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Effect of nitrogen source and acclimatization on specific growth rates of microalgae determined by a high-throughput in vivo microplate autofluorescence method

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Abstract Specific growth rates (SGR) of freshwater algae species (*Chlorella vulgaris*, *Auxenochlorella protothecoides*, and *Chlorella sorokiniana*) and the marine species *Nannochloropsis oculata* on various nitrogen sources (ammonium carbonate, ammonium chloride, sodium nitrate, and urea) could be determined by in vivo chlorophyll-a autofluorescence. These preferences could be determined before large pH changes occurred in the media, with no significant difference ($P>0.05$) between buffered and non-buffered media. In all algal species, acclimatization was observed with no significant difference ($P>0.05$) between SGRs of the second and third cultivations. ANOVA of SGRs in the acclimatized second and third cultivations revealed preferences for nitrogen sources among most of the algae; *C. vulgaris* preferred sodium nitrate over other nitrogen sources, *A. protothecoides* adapted to urea after no growth in the first cultivation, and the SGRs of *N. oculata* showed an aversion for sodium nitrate over other nitrogen sources ($P<0.05$).

Keywords Adaptation · *Auxenochlorella* · *Chlorella* · *Nannochloropsis* · pH changes · Industrial wastewater

Introduction

Atmospheric CO_2 and global temperatures continue to rise from human influence and largely from the combustion of fossil fuels (USEPA 2010). Meanwhile, algal biotechnologies have demonstrated the potential to produce drop-in petroleum replacements with the possibility of reducing these fossil fuel combustion emissions. Nitrogen supply constitutes a major cost of large-scale algal production facilities and is an important factor influencing the economic feasibility of commercial algal production (Peccia et al. 2013; Sturm and Lamer 2011). A more efficient use of nitrogen by choosing the appropriate nitrogen source may lead to more efficient and economical production of algae for high-value products. "Technical wastewaters," i.e., industrial side streams or by-products that could possibly be used as substrates for other biological processes, often contain significant amounts of nitrogen. These technical wastewaters could constitute an attractive alternative for nitrogen fertilizers in algal productions, while simultaneously treating water. Treating industrial wastewaters with algae requires a rapid screening technique to screen growth rates of numerous algae containing high-value product grown at various wastewater concentrations. Such a technique must be able to accommodate the large physiochemical changes caused by nitrogen assimilation.

It is well known that most algae have the ability to utilize various forms of nitrogen, following a variety of pathways ultimately leading to the assimilation of ammonium into amino acids (Hodson and Thompson 1969; Solomon and Glibert 2008; Perez-Garcia et al. 2011). Technical wastewaters may have vastly different compositions which can include nitrogen sources such as ammonium, nitrate, urea, as well as amino acids in varying concentrations. Nitrate is primarily used in algal media because it is not evaporated like ammonium and urea nitrogen sources during autoclavation applied during preparation of the media for the algal cultivation. Therefore, ammonia and urea must be added aseptically post autoclavation (Harisson and Berges 2005). Nevertheless, in most algal species all nitrogen sources must be converted to ammonium inside the algal cell before being assimilated into biomass, which requires energy. When nitrate is used as a nitrogen source, the nitrate is first reduced to nitrite by an

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energy-requiring reaction catalyzed by nitrate reductase followed by the reduction to ammonium by ATP-dependent or energy-dependent nitrite reductase, where it is finally synthesized into amino acids (Perez-Garcia et al. 2011). Similarly, urea is converted to bicarbonate and ammonium by the energy-dependent urease (Solomon and Glibert 2008). Alternatively, some species of green algae such as *Chlorella* spp. lack the urease enzyme and rely on two energy-dependent enzymes: urea carboxylase and allophanate (Hodson and Thompson 1969) or urea amidylase (Perez-Garcia et al. 2011). Though ammonium could appear to be favorable by all accounts, other genetic and physiological properties such as light, presence of organic carbon, heavy metals, and active and passive transport of the molecules may result in alternative algal nitrogen preferences. In some cases, excess ammonium can become inhibitory as well (Sunda et al. 2005).

A high-throughput method of algal cultivation is desirable to assess the growth viability of numerous algae on technical wastewaters. High-throughput microplate growth inhibition assays have been used to rapidly determine inhibition of microorganisms from a multitude of toxicological effects by observing specific growth rates determined by optical density or autofluorescence of chlorophyll-a (Eisentraeger et al. 2003). Similarly, algae have been used in microplate assays to determine the sensitivity to contaminants (Blaise and Vasseur 2005; Eisentraeger et al. 2003) with the benefit of low detection ranges using in vivo autofluorescence (Mayer et al. 1997). Furthermore, batch algal cultivations in microplates have the advantage that many measurements can be made, without disturbances and losses by sampling. Larger algal cultivations can be hindered by the high labor intensity and maintaining pure axenic algal cultures during frequent sampling for growth monitoring. Eisentraeger et al. (2003) reported that 24-well and 96-well microplate growth-inhibition assays were nearly identical to results obtained from 100 mL Erlenmeyer flask assays for the algal species *Desmodesmus subspicatus* (formerly *Scenedesmus subspicatus*). However, toxicological screenings rely on comparing specific growth rates after the addition of a toxicant in identical media, which includes identical nitrogen sources (Eisentraeger et al. 2003).

Different nitrogen sources result in large physiochemical changes in the culture due to various modes of nitrogen assimilation. In determining the best algal strain to be grown on technical wastewaters, it is also important to consider how the algal specific growth rates were affected by the resultant physiochemical changes from the nitrogen composition of the technical wastewaters. In batch cultivations, ammonium (NH_4^+) consumption in algae is generally followed by significant drops in pH due to the consumption of bicarbonate (Mayer et al. 1997); alternatively, nitrate (NO_3^-) consumption in batch cultivations results in elevated pH from bicarbonate production (Grobbelaar 2004). This effect makes the comparison of specific growth rates of algae grown on different nitrogen sources difficult by necessitating pH control and, therefore, larger and more time-consuming methods. However, an alternative to pH control may be to exploit the low detection limit of in vivo autofluorescence of algae during exponential growth inside a microplate, before large physiochemical changes occur in the culture.

The aim of this research was to determine the preference of nitrogen source for algal growth for four industrially important microalgae by using a microplate screening method. Additionally, the adaptation potential to various nitrogen sources upon successive cultivations was investigated. Furthermore, we investigated the viability of microplate screening using in vivo chlorophyll-a autofluorescence. The pH changes and time of occurrence were tested in the media with the different nitrogen sources.

Materials and methods

Four species of algae were used in this study: the freshwater algae *Chlorella vulgaris*, *Auxenochlorella protothecoides*, *Chlorella sorokiniana*, and the marine alga *Nannochloropsis oculata* (details in Table 1).

Freshwater algae were grown in modified Woods Hole medium (MWC; Guilard and Lorenzen 1972) containing selenium. The saltwater *N. oculata* was grown on an artificial seawater L1 medium (Guillard 1975; Table 1). The media were modified to contain one of four different nitrogen

Table 1 The details of the four algal species selected for this study

Species	Culture collection	Strain no.	Media (habitat)	Stock nitrogen source
vulgaris	SCCAP	K-1006	MWC+Se (fresh)	Sodium nitrate
protothecoides	CCAP	211/8D	MWC+Se (fresh)	Ammonium carbonate
sorokiniana	SAG	1.80	MWC+Se (fresh)	Sodium nitrate
oculata	SCCAP	K-1281	L1 (marine)	Sodium nitrate

SCCAP Scandinavian Culture Collection of Algae and Protozoa, *CCAP* Culture Collection of Algae and Protozoa, *SAG* Sammlung von Algenkulturen (Culture Collection of Algae)

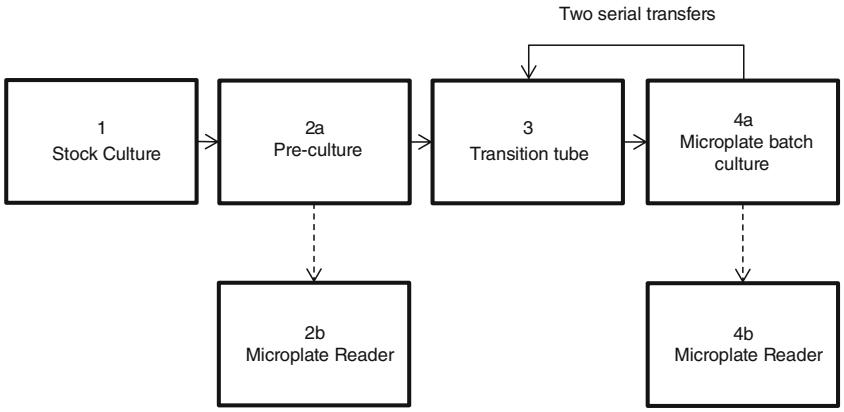

Fig. 1 Workflow: *1* stock cultures received from various culture collections were used to inoculate pre-cultures. *2a* Pre-cultures were grown in 20-mL tissue culture flasks in $NaNO_3$ media on a linear shaking with fluorescent light (60 μmol photons m^{-2} s^{-1}); once green cells became apparent, 2 mL of pre-culture was measured for fluorescence cell concentration. *3* Each strain was then diluted with media of each nitrogen source (ammonium carbonate, ammonium chloride, sodium nitrate, and urea) inside a 15-mL axenic test tube to a desired concentration of 500 RFU. *4a* Contents of the transition tube were split into triplicates inside a microplate and placed on the orbital shaking table with fluorescent light (60 μmol photons m^{-2} s^{-1}). *4b* A minimum of four measurements with 1.5- to 2-h intervals during exponential growth were taken on the microplate reader. Two serial transfers were made of the algae during exponential growth: undergoing dilution in the transition tubes and returning to new microplates for subsequent cultivations

sources at 1 M nitrogen: 85.0 g $NaNO_3$, 48.0 g $(NH_4)_2CO_3$, 53.5 g NH_4Cl, and 30.0 g $CO(NH_2)_2$. The pH was adjusted to 7.5 for all nitrogen source media, where CO_3^{2-} functions as the buffering agent.

Stock cultures were grown on sodium nitrate (1 M) with the exception of *A. protothecoides*, which was grown on ammonium carbonate (1 M) because of its inability to grow on nitrate (0.5 M; Table 1). Subsequent pre-cultures were grown on each nitrogen source, except for *A. protothecoides*, which was grown on ammonium carbonate, as opposed to sodium nitrate. The pre-cultures were grown at 60 μmol photons m^{-2} s^{-1} fluorescent light at a constant temperature of 20 °C on a horizontal shaker table (Gerhardt UK Ltd., UK) until dense green autotrophic seeds in the exponential growth phase were observed, which were then used as inoculum (inoculum to experimental culture ratio of 1:100).

Scaled-up experimental setup for pH monitoring In order to measure the pH change during algal growth, duplicate 300 mL Erlenmeyer flask (100 mL working volume) batch algal cultures were exposed to 60 μmol photons m^{-2} s^{-1} fluorescent light on a linear shaker table. Two-milliliter sample volumes were extracted from the flasks and placed inside a microplate for fluorescence measurements throughout the growth cycle. Subsequently, the pH was measured for each sample.

Microplate batch culture experimental setup Semi-continuous microplate batch cultures for each of the four algae were grown in a Costar 24-well, clear, flat-bottom microplate well (Corning) of the four different nitrogen sources (ammonium carbonate, ammonium chloride, sodium nitrate, and urea) in triplicates. Each plate contained a single culture grown on each of the four nitrogen sources with triplicates under equal light conditions. Microplates were mixed by shaking on an orbital shaker (Kühner Shaker X, Adolf Kühner AG, Switzerland) at 175 rpm (50 mm throw) modified to permit fluorescent light from beneath the microplate in a 24 h constant light regime. The light remained stationary, while a modified frame holding the microplates rotated above, resulting in an average light intensity of 60 μmol photons m^{-2} s^{-1} (Fig. 1).

A successive cultivation experiment was set up for disclosing possible adaptation tendency of the algae to a specific nitrogen source. During the successive cultivation experiment, algae were initially transferred into fresh media containing each nitrogen source where they were incubated until they were at exponential growth or at the early stationary phase. Then, a new batch was set up, inoculated from the previous cultivation. Twenty microliters of the algal culture was used as inoculum and was transferred into fresh media for a total volume of 2 mL. Inoculum was transferred to three wells for replication of the data. Each microplate cover was discarded and replaced with a Breathe-Easy (Diversified Biotech, USA) gas-permeable sealing membrane, which permits gas exchange while minimizing contamination.

Analyses In vivo cell concentration was measured with a Synergy Mx multimode microplate reader (BioTek Instruments, Inc., USA) measuring the autofluorescence of chlorophyll-a using an excitation wavelength of 440 nm and an emission wavelength of 690 nm. Fluorescence measurements were reported as relative fluorescence units (RFU). Fluorescence measurements were only deemed valid as a proxy for cell number during exponential growth. Actual cell concentrations were measured using a cell counter (Coulter

Z2, Beckman, Germany) at the beginning and end of each microplate experiment.

Both pH measurements and autofluorescence values (RFU) were determined for all experiments. pH was measured using a FiveEasy plus pH meter (Mettler-Toledo).

Specific growth rate calculation The specific growth rate (μ) was calculated by a linear transformation of the fluorescence (RFU) values, where μ_{max} was the slope of linearized exponential growth equation $F_t=F_0e^{\mu t}$ (F_t RFU after t days, F_0 initial RFU). The exponential growth period was evaluated visually in a plot of RFU vs. time. A minimum of three data points in the exponential phase were used for the determination of the specific growth rate.

Statistics A two-way ANOVA with comparison was executed on the specific growth rate data for microplates, and a t test was executed on specific growth rate data of flask experiments using SigmaPlot 11.0 statistical software. The two-way ANOVA with comparison was performed with cultivation period and nitrogen source as treatment variables. The null hypothesis of the two-way ANOVA and t test was rejected for P values less than 0.05. All results are given as the average± standard deviation. The reported specific growth rates were the mean of independent replicates (n=3)±standard deviation (SD) for microplate measurements. Graphical reports of fluorescence (RFU) and pH for 300-mL Erlenmeyer flask cultures were the mean of two independent replicates (n=2).

Results

In vivo autofluorescence detection and pH influence on specific growth rate

In vivo chlorophyll-a autofluorescence was directly proportional to cell number in all the tested algal species at low concentrations (<15,000 RFU; Fig. 2). For the *Chlorella* species in this experiment, there is a direct linear correlation of RFU to cell number for all points tested. For *N. oculata*, a linear fit of all five data points reveals an r^2=0.977; however, if a linear fit is applied to the first four data points (<15,000 RFU), an r^2=0.999 is achieved. For *C. vulgaris*, the r^2 for the first four, five, and all six points are 0.999, 0.999, and 0.997, respectively. When fitted with a second-order polynomial on all data points, *N. oculata* and *C. vulgaris* had r^2 values of 1.0 and 0.999, respectively (Fig. 2). For *N. oculata*, it has been shown that all specific growth rates can be determined before 15,000 RFU, using 1.5- to 2-h sampling intervals, where a more accurate correlation to cell number can be made using the four-point linear correlation (Fig. 3). *C. vulgaris* can be correlated directly with precision at any level within the limits of the fluorescence detection. The same correlations were observed for *A. protothecoides and C. sorokiniana* as well (data not shown).

A comparison of pH and cell concentration (RFU) for *A. protothecoides* demonstrates that a growth curve was evident before (<4 days) large pH changes occurred in both ammonium chloride media and buffered ammonium carbonate media. These two nitrogen sources show a pH decrease after day 4 with distinctly different pH consequences, where ammonium chloride media dropped from pH 7.5 down to pH 3.7 at day 9, while ammonium carbonate only decreased slightly (Fig. 4). The specific growth rates of the flask culture for ammonium carbonate and ammonium chloride were 0.88 ±0.03 and 0.93±0.10 day^{-1}, respectively; however, there was no significant difference between specific growth rates (P= 0.543). Fluorescence data beyond day 4, which had reached the limits of detection of in vivo autofluorescence, were not included.

Acclimated specific growth rates

Acclimatization to the new environment was evident through statistically different specific growth rates of the algae from

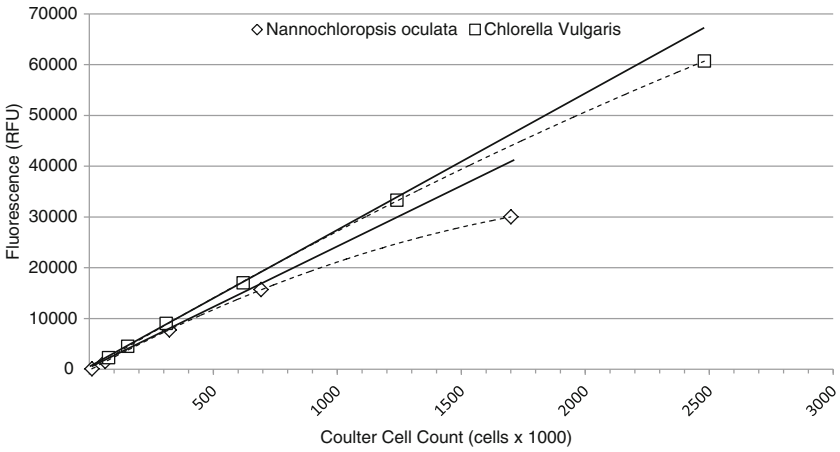

Fig. 2 Fluorescence vs. coulter cell number for *C. vulgaris* and *N. oculata*. The *solid lines* represent the linear regression of the first five points for *C. vulgaris* and the linear regression of the first four points for *N. oculata*. The *curved dotted lines* represent the a second-order polynomial regression trend for each alga

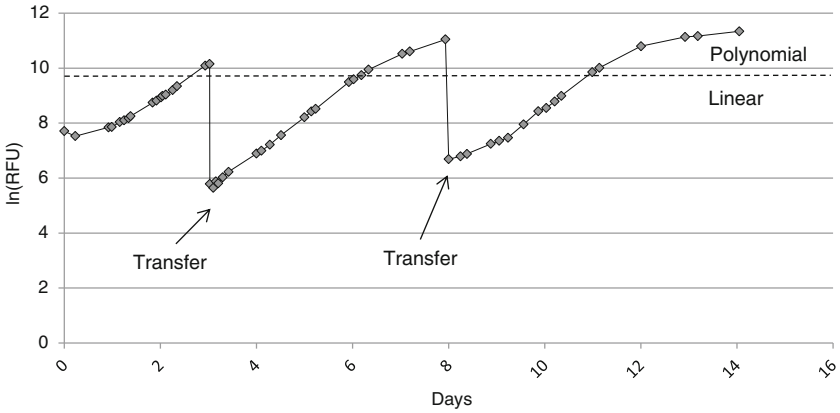

Fig. 3 The natural logarithm of fluorescence (RFU) of *Nannochloropsis oculata* cultivated in media containing ammonium chloride over two serial transfers (*arrows*). During all growth cycles, biomass and subsequently specific growth rate could be determined before 15,000 RFU (*above dashed line*). The microalgae were cultivated until the upper fluorescence limit was reached in the last transfer

the first to second cultivation, followed by consistent statistical similarity between specific growth rates from the second to third cultivation. *C. vulgaris* grown on ammonium chloride and sodium nitrate had significantly higher specific growth rates in the second cultivation when compared to the first ($P<0.05$). *A. protothecoides* had significantly lower ($P<0.05$) specific growth rates in the first cultivation compared to the second cultivation for ammonium carbonate, ammonium chloride, and urea, where there was no growth on urea in the first cultivation and no growth on sodium nitrate throughout the experiment. The specific growth rate of *C. sorokiniana* was significantly higher in the first cultivation compared to the second cultivation only for ammonium carbonate ($P<0.05$), while all other sources exhibited no significant difference ($P>0.05$). *N. oculata* had significantly lower specific growth rates in the first cultivation compared to the second cultivation ($P<0.05$). There was no significant difference ($P>0.05$) in specific growth rate between the second and third cultivations grown on each respective nitrogen source for all of the algae.

The two-way ANOVA showed that there were no significant interactions between the second and third cultivation data for any type of nitrogen source ($P>0.05$); however, there was a significant interaction when the first cultivation data was included in the test for *A. protothecoides* and *N. oculata*. Without significant interaction of these two variables, an evaluation of the preferred nitrogen source could be made starting at the second cultivation of algal growth in most of the algae.

Nitrogen preference

A nitrogen source preference or aversion for most algal species was evident in the performed experiments after omitting the first cultivation of growth. Specific growth rates of all four algae grown in the four media can be seen in Fig. 5.

In *C. vulgaris*, the specific growth rates of the algae grown on sodium nitrate were significantly higher than the specific growth rates of the algae grown on urea, ammonium carbonate, and ammonium chloride ($P<0.05$). There was no significant difference in specific growth rates of *C. vulgaris* grown on ammonium carbonate and ammonium chloride ($P>0.05$). Despite there being no significant difference ($P>0.05$) between ammonium carbonate and urea, ammonium in the form of ammonium chloride had a significantly higher ($P<0.05$) specific growth rate than urea.

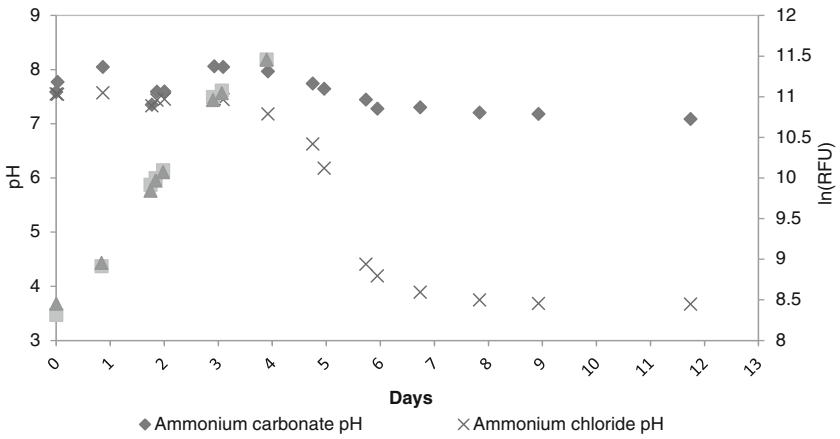

Fig. 4 Time course, pH, and the natural logarithm of the fluorescence (RFU) of *A. protothecoides* grown in ammonium carbonate and ammonium chloride

A. protothecoides showed no growth at all on sodium nitrate, and all remaining nitrogen sources had significantly ($P<0.05$) higher specific growth rates. Although *A. protothecoides* showed no growth on sodium nitrate as a nitrogen source, the cells remained alive and green in microscopic examinations (data not shown). Furthermore, there were also significantly higher specific growth rates when the algae were grown on ammonium chloride compared to urea ($P<0.05$); however, there was no overall significant difference between specific growth rates when ammonium carbonate or urea was used as the nitrogen source ($P>0.05$).

In general, *C. sorokiniana* did not show a preference for any of the nitrogen sources with significantly similar ($P>0.05$) specific growth rates for all nitrogen sources after omitting the first cultivation of growth; however, it showed a significant preference for urea over sodium nitrate in the second cultivation, explained by the significant increase in specific growth rate ($P<0.05$). The significantly higher specific growth rate of urea over sodium nitrate was most likely due to the erratic results between triplicates of the specific growth rate of *C. sorokiniana* grown on urea in the second cultivation of growth (SD=0.63), which subsided in the third cultivation (SD=0.14).

Finally, after removing the first cultivation of growth from the ANOVA test, the specific growth rates of *N. oculata* grown on sodium nitrate were significantly lower ($P<0.05$) than the ones when grown on all other nitrogen sources. Between ammonium carbonate, ammonium chloride, and urea, there was no statistical difference ($P>0.05$) between specific growth rates.

pH assay in microplates

There was no significant difference between the specific growth rates of *C. vulgaris*, *A. protothecoides*, *C. sorokiniana*, and *N. oculata* in cultures of ammonium chloride and ammonium carbonate in the second and third cultivations after acclimatization ($P<0.05$; Fig. 5).

Discussion

The four microalgae species were successfully cultivated on all nitrogen sources tested with only one culture test without growth. Differences in growth and adaptation

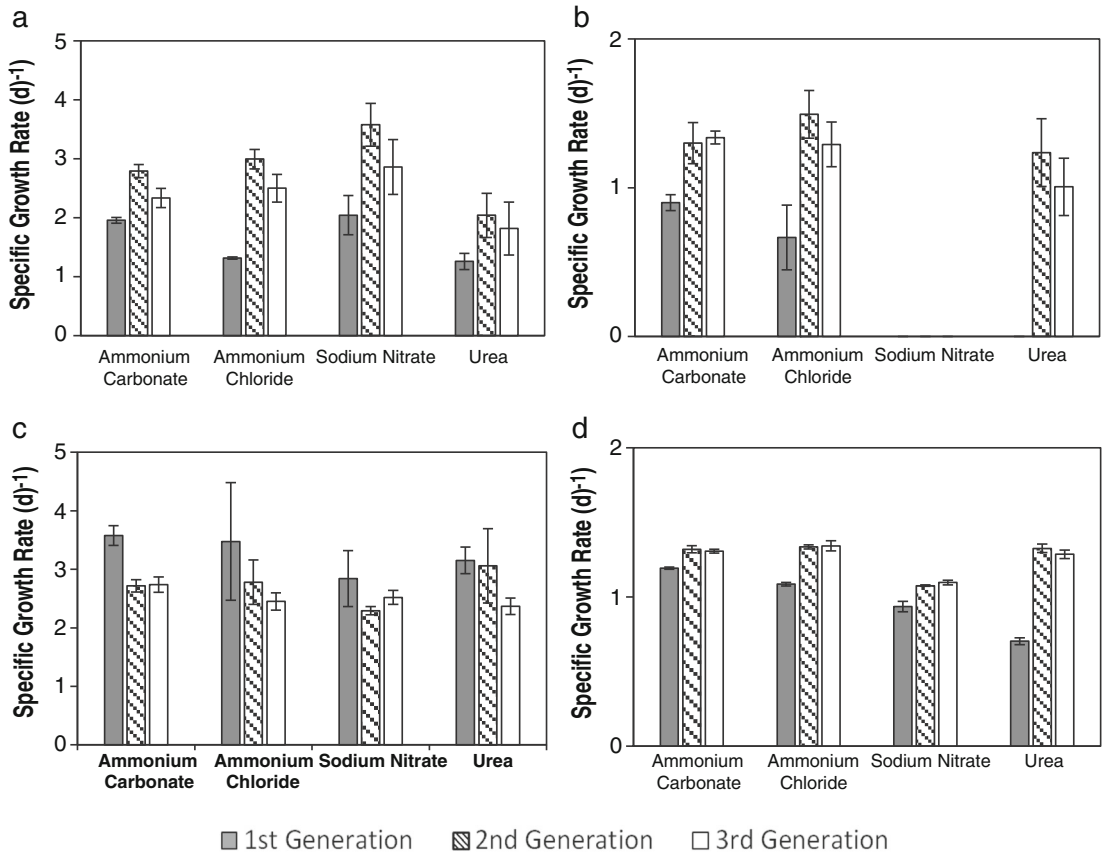

Fig. 5 Specific growth rate (day^{-1}) of **a** *Chlorella vulgaris*, **b** *Auxenochlorella protothecoides*, **c** *Chlorella sorokiniana*, and **d** *Nannochloropsis oculata* grown over three cultivations on the four different nitrogen sources (ammonium carbonate, ammonium chloride, sodium nitrate, and urea). The nitrogen source concentration was 1 M nitrogen. Note the *different scales* of the *y*-axis

were observed and could be measured before substantial changes in pH occurred.

Early detection with in vivo autofluorescence before changes in pH For *N. oculata*, a comparison of the five-point linear fit and four-point linear fit suggests the use of the four-point linear fit below values of 15,000 RFU. It has also been demonstrated that several points in exponential growth can be determined below 15,000 RFU, using 1.5- to 2-h sampling intervals. Furthermore, in *N. oculata*, values above 15,000 RFU implicate some type of limitation of the correlation, where autofluorescence is no longer a direct proxy for cell number and can result in a non-linear correlation (Wood et al. 2005) which may be caused by the possible self-shading of chlorophyll-a. Alternatively, if exponential growth values occurred at higher cell concentrations, cell counting would be required for each of the screened algal species to determine a specific non-linear correlation to cell number due to changes in the chlorophyll-a content for each screened species at a given light condition, which can change the concentration of chlorophyll-a (MacIntyre and Cullen 2005). In *Nannochloropsis* spp., chlorophyll-a can range from 0.18 to 0.46 % of the biomass (Rebolloso-Fuentes et al. 2001). Depending on culture conditions, *C. vulgaris* can contain 1.57 to 2.16 % chlorophyll-a (Sharma et al. 2012). The polynomial fit of *C. vulgaris* had a higher r^2 value than the linear fit for all six points, again demonstrating the polynomial trend evidenced in *N. oculata*. This may be a possible concern in the screening of other species, as well as screenings at low light, where the amount of chlorophyll-a is expected to increase (MacIntyre and Cullen 2005).

Similarly, the lack of significant differences ($P<0.05$) between ammonium carbonate and ammonium chloride nitrogen sources when comparing the second and third cultivation data in the microplate experiments confirms that early autofluorescence detection can be implemented before a significant pH change (<70,000 RFU). Mayer et al. (1997) discussed similar results, where low density detection of exponential growth, using in vitro autofluorescence of chlorophyll-a, in *Selenastrum capricornutum* was possible before the onset of large physiochemical changes (e.g., pH). Typically, utilization of ammonia will result in lower pH and potential inhibition of algal growth. In this study, pH in green 100 mL algal cultures was as low as 3.68. Shi et al. (2000) reported that the pH in heterotrophic cultures of *Chlorella protothecoides* grown on ammonium was reduced to below 4.0, which resulted in the death of the culture.

Effect of acclimatization and nitrogen source on specific growth rates In this work, it was evident that after two successive cultivations, acclimatization to new media or the new microplate environment was achieved when cultivating algae at new conditions or new media composition. However, Wood et al. (2005) mentioned that complete acclimatization can take several cultivations especially for in vivo fluorescence, where the expression of chlorophyll molecules was highly sensitive to changing light sources. Despite the sensitivity of chlorophyll expression to new light conditions, the relative chlorophyll-a content used to measure growth rate during exponential growth remained unchanged and viable for specific growth rate determinations. Furthermore, significant differences ($P<0.05$) from the first cultivation compared to the second showed that the first cultivation had little reliability for nitrogen source comparisons. Adaptation to new culture conditions can be an important aspect for determining media preference, especially in the case of macronutrients. Hildebrand (2005) showed that ammonium transporters in the diatom *Cylindrotheca fusiformis* were upregulated when grown on nitrate, an effect also observed during nitrogen limitation. Such an effect may cause an uncharacteristic preference for ammonium over other nitrogen sources and should be considered during future screenings. Unlike most *Chlorella* species, *C. vulgaris* K-1006 showed a significant preference for nitrate in the second and third cultivations after acclimatization. In general, green algae show a distinct preference for ammonium when exposed to ammonium and nitrate simultaneously (L'Helguen et al. 2008; Hyenstrand et al. 2000; Maguer et al. 2007). This is usually due to the downregulation enzymes for nitrate assimilation (Syrett and Morris 1963; Bates 1976; Caperon and Ziemann 1976; Terry 1982). A predictive model for marine phytoplankton by Flynn et al. (1997) suggests that, in the presence of nitrate, there is also a depression of ammonium transport. Similarly, Caperon and Zieman (1976) showed that the presence of nitrate in the incoming media of a continuous culture of *Monochrysis lutheri* depressed the specific uptake rate of ammonium. It is possible that there was an inhibitory effect of nitrate on ammonium uptake; however, this was reported to occur at high nitrate concentrations (Caperon and Ziemann 1976; Dortch 1990). Dortch and Conway (1984) observed that, in the diatoms *Skeletonema costatum* and *Chaetoceros debilis*, a simultaneous addition of ammonium and nitrate would reduce uptake rates for each compared to the uptake rates of an addition of each nitrogen source, separately. They also observed inhibition of ammonium uptake in *S. costatum*, when the culture was preconditioned on nitrate. Furthermore, in some algal cells grown on nitrate, nitrogen uptake can exceed specific growth rate because of the rate-limiting enzymatic reactions of nitrate and nitrite reductase, which results in accumulation of the nitrogen source (Dortch 1982; Needoba and Harrison 2004), and it is unknown whether such pools of nitrate persist into the later cultivations inhibiting ammonium assimilation, which may be in effect in *C. vulgaris*.

The significant ($P>0.05$) aversion for nitrate found in *A. protothecoides* was consistent with reports that no species in the subgenus *Auxenochlorella* can utilize nitrate, even

during heterotrophy (Shihira and Krauss 1965). However, Shi et al. (2000) reported that *C. protothecoides* CS-41 did have a preference for nitrate and urea over ammonium expressed as specific growth rates; however, this was during heterotrophic conditions. Shen et al. (2010) also reported heterotrophic growth of *C. protothecoides* on potassium nitrate. However, the preference was due to the deleterious effects of the lowering pH in the ammonium samples, rather than nitrogen source preference.

In the case where *A. protothecoides* apparently adapted to urea, the ammonium in the stock culture may have downregulated enzymes, such as urea amidolyase, since ammonium is energetically favorable to other nitrogen sources, thereby necessitating a long acclimation period to the new media of one cultivation period. *A. protothecoides*, having shown adaptation to the urea starting in the second cultivation, did so due to a possible downregulation of urea-metabolizing enzymes in an ammonium carbonate stock culture. Solomon and Gilbert (2008) showed that ammonium may downregulate urease in dinoflagellates; however, *Chlorella* spp. metabolize urea via the enzyme urea amidolyase (Perez-Garcia et al. 2011), which may be subjected to downregulation, as well, as a possible explanation. Alternatively, Solomon and Gilbert (2008) showed that the presence of nitrate or urea can upregulate urease activity in dinoflagellates. Upregulation of urea-reducing enzymes from the presence of nitrate may be a possible explanation for why other algal strains in the study, preconditioned on nitrate media, grew on urea starting in the first cultivation.

Unlike the other alga, *C. sorokiniana* underwent an experimental mishap, where the pH was not adjusted to pH 7.5 in the ammonium carbonate media, and the experiment was repeated for another three cultivation cycles, with the pH adjusted, for a total of six cultivations inside the microplate. Consistent with the mishap, only the first cultivation of *C. sorokiniana* grown on ammonium carbonate media had a significant difference ($P<0.05$) to the second cultivation, where the other nitrogen sources had no significant difference ($P>0.05$) between the first and second cultivation specific growth rates. This suggests that acclimatization had already occurred in the ammonium chloride, sodium nitrate, and urea media due to the prolonged acclimation period to the nitrogen sources and microplate environment before the experiment. Incidentally, the highest observed average specific growth rate throughout the experiment was 3.58 day^{-1}, which occurred in the first cultivation of *C. sorokiniana* growth on ammonium carbonate, where the previously reported highest specific growth rate for *C. sorokiniana* was 6.48 day^{-1} (Sorokin 1959).

The significant aversion observed in *N. oculata* does not agree with results by Dong et al. (2000), where the highest specific growth rate occurred with nitrate as a nitrogen source. Urea uptake rates can be higher than nitrate uptake rates in many marine dinoflagellates and in some phytoplankton (Solomon and Glibert 2008). The dinoflagellate *Lingulodinium polyedrum* had higher urea uptake rate than both ammonium and nitrate (Kudela and Cochlan 2000). These higher uptake rates may be due to more concentrated internal pools of ammonium and urea, and urease activity increasing the availability of ammonia as seen in marine dinoflagellates (Solomon and Glibert 2008). Davis et al. (1953) found that the organic nitrogen sources urea and glycine were preferable compared to potassium nitrate for growth of *Chlorella pyrenoidosa*, confirming that urea can also be favored over nitrate.

Conclusions

The results of this study suggest that not all algae have the same nitrogen preference. Among the studied strains/species, the highest specific growth rates in microplates were observed in *C. vulgaris* and *C. sorokiniana*. In general, adaptation to the new environment and nitrogen source took one cultivation before consistent specific growth rates could be observed for comparison. Moreover, it has been shown that microplate batch cultures were adequate to optimize media through comparison, exemplified by these nitrogen source comparisons. This method may be highly suitable for determining algae to grow on technical wastewaters containing different mixtures of nitrogen sources, organic carbon, and micronutrients, which can play an important role in nitrogen uptake.

In vivo autofluorescence most likely has the added benefit of accurately depicting the specific growth rate in batch cultures before physiochemical changes in the media can influence the specific growth rate. This early detection of the specific growth rate by fluorescence before pH changes may have the added benefit of being more analogous to scaled-up experiments and industrial processes with pH control.

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