Ammonia Inhibition in Oleaginous Microalgae

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ABSTRACT

Waste-streams containing nitrogen (commonly as $\text{NH}_3/\text{NH}_4^+$) have been promoted as a means to lower the energy burden and improve the overall sustainability of microalgae-based fuel and chemical production. However, beyond a concentration threshold, ammonia (NH_3) is toxic to many microalgae. This study investigated the ammonia tolerances of oleaginous microalgae. Four microalgae that are often considered in biofuel production studies (*Neochloris oleoabundans*, *Dunaliella tertiolecta*, *Chlorella sorokiniana* and *Nannochloropsis oculata*) were grown in batch reactors maintained at 10 different NH_4Cl concentrations at a constant $\text{pH}=8$. Growth rates and lipid profiles were monitored. Ammonia acted as an inhibiting substrate for *N. oleoabundans* and *D. tertiolecta* at 2.3 and 3.3 mg L^{-1} NH_3 , respectively. Growth rates for *C. sorokiniana* and *N. oculata* were largely unaffected by ammonia concentrations. *D. tertiolecta* demonstrated significant neutral lipid alterations during ammonia inhibition.

Keywords: biofuels, ammonia, nitrogen, nitrogen recycle, bioenergy, wastewater

1. INTRODUCTION

Biofuel sustainability may be challenged by the energy burden associated with the production of nitrogen fertilizers needed for microalgae cultivation. Nitrogen demand is dependent on the type of biofuel feedstock, with 1.9 g N MJ^{-1} of fuel required for corn derived ethanol and 0.18 g N MJ^{-1} for soy-based biodiesel [1,2]. These nitrogen demands are low when compared to biofuels from microalgae, which have projected N demands ranging from 6.6 – 46.9 g N MJ^{-1} biodiesel [3]. The nitrogen required for microalgae cultivation represents a major energy burden, accounting for an estimated 45% of the total energy input [4,5]. One energy saving approach that is commonly outlined in life cycle analyses includes the utilization of N-containing waste

streams such as secondary wastewater treatment plant effluent, anaerobic digester centrate, dairy waste runoff or post lipid-extracted biomass [6-8]. Nitrogen in these waste streams is recovered as $\text{NH}_3/\text{NH}_4^+$, a common byproduct of biomass degradation.

Due to fewer biochemical steps for incorporation of $\text{NH}_3/\text{NH}_4^+$ into amino acids and the low molecular weight, it is often assumed that microalgae will demonstrate preferential uptake of $\text{NH}_3/\text{NH}_4^+$ over other forms of nitrogen [9]. However, experimental data points to a complexity of nitrogen assimilation that does not allow for a universal preference statement. In many phototrophic microorganisms, unionized ammonia can interfere with autotrophic metabolism [10,11], either by ligating to the photosystem II oxygen evolution reaction core thereby creating increased photo sensitivity that eventually results in increased photosystem damage [12,13], and/or by uncoupling photophosphorylation by reducing the pH gradient need to power the conversion of ADP to ATP [14]. Nitrogen substrate affinities within an organism may also be impacted by available N concentration, growth phase and light regimes [15-19].

Nitrogen preferences or $\text{NH}_3/\text{NH}_4^+$ inhibition are not well documented in common strains of oleaginous microalgae at concentrations relevant for biofuel production. In this study we investigated the ammonia tolerances of the oleaginous microalgae *Nannochloropsis oculata*, *Dunaliella tertiolecta*, *Neochloris oleoabundans* and *Chlorella sorokiniana*. Tolerance is assessed by inhibition of growth and neutral cellular lipid production and characterization as a function of nitrogen type (NO_3^- or $\text{NH}_3/\text{NH}_4^+$) and concentration. This work directly addresses critical assumption in many life cycle analyses and microalgae biorefinery schemes' surrounding nutrient recycling for microalgae derived biofuel production, and identifies relevant free ammonia concentrations and oleaginous microalgae that can be used for production in systems with high $\text{NH}_3/\text{NH}_4^+$ content.

2. METHODS

2.1. Species and Media Selection

Four microalgae species were chosen based upon their elevated lipid content and rapid growth rates [20]. All species were obtained from the microalgae library at the University of Texas at Austin and include the marine isolates *Nannochloropsis oculata* (UTEX LB 2341) and *Dunaliella tertiolecta* (UTEX LB 999), and the freshwater isolates *Neochloris oleoabundans* (UTEX 1184) and *Chlorella sorokiniana* (UTEX 1602). Freshwater species were grown in a modified Bold 3N media [21] with $1/3^{\text{rd}}$ K_2HPO_4 and KH_2PO_4 buffer concentrations to prevent phosphate interference with ammonia measurements. Marine species were grown in enriched seawater Erdschreiber's Media [22]. In both media, the nitrogen concentrations were modified for experimental conditions as outlined below.

2.2. Growth Conditions

Growth experiments were conducted in 1L Erlenmeyer flask batch reactors that were sparged via porous stone diffusers with air treated by an activated carbon filter (Koby Inc, MA, USA) and a 0.45 μm pore-size polycarbonate membrane. Air-flow was metered by mass flow controllers (Cole-Parmer Instrument Company, IL, USA) at a rate of $0.6 \text{ L air min}^{-1} \text{ L}^{-1}$. Reactors were well mixed using magnetic stir bars or an orbital mixer at 200 rpm and were exposed to fluorescent light with a photosynthetic flux density of $240 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Thermo Fisher Scientific, MA, USA) and a 14/10 light/dark cycle. Seed cultures were grown with NO_3^- enriched media for 7 to 10 days, after which biomass was harvested through centrifugation (RC-6 Plus, ThermoScientific, DE, USA) at 5000 g for 5 minutes, and cells were washed with

nitrogen-free growth media. Seed cultures were enumerated by light microscopy using a hemacytometer counting chamber (Hausser Scientific, PA, USA). Inocula for all *N. oleoabundans*, *C. sorokiniana*, and *N. oculata* experiments were 1.0×10^6 cells into 600 mL of media. Inocula for the *D. tertiolecta* experiments were 5.0×10^5 cells into 600 mL of media due to the large size of the cells, which increase the initial optical density.

Each experimental run lasted 10 days and biological triplicates were performed. Ammonium chloride was added to the microalga growth media to form ten different concentrations that were maintained throughout the growth cycle. Concentrations included the following: 10, 20, 50, 100, 200, 300, 400, 500, 750, 1000 mg L⁻¹ NH₄Cl. To control the concentration of free ammonia (NH₃), experimental media contained 50mM HEPES (American Bioanalytical, MA, USA) buffering agent adjusted to a pH of 8. At pH 8, the NH₃ (NH₄⁺) concentrations were: 0.2 (3.2), 0.3 (6.4), 0.8 (16.1), 1.7 (32.2), 3.3 (64.4), 6.7 (128.8), 8.4 (161.0), 12.5 (241.5), 16.7 (321.9) mg L⁻¹. Control experiments revealed that HEPES did not act as an N-source for these microalgae. HEPES and no HEPES controls were also tested with both *N. oculata* and *D. tertiolecta* and no growth inhibition due to HEPES was observed.

Inorganic nitrogen was converted to ammonia and measured with a two-step process using the Total Inorganic Nitrogen Pretreatment Reagent Set and Nitrogen-Ammonia Reagent Set, TNT, AmVer (Salicylate), High Range (HACH, CO, USA). Calibration curves relating absorption and NH₄⁺ + NH₃ concentrations were created to monitor nitrogen concentrations. Ammonium concentrations were calculated using NH₃ / NH₄⁺ equilibria. To maintain constant total nitrogen concentrations and balance between NH₄⁺ and NH₃, pH was closely monitored and nitrogen was replenished as needed with a filter sterilized NH₄Cl solution. “The reactor pH values, including variation throughout growth experiments, are presented in **Table S2.**”

2.3. Sampling and Analysis

Samples were taken every other day in the initial stage of the experiments and daily during the mid-exponential phase (days 5-10). Five milliliters (5 mL) were collected and tested for pH to insure system stability and constant free ammonia concentration. Cell density was measured in triplicate through photospectroscopy (Helios Delta, Thermo Fisher Scientific, MA, USA) at a wavelength of 655 nm. To create cell concentration versus absorbance ratios, samples of known absorbance were checked for cell numbers using light microscopy and hemacytometer-based counting. In addition to growth, total lipids and fatty acid characterization was performed. Total cellular lipid content was determined using cells harvested after the 10th day of the experiments (late log phase), collected via centrifugation, freeze dried and stored in a -80 °C freezer until lipid extraction. Neutral lipids were extracted using a modified Folch method with 2:1 chloroform: methanol solution [23]. Total lipids were then transesterified, and fatty acid methyl esters (FAME) [24] were analyzed as an indicator for triacylglyceride content via gas chromatography (HP 6890 A, Agilent Technology, CA, USA) equipped with a SP-2560 Capillary Column (Sigma-Aldrich, MO, USA). Peaks were identified and quantified via standardization using a Supelco® 37 component FAME mix (Sigma Aldrich, MO, USA).

2.4. Inhibition Kinetics Modeling

After completion of growth experiments, linear regression over the mid-exponential growth phase was used to estimate growth rates at each ammonia concentration. Data points from replicate samples were aggregated for a single linear regression for model fitting. Characteristic

growth experiment profiles are provided in **Figure S1**. Inhibition kinetics were evaluated using the Monod-based substrate inhibition model developed by Han and Levenspiel [25] equation 1:

$$R_C = k(1 - C_s/K_i)^n \frac{C_s}{C_s + K_m(1 - C_s/K_i)^m} \quad (1)$$

Where R_c is the cell growth rate (cells time⁻¹), k_{MAX} is the maximum growth rate (cells time⁻¹), C_s is the substrate concentration (mg L⁻¹), K_i is the inhibition constant (mg L⁻¹), K_m is the Monod half saturation concentration of growth kinetics (mg L⁻¹) and m and n are unit-less constants that account for observed changes to k and K_m .

2.5. Statistics

All growth experiments were conducted in biological triplicate. The resulting algal concentration data for each treatment was aggregated and linearly regressed to obtain growth rates and standard deviations for each species and each NH₄⁺/NH₃ concentration. Inhibition parameters from eq. 1 were determined by optimized model fit to the observed data through minimizing residuals via the sum of least squares. Goodness of fit was evaluated through the root mean square deviation (RMSD). All lipid analysis was performed in triplicate and standard deviations were calculated for each lipid fraction. T-tests with reported p-values were used to compare lipid content among the different species and different nitrogen conditions.

3. RESULTS AND DISCUSSION

3.1 Effects of Ammonia on Growth

The experimental conditions presented in this study include free-NH₃ concentrations ranging from 0.2 - 16 mg L⁻¹ and control cases with NO₃⁻. At pH 8, these NH₃ concentrations would correspond to 3.4 - 339 mg L⁻¹ NH₄⁺ + NH₃ and bracket typical municipal and industrial wastewater stream concentrations [26]. Ammonia toxicity in photosynthetic organisms appears to primarily occur through disrupting the thylakoid transmembrane proton gradient. As bulk concentrations increase, NH₃ freely diffuses across cell membranes, leading to increased flux through the chloroplast membrane into the thylakoid lumen. In the acidic environment of the thylakoid, NH₄⁺ forms, thereby decreasing the transmembrane proton gradient needed to power ATP to ADP conversion [11,13,14]. An additional mechanism of toxicity may stem from increased NH₃ concentrations in the chloroplast that promote NH₃ ligation to the organo-metallic reactor core in the D1 protein subunit of the oxygen-evolving complex. Once ligation occurs, the subunit is damaged due to destabilized electron transfer, effectively halting PSII operations [12,13,27]. Though ascertaining the primary mechanism of inhibition is beyond the scope of this study, each of the above mechanisms would inhibit growth and possibly effect cell physiology in ways important to biofuel production.

Of the four species investigated in this study, two showed growth inhibition: *N. oleoabundans* and *D. tertiolecta* (**Fig.1 A and B**). Nonlinear regression using the Han and Levenspiel equation (**eq. 1**), with parameters summarized in **Table 1**, confirmed that growth dependence on NH₄⁺ followed substrate inhibition kinetics. *N. oleoabundans* showed 50% growth inhibition (IC₅₀), occurring near 8 mg L⁻¹ NH₃. At optimal NH₄⁺ / NH₃ concentrations, observed growth rates for *N. oleoabundans* did not significantly differ from growth on NO₃⁻,

with maximum growth rates of $1.2 \pm 0.3 \times 10^5$ cells hour⁻¹ on $\text{NH}_4^+ / \text{NH}_3$ and $1.4 \pm 1.1 \times 10^5$ cells hour⁻¹ with 547 mg L⁻¹ NO_3^- ($p = 0.05$). Inhibition behavior in *N. oleoabundans* was also observed by Li et al. (2008), who showed a ~50% reduction in biomass production with 90 mg L⁻¹ NH_4^+ grown with 5% CO_2 compared to growth on NO_3^- , though pH was not reported. *D. tertiolecta* had an IC_{50} occurring at 8.4 mg L⁻¹ NH_3 . As with *N. oleoabundans*, *D. tertiolecta*'s maximum growth rate on $\text{NH}_4^+ / \text{NH}_3$ ($1.4 \pm 0.3 \times 10^4$ cells hour⁻¹) was not different ($p = 0.5$) than the growth rate observed on NO_3^- ($1.6 \pm 0.19 \times 10^4$ cells hour⁻¹ at 143 mg L⁻¹ NO_3^- mg L⁻¹). These inhibition concentrations for both species are significant as they occur near or before stoichiometric nitrogen demands for maximum culture densities, and can act as a limiting factor preventing acceptable biomass production. Assuming an average molecular formula $\text{C}_{106}\text{H}_{181}\text{O}_{45}\text{N}_{16}\text{P}$ [28] for microalgae and a low range final microalgae culture density of 1 g L⁻¹ dry mass results in an N-demand of 118 mg L⁻¹ $\text{NH}_4^+/\text{NH}_3$. At this concentration and an operating pH 8, the NH_3 concentration would be roughly 5.6 mg L⁻¹; well into the inhibition zone for both species, which begin at 2.3 and 3.3 mg L⁻¹ NH_3 for *N.oleoabundans* and *D. tertiolecta*, respectively.

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Table 1. Summary of growth parameters for NH₃ inhibited alga

Parameter	<i>N. oleoabundans</i>	<i>D. tertiolecta</i>
k_{MAX}	2.9 x 10 ⁵	2.5 x 10 ⁴
<i>n</i>	2.2	1.3
<i>m</i>	-0.14	0.44
K_m	2.6	0.6
K_i	16.4	16.4
IC₅₀	7.7	8.4
IC₁₀₀	15.9	16.4
R_{C,NH4+}	1.2 x 10 ⁵ ± 1.1 x 10 ⁴	1.4 x 10 ⁴ ± 2.9 x 10 ³
R_{C,NO3-}	1.41 x 10 ⁵ ± 1.1 x 10 ⁴	1.6 x 10 ⁴ ± 5.4 x 10 ³

210 k_{MAX} (calculated maximum growth rates), R_{C, NH4+} and R_{C, NO3-} (growth rates for NO₃ and NH₄, respectively) reported as cells
 211 hour⁻¹. K_m (Monod half-saturation concentration), K_i (inhibition concentration), IC₅₀ (50% growth inhibition concentration), IC₁₀₀
 212 (complete growth inhibition concentration) reported as mg L⁻¹. *m* and *n* are unitless constants that account for observed changes
 213 to *k* and *K_m*. Parameters k_{max}, *m*, *n*, K_m and K_i were optimized for best fit.
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215 The two remaining species of this study, *C. sorokiniana* and *N. oculata*, did not show growth
 216 inhibition due to NH₃ over our experimental concentrations (**Fig.1 C and D**). This lack of
 217 inhibition (ANOVA *p* > 0.4) has been previously demonstrated in another *Chlorella* species, *C.*
 218 *vulgaris*, which maintained comparable growth rates up to 1000 mg L⁻¹ NH₄⁺ at neutral pH (4.7
 219 mg L⁻¹ NH₃) [29]. However, each species did express an apparent growth preference for N-
 220 sources. The growth rate of *C. sorokiniana*, on 123 mg L⁻¹ as NO₃⁻N was 1.75 times greater than
 221 the average growth (2.3 ± 0.2 x 10⁵ cells hour⁻¹) over experimental NH₄⁺ / NH₃ concentrations
 222 (*p* = 0.02). On the other hand, *N. oculata* showed a clear substrate preference for NH₄⁺ / NH₃ as
 223 a N source with an average growth rate outpacing nitrate growth by 4.5 times, at 1.2 ± 0.1 x 10⁵
 224 cells hour⁻¹.
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Figure 1. Growth rate versus free NH_3 curves for each species: (A) *N. oleoabundans*, (B) *D. tertiolecta*, (C) *C. Sorokinana*, and (D) *N. oculata*, represented by shaded circles, reported as cells hour^{-1} over NH_3 concentrations. The error bars indicate the standard deviation of the slope from linear regression for each growth rate experimental condition. The output of the substrate inhibition model using eq 1 is represented by the dashed line. The Root Mean Squared Deviation (RMSD) was used to evaluate the goodness of fit of the curve fitting.

Responses to NH_3 may provide some insight into the toxicity mechanism as reflected in the expression of inhibition and the resulting model constants. As PSII is damaged, substrate (light)

utilization would be reduced. Based on comparisons of unitless constants n and m , Han and Levenspiel categorized six common growth inhibition patterns [25]. By utilizing this framework, noncompetitive inhibition was observed in the growth inhibition model of *D. tertiolecta*, with the unitless constants $n > m > 0$. For the other test species that showed inhibition, *N. oleoabundans*, the values for the unitless constants from **eq 1** indicate a general inhibition ($n > 0$, $m < 0$). While the above results found different NH_3 tolerances among different taxa, current literature suggests toxicity responses to NH_3 in seawater and freshwater may not be shared within a genus. Collos & Harrison [30] reviewed the effects of ammonia at low free ammonia concentrations and demonstrated variable toxicity responses existed within genera.

3.2 Effects of Ammonia on Lipid Profile and Cellular Content

Due to the focus on oleaginous microalgae, metabolite measurements were restricted to neutral lipid content and character. Aside from the implications on productivity from cellular lipid content, lipids chain length and degree of unsaturation can affect physical and chemical biofuel characteristics such as viscosity, density, cold flow properties, oxidative stability and cetane number [1]. The central comparisons for lipid content and character for each species were made through growth media containing NO_3^- as the N source versus growth media containing $\text{NH}_4^+ / \text{NH}_3$ as the N source. For the $\text{NH}_4^+ / \text{NH}_3$ cases, two NH_3 concentrations were considered and are denoted as LOW and HIGH. Ammonia concentrations for the LOW conditions were 3.3 (63), 3.3 (63), 3.2 (63), and 3.3 (63) $\text{mg L}^{-1} \text{NH}_3 (\text{NH}_4^+)$ for *N. oleoabundans*, *D. tertiolecta*, *C. sorokiniana*, and *N. oculata*, respectively and corresponded to concentrations that were not inhibitory to these taxa. The NH_3 levels for the HIGH conditions were 16.0 (314), 11.5 (236), 12.8 (315), and 16.3 (315) $\text{mg L}^{-1} \text{NH}_3 (\text{NH}_4^+)$ for *N. oleoabundans*, *D. tertiolecta*, *C.*

sorokiniana, and *N. oculata*, respectively and for the taxa that showed inhibition, corresponded to concentrations at least 3.5 times the concentrations where inhibition begins and 1.4 times the IC_{50} .

3.2.1 FAME concentration

Table 2 (see **Table S1** for reported standard deviations) indicates that lipid content for *N. oleoabundans*, *C. sorokiniana* and *N. oculata* were not significantly different between NO_3^- and HIGH and LOW NH_3 conditions with average total neutral lipid content reported as fatty acid methyl esters (FAME), measuring 7.2%, 7.3% and 17.2% respectively. These values are similar to total FAME values reported in prior studies. Rismani-Yazdi [31] and Soh [32] both used an identical *N. oleoabundans* strain and reported FAME values between 5% and 10%. Similarly, lipid content with *N. oculata*, had reported values of 20% and 18% neutral lipid content. The 7% FAME value for *C. sorokiniana* is within the 6.6% to 12% range previously reported in the literature [32-34]. The remaining species, *D. tertiolecta*, showed statistically significant changes to the lipid content between the conditions. For *D. tertiolecta* grown on NO_3^- , lipid content was 6.3 %, LOW NH_3 was 8.5% and HIGH NH_3 was 11.0% dry biomass (NO_3^- vs LOW: $p = 0.02$, NO_3^- vs HIGH: $p = 0.0007$, LOW vs HIGH: $p = 0.01$).

3.2.2 FAME characteristics

The variable nitrogen conditions also resulted in minor changes in lipid profiles for *N. oleoabundans*, *C. sorokiniana* and *N. oculata*. For *N. oleoabundans*, the fresh water species that exhibit growth inhibition when exposed to high levels of free ammonia, the HIGH NH_3 condition resulting in a small (~2%) but significant ($p < 0.01$) decrease in polyunsaturated fatty

acids (PUFA) and an equally small ($p = 0.01$) increase in monounsaturated fatty acids (MUFA). Other significant alterations occurred with C18 lipids, with the change from NO_3^- to NH_3 and LOW to HIGH NH_3 resulting in a reduction of C18:3 ($\text{NO}_3^- > \text{LOW} > \text{HIGH}$: $p \leq 0.01$), and the transition from NO_3^- to NH_3 resulting in an increase in C18:2 content ($p \leq 0.01$). While not showing any growth inhibition when exposed to high levels of NH_3 , *C. sorokiniana* demonstrated a small, but statistically significant increase in MUFAs, (all cases: $p < 0.02$). This increase was concomitant with an increase in C18:1 content (all cases: $p < 0.03$) from 5% with NO_3^- to 9% with LOW NH_3 and finally 13% under HIGH NH_3 concentrations. Additionally, *C. sorokiniana* responded to the change of N-source by increasing C18:2 content.

D. tertiolecta showed the most significant changes to the lipid profile between the HIGH and LOW NH_3 condition (**Table 2**). There was a significant ($p < 0.01$) transition toward increased overall lipid saturation during NH_3 inhibition, changing from a saturation/unsaturation ratio of 0.6 while grown with LOW free NH_3 to 1.3 when growth is inhibited by HIGH free NH_3 . PUFA content was greatly reduced from 52% and 47% of the lipid content for growth on NO_3^- and LOW free NH_3 , respectively to a HIGH free NH_3 content of 8% (HIGH vs NO_3^- : $p < 0.01$, HIGH vs LOW: $p = 0.02$), representing a near 6 times decrease. MUFA increased 3 times during NH_3 induced growth inhibition, changing from ~12% lipid content for LOW NH_3 to 35% for HIGH NH_3 ($p < 0.01$). *D. tertiolecta* also showed a decrease in average chain length when exposed to high ammonia concentrations. This is due to the decrease in C18:3 (LOW vs HIGH: $p = 0.01$, NO_3^- vs HIGH: $p = 0.01$) and an increase in C16:1 (LOW vs HIGH and NO_3^- to HIGH: $p < 0.01$).

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Table 2. Summary of FAME comparisons.

	<i>N. oleoabundans</i>			<i>D. tertiolecta</i>			<i>C.sorokiniana</i>			<i>N. oculata</i>		
	NO ₃	LOW	HIGH	NO ₃	LOW	HIGH	NO ₃	LOW	HIGH	NO ₃	LOW	HIGH
C8:0	-	-	-	-	-	-	-	-	-	0.3	0.3	0.1
C10:0	-	0.5	0.3	0.5	0.4	0.6	-	-	0.2	0.4	0.6	0.2
C12:0	-	0.1	0.2	0.4	0.6	0.4	0.1	0.8	0.2	0.6	0.4	0.4
C14:0	0.6	0.7	0.4	0.6	0.7	<u>4.7</u>	0.3	2.4	0.2	7.2	10.9	5.6
C14:1	-	-	-	-	-	-	-	-	-	0.1	0.6	0.1
C16:0	26.3	24.9	26.0	27.3	32.3	44.3	30.7	18.5	26.9	32.0	21.4	34.8
C16:1	1.7	1.3	1.0	0.8	0.9	26.5	0.9	1.4	1.3	28.4	30.2	19.6
C18:0	3.6	4.4	4.1	4.9	5.6	5.7	3.2	2.9	3.3	3.3	2.5	4.4
C18:1	5.4	6.0	8.3	9.6	10.0	<u>8.4</u>	5.5	9.6	13.5	4.3	5.5	5.7
C18:2	27.1	30.7	32.0	8.0	7.6	2.0	21.5	37.8	30.9	3.5	4.1	3.7
C18:3	35.2	31.0	27.2	44.7	38.4	<u>1.9</u>	37.1	24.3	22.6	0.4	1.4	8.6
C20:0	0.2	0.4	0.3	0.3	0.6	0.7	0.5	-	-	-	0.1	0.5
C20:1	-	-	-	0.5	1.5	0.2	-	0.1	-	-	0.1	0.5
C20:2	-	-	0.2	2.1	1.4	-	-	2.2	0.6	0.5	0.1	3.7
C20:3	-	-	-	0.3	-	0.4	-	-	-	0.7	0.5	0.2
C20:4	-	-	-	-	-	-	0.1	-	-	-	-	-
C20:5	-	-	-	-	-	4.1	-	-	-	18.3	21.2	11.9
PUFA %	62.3	61.7	59.5	55.1	47.4	8.5	58.7	64.3	54.2	23.3	27.3	28.1
MUFA %	7.0	7.3	9.3	10.9	12.4	35.1	6.4	11.1	14.8	32.8	36.4	25.9
SATURATION	30.7	31.0	31.2	34.0	40.2	56.5	34.9	24.6	31.0	43.8	36.2	46.0
Total FAME	7.7	6.8	7.1	6.3	8.5	11.0	7.3	7.5	7.2	18.8	16.0	16.8
Avg. Chain Length	17.4	17.4	17.4	17.5	17.3	16.4	17.4	17.6	17.4	16.8	16.9	17.1
SAT/UNSAT	0.45	0.45	0.45	0.57	0.67	1.3	0.45	0.45	0.54	0.78	0.63	0.85

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Numbers reported as percentages. *Total FAME* is the reported dry cell mass percentages of lipids (mg lipid per mg dry biomass). SAT/UNSAT is the ratio of saturated FAME to unsaturated FAME. **Bold** numbers are significant ($p \leq 0.05$) with respect to NO₃ (NO₃ vs LOW, NO₃ vs HIGH). Underlined numbers are significant with respect to LOW (LOW vs HIGH).

311 We did not observe a unified lipid response from the species investigated during exposure to
312 ammonia verses nitrate, whether or not inhibition occurred. However, the changes in lipid

character might have implications on the produced biofuel's performance. Longer chain lengths (heavier macromolecules) increase the density, viscosity and cetane number of the resulting biodiesel [35]. Additionally, highly saturated lipids increase the produced fuels stability as they are less susceptible to auto-oxidation, but also reduce the fuel's temperature dependent properties such as cloud (below which the fuel will form waxes in solution) and pour (below which the liquid loses flow characteristics) points [36]. The greatest changes in lipid characteristics due to growth in NH_4^+ were observed for *D. tertiolecta*. The changes associated with growth on NH_4^+ are largely beneficial for fuel characteristics as increased saturation will decrease the cetane number, increase fuel stability and increase resistance to oxidation during storage. However, the substantial reduction in PUFA could minimize the positive effects on cetane number and fuel stability.

4. CONCLUSIONS

Microalgae growth schemes involving NH_4^+ containing waste streams have been suggested to reduce energy input from nitrogen fertilizers to support cell biosynthesis. Ammonia was shown to inhibit growth in two of the four microalgae species tested at concentrations that would not permit batch cultures to reach full density. These observations call into question many assertions regarding microalgae life cycle analysis and the corresponding energy input reduction strategies, as a significant degree of variability and nutrient sensitivity occurs among oleaginous microalgae.

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