclusions

Successful cultivation of marine microalgae utilizing shale gas flowback water and
municipal AD effluent as sources of water and nutrients is dependent on the algal strain. In
particular, cultivation of *N. salina* and *D. tertiolecta* in this unsterilized medium is feasible and
the obtained algal growth, lipid content, and fatty acids were comparable to values achieved in
sterilized commercial nutrients and salts at the same initial inorganic nitrogen, salinity, and pH
levels. Further research in the continuous culture of microalgae utilizing this medium is needed
as it is uncertain if uncharacterized constituents will accumulate to inhibitory levels.

Acknowledgements

This project was supported by the U.S. Department of Energy (Cooperative Agreement
No. DE-FE0002546). The authors would like to thank Mrs. Mary Wicks and Stephen Y. Park
(Department of Food, Agricultural and Biological Engineering, OSU) for providing a thorough
review with thoughtful comments.

References

1. APHA, 2005. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, D.C.
2. Blauch, M.E., Myers, R.R., Moore, T., Lipinski, B.A., Houston, N.A., 2009. Marcellus shale post-frac flowback waters-Where is all the salt coming from and what are the implications? Society of Petroleum Engineers.
3. Boussiba, S., Vonshak, A., Cohen, Z., Avissar, Y., Richmond, A., 1987. Lipid and biomass production by the halotolerant microalga *Nannochloropsis salina*. Biomass 12, 37-47.
4. Bucy, H., Marchese, A.J., 2012. Oxidative stability of algae derived methyl esters. J. Eng. Gas Turb. Power 134, 092805.
5. Cai, T., Ge, X., Park, S.Y., Li, Y., 2013a. Comparison of *Synechocystis* sp. PCC6803 and *Nannochloropsis salina* for lipid production using artificial seawater and nutrients from anaerobic digestion effluent. Bioresour. Technol. 144, 255-260.
6. Cai, T., Park, S.Y., Racharaks, R., Li, Y., 2013b. Cultivation of *Nannochloropsis salina* using anaerobic digestion effluent as a nutrient source for biofuel production. Appl. Energ. 108, 486-492.
7. Chen, H., Lao, Y.M., Jiang, J.G., 2011a. Effects of salinities on the gene expression of a (NAD⁺)-dependent glycerol-3-phosphate dehydrogenase in *Dunaliella salina*. Sci. Total Environ. 409, 1291-1297.
8. Chen, M., Tang, H., Ma, H., Holland, T.C., Ng, K.S., Salley, S.O., 2011b. Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresour. Technol. 102, 1649-1655.

- 1 9. Eustance, E., Gardner, R.D., Moll, K.M., Menicucci, J., Gerlach, R., Peyton, B.M., 2013.
2 Growth, nitrogen utilization and biodiesel potential for two chlorophytes grown on
3 ammonium, nitrate or urea. J. Appl. Phycol. 25, 1663-1677.
- 4 10. Ferrell, J., Sarisky-Reed, V., 2010. National algal biofuels technology roadmap.
- 5 11. Fogg, G.E., Thake, B., 1987. Algal cultures and phytoplankton ecology. The University of
6 Wisconsin Press, Madison, Wisconsin.
- 7 12. Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and
8 purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.
- 9 13. Gaudlip, A.W., Paugh, L.O., 2008. Marcellus Shale Water Management Challenges in
10 Pennsylvania. Society of Petroleum Engineers. SPE 119898.
- 11 14. Gibor, A., 1956. The culture of brine algae. Biol. Bull. 111, 223-229.
- 12 15. Giordano, M., 1997. Adaptation of *Dunaliella salina* (Volvocales, Chlorophyceae) to growth
13 on NH₄⁺ as the sole nitrogen source. Phycologia 36, 345-350.
- 14 16. Gregory, K.B., Vidic, R.D., Dzombak, D.A., 2011. Water management challenges associated
15 with the production of shale gas by hydraulic fracturing. Elements 7, 181-186.
- 16 17. Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana*
17 Hustedt, and *Detonula confervacea* (Cleve). Can. J. Microbiol. 8, 229-239.
- 18 18. Hayes, T., 2009. Sampling and Analysis of Water Streams Associated with the Development
19 of Marcellus Shale Gas. Marcellus Shale Coalition.
- 20 19. Horiuchi, J.I., Ohba, I., Tada, K., Kobayashi, M., Kanno, T., Kishimoto, M., 2003. Effective
21 cell harvesting of the halotolerant microalga *Dunaliella tertiolecta* with pH control. J. Biosci.
22 Bioeng. 95, 412-415.

20. Hulatt, C.J., Lakaniemi, A.M., Puhakka, J.A., Thomas, D.N., 2012. Energy demands of nitrogen supply in mass cultivation of two commercially important microalgal species, *Chlorella vulgaris* and *Dunaliella tertiolecta*. Bioenerg. Res. 5, 669-684.
21. Klausmeier, C.A., Litchman, E., Daufresne, T., Levin, S.A., 2004. Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. Nature 429, 171-174.
22. Knothe, G., 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process. Technol. 86, 1059-1070.
23. Krienitz, L., Wirth, M., 2006. The high content of polyunsaturated fatty acids in *Nannochloropsis limnetica* (Eustigmatophyceae) and its implication for food web interactions, freshwater aquaculture and biotechnology. Limnologica 36, 204-210.
24. Li, Y., Watkins, B.A., 2001. Analysis of fatty acids in food lipids. Current protocols in food analytical chemistry UNIT D1.2, D1.2.1-D1.2.15.
25. Ma, X., Zhou, W., Fu, Z., Cheng, Y., Min, M., Liu, Y., Zhang, Y., Chen, P. et al., 2014. Effect of wastewater-borne bacteria on algal growth and nutrients removal in wastewater-based algae cultivation system. Bioresour. Technol. 167, 8-13.
26. McLachlan, J., 1964. Some considerations of the growth of marine algae in artificial media. Can. J. Microbiol. 10, 769-782.
27. Piloto-Rodríguez, R., Sánchez-Borroto, Y., Lapuerta, M., Goyos-Pérez, L., Verhelst, S., 2013. Prediction of the cetane number of biodiesel using artificial neural networks and multiple linear regression. Energ. Convers. Manage. 65, 255-261.
28. Sheehan, J., Dunahay, T., Benemann, J., Roessler, P., 1998. A look back at the US Department of Energy's aquatic species program: biodiesel from algae. National Renewable Energy Laboratory Report NREL/TP-580-24190.

29. Sheets, J.P., Ge, X., Park, S.Y., Li, Y., 2014. Effect of outdoor conditions on *Nannochloropsis salina* cultivation in artificial seawater using nutrients from anaerobic digestion effluent. *Bioresour. Technol.* 152, 154-161.
30. Soderberg, R.W., Meade, J.W., 1991. The effects of ionic strength on un-ionized ammonia concentration. *The Progressive Fish-Culturist* 53, 118-120.
31. Takagi, M., Karseno, Yoshida, T., 2006. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. *J. Biosci. Bioeng.* 101, 223-226.
32. Tang, H., Abunasser, N., Garcia, M.E.D., Chen, M., Ng, K.S., Salley, S.O., 2011. Potential of microalgae oil from *Dunaliella tertiolecta* as a feedstock for biodiesel. *Appl. Energ.* 88, 3324-3330.
33. U.S. Energy Information Administration, 2013. Shale Gas Resources: An Assessment of 137 Shale Formations in 41 Countries Outside the United States. EIA report.
34. Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., Ruan, R.R., 2010. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. *Bioresour. Technol.* 101, 2623-2628.
35. Zhu, C.J., Lee, Y.K., 1997. Determination of biomass dry weight of marine microalgae. *J. Appl. Phycol.* 9, 189-194.

Table 1. Chemical composition of shale gas flowback water and anaerobic digestion effluent.

Parameter	Units	Flowback water	AD effluent
TDS	mg L ⁻¹	41714 ± 1734	9291 ± 54
Salinity	parts per thousand	45.4 ± 0.28	5.25 ± 0.07
pH	no units	6.56 ± 0.22	8.60 ± 0.01
NH ₄ -N	mg L ⁻¹	46.58 ± 0.66	1447 ± 46
NO ₂ -N	mg L ⁻¹	< 10	< 10
NO ₃ -N	mg L ⁻¹	< 10	< 10
PO ₄ -P	mg L ⁻¹	< 10	105.8 ± 1
Cl	mg L ⁻¹	23787 ± 1021	336.9 ± 49
Na	mg L ⁻¹	11455 ± 213	130.0 ± 1
Mg	mg L ⁻¹	3575 ± 386	19.54 ± 4
K	mg L ⁻¹	635.0 ± 12	< 10
Ca	mg L ⁻¹	471.8 ± 12	83.33 ± 2
Br	mg L ⁻¹	191.7 ± 15	< 10
SO ₄	mg L ⁻¹	159.9 ± 3	55.03 ± 3
Li	mg L ⁻¹	20.90 ± 0.01	< 10
F	mg L ⁻¹	< 10	< 10

TDS = total dissolved solids

Table 2. Experimental design for evaluating microalgal growth in flowback water supplemented with different loadings of anaerobic digestion effluent.

AD effluent loading (% v/v)	*NH ₄ -N (mg L ⁻¹)	*PO ₄ -P (mg L ⁻¹)	N/P Ratio
2	75	2	35
4	103	4	24
6	132	6	21
10	190	11	18

* total amount in both the AD effluent and the flowback water

Table 3. Experimental design for evaluating the effect of different nutrient and salinity sources on microalgal growth.

Nutrient source	Salinity source	NH ₄ -N (mg L ⁻¹)	NO ₃ -N (mg L ⁻¹)	Total nitrogen (mg L ⁻¹)
6%AD	FW	132		132
6%AD	CS	132		132
f/2	FW	47	85	132
f/2	CS	47	85	132

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

Table 4. Effect of algal cultivation and growth media on pH.

Strain	Treatment	Initial pH	*Final pH	Δ pH
Experiment 1: 250-mL flasks, 29 to 55 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 150 rpm				
<i>N. salina</i>	2% AD+FW	7.28	8.84 ± 0.04	1.56 ± 0.11
	4% AD+FW	7.46	8.84 ± 0.09	1.38 ± 0.09
	6% AD+FW	7.60	8.83 ± 0.03	1.24 ± 0.02
	10% AD+FW	7.71	8.35 ± 0.11	0.64 ± 0.17
<i>D. salina</i>	2% AD+FW	7.23	7.43 ± 0.03	0.20 ± 0.06
	4% AD+FW	7.50	7.38 ± 0.11	-0.12 ± 0.22
	6% AD+FW	7.62	7.57 ± 0.04	-0.05 ± 0.06
	10% AD+FW	7.70	7.93 ± 0.04	0.23 ± 0.05
<i>D. tertiolecta</i>	2% AD+FW	7.30	7.18 ± 0.07	-0.12 ± 0.04
	4% AD+FW	7.52	7.42 ± 0.07	-0.09 ± 0.07
	6% AD+FW	7.66	7.29 ± 0.15	-0.36 ± 0.16
	10% AD+FW	7.77	7.46 ± 0.09	-0.31 ± 0.09
Experiment 2: 250-mL flasks, 29 to 55 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 150 rpm				
<i>N. salina</i>	6% AD+FW	7.65	8.25 ± 0.04	0.60 ± 0.04
	6% AD+CS	7.61	6.60 ± 1.73	-1.01 ± 1.73
	f/2+FW	7.62	9.00 ± 0.08	1.38 ± 0.08
<i>D. tertiolecta</i>	6% AD+FW	7.65	7.27 ± 0.06	-0.38 ± 0.06
	6% AD+CS	7.61	6.71 ± 0.28	-0.90 ± 0.28
	f/2+FW	7.62	7.74 ± 0.07	0.12 ± 0.07
Experiment 3: 1-L reactors, 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, mixing via ambient air				
<i>N. salina</i>	6% AD+FW	7.98	7.70 ± 0.15	-0.28 ± 0.15
	f/2+CS	7.98	8.83 ± 0.71	0.85 ± 0.76
<i>D. tertiolecta</i>	6% AD+FW	7.97	6.77 ± 0.17	-1.20 ± 0.17
	f/2+CS	8.00	8.28 ± 0.08	0.28 ± 0.08

AD = anaerobic digestion effluent loading; FW = flowback water; f/2 = commercial nutrients; CS = commercial salts; * Final pH measured on Day 18, 19, and 12 for Experiment 1, 2, and 3, respectively.

Table 5. Effect of nutrient and salinity source on microalgal lipid content and fatty acid profiles.

	<i>N. salina</i>		<i>D. tertiolecta</i>	
	f/2+CS ^a	6%AD+FW ^b	f/2+CS	6%AD+FW
Lipid content (g g ⁻¹ AFDW)	0.302 ± 0.029	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 SFA ^c (g g ⁻¹ TFAs)	0.414 ± 0.005	0.362 ± 0.004	0.330 ± 0.014	0.341 ± 0.023
Total MUFA ^d (g g ⁻¹ TFAs)	0.389 ± 0.005	0.430 ± 0.005	0.171 ± 0.011	0.174 ± 0.025
Total PUFA ^e (g g ⁻¹ TFAs)	0.195 ± 0.001	0.209 ± 0.002	0.499 ± 0.025	0.484 ± 0.048
Total UFA ^f (g g ⁻¹ TFAs)	0.586 ± 0.005	0.638 ± 0.004	0.670 ± 0.014	0.659 ± 0.023
BAPE ^g	66.05 ± 0.30	76.76 ± 0.52	91.52 ± 4.58	87.66 ± 8.97
Cetane number	58.39 ± 0.13	57.32 ± 0.08	45.93 ± 0.93	46.50 ± 1.68

^a f/2+CS = commercial nutrients and salts

^b 6%AD+FW = flowback water with 6% anaerobic digestion effluent loading

^c SFA = saturated fatty acids

^d MUFA = monounsaturated fatty acids

^e PUFA = polyunsaturated fatty acids

^f UFA = unsaturated fatty acids

^g BAPE = bis-allylic position equivalent

Figure Captions

Figure 1. Effect of anaerobic digestion effluent loadings on growth of (a) *D. salina*, (b) *D. tertiolecta*, and (c) *N. salina* in the flowback water.

Figure 2. 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.

Figure 3. Effect of different nutrient and salinity sources on (a) specific growth rate and (b) average biomass productivity for *N. salina* and *D. tertiolecta*.

Figure 4. Comparison of (a) specific growth rate and (b) average biomass productivity between *N. salina* and *D. tertiolecta* with different nutrient and salinity sources cultivated in 2-L reactors.

Figure 5. Comparison of fatty acid profiles between (a) *N. salina* and (b) *D. tertiolecta* using media with different nutrient and salinity sources.

Figure 1. Effect of anaerobic digestion effluent loadings on growth of (a) *D. salina*, (b) *D. tertiolecta*, and (c) *N. salina* in the flowback water.

Figure 2. 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.

Figure 3. Effect of different nutrient and salinity sources on (a) specific growth rate and (b) average biomass productivity for *N. salina* and *D. tertiolecta*. 6% AD+FW: 6% anaerobic digestion effluent in flowback water, 6% AD+CS: 6% anaerobic digestion effluent and commercial salts, f/2+FW: flowback water with commercial nutrients.

Figure 4. Comparison of (a) specific growth rate and (b) average biomass productivity between *N. salina* and *D. tertiolecta* with different nutrient and salinity sources cultivated in 2-L reactors. f/2+CS: commercial nutrients and salts, 6%AD+FW: flowback water with 6% anaerobic digestion effluent loading

Figure 5. Comparison of fatty acid profiles between (a) *N. salina* and (b) *D. tertiolecta* using media with different nutrient and salinity sources. f/2+CS: commercial nutrients and salts, 6%AD+FW: flowback water with 6% anaerobic digestion effluent loading.