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Experimental assessment of phycoremediation potentials and biomass quality of *Chlorococcum humicola* (Nageli) rabenhorst

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Abstract
Experimental assessment of growth, productivity, nutrient removal efficiency, biomass quality, and yield of an indigenous fast-growing green algae *Chlorococcum humicola* at varying nutrient regimes can help understand its usefulness in the phycoremediation industry, which is essential to overcome the environmental and bioresource crises for a sustainable future world, especially to fulfil the goals of SDG 30. In this context, *C. humicola* was examined under varying N, P, NP, and N:P in Bold's Basal medium. The highest yield and productivity occurred in N-varied treatments (2– 3 × N with N:P = 3.42–5.13). The highest N removal occurred in the same N- varied treatments. A low N :P ratio, achieved by increasing P, favoured both N and P removal. Algal protein content was directly proportional to the amount of nitrogen in the medium. The highest protein content was present at 4 × N in the medium. N starvation stimulated an increase in lipid content. Overall, the experiment constitutes a model to identify the optimum nutrient requirements for maximum phycoremediation potential and quality biomass productivity of *C. humicola*.

Keywords: Biochemical profile, microalgae, nutrient removal, productivity, biomass

Introduction
The rapid urbanization and industrialization of the 21st century have led to increased carbon emissions, depleted energy and water resources, and heightened water pollution due to industrial wastewater discharge, which poses serious environmental and health risks(Tripathi, Gupta, and Thakur 2019) [54]. This wastewater, often high in nitrogen and phosphorus, contributes to eutrophication. Recently, wastewater treatment has shifted towards resource recovery, focusing on reclaiming water, nutrients, and biomass (Puyol *et al*. 2017) [41]. With the global population projected to rise to 9 billion by 2050, the demand for cleaner water and sustainable energy is growing (Shahid *et al*. 2020) [50]. While traditional physical, chemical, and biological treatments exist, no single method fully addresses all wastewater challenges (Grandclément *et al*. 2017) [24]. Sustainable approaches now emphasize water recycling and carbon retention. Microalgae-based technologies offer a promising solution, as microalgae can grow rapidly on non-arable land, sequester CO_2, and provide effective nutrient removal, especially nitrogen and phosphorus (Renuka *et al*. 2015; Sakarika *et al*. 2019) [43, 46].
Microalgae, adaptable and efficient photosynthetic organisms, require minimal resources for cultivation and are effective in eutrophic waters (García *et al*. 2019 [22]; Ray, Santhakumaran, and Kookal 2021) [42]. Understanding their nutrient uptake is key to optimizing biomass productivity, making them suitable for integration with large-scale wastewater treatment (Zhu *et al*. 2017) [61]. Studies highlight the potential of green algae as a biomass source for bioactive compounds and renewable energy (Santhakumaran, Ayyappan, and Ray 2020) [47]. Certain non-toxic, fast-growing local algae strains, identified in eutrophic waters in Kerala, could offer a low-cost approach for freshwater management and biomass generation (Ray, Santhakumaran, and Kookal 2021) [42]. Factors influencing nutrient removal include algal species, nutrient type, and initial algae concentration ((Dayana Priyadharshini *et al*. 2021) [15]).
This study aims to optimize and evaluate the nutrient removal, water quality improvement, and biomass production of a local green microalgal species in eutrophicated waters. Specific objectives include (1) conducting culture trials with varied N & P concentrations, (2) assessing nutrient removal rates and nutrient removal efficiency concerning different nutrient concentrations (3) assessing biomass quality concerning nutrient levels

Materials and Methods
Microalgal strain
The algae selected for the present study was a molecularly characterized species kept as pure cultures in the algal repository at the School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala. The alga was morphologically and molecularly characterized (MK015655.1). The cellular details of all the species were obtained using a digital compound microscope (Carl Zeiss, model Axio Imager M2). Microphotographs were obtained using a digital camera (Axiocam 305) equipped on a microscope. Morphological measurements (n = 30) were obtained from microphotographs using Zen BLUE 3.6 software. The details are shown in Figure 1.

Experimental design
Three distinct experiments were conducted to examine how altering the nitrogen-to-phosphorus (N:P) ratio by varying nitrogen (N), phosphorus (P), or both simultaneously in BBM medium affects algal parameters. In the first

experiment, the N:P ratio was increased by raising nitrogen (as NaNO3) while keeping phosphorus constant at BBM levels. The second experiment varied the N:P ratio by adjusting phosphorus (as KH2PO4) while keeping nitrogen constant at BBM levels, thereby lowering the N:P ratio. The third experiment assessed the effects of a constant N:P ratio on algal parameters by varying nitrogen and phosphorus in BBM. Each experiment was conducted in triplicate. Four treatments were tested in the first and second experiments, whereas only two treatments were used in the third. Details of the experiments and treatments are shown in Table 1.

Experimental conditions

The pH of the culture medium was 7.3, adjusted using 0.1M HCl/NaOH with the support of a pH meter (Hanna HI 1131). The experimental cultures were maintained under the light intensity of 6000 lux, 16-hour light and 8-hour dark cycle, at 24°C. The cultures were maintained in 1L Erlenmeyer flasks containing 1000 ml of culture medium. In all cases, at the beginning, approximately 200 mg of wet biomass (measured using an electronic balance - Sartorius BSA224S-CW) was added to the medium as seed culture. The initial cell densities of algae in the culture media were determined using a hemocytometer. The initial cell densities for the algae is 1.43 x 10^5 cells per ml. All three culture flasks (triplicate cultures) of a treatment for each alga had approximately similar initial cell densities. The cultures received continuous and filtered aeration through 5mm plastic tubes connected to an air pump (Hailea HAP 80 80L/M 60W). The duration of cultures was for twenty days.

Microalgal growth-assessment

Every third day, the growth rate was measured using a haemocytometer (from 1 ml of media pipetted out after the manual mixing) to ensure normal growth of the algae and the absence of contamination. The entire biomass of a flask was extracted after 20 days of incubation using an Eppendorf centrifuge set to 8000 rpm and 20°C for 10 minutes. The biomass yield was calculated as percentage increase of biomass per liter each day, per the equation 1 (Santhakumaran *et al*. 2019) [48]

% Increase in Biomass yield of algae ($L^{-1}day^{-1}$) =

$$\frac{\text{Final dry weight (mg) x100}}{\text{Initial dry weight (mg) x Volume of culture(L) x total number of culturing days}}$$

The volumetric biomass productivity $P_{Biomass}$ of algae was calculated as per the equation 2 (Hempel, Petrick, and Behrendt 2012) [27]

Biomass Productivity (mg L^{-1} day^{-1}) = $(X_2-X_1).(t_2-t_1)^{-1}$ (2)

Where X_1 and X_2 were the biomass dry weight concentrations (mg L^{-1}) on days t_1 (starting point of cultivation) and t_2 (end point of cultivation), respectively.

Measurement of N and P in the medium

Total Phosphorus (TP) was calculated using the Vanadium Molybdate Yellow method in accordance with standard guidelines (APHA 1995), and Total Nitrogen (TN) was measured as nitrate nitrogen using the HANNA (HI 5315) probe. The following equation 3 and 4 were used to compute the removal rate (mg L^{-1} day^{-1}) and removal efficiency (%) for nutrients (Eladel *et al*. 2019) [18](Eladel *et al*. 2019) [18]

Removal efficiency = $C_i - C_H / C_i \times 100$ (3)

Removal rate = $C_i - C_H / t_H - t_0$ (4)

where C_i is the initial concentration (as mg L^{-1}) at time (t_0), while C_H is the concentration after time (t_H).

Determination of crude protein, carbohydrate, and lipid content of microalgal biomass

The crude protein content in the samples was estimated per the Micro-Kjeldahl method (Trivedy and P. K. Goel 1984) [55] using a semi-automated Kjeldahl apparatus (Tulin Equipment Chennai). The carbohydrate extraction and assessment were done using Dubois's method (DuBois, Gilles, and J.K. Hamilton, P.A. Rebers 1956) [16]. The extraction of crude lipid from the lyophilized algal biomass was carried out using Bligh and Dyer's method (Bligh, E.G. and Dyer 1959) [9]. The percentage of total lipids in algae (TL) (%) was calculated using the following Equation (5) (Joseph and Ray 2024) [28], and the lipid productivity (LP) (mg/ L/day) was calculated as per Equation (6) (Matos *et al*. 2015) [35].

$$TL\,(\%) = \frac{\text{Weight of Lipid (mg) x100}}{\text{Weight of sample (mg)}}$$

$LP = BP \times TL/100$ (6)

where BP = biomass productivity and TL = total lipid content

Statistical Analysis

The statistical analysis and the data processing were performed using SPSS version 21, Microsoft Excel and the GraphPad Prism 5.0 software. All the experiments were conducted in triplicates. Results are represented as mean ± standard deviation. Percentages are expressed as weight/weight on a dry biomass basis. One-way analysis of variance (ANOVA) and Tukey post hoc analysis was done to compare the differences between media composition and algal species regarding biomass yield, biomass productivity, Nitrogen and Phosphorus removal rate and removal efficiency, total carbohydrate, total protein, total lipid and pigment content.

Result and Discussion

Taxonomic identity of the Algae

Chlorococcum humicola (Nageli) Rabenhorst.
Phylum: Chlorophyta
Class: Chlorophyceae
Order: Chlamydomonadales
Family: Chlorococcaceae
Genus: *Chlorococcum*
Species: C. *humicola*

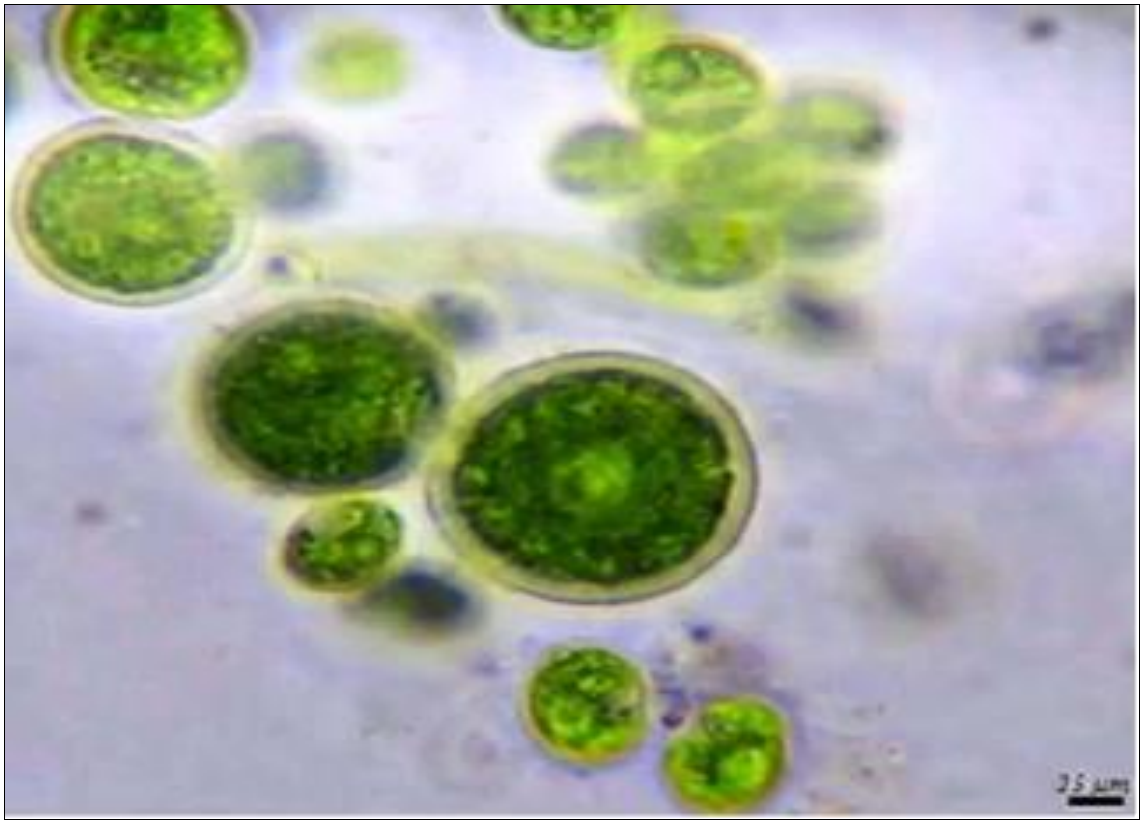

Fig 1

Table 1: The biomass yield and biomass productivity

Media conc/Algae*	Biomass yield	Biomass Productivity	N Removal		P Removal	
			NRR	NRE	PRR	PRE
Experiment 1 (Variation in N content at BBM level of P & K)						
BBM1/2N	150.07±4.34	48.83±1.16	0.928±0.024 0.024[e]	88.210±1.694	1.45±0.035 0.035[a]	57.06±2.58
BBM2N	265.19±9.47	88.65±2.06	2.610±0.241 0.241[c]	66.090±2.006	1.357±0.035 0.035[a]	51.77±0.69
BBM3N	345.45±3.71	110.86±3.77	4.309±0.193 0.193b	75.529±2.501	1.309±0.015 0.015[a]	50.04±2.92
BBM4N	249.48±4.54	89.57±4.54	4.691±0.107 0.107a	65.785±0.949	1.370±0.026 0.026[a]	50.00±1.55
Experiment 2 (Variation in P at BBM level of N content)						
BBM1/2P	183.09±4.46	59.20±3.56	1.795±0.101 0.214[a]	88.626±0.546	0.79 ±0.101[d] 1.224	39.81±1.224
BBM2P	328.63±3.55	105.50±3.01	1.930±0.053 0.053[a]	91.337±1.158	1.538±0.43 0.43[a,b]	35.63±1.39
BBM3P	291.74±4.58	95.02±2.53	1.895±0.106 0.106[a]	91.059±1.090	1.791±0.010 0.010[a]	26.66±2.079
BBM4P	216.75±5.03	70.42±1.06	1.870±0.021 0.021a	89.365±1.579	1.876±0.017 0.017[a]	22.36±2.479
Experiment 3 (Variation in N and P together)						
BBM1/2NP	130.64±3.48	41.71±0.91	0.938±0.036 0.036[c]	84.354±2.513 2.513	0.110±0.010 0.010[b] ±1.544[a]	10.03±1.94 0.010[b]
BBM2NP	239.85±3.97	78.96±2.51	3.212±0.102 0.102[a]	82.709±2.062 2.062	0.112±0.007 0.007[b] 0.110[a]	3.71±1.234 1.234[c] 0.007[b]
Control (Normal BBM)						
BBM	192.37±7.88	64.40±2.20	1.815±0.101 0.101[d]	89.429±1.505	1.120±0.110 0.110[c],	38.83±1.25

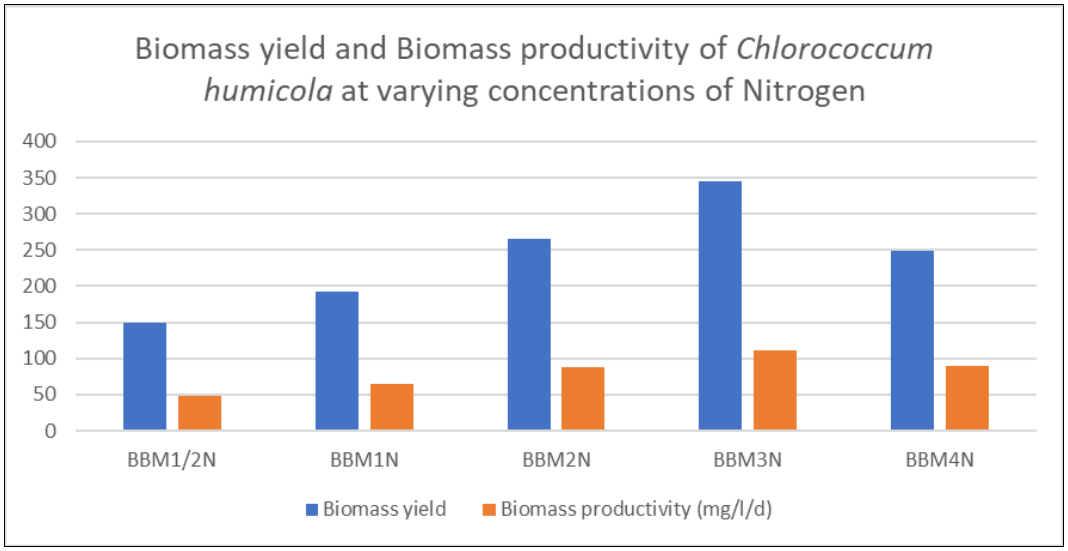

Fig 2: (a)

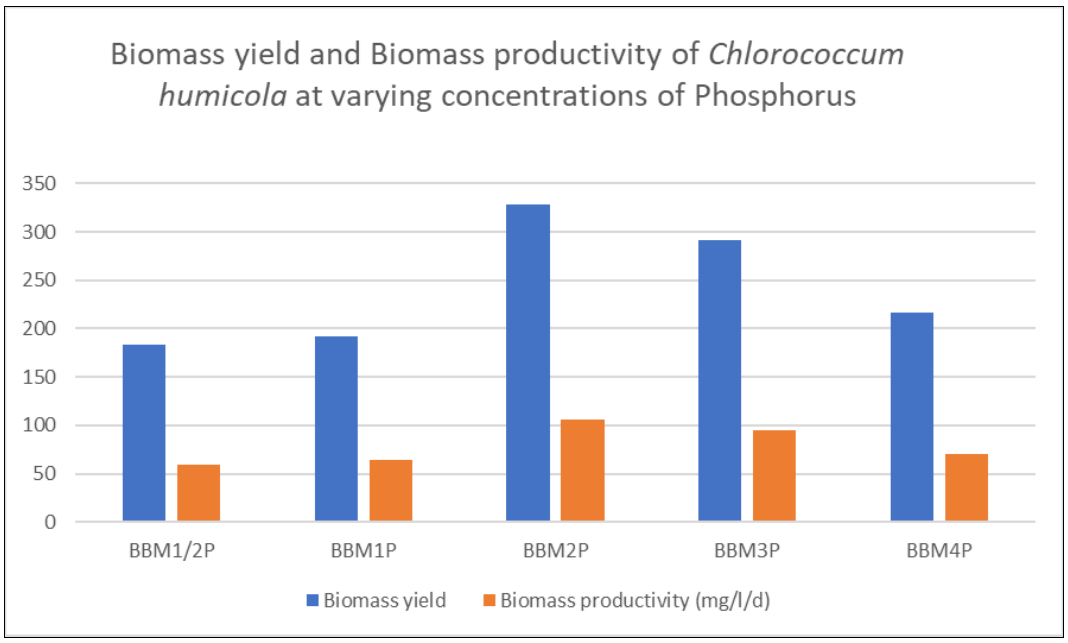

Fig 2: (b)

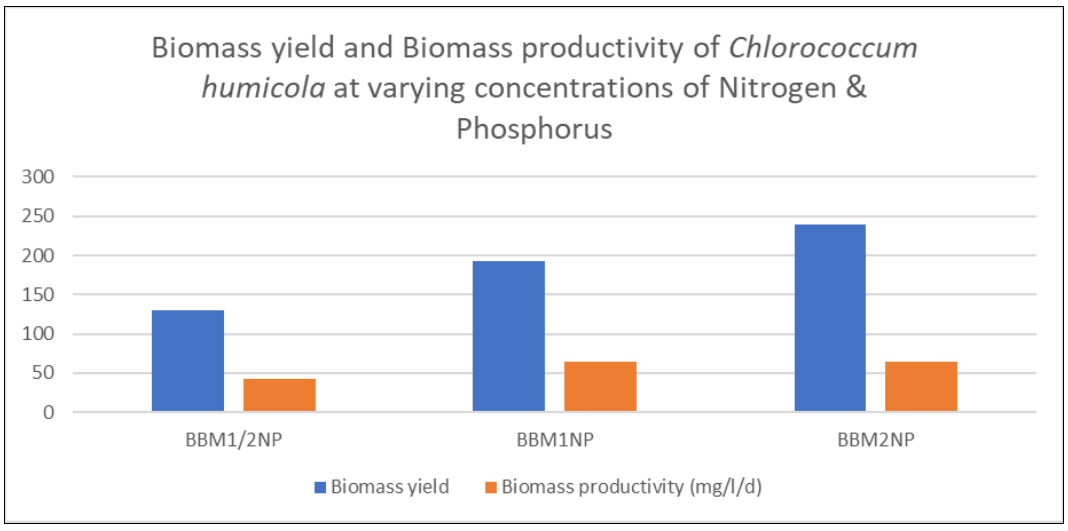

Fig 2: (c)

The biomass yield and productivity details are given in table 1 and figs. 2 a,b and c respectively. In varying concentrations of Nitrogen, the biomass yield and biomass productivity of *C. humicola* ranged from 150.07±4.34%

increase and 48.83±1.16 mg L^{-1} day^{-1} (BBM1/2N) to 345.45±3.71% increase and 110.86±3.77 mg L^{-1} day^{-1} (BBM3N). When the concentration of N is increased above 3N, the biomass yield was found to be significantly reducing, showcasing the intolerance of the algae to grow in very high concentration of N. Statistical analysis has shown that there was a significant difference ($p < 0.05$) in the biomass yield and biomass productivity of *C. humicola* at varying concentrations of Nitrogen.

In varying concentrations of Phosphorus, the biomass yield and productivity of *C. humicola* ranged from 183.09±4.46 % increase and 59.20±3.56 mg L^{-1} day^{-1} (BBM1/2P) to 328.63±3.55 % increase and 105.50±3.01 mg L^{-1} day^{-1} (BBM2P). When the concentration of P is increased above 2N, the biomass yield and productivity was found to be significantly reducing, showcasing the intolerance of the algae to grow in higher concentrations of P beyond 2P. Statistical analysis has shown that there was a significant difference ($p < 0.05$) in the biomass yield and productivity of *C. humicola* at varying concentrations of Phosphorus.

In varying concentrations of Nitrogen and Phosphorus, the biomass yield and productivity of *C. humicola* ranged from 130.64±3.48 % increase and 41.71±0.91 mg L^{-1} day^{-1} (BBM1/2NP) to 41.71±0.91% increase and 239.85±3.97 mg L^{-1} day^{-1} (BBM2NP). Statistical analysis has shown that there was a significant difference ($p < 0.05$) in the biomass yield and productivity of *C. humicola* at varying concentrations of nitrogen and Phosphorus.

It is clear from Figs 2 a,b and c that the algal response varied to variations in N and P, which is observed previously as well (Mahmoud *et al*. 2015) [32]. The algal growth and yield are also related to the total amount of N or P in the medium (Figler *et al*. 2021) [21]. In the first experimental trial of all three experiments, N-limited, P-limited and N & P limited treatments (half the amount of N, P, and N & P in BBM), the species' biomass yield and productivity became lower than that of the control as expected for green algae in general. However, on increasing N in the medium (3-4 times N in the BBM and N:P ratio 5.13 - 6.84), the biomass yield of *the algae* showed a decreasing trend, reaching the lowest at BBM4N (164.64 ppm NO_3-N). The observation was similar to that of *Coelastrum morus*, where an increase in the N:P ratio of five or more is favorable for its biomass yield (Figler *et al.* 2021) [21].The present findings supports the previous literature data that higher N:P ratios are better for microalgal growth (Mayers, Flynn, and Shields 2014) [36] in the cases of higher N content but up to certain limits for specific species. In the second experimental trial of N:P lower than one due to an increase in P content, *C. humicola* shows an increase in productivity in BBM 2P (Fig. 2 b), which reveals the capacity of the species to grow at N:P (0.98) lower than that of BBM but with an excess of P. Therefore, *C. humicola* may be further experimented with for industrial wastewaters with high P content and low N:P ratios. *C. humicola* showed a significant steady increase in biomass from ½ to 2 N and P in the medium. Therefore the species may be considered algae tolerant to increased N and P nutrients.

Nutrient Removal Rate (N and P) and Nutrient Removal Efficiency (N and P)

Nitrogen removal rate (NRR) and nitrogen removal efficiency (NRE)

The details of the NRR and NRE of *C. humicola* are in **Table 1**. It is clear from **table 1** that the NRR of *C. humicola* increases with an increase in N concentration however NRR remains the same without any significant difference when the concentration of P is varied in the medium. The NRR also increases with an increase in N and P concentrations in the medium.

The NRE decreased significantly with an increase in N concentration from 41.16 ppm nitrate N to 164.64 ppm nitrate N. The NRE remained the same without any significant difference as the phosphorus concentration increased from 33.32 to 173.14 ppm. However, when the concentration of N and P increased without a change in N:P, the NRE was found to be significantly decreasing as is visible from Table 1.

High nitrogen (N) levels in water are a significant pollution problem, particularly in natural water bodies within intensive agricultural areas (Mali, Sanyal, and Bhatt 2015) [33], leading to serious ecosystem damage (Malik *et al*. 2020) and posing risks to human health (Clements, Lee, and Bloomer 2014) [13]. Nitrogen is also an essential and costly fertilizer critical to global agricultural productivity (Mohammed *et al*. 2022) [37]. Furthermore, N is a vital nutrient for microalgal growth and production (Daneshvar *et al*. 2019) [14]. Phycoremediation of nutrient-rich agricultural and industrial wastewaters high in N can allow for the recovery of lost nitrogen as a valuable biomass resource (Ummalyma *et al*. 2022) [57], given the availability of suitable microalgal species with high-quality biomass adapted to specific geographic zones and nutrient profiles of wastewaters. This study therefore examines the Nitrogen Removal Rate (NRR) and Nitrogen Removal Efficiency (NRE) of *C. humicola* under different nitrogen and phosphorus concentrations, both individually and combined providing a model system to identify algal species suitable for phycoremediation. In the experimental media (BBM), nitrate ions provided as $NaNO_3$ are absorbed into algal cells via 'Nit' or 'permease' receptors on the plasma membrane, facilitating nutrient removal from the media (Sanz-Luque *et al*. 2015) [49].

The study revealed that at constant P, the NRR and NRE of the species tested varied per the amount of N and N:P ratio in the medium. In general, all the species showed the highest NRE at the control and the lowest N content in the medium

It is already known that at a low amount of N in media, NRE is usually almost complete (Shriwastav *et al*. 2014) [51]. However, the NRE by *C. humicola* decreased with further increase in N (and N:P) in the medium (**Table 1**). In general, microalgae utilize N and P together for biomass production. Therefore, an excessive supply of any one major nutrient element can lead to the total removal of the other essential nutrients for growth from the waters (Beuckels *et al*. 2015) [8] at the beginning, leading to no change in NRE further. In the present study, *C. humicola* showed a significant increase in NRR at an increase of N up to 3 to 4 times in the medium. It suggests that a high N:P ratio with higher N content favors the NRR of by species. Since N:P showed no influence on NRR by certain species previously (Figler *et al*. 2021) [21], the present observation suggests species specificity in NRR by different green algae. It may also be noted that *C. humicola* has an NRR of 4.69±0.71 mg l^{-1} d^{-1} at 164.64 mg l^{-1} N (N:*P* = 6.84) in the medium, which corresponds to an NRE of 65.78±5.56%. Therefore, it can be considered a promising species for further experimentation in treating industrial wastewater rich in N with high N: P. Moreover, the NRR and NRE observed for

the species in the present study remain within the range of algae used in the treatment of dairy wastewater (Vidya *et al*. 2021) [58], aquaculture wastewater (Akmukhanova *et al*. 2022) [1], palm oil mill effluent (Emparan, Harun, and Jye 2019) [19], municipal wastewater (Škufca *et al*. 2021) or rice mill effluents (Umamaheswari and Shanthakumar 2021) [56] as supplemented growth media and can be successfully used in treating such industrial wastewaters. Although the NRR of *C. humicola* was at 4.49 and 4.69 mg l^{-1} d^{-1} at four times N in the medium, the biomass yield remained significantly lower than that at three times N in the medium. Similar observations are also available previously (Choi and Lee 2014) [11]. However, the present study reveals that *C. humicola* may be considered promising species for further investigation in nitrogen removal from eutrophic waters, especially at higher N:P ratios with high N content and P limitations

In *C. humicola*, NRR at two times N and P together was considerably higher than its NRR at two times N alone in the BBM, suggesting that sufficient P availability than an increase in N:P ratio is essential to NRR of such green algal species. Such species specificity in NRR is already known for *Desmodesmus* (G. Li *et al*. 2021)

Phosphorus removal rate (PRR) and phosphorus removal efficiency (PRE)

The details of the PRR and PRE-of *C. humicola* are also listed in Table 1. It is clear from Table 1 that the PRR of *C. humicola* increases with an increase in P concentration from 33.32 to 133.19ppm. From 133.19 to 173.14ppm, the PRR remained same without any significant difference. However, PRR remains the same without any significant difference when the concentration of N is varied in the medium. The PRR remained same without any significant difference when the concentration of N and P is increased in the medium up to BBM2NP.

The PRE-remained the same without any significant difference when the concentration of N is increased from 20.58 to 164.64 ppm, except at 44.16 ppm N where the PRE-decreased significantly. With the increase in P, PRE-remained the same without significant difference from 33.32 ppm P to 93.24 ppm P but decreased significantly when the concentration of P is increased from 93.24 to 133.19ppm and then remained the same without any significant difference till 173.14 ppm P. However, when the concentration of N and P increased without a change in N:P, the PRE-was found to be significantly decreasing both towards lower and higher concentrations of N and P as evident from table 1.

PRR is directly proportional to P in the medium. Maximum PRR of 1.867±0.017 was observed when the amount of phosphorus was four times in the medium. Previously also, researchers have demonstrated increased PRR with increased phosphorus in the media (Figler *et al*. 2021) [21]. In general, the PRR and PRE-of *C. humicola* showed an increase at half the amount of nitrogen than that in the standard BBM (control). This suggests a low N:P ratio from less N content favorable for PRR and PRE-of the species. Since the same species was also showing a high PRR at low N:P from high P content in the medium, it may be suggested that a low N:P ratio is generally favorable to the PRR of this species. In *C. humicola*, PRR remained the same without any significant difference at all the increasing levels of N in the medium. Some green microalgae store phosphate in their cells as volutin granules through a luxury uptake pathway (Bahman *et al*. 2022) [6]; Gupta & Pawar 2018) [26]. Such reserves are used for the prolonged growth of microalgal cells (Tripathi, Gupta, and Thakur 2019) [54]. Therefore, a local variation in P in the growth environment may not represent an immediate response to excessive removal or uptake of P into algal cells. It indicates that this species can remove P from waters with a higher N:P ratio from higher N concentration.

Generally, the low PRR from a high N content or high N:P ratio acts as stress, negatively affecting the phosphorus uptake (Arbib *et al*. 2013) [5].

The Biochemical characterization

Table 2: Protein, carbohydrate, and lipid content of algal biomass

Media conc/Algae*	Protein content	Carbohydrate content	Lipid content
Treatment 1 (Variation in N content at BBM level of P)			
BBM1/2N	19.87±0.971	18.75 ±1.39	34.26±0.60
BBM2N	27.35±1.625	11.25±1.36	29.38±0.86
BBM4N	38.89±1.867	9.27 ±0.67	22.39±1.58
Treatment 2 (Variation in P content at BBM level of N)			
BBM1/2P	22.74±0.967	15.44±1.33	28.79±1.30
BBM2P	26.08±0.648	15.61±0.54	25.51±0.46
BBM4P	25.39±0.458	16.58±0.50	23.96±1.16
Treatment 3 (Variation in N and P together)			
BBM1/2NP	18.72±0.931	18.13±1.23	35.27±0.50
BBM2NP	28.47±0.434	9.84±0.85	25.82±1.27
Control (Normal BBM)			
BBM	24.20±1.618	16.12±1.37	29.68±1.24

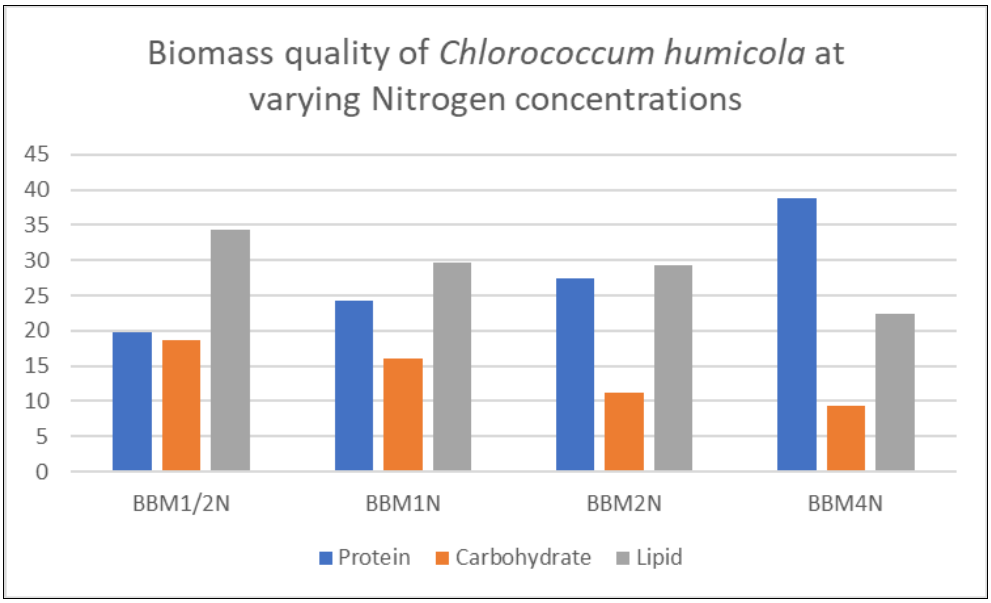

Fig 3: (a)

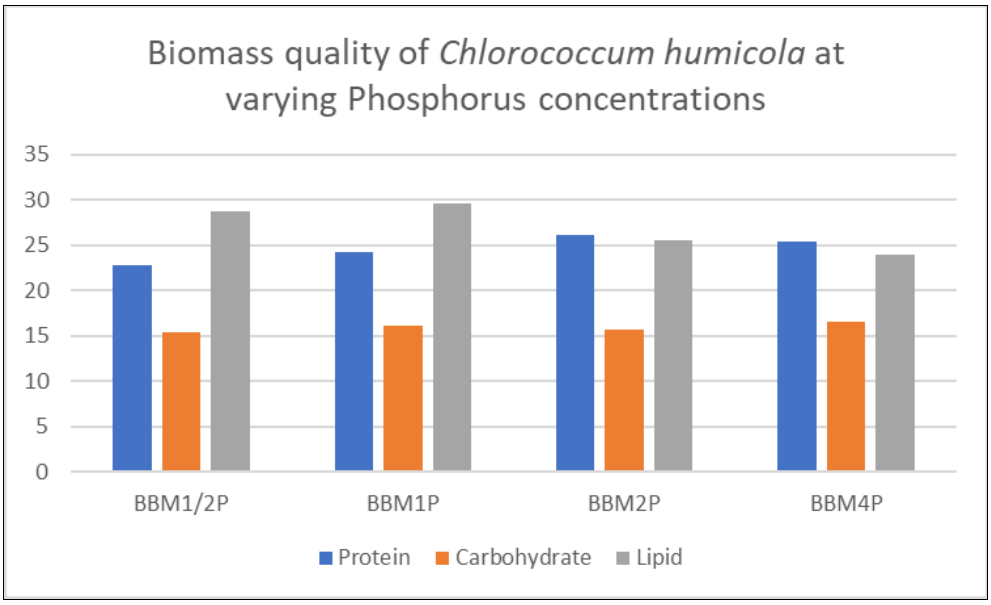

Fig 3: (b)

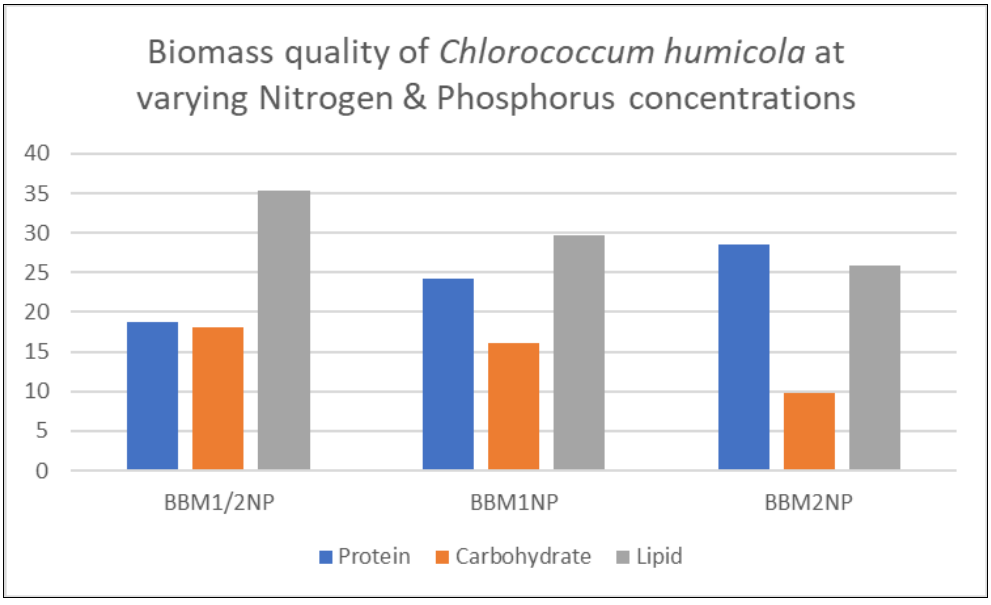

Fig 3: (c)

The details of the protein, carbohydrate and lipid content of *C. humicola* at varying N (Treatment 1) in the medium, at varying amount of P in the medium (Treatment 2) and at varying amount of N&P together in the medium (Treatment 3) are examined in detail (Table 2) and Figs 2 a, b and c. The protein content was directly proportional to the amount of nitrogen in *C. humicola*. The alga has the highest protein content of 38.89% when N was excessive in the medium, at 4 times N of BBM (keeping P at BBM level). The lowest protein content of 18.72% was observed when the amount of N & P was minimal in the medium (BBM1/2NP). However there is no variation in the protein content when the P level was increased from BBM2P to BBM4P.

At varying amounts of N, and varying N & P together, the carbohydrate content of *C.humicola* was inversely proportional to the amount of Nitrogen in the medium (Fig.2). However carbohydrate content did not show any variation when the amount of P was varied in the medium keeping N constant. The highest carbohydrate content of 18.75 ±1.39 % was observed when the medium had the lowest N content and the lowest content of 9.27 ±0.67 % was observed when N content was highest in the medium (BBM4N)

At the varying amounts of N, N, and P together and varying P in the medium, the lipid content was inversely proportional to the amount of N, P and N& P together in the medium for *C. humicola*. The algae showed the highest lipid content at half the amount of N or P or N & P together in the medium. The highest lipid content of 35.27±0.50 % was observed in BBM1/2NP and BBM1/2N without any significant difference. The lowest lipid content was observed in the media with the highest N content.

In general, the protein content was directly proportional to the amount of nitrogen in the species. The positive response of algae in protein content to an increase in N in the medium is well-known (Ansari *et al*. 2017; Fal *et al*. 2022) [3, 20]. But according to Safafar *et al*. (2016) [45] (Safafar *et al*. 2016) [45] the amount of protein in microalgae varies depending on the species and is regulated by growth stage, photosynthetic rate, and nitrogen availability.The protein content of the species was also measured at varying amounts of P in the medium (keeping N constant at the BBM level). In general, an excessive amount of P in the medium is useful to the growth and yield of algae (El-Sheek and Rady 1995; Richmond 2004) [17, 44]. However, a deficiency of P in the medium may cause a relative increase in carbohydrate content in algal cells (Baiee and Salman 2016) [7]. The protein content of the *C. humicola* also decreased significantly from that of the control at BBM1/2P. Moreover, the protein content of *C. humicola* remained the same as that of BBM level at 2-4 times P in the medium. In the present study, BBM levels of P were found optimum for the protein content of the algae. The protein content of *C. humicola* was significantly lower than that of many species reported in the literature at all concentrations of P except at control, revealing its weakness as a protein-yielding species.The protein content at half the NP of that of the BBM level was significantly lower than that at the normal BBM level of NP. Previous studies also reveal that certain microalgae show a decrease in protein content in response to starvation (Chokshi *et al*. 2016; Pacheco *et al*. 2021) [12, 39]. However, at double the NP level, the protein content of *C. humicola* showed a significant increase (Fig.3 c) revealing species-specific responses to the rise of nutrients in the growth medium. In general, the present study also revealed that *C. humicola* can produce protein-rich biomass especially if they are cultured without stress, which agrees with previous reports (Amorim *et al*. 2021) [2].

In general, nitrogen starvation cause algae to show decrease in nitrogenous content in the biomass (Pancha *et al*. 2014) [40] and a general increase in its carbohydrate content (Brányiková *et al*. 2011). *C. humicola* showed a significant decrease in total carbohydrate content at two-times N in the medium than that at the control. Still, it remained constant without any significant difference at four-times N in the medium. It is well-known that some species of algae usually manufacture large quantities of polysaccharides, while others do so only when subjected to cellular stress conditions (Moreira *et al*. 2022) [38]. It is already known that P deprivation in the medium can induce an increase of carbohydrate content in certain species of algae (Brányiková *et al*. 2011; Markou, Angelidaki, and Georgakakis 2012) [34]. But in the present study, the carbohydrate content remained the same without any significant difference in all concentrations of phosphorus showing species-specific characteristics under all varied Phosphorus conditions in the media. At varying amount of NP together in the medium *C. humicola* showed the same carbohydrate content without any significant difference from the control at half NP level in the medium. At two-times NPK in the medium, *C. humicola* and *Curvastrum pantanale* showed a significantly lower carbohydrate content than that at its control. These observations also reveal the previous reports that the carbohydrate profile is impacted by nutrient starvation (González-Fernández and Ballesteros 2012) [23] in microalgae especially in the case of Nitrogen.

In general, algae show higher lipid yield at low N content in the medium (Y. Li *et al*. 2008). According to Griffith *et al*. (2014) (Griffiths, Van Hille, and Harrison 2014) [25], lipid accumulation can be negatively impacted if N availability is critical in the medium. But *C. humicola*, lipid content was decreasing from control towards an increase in N in the medium. Therefore, the exact relationship between lipid content and N availability in the medium in specific species of algae need to be worked out through further experimental studies In *C. humicola* the lipid content at half the amount of P and K in the medium, at the control and two times P and K in the medium was significantly higher than that at 4 times P and K in the medium. In previous studies, P deprivation has induced excessive lipid content in certain species of algae (Xin *et al*. 2010) [59],(Yaakob *et al*. 2021) [60]. But the present study reveals that for *C. humicola* P levels upto 2P will not have a significant influence in the lipid content of the alga. *C. humicola* showed significantly lower lipid content at two times NPK than that at the control. The third experimental studies in general reveal that when nutrients are in balance, a general deprivation of N & P as a stress can induce more lipid production in all the five species tested. Previously also researchers have reported that environmental stress of diverse kinds including nutrient deprivation induce lipid production in algae (Sun *et al*. 2018) [53].

Conclusion

Green algae with a high global biological diversity and biomass potential have a range of industrial applications

such as phycoremediation of wastewaters, nutrient recovery, and bioresource production for food, feed, nutraceutical, and medicinal properties, which can be combined in many ways in industrial processes. The prospects of such algal industries depend on deep experimental assessment of local strains regarding their industrial potential. Then only sufficient candidate species can be added for treating specific wastewaters concerning diverse nutrient or other environmental regimes. The present comprehensive experiment with *C. humicola* involving three experiments and diverse treatments at each experimental level for testing specific amounts of N or P or NP in the medium remains a model for the preliminary testing of algae for assessing their phycoremediation potential combined with biomass quality and yield. In the present assessment, *C. humicola* was proved to be a promising species concerning the removal of N and P for further industrial trials in treating eutrophic wastewater combined with simultaneous production of valuable biomass resources.

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