



Microalgae Growth and Phosphorus Uptake of *Chlamydomonas Reinhardtii* 11/32C under Different Inorganic Nitrogen Sources

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Abstract: Microalgae have been proven to be effective in utilizing nitrogen (N) and phosphorus (P) from a wide range of wastewater sources. This ability enhances the potential role that microalgae may have not only in wastewater bioremediation, but also in algal biomass production as an alternative feedstock for biodiesel and bio-fertilizer production. To investigate the ability of microalgae at recovering nutrients, the microalga strain *Chlamydomonas reinhardtii* 11/32C was selected to determine P uptake rates. Results shown that *C.reinhardtii* 32C cultivated in combination of NO_3^- and NH_4^+ as nitrogen sources was able to uptake $0.067 \text{ mg P l}^{-1}\text{d}^{-1}$. Combination of both nitrogen can produce specific growth rate of 0.128 d^{-1} and $89 \text{ mg VSS l}^{-1}\text{d}^{-1}$ of biomass dry weight. These value is the highest compare to *C.reinhardtii* 32C cultivated in NO_3^- or NH_4^+ . These findings are fundamental to understand and plan future studies for cultivation conditions to induce luxury nutrient uptake by selected microalga.

Keywords: Algal growth, *C. reinhardtii*, P uptake

1. Introduction

Eukaryotic microalga is a simple multicellular microorganism which can grow in severe environmental condition [1]. Microalgae provide an essential characteristic in their multi-purpose function, for instance, in tertiary wastewater treatment, food production, feed and chemicals [2]. The major problem of wastewater contains a high concentration of nutrients, mainly N and P in which require high cost for a chemical treatment to remove them during wastewater treatment [3]. Microalgae provide a sustainable system for nutrient removal because of their ability to assimilate nutrient and organic carbon. The algae have their potential feature in bioremediation options and also in algal biomass production as an alternative feedstock for bio-fertilizer production [4].

In the previous studies, microalga showed to be able to remove nutrient from various wastewater sources. *Chlorella* sp and *Chlorella vulgaris* removed N and P from wastewater by a removal efficiency from 76 to 83% for N and from 63 to 75% for P in digested manure [5]; 55 to 88% for N and 12 to 100% for P in municipal wastewater [6]; and 30 to 95% for N and 20 to 55% for P in industrial wastewater [7]. Furthermore, microalgae have been employed as a bio-fertilizer. Faheed and his co-workers [8] observed that dried microalgae could act as a soil additive which improves plant nutrients.

Another study also examines the effect of blue-green algae as a bio-fertilizer to enhance yield and mineral composition of the rice plant. This application can improve N soil status through atmospheric N fixation [9].

As wastewater may contain different forms of N sources (e.g. Nitrate, Ammonium), it is crucial to understand the effect of various N sources on microalgae growth and nutrient uptake rate [10]. As observed by Tam and Wong [11] and Larsdotter [12], Ammonium is the preferred compound of N source for microalgae as a redox reaction is not incorporated in its nutrient assimilation [13]. However, Fernandez and Galvan [14] have studied that nitrate is more abundant in the environment mainly in natural soils, and its utilization became fundamental pathways in incorporating the macronutrient of N in living organisms.

This study was conducted to investigate the ability of green microalgae *C. reinhardtii* 32C in recovering nutrients primarily to determine N and P uptake rates under different of nitrogen sources (e.g. NO_3^- , NH_4^+ , NO_3^- , NH_4^+). The results of this study are essential to conducting further research for enhancing luxurious nutrient accumulation in algal cells by applying a wide range of environmental factors and selected nitrogen sources.

2. Materials and Method

The algae cultivated in Bold's Basal Medium (BBM) and placed in a 2.5 litre of tubular photobioreactors (PBRs). Three PBRs were set containing (i) NO_3^- , (ii) NH_4^+ and (iii) a mix of NO_3^- and NH_4^+ as nitrogen sources in BBM used for these experiments (Fig. 1). The algae sample were taken for every two days over the cultivation time of 14 days. Then the sample was analyzed for TSS, VSS, organic-N, and organic-P. The remaining nutrient concentration in the solution was also analyzed for NO_3^- , NH_4^+ and PO_4^{3-} concentration. NO_3^- was analyzed by using ion chromatography (850 Professional IC Metrohm) and the other parameters were analyzed according to standard methods for the examination of water and wastewater (APHA, 2012) [15] as listed in Table 1.

Table 1 - Standard methods for wastewater sample examination

No	Parameters	Method Code*
1	TSS	2540 D,E
2	VSS	2540 D,E
3	Org-N	TKN-4500-Norg
4	Org-P	4500-P, B
5	NH_4^+	4500-NH ₃ B,C
6	PO_4^{3-}	4500-P, E

*Standard methods for the examination of water and wastewater (APHA, 2012), unless otherwise stated



Fig. 1 - *C.reinhardtii* 11/32C cultivation with different nitrogen sources: NO_3^- (left), NH_4^+ (center), and a mix of NO_3^- and NH_4^+ (right)

25 gr l⁻¹ of NaNO₃ and 10 gr l⁻¹ of NH₄Cl used as N sources and followed by 1.41 mg of K₂HPO₄ and 3.29 mg KH₂PO₄ used as P sources. The following environmental conditions were set to these experiments: 100 mg N l⁻¹, 1 mg P l⁻¹; photoperiod 12 hr light; 12 hr dark; and light intensity 250 μE/m²s.

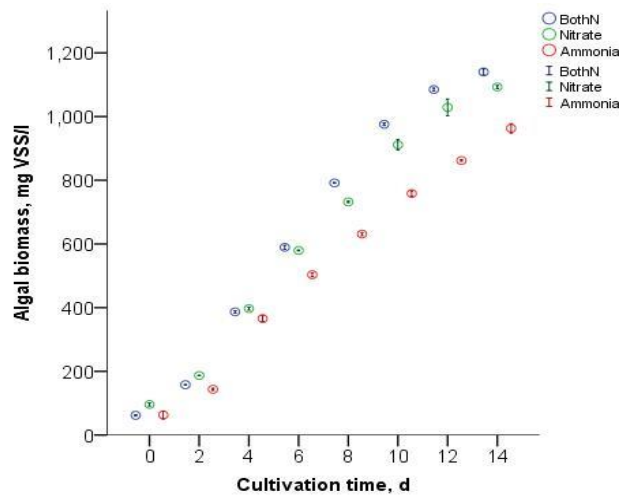
3. Results

3.1 Effect of Inorganic Nitrogen Source on Algae Growth and Biomass Productivity

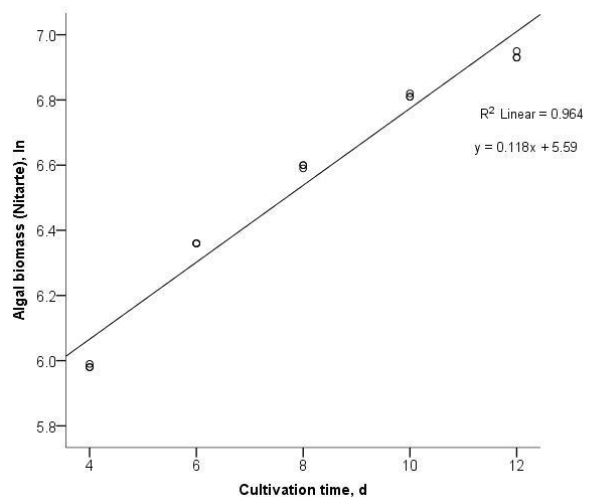
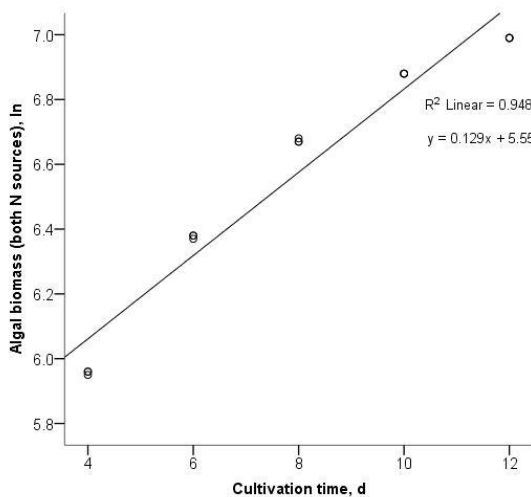
Different N sources (e.g., (1) NO₃⁻ and NH₄⁺; (2) NO₃⁻; and (3) NH₄⁺) were used to investigate the effect of N source on the algal growth rate, P uptake and biomass production. As shown in Fig. 2, algal growth rate and biomass productivity increased during the exponential growth phase (between days 4 and 12). The exponential growth phase was confirmed by linear correlation of transformed data (Ln VSS v time) with a resulting R² value of 0.99, which gave confidence when calculating specific algal growth rates.

Results showed that there was no significant difference in microalgae growth and biomass production under different N sources (*p*>0.05). However, specific algal growth rates and biomass productivity cultivated in a media containing both NO₃⁻ and NH₄⁺ (experiment 1) was always higher than the figures reported for NO₃⁻ and NH₄⁺ on their own (experiments 2 and 3, respectively). The specific algal growth rates were 0.128 d⁻¹, 0.117 d⁻¹, and 0.106 d⁻¹ for experiments 1, 2, and 3, respectively.

Algal biomass productivity for experiment 1 (NO₃⁻ and NH₄⁺ mix) was 89 mg VSS l⁻¹ d⁻¹, while the figures for algae grown in media containing only NO₃⁻ or NH₄⁺ were 79 and 62 mg VSS l⁻¹ d⁻¹, respectively. In practical terms, it seems that *C.reinhardtii* 11/32C became better when using a combination of NO₃⁻ and NH₄⁺. Effect of six different nitrogen source and concentration were also tested on microalgae growth of *Scenedesmus bijugatus*. The algae grew well in all nitrogen sources, but maximum growth was reported for KNO₃ [16], similarly in *Botryococcus braunii* [17]. NH₄⁺ also showed to trigger the optimum growth and lipid content of marine microalgae of *Ellipsoidion sp.* during pre-logarithmic phase [18].

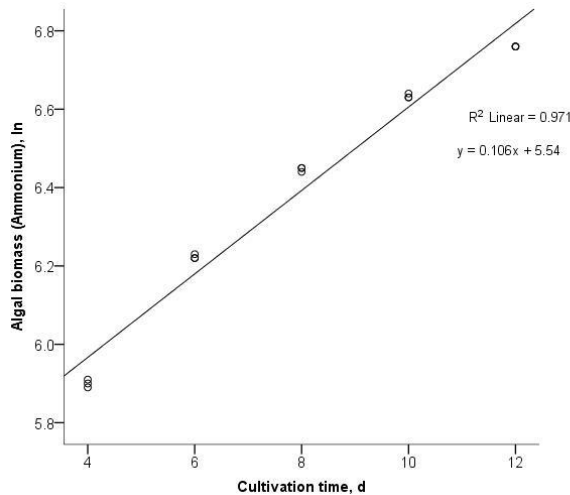


(a) Algal biomass concentration of *C.reinhardtii* 11/32C in different N sources



(b) The specific growth rate of *C.reinhardtii* 11/32C in both N sources

(c) The specific growth rate of *C.reinhardtii* 11/32C in NO_3^-



(d) The specific growth rate of *C.reinhardtii* 11/32C in NH_4^+

Fig. 2 - Algal biomass and specific growth rate of *C.reinhardtii* 11/32C using different nitrogen sources

Fig. 3 shows that *C.reinhardtii* 11/32C preferred to take up NH_4^+ over NO_3^- with a total ammonium removal from the media of 97%. However, the specific growth rate and biomass productivity of *C.reinhardtii* 11/32C grown found in experiment 3 is lower than those found in experiments 1 and 2. Also, algae have shown to consume less NO_3^- from the media with an average N reduction of 45%. This study thus has been demonstrated that *C.reinhardtii* 11/32C produced a higher specific growth rate and biomass productivity when cultivated with a mix of NO_3^- and NH_4^+ in the culture media (Experiment 1). That could imply the convenience of using the effluent from a conventional activated sludge process (i.e., no extended aeration for full nitrification) as the perfect culture media concerning algal biomass production.

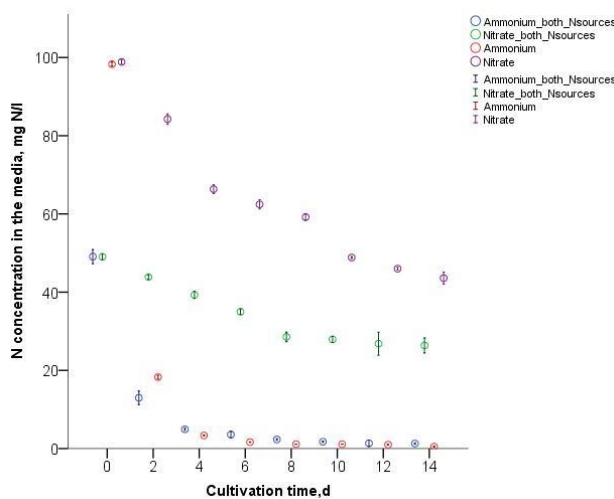


Fig. 3 - Nitrogen species concentration in the culture media over time

In terms of P assimilation, the concentration of PO_4^{3-} declined rapidly within the first two days of the cultivation period in all the media tested independently of the N source used (Fig. 4); the microalgae were able to remove more than 99% of PO_4^{3-} in all three experiments. It is because P is an essential nutrient for algal growth and metabolism that needed for producing cellular constituents such as phospholipids, nucleotides and nucleic acids [19]. The N concentration supply influences P storage in the algal biomass, and it is shown that P uptake by *C. reinhardtii* 11/32C was high due to high concentration of N in the medium, and the trend was similar with the P uptake by *Chlorella* and *Scenedesmus* [20]. PO_4^{3-}

concentration was reduced from approximately 1 mg P l⁻¹ to 0.012 - 0.063 mg P l⁻¹ during the first four days and then completely removed below the analytical detection limit by day 14.

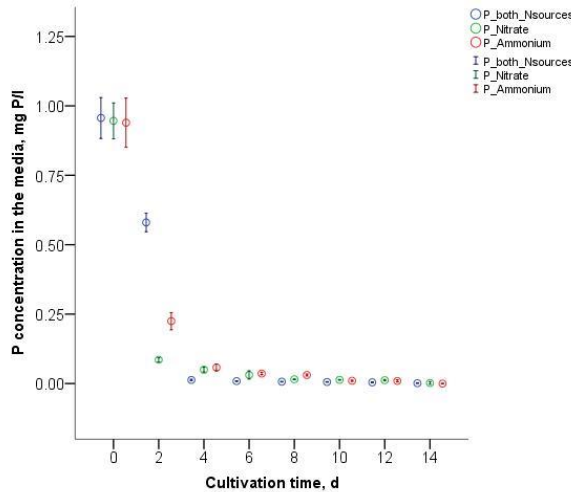


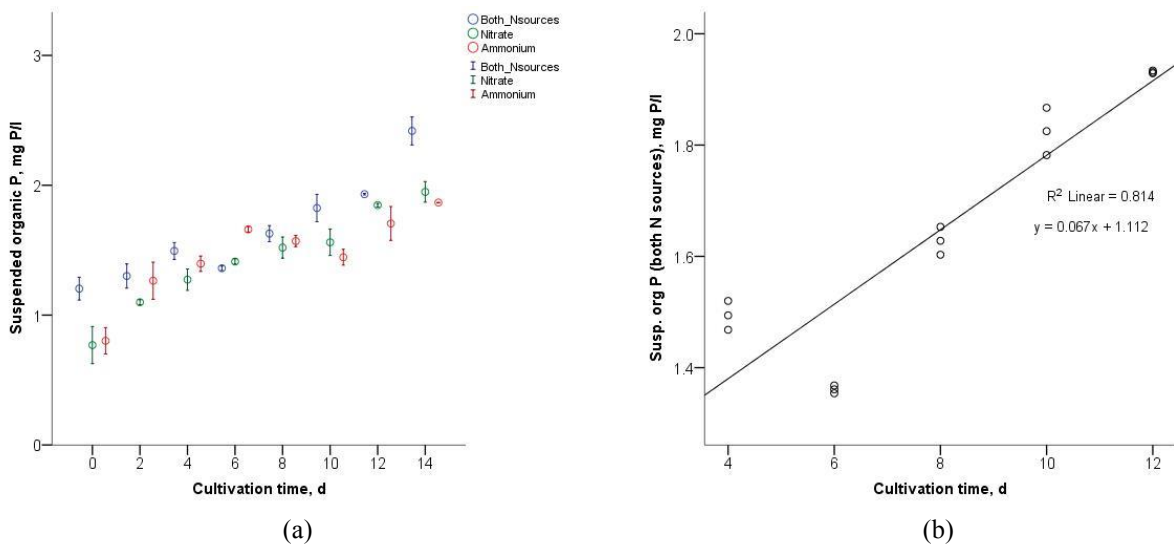
Fig. 4 - Remaining P concentration under different nitrogen sources

3.2 Effect of Different Inorganic Nitrogen Sources on P Uptake Rate of *C.reinhardtii* 11/32C

P uptake by *C.reinhardtii* 11/32C during the exponential growth phase occurred from day 4 to 12 (Fig. 5). The net P uptake rate was calculated from data reporting suspended organic P in the media over time. Exponential regression coefficients (R^2) for the three experiments testing different nitrogen sources were 0.8, 0.9, and 0.5, for experiments 1, 2 and 3, respectively. The combination of NO_3^- and NH_4^+ as N source influenced higher P uptake and produced higher concentrations of suspended organic P than using NO_3^- and NH_4^+ on their own, although a short lag phase was found from day 4 to 6.

The effects caused by switching NH_4^+ utilization in the first four days for NO_3^- afterwards enhanced net P uptake rates (Fig. 5(b)). This stressful condition triggered faster P uptake by microalgae, which continue day 14. After day 4, P uptake rate in Experiment 1 was 0.067 mg P l⁻¹d⁻¹, which is higher than P uptake rates found for experiments using NO_3^- or NH_4^+ ; P uptake rates for tests 2 and 3 were 0.065 and 0.020 mg P l⁻¹d⁻¹ respectively.

Interestingly, when the content of P was plotted against the corresponding N content in dry biomass, P content varied from 0.2 to 2.3%. The higher P content was about 1.8 to 2.3% when algae were cultivated in the media containing both NO_3^- and NH_4^+ and it corresponded to N content ranging from 3.0 to 4.2% (Fig. 6). From that, it is possible to infer that by using a media combining NO_3^- and NH_4^+ as a nitrogen source, Ammonium is rapidly consumed by microalgae and then the shift into nitrate as N source accelerates P uptake and in-cell storage.



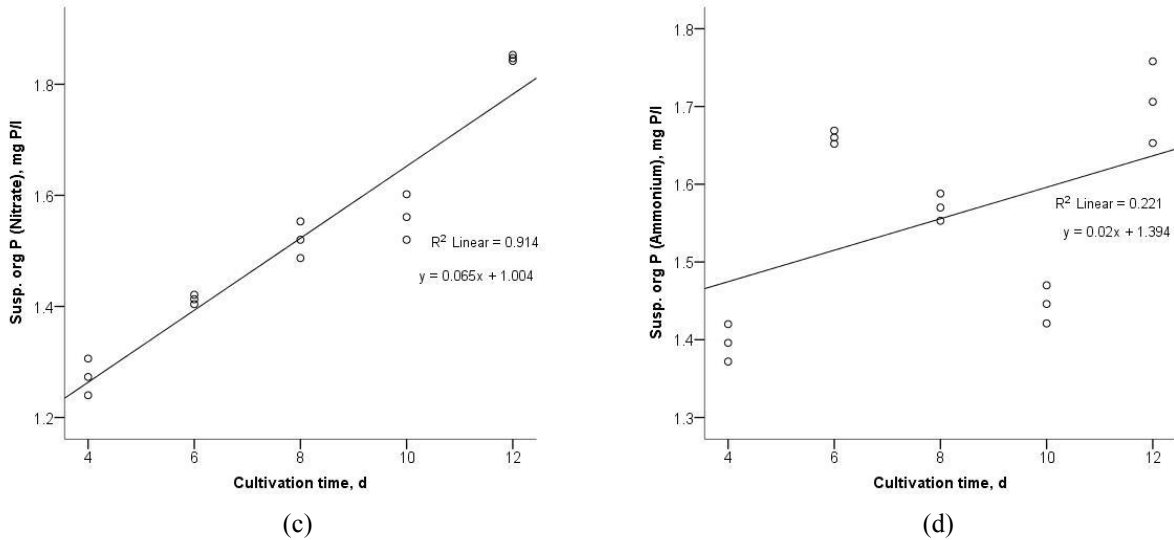


Fig. 5 - Suspended organic P over 14 days (a) and in exponential phase (b-d)

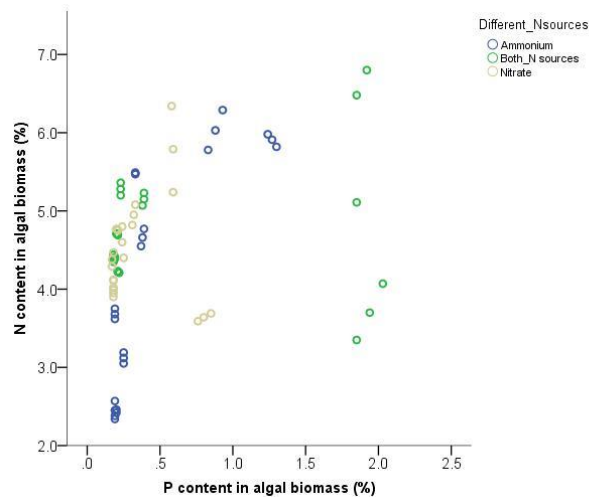


Fig. 6 - Phosphorus vs Nitrogen content in algal biomass (% dry weight)

4. Discussion

This study aimed to identify favourable N sources for luxury P uptake and algal biomass production using *C.reinhardtii* 11/32C as a model organism. In the photosynthetic process, the utilization of HCO_3^- by microalgae will result in releasing hydroxyl ions ($\text{HCO}_3^- \rightarrow \text{CO}_2 + \text{OH}^-$) [21]. As a typical response to inorganic carbon assimilation by microalgae, the pH increases as the photosynthetic activity progresses [10, 22]. Furthermore, regarding the presence of total ammonia in aqueous solution, the equilibrium between NH_4^+ and NH_3 is strongly pH-dependent [23]. The shift of NH_4^+ to NH_3 occurs due to changes in pH above 8, which displaces the equilibrium from NH_4^+ to NH_3 [22]. The uncharged NH_3 is most toxic because this form more soluble and readily transfer through the cell membranes than NH_4^+ [23]. It is also well studied that NH_4^+ inhibits microalgal growth at a concentration higher than 50 mg N l^{-1} [24] or even at 28 mg N l^{-1} [25], due to toxic effects caused by unionized ammonia (NH_3) [26].

Since NH_3 is gas, it is potentially volatile and can be lost to the atmosphere. However, at the pH above eight, only 10% of total ammonia is present as a toxic form of NH_3 and 90% as NH_4^+ [27]. It indicates that microalgae can assimilate more N in forms of NH_4^+ for their growth. The preference of NH_4^+ over the other nitrogen sources also conveyed in Fig. 3 that shows 97% of NH_4^+ utilized by *C.reinhardtii* 11/32C. Valero and Mara [28] reported that NH_3 volatilization was not the most critical mechanism on N removal process, because the NH_3 and total N were removed mostly through microalgal biological uptake. They also confirmed that the removal rate of NH_4^+ is faster than NH_3 volatilization [29].

Measurements of pH values during this study confirmed that the pH of the algae culture was as high as 9.6 when NH_4^+ was used as a sole nitrogen source. When other N sources were used, including a mix of NO_3^- and NH_4^+ and NO_3^- , the recorded pH was 9.8 and 9.7, respectively. Higher pH cause the algal growth rate and biomass production of

C.reinhardtii 11/32C in NH_4^+ was lower than in the combination of NO_3^- and NH_4^+ or NO_3^- . Similar effects were also observed in *Chlorella sorokiniana* [30] and *Scenedesmus bijugatus* [16].

Moreover, it was demonstrated that *C.reinhardtii* 11/32C preferred NH_4^+ over NO_3^- in which NH_4^+ removal was higher than NO_3^- . Consequently, this preference for Ammonium at the concentration levels of the tests conducted did not prove to have a significant inhibitory effect on algal growth. In addition to different nitrogen sources, P uptake was better by microalgae when a combination of NO_3^- and NH_4^+ were used. It might be caused by the stress effect of changing N source from ammonia to nitrate from the media. In contrast, P content in the algae grown in NH_4^+ only was also higher than the figures found when the culture media contained only NO_3^- . It is because the algae using NH_4^+ produced less biomass than when using NO_3^- and it may influence higher amounts of P content in algal cells.

Interestingly, P luxury uptake was also observed in *C.reinhardtii* 11/32C cultivated in the media containing both NO_3^- and NH_4^+ . The resulting content of P found in the harvested algal biomass was 2.1%, and it is higher than the typical amount of P reported in microalgae ($\approx 1\%$ P). Hence, the combination of NO_3^- and NH_4^+ has proved to be the best N source for enhancing algal growth and luxury P uptake in *C.reinhardtii* 11/32C.

5. Conclusion

C.reinhardtii 11/32C revealed to be able to assimilate several inorganic nitrogen sources for its growth, biomass production, and P uptake. In the present study, a combination of NO_3^- and NH_4^+ for algal growth was found to be the most appropriate nitrogen source for *C.reinhardtii* 11/32C, which produced higher intracellular P uptake and algal biomass productivity. The combination of NO_3^- and NH_4^+ also triggered the luxurious of P uptake, which P content in algal biomass was 2.1%.

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