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Nitrogen uptake by the macro-algae *Cladophora coelothrix* and *Cladophora parriaudii*: influence on growth, nitrogen preference and biochemical composition

Michael E. Ross [a,b], Katharine Davis [a], Rory McColl [a], Michele S. Stanley [b], John G. Day [b], Andrea J.C. Semião [a,*]

[a] School of Engineering, The University of Edinburgh, Edinburgh, EH9 3FB, UK

[b] Scottish Association for Marine Science (SAMS), Scottish Marine Institute, Oban, Argyll, PA37 1QA, UK

Corresponding author:

* asemiao@staffmail.ed.ac.uk

E-mail addresses:

michael.ross@sams.ac.uk

kathzd92@gmail.com

rorymccoll@yahoo.com

michele.stanley@sams.ac.uk

john.day@sams.ac.uk

Abstract

The capacity of macro-algae to remove nutrients means they have the potential to concomitantly bioremediate polluted waters and generate exploitable biomass. The influence of different nitrogen (N) regimes on growth, biochemical composition and bioremediation capacity was studied for two species of the macro-alga Cladophora. These were incubated in media containing four single N sources, ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and urea ($CO(NH_2)_2$), each with four nitrogen/phosphorous (N/P) ratios, followed by equimolar dual mixtures of these N sources at two selected N/P ratios. There were clear differences in growth between species, depending upon the nutrient regime. In every instance, the daily growth rate (DGR) of *Cladophora parriaudii* (4.75 – 11.2%) was higher than that of *Cladophora coelothrix* (3.98 – 7.37%) with significance when either NO_2^- (p = 0.025) or urea (p = 0.002) were the employed N form. Differences in algal productivity were reflected in the corresponding N-uptake, whereby *C. parriaudii* consistently removed more N than *C. coelothrix*. There were significant differences in growth (p = 0.005) when *C. parriaudii* was cultivated in a single and multi-N source medium: NH_4^+ was preferentially removed from the medium, whereas urea was typically removed secondarily. However, the presence of urea in the medium enhanced the uptake of the other co-existing N forms and resulted in an increased DGR and yielded a biomass rich in carbohydrates. The relative composition of *C. parriaudii* varied depending upon N/P ratio of the medium, with the final proportion of protein and carbohydrate ranging from 5-15% and 36-54% per unit dry weight, respectively. Results from this study demonstrated that algal strain selection is key to treating waste-streams with specific N profiles. Additionally, the biochemical profile of the biomass produced is dependent on the alga and the N regime, providing the potential for designing processes with specific properties and products.

Keywords: *Cladophora*, alga, bioremediation, nitrogen removal, growth rate, biochemical composition

Author Contributions:

M.E.R. is responsible for the integrity of the work as a whole, all named authors contributed materially to the execution of the final manuscript and were involved in the inception, data acquisition, analysis and interpretation, drafting and editing the manuscript.

1. Introduction

Nitrogen is typically present in wastewater effluents as either dissolved inorganic nitrogen (DIN), *i.e.* ammonia (NH_3), ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-), or as dissolved organic nitrogen (DON), *i.e.* urea ($CO(NH_2)_2$) [1]. Since some of these substances are hazardous and can have an adverse effect on the aquatic environment, the Water Framework Directive (Directive 2000/60/EC) has issued a variety of directives to improve water quality across Europe, which include the Nitrates Directive 1991 and the Dangerous Substances Directive 2006/11/EC. The development and implementation of novel or innovative biological wastewater treatment (WWT) technologies that can remove these pollutants and reduce their potentially deleterious effects is therefore of vital ecological and political importance.

Macro-algae are a diverse collection of macroscopic aquatic organisms the best known of which are the common green, brown and red seaweeds. Some species have high growth rates of 7.1-11.7% per day [2], good nitrogen uptake capacities between 19-96.6% [3] and beneficial utilization of CO_2 [4]. In addition, the biomass produced by wild harvest or via cultivation could be used as a foodstuff, fertiliser, high-value product, or for conversion into biofuels. These features have stimulated interest in exploring their potential utilisation for bioremediation in biological WWT, *e.g.* in inland and offshore aquaculture systems, including marine integrated multi-trophic aquaculture (IMTA) [5]. Aquaculture is the fastest growing animal food production sector worldwide, therefore improving sustainability through the effective treatment of its waste products is of the utmost importance [5]. In these systems, the production of biomass could concomitantly offer an economic return or negate some of the operational costs of WWT [6]. However, wastewater properties,

including nutrient characteristics, can be highly variable [7], which will influence algal growth rates, biomass production, nutrient uptake and algal composition. Therefore, understanding and optimising these parameters, as well as selecting robust species that maintain good performance, despite potential fluctuations in WWT conditions, are prerequisites for their successful implementation [8].

In an aquaculture WWT scenario, nutrients will be supplied continuously with N concentrations reported in the range of 50-124,900 µM [6, 9]. WWT with algae typically employs outdoor raceway ponds or high-rate algal ponds (HRAPs) with a treatment time of several weeks [10]. However, many studies involving N-uptake by macro-algae are unsuitable for assessing their potential as a tool for nutrient removal, given their experimental design and provision of conditions being unrepresentative of real conditions: these include the supply of low N concentrations (<35 µM) and/or the use of short incubation times (<24 h), with no data provided on algal growth rates [11, 12]. The determination of algal growth is one of the most fundamental parameters in phycological research, used for the comparison between treatments, assessment of biomass production and optimisation of culture conditions [13]. In addition, studies often employ the use of an algal inoculum that has been pre-acclimated in conditions designed to lower their internal nutrient reserves [14]. The subsequent re-introduction to a N replete medium will result in "surge uptake", where N is removed at a transiently enhanced rate due to the replenishment of internal N pools [15]. This form of uptake may indicate how well suited an individual species is at exploiting pulses of nutrients in an otherwise oligotrophic environment [1], but will be of reduced value in a bioremediation context [10].

Furthermore, the relationship between algal growth and nutrient uptake is very complex and requires further research. Macro-algal nutrient uptake is dependent upon N concentration and typically follows a Michaelis-Menten-type curve, whereby N removal follows a linear trend with increasing external N concentration until a plateau is reached [3, 16]. The rate and maximum amount of N removal achieved is dependent upon biological, physical, and chemical factors, which includes the form of N present [16, 17]. For example, *Gracilaria vermiculophylla* removed 100% of NH_4^+ in contrast with ~40% of NO_3^- [14]. A similar trend was also observed when cultivated in N mixtures, where NH_4^+ is generally removed ahead of NO_3^- [16, 18], or that NO_3^- uptake was suppressed when NH_4^+ was present [19]. Nitrogen source preference by macro-algae is complex and may be determined by: species-specific morphology, shore-line position, nutritional or life-history, the physiological capability and energetic requirement to uptake and assimilate certain nitrogen forms [1, 19, 20].

Fundamentally, mechanisms for N uptake are dependent on the N form present and are geared towards the same objective, namely: an N form enters the cell across the cell membrane, passively or actively, whereupon it is reduced to NH_4^+ and amino acids, before assimilation into macromolecules, like proteins, for growth [16]. Assimilation of NO_3^- occurs by sequentially reducing NO_3^- to NO_2^-, followed by NO_2^- to NH_4^+, via the action of N reducing enzymes [10]. Urea is converted into NH_4^+ by urease [21]. However, there is a dearth of studies involving macro-algal removal of other N forms that are also present in wastewaters, such as NO_2^- and urea, as well as mixtures containing these N forms [12, 22]. These N sources are components of the overall dissolved N pool and may play an important role in the algal N budget [12] and also the efficiency of nutrient removal.

In addition to growth rate and nutrient uptake capacity, macro-algal biochemical composition is also affected by nutrient regime. Furthermore, the composition of biomass is important to determine in order to assess their suitability for commercial applications. Several studies have demonstrated seasonal changes in macro-algal chemical composition elicited by differences in environmental conditions as well as nutrient supply [23]. It has been shown experimentally that the tissue N content of macro-algal biomass increases with increasing concentrations of N in their growth medium [24], in contrast with an accumulation of polysaccharides under N-limitation [25]. However, there is still a lack of publically available data on the influence of nutrient regimes in regards to nutrient type, concentration and N/P ratio on macro-algal growth, N uptake, and biochemical composition.

The primary aim of this study was to examine the ability of two robust marine species of algae from the genus *Cladophora* for N removal when subjected to different nutrient regimes and their influence on growth and biochemical composition, with a view for future use in bioremediation. *Cladophora* was selected as a model because of the high growth rates and propensity to bloom in nutrient rich conditions. They are also renowned for their tolerance to a wide range of abiotic factors and resistance to grazers. In addition, *Cladophora* are globally distributed in fresh-, brackish-, and marine-waters: this ubiquity means that local strains or species could be employed, on a case-by-case basis, avoiding the introduction of non-native or alien species [26-28]. These features make *Cladophora* a suitable candidate for investigation into a broad range of applied biotechnological applications, including bioremediation [8]. To explore this potential, cultivation medium was formulated with four different N/P ratios, in order to simulate those found in wastewater streams [29], with nitrogen supplied in both organic and inorganic forms found in wastewaters, specifically NH_4^+, NO_2^-, NO_3^- and urea. In addition, N forms were supplied

either singly or dually in equimolar concentrations to elucidate if there was preferential N uptake. This is the first study of its kind to cultivate and characterise algae under the conditions described above. Algal performance was assessed on the basis of growth, nitrogen removal and biochemical composition of the biomass produced.

2. Materials and Methods

2.1. Macro-algal strains and culture conditions

Algal strains studied were obtained from the Culture Collection of Algal and Protozoa (CCAP), at the Scottish Association for Marine Science (SAMS, UK). These included two marine isolates of the genus *Cladophora*: *Cladophora coelothrix* CCAP 505/10 and *Cladophora parriaudii* CCAP 505/09. Cultures were grown in a nutrient enriched media prepared as follows for all experiments: 33.5 g L^{-1} artificial seawater (Instant Ocean, UK), pH adjusted to 7.95 ± 0.08 with buffering provided by the addition of 2 mM Tris-HCl; trace metals (initial concentration = 11 μM Na_2EDTA, 11 μM $FeCl_3 \cdot 6H_2O$, 40 nM $CuSO_4 \cdot 5H_2O$, 76.5 nM $ZnSO_4 \cdot 7H_2O$, 42 nM $CoCl_2 \cdot 6H_2O$, 910 nM $MnCl_2 \cdot 4H_2O$, 25 nM $Na_2MoO_4 \cdot 2H_2O$); vitamins (initial concentration = 2.97 nM Thiamine HCl, 0.004 nm Cyanocobalamin, 0.02 nM Biotin); phosphorous was added as dihydrogenphosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) to give an initial P concentration of 80 μM. Nitrogen type and concentration was varied and was supplied as either ammonium chloride, sodium nitrate, sodium nitrite, or urea at molar N/P ratios of 2/1, 12/1, 22/1, and 32/1, equating to initial N concentrations of 160, 960, 1760, and 2560 μM, respectively, with concentrations based upon those found in aquaculture effluents [6, 9]. For the dual nitrogen experiments, N was added in equimolar combinations

at two N/P ratios (*i.e.* 2/1 and 12/1). All chemicals used were obtained from Fisher Scientific, UK.

The cultures were incubated in an illuminated shaker (Sartorius Stedim Biotech, Germany) at 100 rpm at a temperature of 24°C, under an 18/6 h (Light/Dark) photoperiod with 30-40 μmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR: 400-700) (LM-100 Light Meter, Amprobe, Germany). The algae studied were pre-acclimated in their respective experimental conditions and medium for seven days, after which time 35.7 mg fresh weight (FW) sub-samples were inoculated into triplicate 100 mL flasks containing 50 mL of the experimental medium and then incubated, as outlined above, for 14 days. Additional flasks containing the experimental media only acted as negative controls, whereas those containing biomass and f/2 medium served as a positive control.

2.2. Measurement of biomass and its constituents

Cultures were sampled six times during the study period: days 0, 2, 5, 9, 12, and 14 whereby they were removed aseptically in a laminar flow cabinet (MSC Advantage, Thermo Scientific) for FW determination and water chemistry analysis. On each sampling day, FW was determined by placing the biomass into a reticulated spinner (Chef'N Salad Spinner), spinning for 90 s to remove residual water and weighed gravimetrically in an analytical balance (PS-60, Fisher Brand, UK) [13]. Biomass was then returned to its original, corresponding flask and cultivated under the conditions described above. After the experimental period, the algal cultures were harvested and rinsed for 3-5 seconds with deionised water, from a wash bottle, to remove extracellular salts and nutrients, FW was

determined, the samples frozen at -18°C and then freeze-dried overnight or until a <5% variation in mass was achieved (Modulyo 4K Freeze-Dryer).

Biochemical composition analysis was performed on lyophilised material. Protein was extracted using hot-TCA and an overnight incubation in Lowry Reagent D, and quantified using the Lowry assay, as described by Slocombe *et al.* [30]. Total soluble carbohydrate of the biomass was extracted and quantified using a modified phenol-sulphuric acid method derived from that of Dubois *et al.* [31]. Biomass (5 mg) was hydrolysed with 2.5 mL 1M H_2SO_4 and autoclaved at 121°C for 15 min (TCR/40/H, Touchclave-R, LTE Scientific, UK). Hydrolysates were cooled to room temp (RT) and centrifuged for 2 min at 15,600 g (5414 Microcentrifuge, Eppendorf®). The assay was performed on 30 µL of supernatant with the direct addition of 0.5 mL of 4% phenol (w/v), followed by 2.5 mL of conc. H_2SO_4 (> 96%). The reaction was cooled to room temperature and read at 490 nm (Heλios Gamma UV-vis spectrophotometer, Thermo Scientific), which was calibrated against a 0-5 mg L^{-1} glucose calibration curve. Blanks were composed of deionised water in place of the hydrolysate.

2.3. Water chemistry analysis

For each sampling day analysis of nutrients present in solution was performed. Soluble NO_2^- and NO_3^- were measured using Ion Chromatography (883 Basic IC Plus, Metrohm, UK) [13]. Total Ammonia Nitrogen (TAN) and phosphate (PO_4^{3-}) were determined colourimetrically using proprietary test-kits (1.00683.0001 and 1.14848.0001, Spectroquant®) and following the manufacturer's instructions.

Urea was determined in water samples using a method derived from that of Mulvenna and Savidge [32]. Urea reagents were freshly prepared on each day of analysis and were: Urea Reagent A (1.7 g of diacetylmonoxime in 46 mL dH$_2$O, made up to 50 ml with thiosemicarbazide solution: 0.475 g in 100 mL dH$_2$O), and Urea Reagent B (31.26 mL dH$_2$O was acidified with 40 mL conc. H$_2$SO$_4$, once cooled 66 µL of ferric (III) chloride solution is added: 0.15 g in 10 mL dH$_2$O). Samples were diluted with deionised water to fall within the calibration range, and 2 mL of sample was added to a reaction tube. To this, 143 µL of Urea Reagent A was added and immediately mixed (Vortex 3, IKA®), followed by 458 µL of Urea Reagent B and mixed again. Teflon-lined screw-caps were loosely added and the samples were incubated for 30 mins at 85°C (Gallenkamp Plus II Incubator, Sanyo, UK). Samples were quickly cooled in a water bath for a total of 10 min, replacing the water after 5 minutes. The samples were then read in a spectrophotometer at 520 nm (Heλios Gamma UV-vis spectrophotometer, Thermo Scientific) and compared against a 0-5 mg L^{-1} urea calibration curve. Blanks were composed of deionised water in place of the sample.

2.4. Data analysis

Algal growth is expressed as daily growth rate (DGR) and was determined using the equation (1) as recommended by Yong *et al.* [33]:

$$DGR = \left[\left(\frac{W_t}{W_0}\right)^{1/d} - 1\right] x \; 100 \qquad (1)$$

Where W_t and W_0 are the final and initial FW mass, and *d* is the time in days.

Protein and carbohydrate content of the biomass is portrayed as % DW. Data on water chemistry were converted to molar N form and expressed either as total N uptake (µM), or as a proportion of N removed from the growth medium (%), both relative to values obtained from corresponding negative controls. All experiments were performed in triplicate and the experimental error was calculated and expressed as one standard deviation (SD). Within a single N/P ratio, comparison of DGR, nutrient uptake, and biochemical composition was performed using a 2-sample t-test. A one-way ANOVA with Tukey's post hoc analysis was used to determine differences in DGR of *C. parriaudii* between multiple N sources provided at the same concentration. Relationships between DGR and N removal, and protein to carbohydrate content were described using Pearson's correlation, r. Levels of significance were set at $p < 0.05$; all statistical analyses were performed using Minitab® Statistical Software version 17.

3. Results and discussion

In this study, two species of *Cladophora* were investigated for N removal, growth rate, and biochemical composition after 14 days incubation under different nutrient regimes that were designed to simulate a broad range of wastewaters. The only experimental variable was nitrogen addition, with growth media formulated with four different N/P ratios and four different sources of nitrogen: NH_4^+, NO_2^-, NO_3^-, or urea. This study was performed in two main sections. Firstly, N sources were added singly for comparison between species on a fundamental level. Secondly, media was formulated with dual nitrogen sources in equimolar concentrations, provided at a 2/1 and 12/1 N/P ratio, to elucidate the N source preference of *C. parriaudii*. The removal of N was attributed to the algal strains studied by

comparison against negative control flasks where nutrient removal was not observed (see Supplementary Data Fig. S1). In addition, several samples, across different nutrient regimes and sampling days, were analysed for the presence of other nitrogen forms: in no instance was any other form of N present than that which was originally added (data not shown). Positive control cultures grew consistently throughout the experimental time-frame, indicating that no unexpected changes in growth occurred (Supplementary Data Fig. S2). In addition, PO_4^{3-} removal was never greater than 53%, therefore, neither species of *Cladophora* would have suffered from P-limitation (Supplementary Data Fig. S3 and S4). The addition of Tris-HCl buffered the cultivation media, which prevented excessive shifts in pH caused by algal growth, with final pH values (*i.e.* measured on day 14) of 8.18 ± 0.08 and 8.4 ± 0.25 measured for *C. coelothrix* and *C. parriaudii*, respectively.

3.1. Uptake of single nitrogen sources

Experiments were performed in order to determine whether two closely related species of *Cladophora* are able to sequester different N sources indiscriminately, across a range of concentrations, and if this influences growth. Single sources of N were added at four different N/P ratios, with the DGR and N uptake determined (Fig. 1 and 2).

Both species studied exhibited growth and N uptake irrespective of nutrient regime. *C. parriaudii* had a higher DGR (Fig. 1) and N-uptake (Fig. 2) than *C. coelothrix* under all nutrient regimes tested. Differences in DGR were statistically significant at 12/1 NO_2^- (p = 0.025) and for all urea concentrations tested (p = 0.002 – 0.049) (Fig. 1). A reduction in growth rate for both species with increasing concentrations of NH_4^+ was observed (Fig. 1a). This was most likely due to the increasing concentration of the toxic un-ionised NH_3 form

that exists in equilibrium with NH_4^+ [34]. Previous studies have reported toxic effects of NH_3

with algal cultures at concentrations ranging from 2.34 - 2000 µM [35, 36], and NH_3

concentrations in this study fell within this range, varying between 0-250 µM. Disparities in

growth between the two species were generally reflected in the amount of N removed (Fig.

2). For example, at a 12/1 ratio with NO_2^-, DGR for *C. parriaudii* was 8.6% as opposed to 5%

for *C. coelothrix*, with corresponding NO_2-N uptake of 538 (± 79.9) µM and 193 (± 80.3) µM,

respectively (Fig. 1b and 2b). Overall, differences in DGR and N uptake observed may be

accounted for by interspecific variations in physiology, capacity to utilise N forms, growth

strategy, as well as morphology [20, 37]. For instance, Luo *et al.* [37] reported that *Ulva*

prolifera had higher rates of growth and nutrient uptake than *Ulva linza* across a range of

temperatures, irradiances, and N concentrations, most likely as a result of them having a

different thallus morphology. A similar explanation may be valid in this case: Ross *et al.* [13]

noted that *C. parriaudii* grows rapidly outwards in a loose skein, whereas *C. coelothrix* grows

slowly in tightly knit clusters. The denser growth strategy exhibited by *C. coelothrix*, as

opposed to *C. parriaudii*, will likely enhance the effects of growth-limiting factors, such as

self-shading and low nutrient availability within the tightly knit colony, as observed in other

members of Cladophoraceae [38, 39].
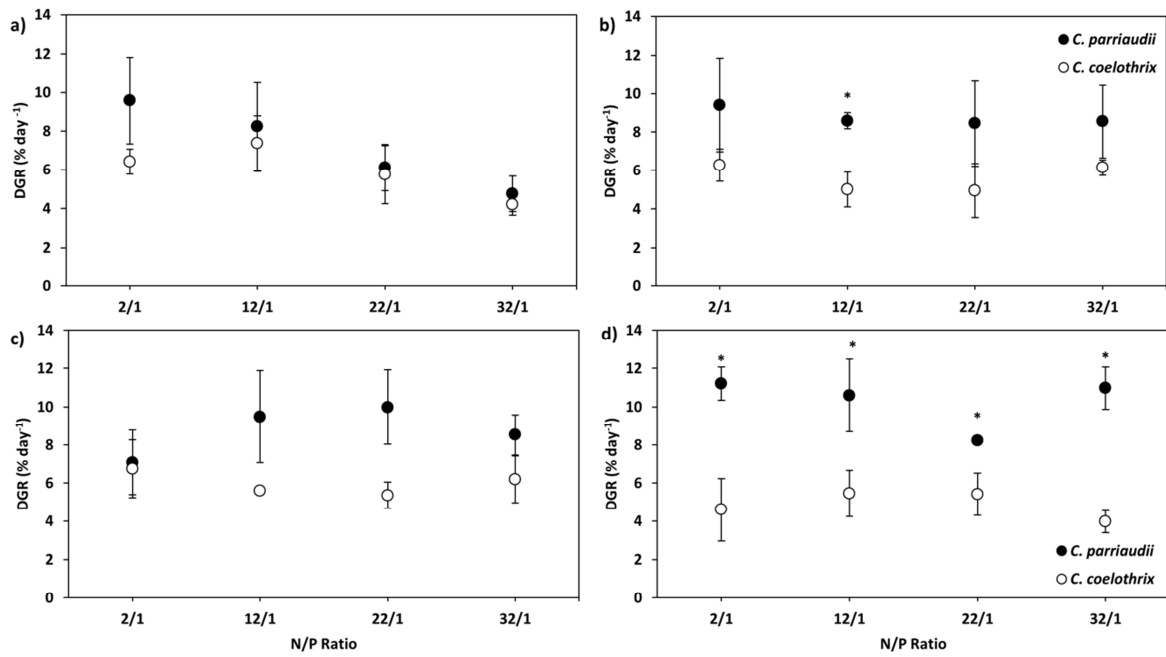
Fig. 1. The DGR of *C. parriaudii* (black) and *C. coelothrix* (white) cultivated for 14 days in different media formulations with N/P ratios of 2/1, 12/1, 22/1, and 32/1, equivalent to initial N concentrations of 160, 960, 1760, and 2560 µM, respectively. N was supplied as NH_4^+ (a), NO_2^- (b), NO_3^- (c), or urea (d) (*n* = 3, *error bars* = 1 SD). Statistically different DGR between species are indicated * (*p* <0.05).
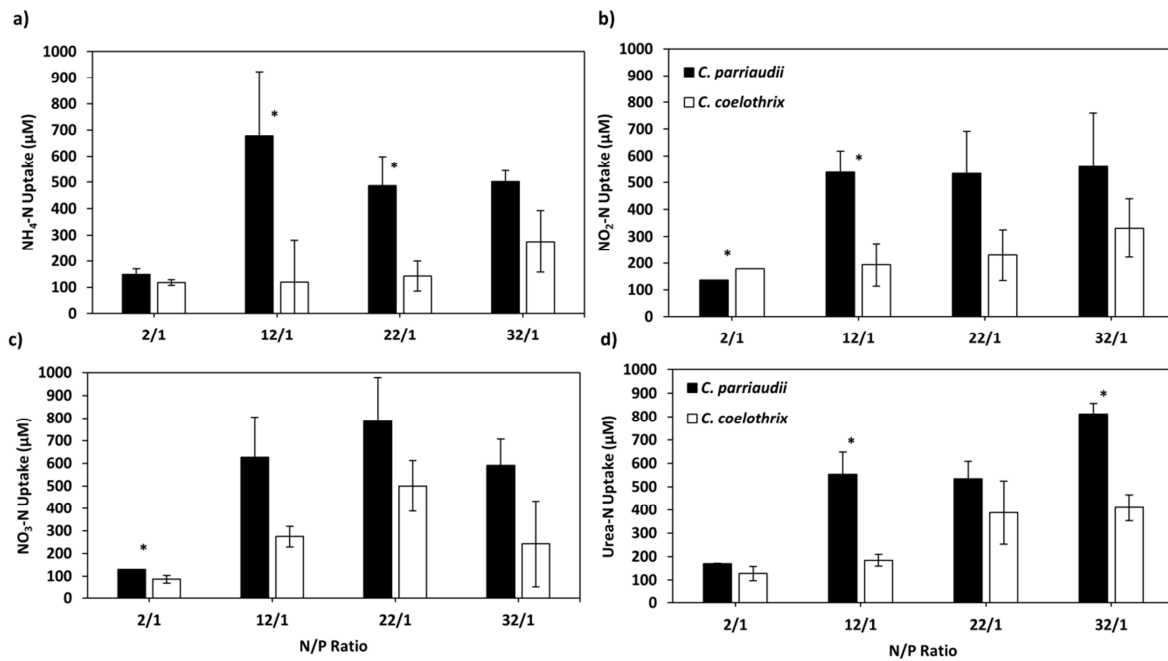
Fig. 2. The total N uptake by *C. parriaudii* (black) and *C. coelothrix* (white) when cultivated

for 14 days in different media formulations with N/P ratios of 2/1, 12/1, 22/1, and 32/1,

equivalent to initial N concentrations of 160, 960, 1760, and 2560 µM, respectively.

Nitrogen was supplied as NH_4^+ (a), NO_2^- (b), NO_3^- (c), or urea (d) (*n* = 3, *error bars* = 1 SD).

Statistically different total N uptake between species are indicated * (*p* <0.05).

Greater differences in performance between the two species occurred when cultivated on

urea. Both the highest and lowest DGR was achieved with 11.2% by *C. parriaudii* and 3.98%

by *C. coelothrix*, respectively. This indicates that two closely related species of *Cladophora*

have different capacities for N source utilisation. Literature on macro-algal urea removal is

sparse in comparison to NO_3^- or NH_4^+ [1]. However, Phillips and Hurd [1] observed negative

values for urea uptake by inter-tidal macro-algal species during winter conditions, but noted

uptake saturation during summer months with increasing urea concentrations. The authors

hypothesized that the energetically expensive enzyme urease, required to convert urea to

NH$_4^+$, may be down-regulated during winter months when abiotic conditions are sub-optimal for growth. A similar concept may apply here, where there was a strongly positive correlation between the DGR and N uptake by *C. parriaudii* at each nutrient ratio, with Pearson's *r* values of 0.645-0.848 and *p* = <0.001 – 0.024. However, no such relationship existed with *C. coelothrix*, *p* = 0.656 – 0.999, which suggests that the growth rate of *C. parriaudii* was strongly influenced by the nutrient regime, whereas the growth of *C. coelothrix* is likely to be more strongly influenced by another parameter, such as light intensity. This suggests that *C. coelothrix* may have under-expressed urease production as a consequence of sub-optimal cultivation conditions and the low rate of urea assimilation that occurred may have been for maintenance or housekeeping purposes.

A common trend in N uptake was not shared between *C. coelothrix* and *C. parriaudii* (Fig. 2), most likely due to the physical and biological factors mentioned above. In the case of *C. parriaudii*, N uptake followed a Michaelis-Menten-type trend, generally plateauing at a ratio of 12/1 and beyond, which is comparable to observations reported by other studies [3, 16]. The kinetics of nutrient uptake were described in detail by Harrison and Hurd [17], defining this plateau as a period of "internally controlled uptake", with the activity of N reducing enzymes limiting the rate of further N uptake.

Due to the lower rates of growth and N uptake, *C. coelothrix* was not investigated further. However, *C. coelothrix* has been reported to be a robust species [13] and merits further study in conditions more representative of WWT, where it may exhibit a higher degree of tolerance and growth in comparison to *C. parriaudii* [40]. In addition, as N removal by *C. parriaudii* plateaued beyond a 12/1 ratio, only ratios of 2/1 and 12/1 were investigated for the remainder of this study.

3.2. Uptake of nitrogen combinations

Combinations of different nitrogen sources were added to the growth matrices to determine whether *C. parriaudii* is capable of complimentary N uptake and what effect this has on DGR (Fig. 3) and total N uptake (Fig. 4). Data regarding N removal at the 2/1 ratio are available as Supplementary Data (Fig. S5): these are not shown, as the trends are similar to the 12/1 ratio, albeit with complete N removal achieved by day 9.

Generally, cultures that have removed the most N also tend to have a higher DGR. The N source appeared to have a greater effect on the DGR of *C. parriaudii* than N/P ratio (Fig. 3): no significant differences in growth were observed between N/P ratios, whilst there was significance in the DGR values within a given N source. For example, the DGR of *C. parriaudii* when cultivated under a 2/1 ratio with urea as the common N source (Fig. 3d) ranged from 7.8-11.2% (p = 0.005).

The inclusion of urea in the media tended to enhance both the growth and N uptake by *C. parriaudii* and may be explained when considering its influence on biochemical composition of the biomass, which will be discussed later. Greater total N removal was achieved by *C. parriaudii* when incubated in NH_4-Urea (79.8%) as opposed to NH_4^+ alone (67.7%) and mixtures of NH_4-NO_2 (32.7%) and NH_4-NO_3 (50.4%) (Fig. 4a). The same trend was observed in media combinations with NO_2^- or NO_3^- as the common N source (Fig. 4b and c). Interestingly, growth and N uptake was greater in urea mixtures in comparison to a single addition of urea: the DGR of single urea at 12/1 was 9.2%; however, this increased to 10.6%, 10.6%, and 11.2% with combinations of NH_4-Urea, NO_2-Urea, and NO_3-Urea, respectively (Fig. 3d). As growth and N removal are strongly related, a corresponding trend was found in urea uptake. The lowest total N removal was observed with a single urea addition (61.4%), as opposed to

combinations containing urea: NH_4-Urea with 79.8%, NO_2-Urea with 77.5%, and NO_3-Urea with 65.2% (Fig. 4d). Overall, these results indicate that *C. parriaudii* is capable of complimentary N uptake and that this can enhance growth. In a previous study, Bracken and Stachowicz [20] reported that of eight local species tested, neither *Cladophora* nor *Prionitis* exhibited the greatest N removal in monoculture. However, *in situ*, they were in fact the most dominant in the Bodega Marine Reserve, California, partly attributed to their ability to utilise multiple nitrogen forms when available.

In contrast with urea, incorporating either NH_4^+ or NO_2^- into a mixture generally resulted in reduced DGR: for example, a treatment of 12/1 NO_2-NO_3 resulted in one of the lowest observed rates of growth (6.9%) and total N removal (47.7%). Both NO_2^- and NH_3 (which co-exists in equilibria with NH_4^+) are toxic to algae [34, 41], and possibly impaired the functionality of the organism.

Differences in algal performance may be a result of an inherent preference for a certain nitrogen form, which can be elucidated by measuring temporal N removal of the component N sources.

3.3. Preferential nitrogen uptake

To determine whether *C. parriaudii* exhibited any preference for a particular N source, the removal of the component N sources from the dual N media was investigated (Fig. 5). The trends between 2/1 and 12/1 N removal preference were very similar; however, as a lesser concentration of N was added in the 2/1 regime, the relationships were achieved more

rapidly making them more difficult to discern. For this reason only 12/1 have been included (Fig. 5), and the 2/1 data is available as Supplementary Data (Fig. S6).

There are clear differences in N uptake preference for *C. parriaudii*: NH_4^+ was removed at a greater rate and to a higher degree than any other N source it was mixed with (Fig. 5a, b, and c). A preference for NH_4^+ over NO_3^- has been reported elsewhere for both eukaryotic algae and cyanobacteria [14, 42, 43]. However, there are very few studies on macro-algal preference in dual N media formulations involving alternative nitrogen sources, such as urea and NO_2^- [12, 22]. This preference for NH_4^+ is presumed to be because it is less energetically expensive to assimilate than the other N sources, as NO_2^- or NO_3^- are more oxidised forms of nitrogen that need to be reduced to NH_4^+, whereas urea requires different enzymes for its assimilation [14, 21, 44]. Additionally, algal-bacterial interactions may also play a role, such as nutrient mineralization [45].
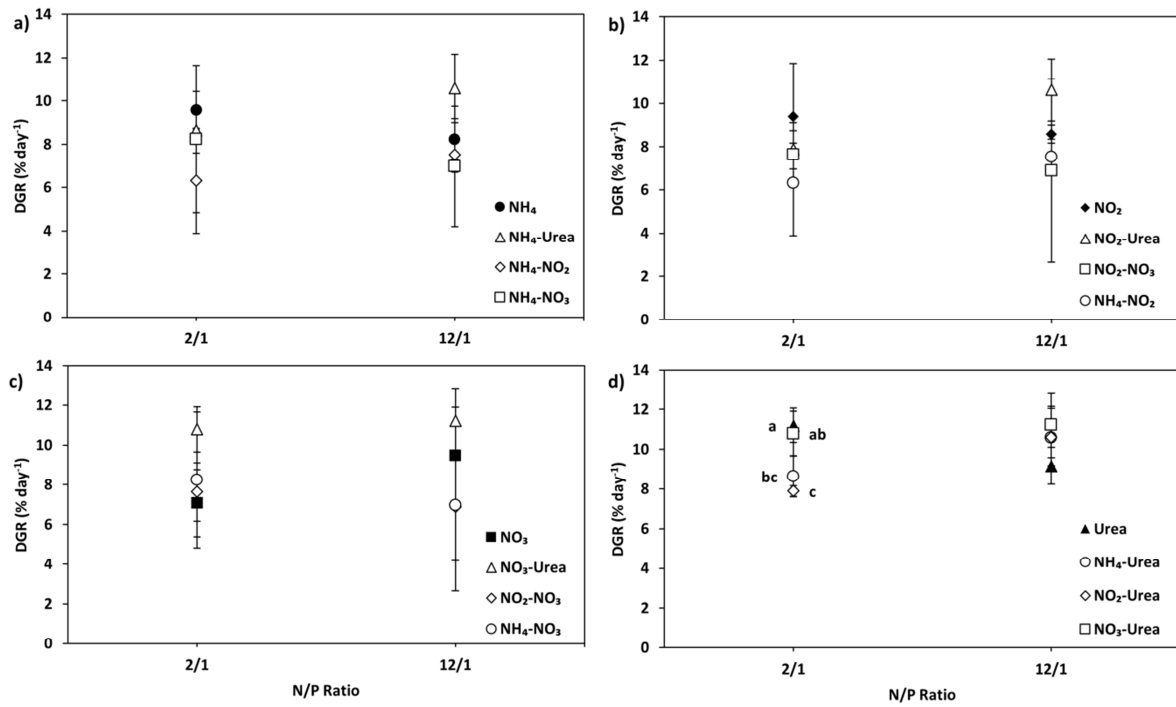
Fig. 3. The DGR of *C. parriaudii* cultivated for 14 days in different media formulations with 2/1 and 12/1 N/P ratios, equivalent to initial N concentrations of 160 and 960 µM, respectively. N was added as NH_4^+, NO_2^-, NO_3^-, or urea, either singularly or dually in equimolar concentrations. The figures have been grouped by common N source as NH_4^+ (a), NO_2^- (b), NO_3^-, (c), and urea (d), with the darkened shape denoting the common type (n = 3, *error bars* = 1 SD). Treatments which do not share a letter are significantly different, whereas, treatments that share a letter or have no letter are not significantly different (p <0.05).
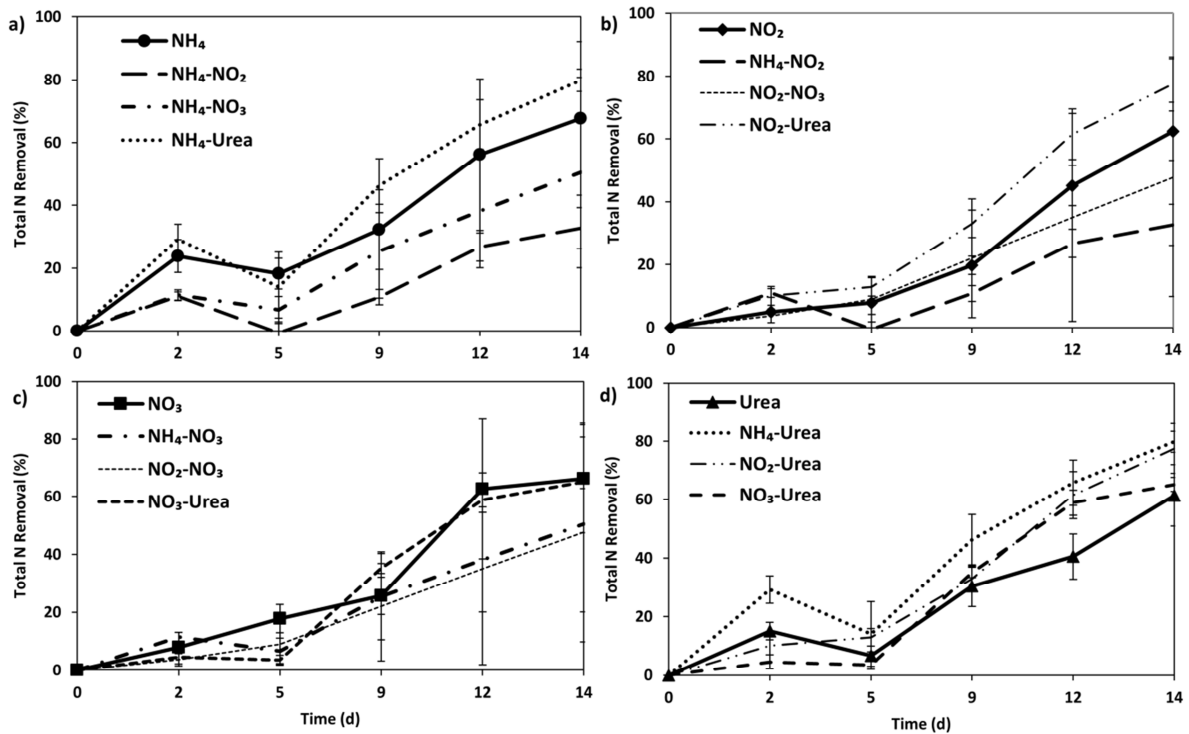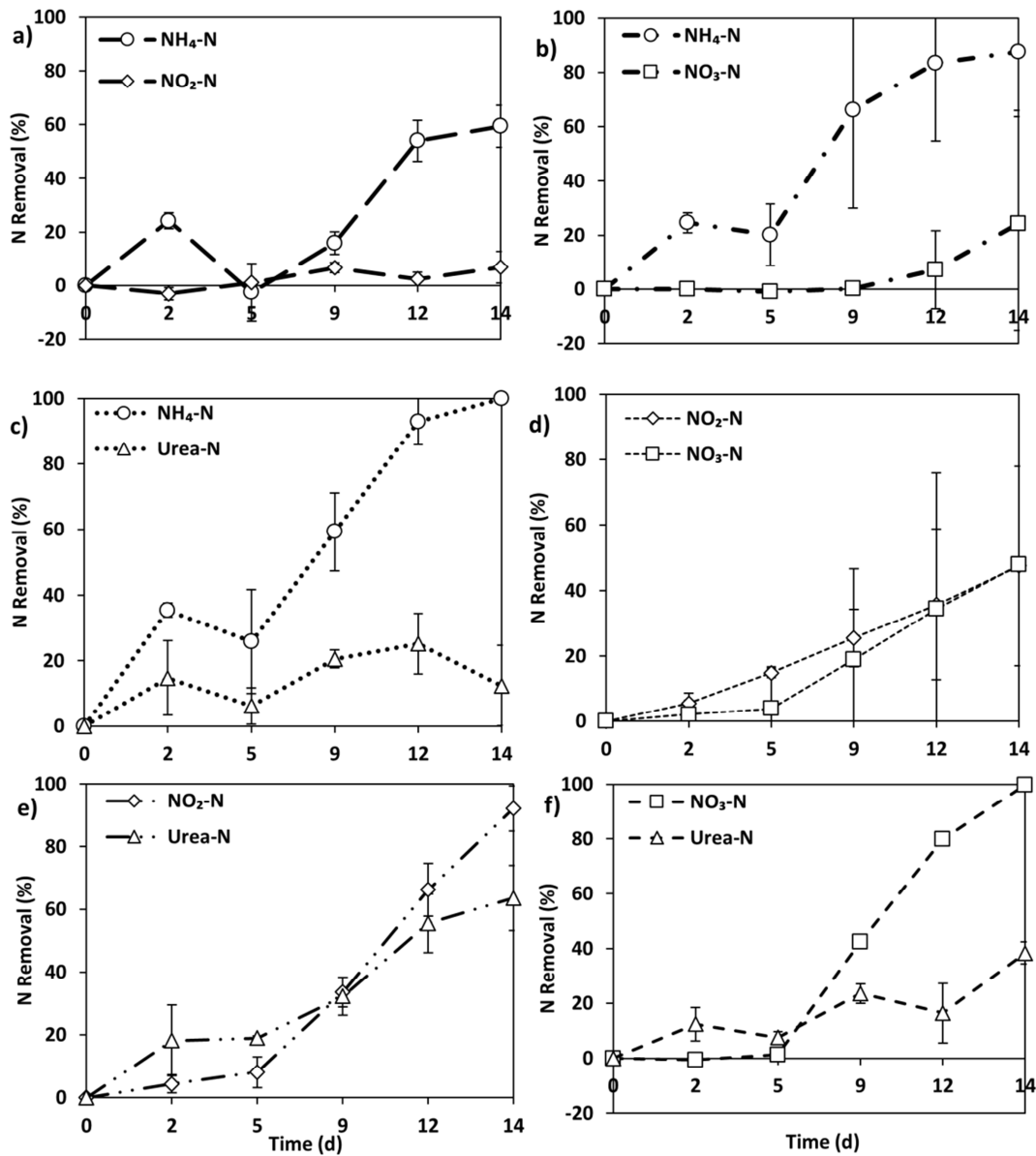
Fig. 4. The temporal total N removal by *C. parriaudii* cultivated for 14 days in different media formulations with a 12/1 N/P ratio, equivalent to an initial N concentration of 960 µM. Nitrogen was added as NH_4^+, NO_2^-, NO_3^-, or urea, either singularly or dually in equimolar concentrations. The figures have been grouped by common N source as NH_4^+ (a), NO_2^- (b), NO_3^-, (c), and urea (d) with the darkened shape and solid line denoting the common type for each corresponding graph (*n* = 3, *error bars* = 1 SD).

Similarities between the removal of NO_2^- and NO_3^- by *C. parriaudii* cultivated in NO_2-NO_3 media were observed, with an overall equal removal of 47% for each, despite a slight initial delay in NO_3^- uptake (Fig. 5d). Furthermore, their removal when in conjunction with other forms, in this case with NH_4^+ and urea, were similar. There was an initial 5-day lag period for NO_2^- and NO_3^- uptake before 92.2% and 99.8% were removed, respectively, when urea was the additional nitrogen type (Fig. 5e and f). However, only 6.8% and 24.3% of NO_2^- and NO_3^-,

respectively, were removed when NH_4^+ was also present in the media (Fig. 5a and b). Raven *et al.* [44] suggested that NO_3^- uptake could be completely suppressed when in the presence of 1000-2000 μM NH_4^+, with the most plausible reason being that NH_4^+ inhibits the biosynthesis of nitrate/nitrite reductase or urease enzymes [21]. In this study, NO_3^- removal only occurred after the concentration of NH_4^+ had reduced to 95-207 μM, typically achieved between days 5 and 9. However, in the NH_4-NO_2 treatment, the concentration of NH_4^+ never went below 154 μM and translated into a low removal of NO_2^- of <7%. This suggests that there is a threshold NH_4^+ concentration above which NO_2^- and NO_3^- removal is suppressed. This was further highlighted at the 2/1 ratio (Fig. S6) where the onset of NO_2^- and NO_3^- removal from the NH_4-NO_2 and NH_4-NO_3 mixtures occurred once NH_4^+ was almost depleted. The presence of NH_4^+ in the media was considered to be the causative agent of the poor removal of NO_2^- and NO_3^-. These virtually identical trends in removal of NO_2^- and NO_3^- were assumed to be due to their chemical similarity and their common metabolic pathway for nitrogen reduction and assimilation.

Fig. 5. The preferential removal of component N sources by *C. parriaudii* cultivated for 14 days in different media formulations with an N/P ratio of 12/1, equivalent to an initial N concentration of 960 µM. Nitrogen was added as NH_4^+ (circle), NO_2^- (diamond), NO_3^- (square), or urea (triangle) in equimolar concentrations. The graphs have been grouped by their dual nitrogen additions as NH_4-NO_2 (a), NH_4-NO_3 (b), NH_4-Urea (c), NO_2-NO_3 (d), NO_2-Urea (e), and NO_3-Urea (f) (n = 3, *error bars* = 1 SD).

A >92% removal of NH_4^+, NO_2^-, and NO_3^- was observed, when added in an equimolar combination with urea; however, the corresponding removal of urea was comparatively low, amounting to 12.4%, 63.6%, and 38.5%, respectively (Fig. 5c, e, and f). This was a somewhat surprising result given the relatively high rate of urea removal when added as a single source (61.4%) (Fig. 4d), and the correspondingly high DGRs when it was incorporated into dual N media (Fig. 3d). Tyler *et al.* [12] observed enhanced uptake of urea in the presence of NH_4^+ by *Gracilaria vermiculophylla* and *Ulva lactuta*, suggesting that each N form has a distinct uptake mechanism allowing their simultaneous removal. This was in contrast with results observed in this study where urea uptake was suppressed in the presence of other N sources, especially NH_4^+. The discrepancy between these findings is likely due to differences in experimental design and cultivation conditions: Tyler *et al.* [12] employed a shorter time-frame and a much greater biomass/nutrient ratio, with an inoculum that was previously maintained in low nutrient seawater, meaning that surge uptake would have played a prominent role in nutrient removal.

3.4. Biochemical composition

It should be noted that, despite being commonly used for biochemical analysis, the colourimetric methods used for biochemical determination in this work have their limitations, for example the non-determination of uronic acids from the carbohydrate assay [23, 30, 31, 46].

A strong inverse correlation between protein and carbohydrate content (r = -0.585, p < 0.001) was observed: generally, algal cultures maintained in a 2/1 ratio had a low protein and high carbohydrate content, whereas at a 12/1 ratio, they had a high protein and

reduced carbohydrate composition (Table 1). At a 2/1 ratio, N was generally completely removed after 9 days (Fig. S5), likely resulting in N-deprivation and hence a lower protein content, whereas at the 12/1 ratio N was never depleted and therefore cultures would not have been N-limited and protein synthesis will have continued (Fig. 4). The influence of nutrient supply on biochemical composition has been observed elsewhere, where macro-algae have been found to accumulate storage polysaccharides under N-deprivation, with a shift towards the synthesis of proteins and pigments when N is sufficient [25, 47]. In this study, the highest protein content at both ratios was found in *C. parriaudii* maintained under NH_4-NO_2, with values of 10.8% and 15% at 2/1 and 12/1, respectively, coincident with the lowest DGR and total N uptake (Fig. 3a and 4a). Meanwhile, the highest carbohydrate content was 54%, achieved in flasks where the alga was cultivated under a 2/1 ratio with NO_2-Urea, where complete N removal was attained after 9 days.

It appears that N uptake, as seen between different N/P ratios, has a greater influence on biochemical composition than N form (Table 1), despite Corey *et al.* [18] finding that the tissue N content of the biomass of *Palmaria palmata* significantly changed from 3.1% to 4.1% when cultivated under NO_3^- or NH_4^+, respectively. In their study, there was however a significantly greater removal of NH_4^+ in comparison to NO_3^- [18], therefore, it seems more likely that an algal species preference for an N form will influence the uptake rate and absolute N removal, which will in turn affect the biomass composition, in contrast with the N form having a direct influence on biochemical composition.

The inclusion of urea in dual N media combinations resulted in carbohydrate rich biomass. For example, three of the four highest yields at a 2/1 ratio (48.4-54%) and the three highest at a 12/1 ratio (40.6-42.8%) were obtained when urea was part of the growth medium

(Table 1). Considering that urea is an organic molecule its influence upon the algal biochemical composition may be slightly more complex. Both Choo *et al.* [48] and Raven *et al.* [49] have described multiple mechanisms for inorganic carbon acquisition by *Cladophora* sp., therefore the capacity to utilise carbon from urea is a distinct possibility. The conversion of urea to NH_4^+, via enzymatic activity, results in the formation of 2 NH_3 and one CO_2 molecule [21]. This CO_2 formation may enhance photosynthetic activity and facilitate carbohydrate construction [50]. This theory of complimentary carbon and N sequestration may also explain the enhanced DGR and N uptake by *C. parriaudii* when cultivated in growth medium containing urea (Fig. 3d and 4d).

Table 1. The protein and carbohydrate content per unit DW of *C. parriaudii* cultivated for 14 days in different media formulations with 2/1 and 12/1 N/P ratios, equivalent to initial N concentrations of 160 and 960 µM, respectively. Nitrogen was added as NH_4^+, NO_2^-, NO_3^-, or urea, singly or dually in equimolar concentrations (n = 3, ± 1 SD). Values highlighted * are significantly different from one another within a nitrogen source ($p < 0.05$).

Nitrogen Source	Protein (% DW)		Carbohydrate (% DW)	
	2/1	12/1	2/1	12/1
NH_4^+	6.2 (± 1.8)*	13 (± 0.7)*	51.1 (± 2)*	31.1 (± 1.3)*
NO_2^-	5.4 (± 1.3)*	10.9 (± 1.2)*	41.7 (± 6.7)	38.5 (± 3.1)
NO_3^-	6.8 (± 1.2)	9.7 (± 0.5)	41.1 (± 2)*	32 (± 1.9)*
Urea	6.5 (± 0.6)	8 (± 0.9)	43.8 (± 11.9)	37.6 (± 3.6)
NH_4-NO_2	10.8 (± 3.3)	15 (± 0.4)	45.6 (± 6.2)	39.2 (± 1.5)
NH_4-NO_3	8.3 (± 2.9)	12.1 (± 1.9)	47.2 (± 5.9)	36.1 (± 6.5)
NH_4-Urea	5.1 (± 0.2)*	11.4 (± 1.8)*	51.5 (± 0.9)	40.6 (± 4.5)
NO_2-NO_3	8.4 (± 3)	11.9 (± 1.1)	38.8 (5.6)	38.2 (± 2.2)
NO_2-Urea	6.5 (± 1.1)*	12 (± 0.9)*	54 (± 8.1)	42.7 (± 2.2)
NO_3-Urea	6.1 (± 0.2)*	10.7 (± 0.4)*	48.4 (± 1.3)	42.8 (± 3.1)

4. Conclusions

There were several key conclusions from this study. Firstly, there were species-specific differences in growth, N-uptake, and ability to utilise certain N forms between two species of *Cladophora*. Furthermore, *C. parriaudii* is capable of complimentary N-uptake and its growth is strongly influenced by N type, rather than concentration, showing a preference towards NH_4^+, potentially due to its reduced form and lower energetic requirement for its assimilation, whereas urea was removed secondarily. However, the presence of urea in the medium enhanced the uptake of other N forms present, possibly due to complimentary carbon sequestration. Whereas, the total amount and rate of uptake of both NO_2^- and NO_3^- were almost identical. Finally, different nutrient regimes elicited a change in the final composition of *C. parriaudii* biomass, with N concentration having a greater influence than N form. This demonstrates that a sea change in algal cultivation practices is required and should include selecting species for biological WWT purposes that are robust, flexible, and can simultaneously utilise multiple carbon and nitrogen forms. Future work on a larger-scale and in conditions more representative of WWT are required and in a broader algal biotechnology context, alternative, cheaper, and more easily assimilable N sources could be employed. Nutrient regimes could be tailored to reduce costs and produce biomass with required commercial attributes.

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