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Cultivating the marine microalga *Nannochloropsis salina* under various nitrogen sources: Effect on biovolume yields, lipid content and composition, and invasive organisms

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ABSTRACT

Algae can be a viable source for biofuel production, but the source of nitrogen used to cultivate could affect algae yields. Here, we observe how various nitrogen treatments can impact the growth and biovolume of microalga *Nannochloropsis salina* as well as invasion of undesired organisms. Invading organisms increase the likelihood of crashes of the desired microalgae culture. Experiments were conducted over 28 days in open aquaria in a greenhouse. We used five different nitrogen treatments; ammonium chloride (NH_4Cl), ammonium hydroxide (NH_4OH), sodium nitrate ($NaNO_3$), urea (CH_4N_2O), and a mixture of all these sources. Highest values for Maximum Sustainable Yield (MSY), a measure of potential harvest rate based on population productivity, were observed in the urea treatment, but cell size was smaller compared to other treatments. Sodium nitrate and the mixture of nitrogen sources also had high MSY values but larger cell sizes, making them the treatments with highest total biovolume. The highest percentages of lipid by weight, but also highest densities of invading organisms were observed in the mixed treatment. Our results suggest that tradeoffs between biovolume and lipid yields as well as culture success can ultimately decide what nitrogen sources to use.

1. Introduction

The current energy crisis results from the excessive consumption of non-renewable fossil fuel and greenhouse gas emissions are causing negative environmental repercussions [1]. Thus, development of modern energy sources like biofuels are important. These are fuels that may be derived from plant oil or animal lipid and their reaction with alcohol to form mono-alkyl ester [2]. Microalgae are at the forefront as a biofuel candidate because they can be grown all year around on non-arable land, they require less space, yields are higher compared to traditional crops, and novel technologies are making rapid improvements [3,4]. However, at this point, microalgae for biofuels are not an economic viable commercial enterprise [5]. Next to using various products from algae,

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lowering the cost of algae lipids is a major goal of current research.

While maximum algae growth occurs typically under ample supply of nutrients and light and optimum temperature conditions, lipid accumulation is enhanced when microalgae cells are stressed. The maximization of oil yield in microalgae strains and cultures through environmental manipulation can be less expensive and easier to achieve in the short-term than alternatives such as gene modification. There is potential to increase yields by manipulating environmental factors, which cause stress for microalgae and induce maximum accumulation of lipids [6]. Sources of stress include manipulating environmental conditions such as salinity [7,8], pH [9,10], temperature [11,12], and nutrients [13,14].

It has been well established, that nitrogen limitation in general is beneficial for increasing lipid accumulation in some microalgae [15,16] and impacts the type of lipids produced [17]. Specifically, nitrogen replete cultures will often produce more triacylglycerols (TAG) in contrast to nitrogen deplete batch cultures, where lipid C16:0 chains are favored [18]. However, lipid productivity is dependent on lipid accumulation as well as microalgae biomass. Wan et al. [16] were able to demonstrate that highest lipid productivity might occur under higher nitrogen concentrations. Not only the amount of nitrogen, but also the source of nitrogen is likely to have an impact on biomass and lipid productivity as well. To date, this topic is not as well studied [13,19,20].

Among 30 microalgae, *Nannochloropsis* spp. was identified to have among the highest biomass and lipid productivity [15]. These are marine microalgae that are tolerant to a large range of environmental conditions. Thus, various *Nannochloropsis* species have become popular model systems. However, to our knowledge, the effects of different nitrogen sources on growth rate and lipid production on *Nannochloropsis* have not been tested. Additionally, despite the general knowledge of contaminating invading organisms being a major challenge [7,15,21,22], little is known about what organisms invade production systems and what environmental conditions might limit their occurrence [8,10].

Here, we test different nitrogen sources (ammonium chloride (NH_4Cl), ammonium hydroxide (NH_4OH), sodium nitrate ($NaNO_3$), urea (CH_4N_2O), and a mixture of all these sources) on growth and lipid accumulation of *Nannochloropsis salina*. Furthermore, we identify organisms that invaded our open cultivation systems in the various treatments. While we found three other studies, that looked at effects of different nitrogen sources on growth of various microalgae [13,19,20], only one study [13] included lipid accumulation and no other studies considered invading organisms or tried to use different nitrogen sources simultaneously (mixed treatment).

2. Methods

2.1. Microalgae cultures and experimental set-up

N. salina (strain 1776) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton. The experiment was conducted in a greenhouse located at New Mexico State University's Fabian Garcia Science Center in Las Cruces, New Mexico, USA. We grew *N. salina* in an outdoors raceway at Fabian Garcia in standard f/2 medium for marine microalgae [23] and subsequently inoculated cultures in a greenhouse where aquaria were subjected to natural light and temperature conditions. The culture medium used during experiments was also f/2 medium, with an alternative source of nitrogen for each treatment. We had a total of five nitrogen treatments (ammonium chloride, NH_4Cl; ammonium hydroxide, NH_4OH; sodium nitrate, $NaNO_3$; urea, CH_4N_2O; and a mixture of all these sources). In order to acquire identical amounts of nitrogen from these different sources we calculated the g mol^{-1} of nitrogen. The standard f/2 medium contains 75 g of sodium nitrate per liter of which 14.0067 g mol^{-1} is nitrogen. Each treatment consisted of this same concentration of nitrogen. This amounted to 41.21 g ammonium chloride, 53.1 cm^3 ammonium hydroxide, and 26.50 g urea per liter of water. The mixture treatment consisted of an equal amount from each treatment. Experiments were conducted in aquaria with 30 L working volume of media and each nitrogen treatment was replicated five times. Aquaria were left open to the environment so that they represent open raceways, typically used for microalgae cultivation and remained susceptible to invading organisms. Salinity was kept between 29 and 33 PSU. Water was circulated using air stone aerators, adding atmospheric CO_2. Three times a week, absorbance readings were taken and samples were collected and preserved using Lugol's solution. Cultures were inoculated with 250 cm^3 of microalgae so that an initial cell density for all tanks was approximately $2.5 \cdot 10^{12}$ m^{-3}. The experiment ran for 28 days from September 27–October 25, 2012. Growth parameters were monitored throughout the duration of the experiment.

2.2. Water quality measurements and nitrogen measurements

Three times per week, during sample collection, we also monitored temperature, pH, and salinity in the aquaria with a Hydrolab (model MS 5, HachHydromet, Loveland CO). At the beginning, middle and end of the experiment, the values were also measured at night to account for day–night fluctuations. Temperature of the aquaria over the duration of the experiment ranged from 16 to 32 °C, salinity from 29 to 33 PSU and pH fluctuated between 7.9 and 9.3. These values are well within the tolerance levels of *N. salina* [8,10] and did not differ among treatments.

Using Colorimeter DR/890 (Hach Company, USA) we monitored concentration levels of detectable nitrate and ammonia in the water. Using NitraVer Reagent, we analyzed in 16 mm test-and-tube vials 1 cm^3 of sample from each tank to measure NO_3–N. Similarly, in test-and-tube vials using Ammonia Reagent we measured 0.1 cm^3 of sample to measure NH_3–N. We took readings until there was no more detectable available nitrogen on day six.

2.3. Cell density evaluation

Cell density was estimated in two ways: (1) Fresh samples were evaluated using optical density of cultures by using a spectrophotometer, at $\lambda = 740$ nm. (2) Samples preserved in

Lugol's solution were taken to the laboratory and processed using a *Benchtop B3 Series FlowCAM* (Fluid Imaging Technologies, Yarmouth, Maine, USA) to monitor growth rates and densities. A *FlowCAM* is a flow cytometer that records, counts, and measures the images of individual particles for a given volume of sample flowing at a constant rate. Samples were diluted so that no more than 30 organisms were counted per frame. Each sample was processed by the *FlowCAM* for 3 min, resulting in >10,000 organisms being counted and measured. Organisms present were grouped into size classes and subsequently number of particles per cubic centimeter. The size classes where based on size parameters of *N. salina*, diatoms, cyanobacteria, flagellate, and microalgae aggregates ("clumps").

The dilution was entered into the *FlowCAM* and particle densities for the different organisms were automatically calculated.

2.4. Fatty acid methyl ester (FAME) analysis

Using optical density we monitored the growth phase of each tank. One week after carrying capacity was reached (day 28) we gathered 1-L samples for subsequent lipid analysis. Lipid content was monitored by transesterification and analysis of fatty acid methyl esters (FAME) by gas chromatography mass spectrometry (GC/MS) for each treatment. *In situ* transesterification followed by GC/MS quantitation of FAMEs as a measure of lipid productivity has emerged as a superior method to gravimetric lipid determination [24–26]. One liter of each culture was centrifuged and the pellet collected and transferred to a 15 cm^3 conical tube. The pellet was then washed with deionized water, shaken and re-centrifuged. This process was repeated five times for each collection to remove salts from the sample prior to analysis. Samples were then lyophilized and tissue placed in a pre-weighed 16 cm^3 glass vial to which 5 $cm^3 \cdot 11$ g L^{-1} potassium hydroxide in methanol was added and allowed to stand for 30 min in a 323 K water bath. An internal standard, glyceryl tritridecanoate, was included in the reaction to monitor conversion efficiency. Each sample vial was vortexed in 10 min intervals. The reaction was quenched after 30 min with 1 cm^3 of 60 g L^{-1} glacial acetic acid. Each sample was back extracted with 2 cm^3 hexane with methyl tricosanoate C23:0, an internal standard for quantification/internal standard calibration. The hexane fraction was further diluted and analyzed via GC/MS under conditions as described by Patil et al. [27].

Briefly, all samples were analyzed with a Hewlett Packard 5890 Gas Chromatograph with a 5972 Mass Selective Detector and 7673 Autosampler. Two microliter injections were loaded onto a 30 m × 0.25 mm diam. (0.25 μm film thickness) Agilent DB-23 capillary column with helium carrier gas. The initial temperature was 353 K and ramped 20 K min^{-1} to 493 K and held for 6 min for a total run time of 13.3 min. Chromatographic signals were matched to a standard mix (Supelco 37 Comp. FAME mix 10 mg cm^{-3} in methylene chloride) and internal calibration was achieved using a C23:0 internal standard spiked at 50 μg cm^{-3}.

2.5. Calculations and statistical analyses

Nannochloropsis growth rate (*r*) was calculated using SPSS 22.0 software [28] from cell counts. We applied more commonly used exponential equations [29]:

$$N_t = N_0 \, EXP \, (r * t)$$

In the equation above, N_t is the population size at time t, N_0 is the initial population size, *r* is the population growth rate, and *t* are the days of our experiment. Data for the time from inoculation until all treatments had approximately reached their maximum densities (day 0–day 21) were used for the calculations.

We also compared growth rates using logistic equations, which better represents microalgae growth data [29]:

$$N_t = K/(1 + (K - N_0)/N_0 * EXP \, (-r * t))$$

K stands for carrying capacity and is the cell density, when the population no longer increases (cell growth approximately equals cell death). It is also referred to as stationary phase. We used data from the entire experiment (day 0–day 28) to calculate growth rate with the logistic equation.

In order to calculate growth rate, we entered the days of our experiment (*t*) and cell densities (N_t) into an SPSS spreadsheet. We conducted nonlinear regressions and set *N* as the dependent variable. The equations above were entered as the model expressions: "*a* EXP (*r**t)" for the exponential and "K/(1 + (K−*a*)/*a**EXP (−*r**t))" for the logistic growth equation. N_0 was simplified to 'a' in our equations. Initial estimates of parameters were set to $a = 5$, $r = 1$, and $K = 70$. Initial estimates of parameter do not impact the model output, unless extreme values that largely deviate from the actual dataset are used. SPSS then conducts an iteration process until Residual Sum of Squares is minimized.

Furthermore, we calculated maximum sustainable yield (MSY), which is a better measure for overall productivity [29]:

$$MSY = r * K/4$$

Nannochloropsis biovolume was calculated for day 28 by applying the volume of a sphere ($V = 4/3 \, ^*\pi \, ^*r^3$) using the area-based radius measured by the *FlowCAM* for each sample. The average volume per cell was then multiplied by the cell density.

One-way ANOVAs were conducted to test the effect of nitrogen source on cell density on day 21, cell size and biovolume as well as density of invading organisms and total FAME content on day 28. We used a significance level of 0.05 and used STATISTICA [30] to run our analyses. We used a Tukey's post-hoc test for comparison of individual treatments.

3. Results

3.1. Nannochloropsis growth rates, densities, and biovolume

Exponential growth rates ranged from 0.051 d^{-1} in the ammonium chloride (NH_4Cl) treatment to 0.096 d^{-1} in the urea

(CH_4N_2O) treatment (Table 1). Estimates using the logistic growth equation were always higher and higher coefficients of determination (R^2) values indicated a better fit using the logistic growth equation as opposed to the exponential growth equation (Table 1). However, ranks using logistic growth rates were almost completely reversed compared to exponential growth estimates (low exponential growth rates equal high logistic growth rates and vice versa) and were lowest in the urea treatment (0.24 d^{-1}) and highest in the ammonium chloride treatment (0.324 d^{-1}) (Table 1). Carrying capacity (K) and Maximum Sustainable Yield (MSY), a product of logistic growth rates and carrying capacity, reflected the order calculated by the exponential growth rates more closely (Table 1).

Cell density on day 21 ranged from $29.7 \times 10^6 \pm 3.3 \times 10^6$ cm^{-3} (mean ± SE) in the ammonium chloride treatment to $74.2 \times 10^6 \pm 9.4 \times 10^6$ cm^{-3} in the urea treatment (Fig. 1). The cell density of the urea treatment was significantly higher than in the other treatments (1-way ANOVA, d.f. = 4, $F = 9.54$, $p = 0.0002$, with Tukey's post-hoc, $p \leq 0.017$).

The average cell diameter at day 28 (last sampling day) for *N. salina* was smaller in the urea treatment (2.9 ± 0.02 μm) than in the other treatments (Fig. 2, 1-way ANOVA, d.f. = 4, $F = 16.65$, $p < 0.0001$, with Tukey's post-hoc, $p \leq 0.0004$).

Biovolume values followed the dynamics of absorbance values closely (data not shown), and not the cell density estimates from the *FlowCAM*. Biovolume was lowest in the ammonium chloride treatments (0.79 ± 0.11 dm^3 m^{-3}) and highest in the treatments with mixed nitrogen sources (1.20 ± 0.04 dm^3 m^{-3}) (Fig. 3, 1-way ANOVA, d.f. = 4, $F = 5.82$, $p = 0.0028$) on day 28. The biovolume in treatments with the mixed nitrogen source was significantly higher than in the ammonium chloride and urea treatments (Fig. 3, Tukey post-hoc $p \leq 0.018$).

3.2. *Invaders*

The densities of invading organisms were estimated for day 28. Invading organisms were significantly more abundant in the mixed nitrogen treatment (mean ± SE: 85,530 ± 27,361.2 cm^{-3}) than the ammonium chloride treatment (7215 ± 2938 cm^{-3}) (1-way ANOVA, d.f. = 4, $F = 3.52$, $p = 0.027$, with Tukey's post-hoc, $p = 0.018$; Fig. 4). Cyanobacteria were most abundant in all treatments. Diatoms and flagellates were absent from the ammonium chloride treatment and no clumping of microalgae was observed in the urea treatment (Fig. 4).

Table 1 – Comparison of exponential growth (0–21 days) and logistic growth (0–28 days) equations and maximum sustainable yield (MSY) in treatments with various nitrogen sources. *r* – estimate for growth rate (d^{-1}); R^2 – coefficient of determination; K – carrying capacity (cells × 10^7 cm^{-3}); MSY – Maximum sustainable yield (cells × 10^7 cm^{-3} d^{-1}).

Treatment	Exponential growth		Logistic growth			MSY
	r	R^2	*r*	K	R^2	
$NaNO_3$	0.075	0.851	0.323	4.65	0.987	0.38
Urea	0.096	0.960	0.240	7.09	0.970	0.43
NH_4OH	0.055	0.775	0.316	2.98	0.972	0.24
NH_4Cl	0.051	0.745	0.324	2.70	0.893	0.22
MIX	0.063	0.860	0.259	4.06	0.960	0.26

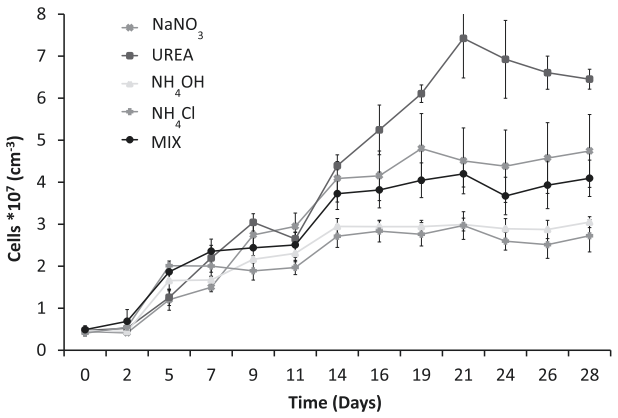

Fig. 1 – *Nannochloropsis salina* growth curves day 0–28 using five nitrogen sources. Error bars represent ± 1 SE (*n* = 5).

3.3. *Lipid accumulation*

Day 28 samples were collected and prepared for fatty acid methyl ester (FAME) quantification by GC/MS. Nitrogen source significantly impacted percent FAME by weight (Fig. 5, 1-way ANOVA, d.f. = 4, $F = 33.40$, $p < 0.0001$). Maximum acyl hydrocarbon content, measured as FAME, was observed in the treatments with mixed nitrogen sources (27.5% ± 0.4%) (mean ± SE) and lowest in the urea treatments (13.2% ± 0.7%). Observed FAME measurement was significantly lower for urea treatment than all others (Tukey's post-hoc, $p \leq 0.0007$) and the mixed nitrogen treatment was significantly higher than the sodium nitrate and the ammonium hydroxide treatments (Tukey's post-hoc, $p = 0.0005$ and 0.0002, respectively). As expected for *N. salina* the observed FAME profile (Fig. 6) shows the dominant acyl hydrocarbons to be 14:0, 16:0, 16:1, and 18:0. The urea treatment shows elevated levels of C18:3n3 and C18:2n6 components.

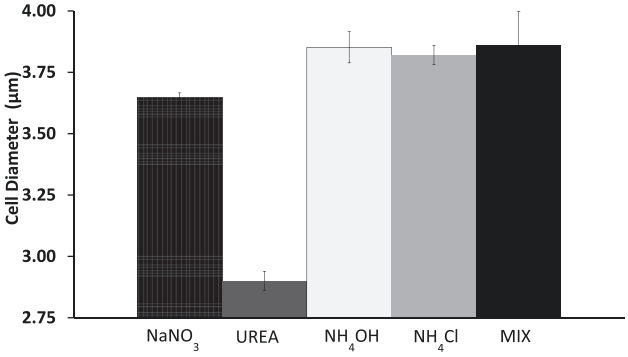

Fig. 2 – *Nannochloropsis salina* average cell area-based diameter in response to different nitrogen sources at sampling day 28 ± 1 SE (*n* = 5).

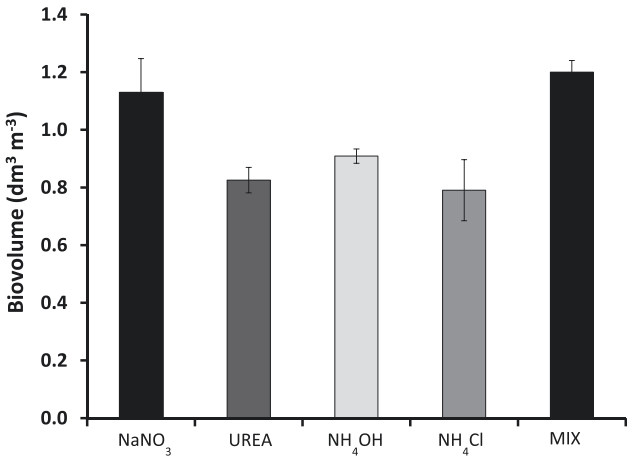

Fig. 3 – Average biovolume of *Nannochloropsis salina* under different nitrogen treatments at sampling day 28 ± 1 SE ($n = 5$).

Assuming percent FAME by weight would be the same than percent FAME by volume, we can multiply percent FAME by weight with total biovolume, to achieve a crude estimate for total lipid ($dm^3\ m^{-3}$) in each sample. Total lipid was highest in the mix treatment (0.33), followed by the nitrate treatment (0.23), and the two ammonium treatments (0.20 and 0.18). The lowest lipid concentration was observed in the urea treatment (0.11) at the end of our experiment.

4. Discussion

Our results suggest that production of *N. salina* (biovolume and lipid accumulation) could increase when grown with various nitrogen sources (ammonium, nitrate, urea) simultaneously. This, however, might also increase the biomass of undesired organisms that could increase the likelihood of a microalgae crash.

We found that the urea treatments had the highest Maximum Sustainable Yield (MSY) values and led to the highest cell densities of *N. salina*. Yet, these positive characteristics were offset by a smaller cell size. The treatments using several nitrogen sources had an intermediate growth rate response and a larger cell diameter. Thus, the mixed treatments led to the highest biovolume (not significantly different from sodium nitrate though). Similarly, Rocha et al. [11] found an increased biomass of *Nannochloropsis gaditana* when combining potassium nitrate with urea. Li et al. [13] found the highest accumulation of biomass for *Neochloris oleoabundans* in the nitrate treatments after 7 days. Biovolume in our nitrate treatment was not significantly different from our mixed treatment after 28 days. Xin et al. [31] attributed lower density of *Scenedesmus* sp. to lower pH values in the ammonium treatments compared to urea or nitrate. Despite a similar finding in *Nannochloropsis* densities, we did not observe a decrease in pH in the ammonium treatments, which might be caused by different uptake of ammonium between the different microalgae species. Overall, we feel confident that the differences in growth we observed are in fact due to different nitrogen sources, since none of the other measured parameters (temperature, pH, salinity) varied significantly among treatments. Furthermore, all invading organisms observed were competitors and we did not observe any microalgae predators. We found that the highest *Nannochloropsis* biovolume as well as highest invader density both occurred in the mixed treatment and do not find any evidence that invaders might have negatively impacted *Nannochloropsis* growth. It seemed surprising that nitrogen was depleted to undetectable limits so quickly and might indicate that microalgae take up nitrogen quickly and continue to grow by using the internally stored nitrogen. Further, we emphasize, that many environmental parameters were not optimized for maximum productivity and this was not the purpose of this experiment. We merely focused on differences in microalgae growth parameters and invaders due to nitrogen source.

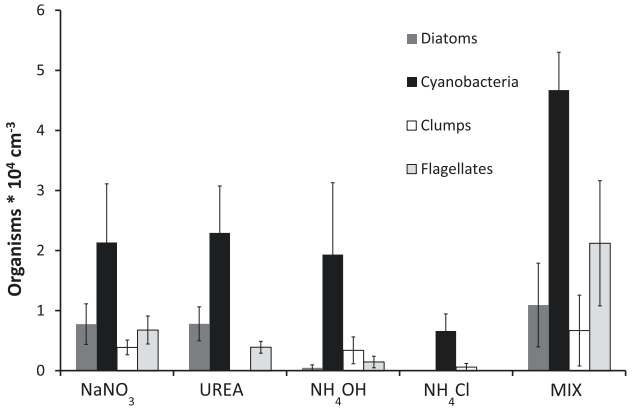

Fig. 4 – Average invading organism density for *Nannochloropsis salina* cultures at sampling day 28 for variable nitrogen sources ± 1 SE ($n = 5$).

Estimates for growth rates using the logistic growth equation were consistently higher than when using the exponential growth equation. That might have been caused by SPSS estimating higher initial cell densities (N_0) when using the exponential growth equation. The coefficients of determination (R^2) values were always higher for the logistic growth rate calculations, indicating that it is indeed the more appropriate formula for calculation of growth rates [29]. However, the logistic growth equation is very sensitive to how fast maximum cell density is reached and less to the overall maximum cell density that can be achieved. For example, in the ammonium chloride treatments, cell density already leveled off after day 14 and carrying capacity (maximum cell density; K) was estimated to be $2.7 \times 10^7\ cm^{-3}$. Although carrying capacity was with $7.09 \times 10^7\ cm^{-3}$ almost three times higher in the urea treatment, cell densities continued to increase until day 21, resulting in a lower growth rate estimate. Thus, Maximum Sustainable Yield (MSY), which multiplies growth rate and carrying capacity, might be the most appropriate response variable to report cell productivity [29].

The densities of undesired organisms and clumping cell aggregates also responded to treatments. They were highest in the mix treatment and lowest with ammonium chloride. The diversity of nitrogen sources in the mixed treatment

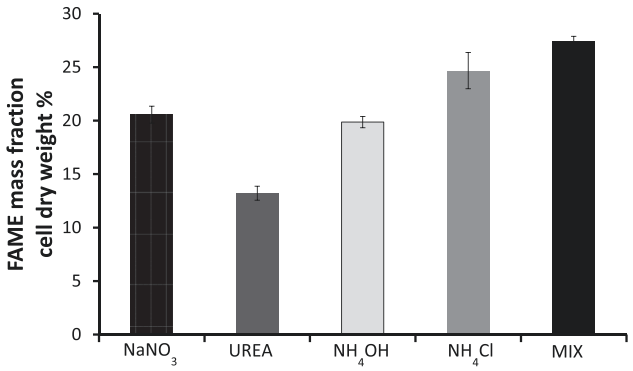

Fig. 5 – FAME content (*per cent* by weight) for *Nannochloropsis salina* cultures at sampling day 28 ± 1 SE ($n = 5$).

seems to have increased the abundance of invading organisms, in particular cyanobacteria. In aquaria with ammonium chloride, invaders seemed to have been inhibited, but that treatment also negatively impacted the growth of *N. salina*. Clumping of microalgae cells might be caused by *N. salina* itself, or could be an indication of bacteria or fungi present in the treatments. Unfortunately, we were unable to consider bacteria, fungi and viruses in our study. Clumping of microalgae was observed in all treatments with the exception of the urea treatment. In general, invading organisms are expected to vary by geographical location and season. This is the third time we are reporting invading organism and all experiments were conducted at the same location – Las Cruces, NM, USA [compare to 8,10]. We find that types of organisms invading as well as abundances are highly variable. However, over the course of the three experiments, diatoms are the most common invading organisms that were found during all seasons. So far, ciliates were only found during spring and summer and were not found in this study, which was conducted during the fall. Cyanobacteria tend to appear in late summer/fall and this is the first time that we are reporting flagellates as invading organisms.

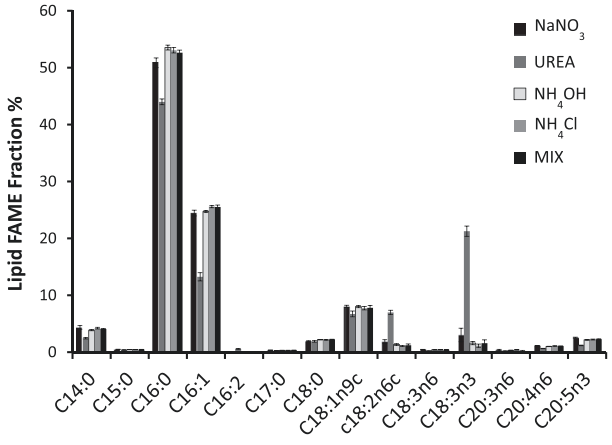

Fig. 6 – Distribution of FAME components for variable nitrogen treatments; ± 1 SE ($n = 5$).

We found the highest lipid accumulation, measured as FAMEs, in the mixed nitrogen treatments (27.5% FAME by weight) for *N. salina*. Li et al. [13] found higher lipid accumulation in *Neochloris oleoabundans* using a nitrate treatment compared to their ammonium or urea treatments. In contrast, we did not find a significant difference between our nitrate and ammonium treatments in terms of lipid accumulation. The fact that Li et al. [13] did not apply a mixed treatment, used a different microalgae, and ended their experiment considerably sooner than we did (7 days vs 28 days) might explain these differences. The concentration of lipid was around or above 20% by weight for all other treatments, except urea, which did not reach values above 13%. The period of highest lipid accumulation of the various treatments may occur at different times. The urea treatment in particular may not have reached the ideal phase for harvest as indicated by their small cell size. However, a delay in harvest will coincide with higher costs to run a production facility and could potentially also carry a higher risk of culture failure. Interestingly, we also find an elevated proportion of C18:3n3 FAME in the urea treatment samples. *N. salina* does not produce C18:n3n3 fatty acid moieties in significant quantity [32] and this FAME signature must therefore derive from one or more invading species. Since we did not find higher numbers of invading organisms in the urea treatment, the difference might lay within the particular species of invader. However, while we are unable to clearly dis-entangle FAME signatures between *Nannochloropsis* and invaders, this is one of the few studies that evaluates and takes invading organisms into consideration at all. All outdoor experiments are bound to have invading organisms but they are missed, if only absorbance values or biomass (weight) are used for microalgae productivity estimates. While several studies have established that nitrogen limitation can increase lipid accumulation in some microalgae [16,33], this study is one of the few studies that demonstrates that nitrogen source itself, can impact lipid accumulation.

5. Conclusions

The results from this study suggest that production of *N. salina* (biovolume and lipid accumulation) could increase when grown with various nitrogen sources (ammonium, nitrate, urea) simultaneously. This, however, might also increase the biomass of undesired organisms that could increase the likelihood of a microalgae crash. Although in this study, none of the tanks crashed. Alternatively, this increased interaction between invading organisms and *N. salina* may have maximized stress induced lipid production. We advocate an approach that provides multiple sources of microalgae available nitrogen which may alter assimilation rates and ultimately microalgae's physiological responses. While other viable approaches to increase production are being advanced, open systems remain a suitable option, with the potential for affordable large scale production. Environmental conditions (e.g., nitrogen source) may be manipulated for maximizing lipid yields.

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REFERENCES

[1] Knothe G. Biodiesel and renewable diesel: a comparison. Prog Energ Combust 2010;36:364–73.
[2] Janaun J, Ellis N. Perspectives on biodiesel as sustainable fuel. Renew Sust Energ Rev 2010;14:1312–20.
[3] Mata TM, Martins A, Caetano NS. Microalgae for biodiesel production and other applications: a review. Renew Sust Energ Rev 2010;14:217–32.
[4] Singh A, Nigam SN, Murphy JD. Renewable fuels from algae: an answer to debatable land based fuels. Bioresour Technol 2011;102:10–6.
[5] Chisti Y. Constraints to commercialization of algal fuels. J Biotechnol 2013;167:201–14.
[6] Lardon L, Helias A, Sialve B, Steyer J, Bernard O. Life cycle assessment of biodiesel production from microalgae. Environ Sci Technol 2009;43:6475–81.
[7] Moazami N, Ashori A, Ranjbar R, Tangestani M, Eghtesadi R, Nejad AS. Large-scale biodiesel production using microalgae biomass of *Nannochloropsis*. Biomass Bioenerg 2012;39:449–53.
[8] Bartley ML, Boeing WJ, Corcoran AA, Holguin FO, Schaub T. Effects of salinity on growth and lipid accumulation of biofuel microalga *Nannochloropsis salina* and invading organisms. Biomass Bioenerg 2013;54:83–8.
[9] Moheimani NR. Inorganic carbon and pH effect on growth and lipid productivity of *Tetraselmis suecicia* and *Chlorella* sp (Chlorophyta) grown outdoor in bag photobioreactors. J Appl Phycol 2013;25:387–98.
[10] Bartley ML, Boeing WJ, Dungan BN, Holguin FO, Schaub T. pH effects on growth and lipid accumulation of biofuel microalgae, *Nannochloropsis salina*, and organisms invading. J Appl Phycol; 2014. in press.
[11] Rocha JMS, Garcia JEC, Henriques MHF. Growth aspects of marine microalga *Nannochloropsis gaditana*. Biomol Eng 2003;20:237–42.
[12] Van Wagenen J, Miller TW, Hobbs S, Hook P, Crowe B, Huesemann M. Effects of light and temperature on fatty acid production in *Nannochloropsis salina*. Energies 2012;5:731–40.
[13] Li Y, Horsman M, Wang B, Wu N, Lan CQ. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Appl Microbiol Biotechnol 2008;81:629–36.
[14] Arudchelvam Y, Nirmalakhandan N. Energetic optimization of algal lipid production in bubble columns: part1: evaluation of gas sparging. Biomass Bioenerg 2012;46:757–64.
[15] Rodolfi L, Zittelli GH, Bassi N, Padovani G, Biondi N, Bonini G, et al. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low cost photobioreactor. Biotechnol Bioeng 2009;102:100–12.
[16] Wan C, Bai FW, Zhao XQ. Effects of nitrogen concentration and media replacement on cell growth and lipid production of oleaginous marine microalga *Nannochloropsis oceanica* DUT01. Biochem Eng J 2013;78:32–8.
[17] Roessler PG. Environmental control of glycerolipid metabolism in microalgae: commercial implications and future research directions. J Phycol 1990;26:393–9.
[18] Pal D, Khozin-Goldberg I, Cohen Z, Boussiba S. The effect of light, salinity, and nitrogen availability on lipid production by *Nannochloropsis* sp. Appl Microbiol Biotechnol 2011;90:1429–41.
[19] Xin L, Hong-ying H, Ke G, Jia Y. Growth and nutrient removal properties of a freshwater microalgae *Scenedesmus* sp. LX1 under different kinds of nitrogen sources. Ecol Eng 2010;36:379–81.
[20] Göksan T, Ak I, Kiliç C. Growth characteristic of the alga *Haematococcus pluvialis* Flotow as affected by nitrogen source, vitamin, light and aeration. Turk J Fish Aquat Sci 2011;11:377–83.
[21] Boussiba S, Vonshak A, Cohen Z, Avissar Y, Richmond A. Lipid and biomass production by the halotolerant microalgae *Nannochloropsis salina*. Biomass 1987;12:37–47.
[22] Sheehan J, Dunahay T, Benemann J, Roessler P. A look back at the U.S. Department of Energy's Aquatic Species Program — biodiesel from algae. Golden CO: National Renewable Energy Laboratory; 1998 July. p. 328. Report NREL/TP-580–24190.
[23] Guillard RRL, Rhyther JH. Studies of marine planktonic diatoms: I. *Cyclotella nanahustedt*, and *Detonula confervacea* (cleve) gran. Can J Microbiol 1962;8:229–39.
[24] Griffiths MJ, van Hille RP, Harrison ST. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. Lipids 2010;45:1053–60.
[25] Bigelow NW, Hardin WR, Barker JP, Ryken SA, MacRae AC, Cattolico RA. A comprehensive GC-MS sub-microscale assay for fatty acids and its applications. J Am Oil Chem Soc 2011;88:1329–38.
[26] Laurens LML, Quinn M, Van Wychen S, Templeton DW, Wolfrum EJ. Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by in situ transesterification. Anal Bioanal Chem 2012;403:167–78.
[27] Patil P, Reddy H, Muppaneni T, Mannarswamy A, Holguin O, Schaub T, et al. Power dissipation in microwave-enhanced in-situ transesterification of algal biomass to biodiesel. Green Chem 2012;14:809.
[28] IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.
[29] Xu Y, Boeing WJ. Modeling maximum lipid productivity of microalgae: review and next step. Renew Sust Energ Rev 2014;32:29–39.
[30] StatSoft, Inc. Electronic statistics textbook. Tulsa, OK: StatSoft; 2013. http://www.statsoft.com/textbook.
[31] Dortch Q. The interaction between ammonium and nitrate uptake in phytoplankton. Mar Ecol Prog Ser 1990;61:183–201.
[32] Volkman JK, Brown MR, Dunstan GA, Jeffrey SW. The biochemical composition of marine microalgae from the class Eustigmatophyceae. J Phycol 1993;29:69–78.
[33] Illman AM, Scragg AH, Shales SW. Increase in *Chlorella* strains colorific values when grown in low nitrogen medium. Enzyme Microb Technol 2000;27:631–5.