Effect of nitrogen and phosphorus removal by two species of Chlorella in allelochemical EMA condition

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Abstract. In order to examine the effect of allelochemical EMA on the absorption of nitrogen and phosphorus by Chlorella vulgaris and Chlorella pyrenoidosa in eutrophic water, Algae were constantly cultured by BG-11 medium with different concentration of allelochemical. The experimental results showed that lower concentrations (0.1mg/L) of EMA have a growth-promoting effect on Chlorella vulgaris, but no obvious effect with higher concentrations. However, EMA has a significant inhibitory effect on Chlorella pyrenoidosa. EMA can also promote the absorption of ammonia nitrogen by two algae. After nine days experiment, The highest ammonia nitrogen removal efficiency occurred in the 1.1 mg/L concentration of EMA, Chlorella vulgaris and Chlorella Pyrenoidosa removal efficiency were 71.89% and 79.06% respectively. High concentration of EMA can inhibit the absorption and utilization of total nitrogen and total phosphorus by two algae. The best removal efficiency of TN were 45.44% (0.1mg/L EMA) and 58.80% (0.1mg/L EMA) respectively. The best removal efficiency of TP were 95.06% (0 mg/L EMA) and 99.70% (0.3mg/L EMA) respectively.

1. Introduction

Aquatic plants can release allelochemical which can inhibit algae growth but no harm to human into water. Previously research identified 14 categories of allelochemicals which contain Phenols, Fatty acid, Terpenes and Alkaloid et al[1]. They will take negative effect in phytoplankton or other plants. With the in-depth study of allelopathy, allelochemicals from more types of plants were identified. The mechanism of allelopathic inhibition to algae growth had also been gradually discovered. Wu [2] found that the effect of allelochemicals is a result of long-term secreted by plants. The concentration of these substances in water will maintain a certain range, and influence algae within a certain range of concentration [3]. But concentration is not the only constraint. The inhibition of different allelochemicals on algae could be combined. The effect apparently higher than individual effect but lower than additivity[4]. Research also proved that allelopathy effect is influenced by the allelochemicals nature and their excretion rate of plant[5].The aqueous extraction which was extracted from Mature Typha angustifolia L. showed effective inhibition on Microcystis and Anabaena [6]. Similar results can be investigated by the extraction of Acorus calamus[7]. Li isolated and identified allelochemical EMA from Phragmites australis which has the inhibitory effect on the C. pyrenoidosa, but the effect on C. vulgaris was not obvious[8]. It could prove that allelochemicals have specificity to target creature. The categories of target species will influence the allelopathy effect. As the harmful algal blooms increase in frequency and the environment-friendly characteristic of allelochemicals,
researchers began to consider using the allochemicals to inhibit algae growth. At present, method which inhibited algae by allelochemicals have been gradually applied to pilot plant test[9], and it is expected to be gradually applied in engineering field. But for now, there is no more in-depth research on how EMA effect the algae on nutrients absorption.

Algae as representative primary productivity, plays an important role in water self-purification. C. vulgaris and C. pyrenoidosa are two kinds of typical green algae. These algae widely distribute in reuse water, ponds, lake, even ocean. The Chlorella has strong adaptability to culture condition which include pH, temperature and salinity et al. When The Chlorella lives in the water bodies which contain nitrogen and phosphorus, the algae will absorb nutrients for its own growth and reproduction. Especially in open water, such as constructed wetlands where excess nitrogen and phosphorus will flow into the water constantly. Nowadays, researchers had realized the importance of nutrients usage to microalgae-based biodiesel production [10]. They also found that the Chlorella has ability to treat wastewater and its production has value in use [11]. Nitrogen in water is an important source of amino acids for algae [12]. And phosphorus can provide materials for the synthesis of ATP or can be stored in algae cell [13]. Once the control of nutrients concentration is improper, nutrients will become the direct factor of algae bloom in water. In this study, two common Chlorella species were selected to experiment and the effects of allelochemicals EMA on the absorption and utilization of nitrogen and phosphorus by these two algae were discussed. The experimental data could be used to investigate the removal and utilization of nitrogen and phosphorus by algae under the influence of allelochemical. And the result could be useful to enhance the ability that algae as a measurement to treat wastewater or harvest algae by-products.

2. Materials and methods

2.1. Resource of algae and allelochemical
The algae were purchased from The Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB), and the allelochemical EMA (2-methylacetoacetate) was 95% pure synthetic compound.

2.2. Algae growth and the growth rate
The density of algae was determined by the optical density of the algae, and the maximum absorption wavelength of the algae was measured before the experiment (C. vulgaris is OD_{650}, C. pyrenoidosa is OD_{680}) . The growth rate was calculated by the following formula.

$$\mu = \frac{\ln OD - \ln OD_0}{T - T_0}$$

OD and OD_0 are the absorbance of algal liquid at days of T and T_0 in the experiment.

The density of algae in algal solution with different dilution ratio was measured by the hemocytometer measurement, and the absorbance of each algal solution concentration was measured too. To ensure the absorbance represents the Algal cell density, the regression curve is established to show the relationship between absorbance and density of algae.

The formula for the regression curve is as follows(A in formula stands for absorbency)

$$C.V \text{ Cell num}(10^5 \text{ ind.}) = 57.086A + 0.0677$$

$$C.P \text{ Cell num}(10^5 \text{ ind.}) = 61.639A + 0.376$$

2.3. Determination of NH_4^+-N, TN, TP
In this experiment, NH_4^+-N was determined by Nessler's reagent spectrophotometry, TN, TP determination using digestion-spectrophotometry (digested by potassium persulfate). sampling 10 mL of algae solution each time after centrifugation, the supernatant was taken for measurement.

Formula for calculating nitrogen and phosphorus absorption rate

$$U = (W_0 - W_1)/(T \cdot G)$$
W₀ is the initial content of the medium, W₁ is the content after the T days’ culture, T is the algal culture time (Days), G is the unitization number of cells of the chlorella (10⁵ ind./mL).

3. Design of inhibition experiment

Seven 500 mL mediums were set for each kind of algae in 7 different concentrations of EMA. The allelochemical concentration of mediums were adjusted to 0 mg/L, 0.1 mg/L, 0.3 mg/L, 0.5 mg/L, 0.7 mg/L, 0.9 mg/L, 1.1 mg/L respectively. Except for the nitrogen and phosphorus content, the others components were in accordance with the BG-11 medium. To simulate eutrophic water quality and provide sufficient nutrients for algae, experiment adjusted the mediums component such as chemical oxygen demand (COD), ammonia nitrogen (NH₄⁺-N), total nitrogen (TN) and total phosphorus (TP) by glucose (C₆H₁₂O₆), ammonium chloride (NH₄Cl) and dipotassium hydrogen phosphate (K₂HPO₄) concentration. The physical and chemical factors in the medium were shown in Table 1.

In the initial stage of the experiment, the initial algal cell density was measured, and equal amount of algae solution was added to each medium. With the relationship between the absorbance and the concentration, the algal density of algae, experiment adjusted the mediums component such as chemical oxygen demand (COD), ammonia nitrogen (NH₄⁺-N), total nitrogen (TN) and total phosphorus (TP) by glucose (C₆H₁₂O₆), ammonium chloride (NH₄Cl) and dipotassium hydrogen phosphate (K₂HPO₄) concentration. The physical and chemical factors in the medium were shown in Table 1.

| Table 1. Initial physical and chemical factors of the medium. |
|------------------|----------------|----------------|----------------|------|------|
| Indicator        | COD            | NH₄⁺-N         | TP             | TN   | pH   | ORP  |
| Concentration    | 100 mg/L       | 30 mg/L        | 1 mg/L         | 30 mg/L | 7.10 | 182.5 |

4. Results and discussion

The optical density curve of C. vulgaris and C. pyrenoidosa was shown in Figure 1. Two species algae in each experimental group entered the stationary phase on the fifth day in the experiment. The growth rate of algae under different allelochemical concentration can be compared by calculating the specific growth rate (μ) on the fifth day of the experiment. On the fifth day, the algal density of C. vulgaris at each concentration was 11.68, 15.93, 11.81, 12.12, 11.87, 11.12 and 10.37 times the initial value. The algal density of C. pyrenoidosa at each concentration was 25.77, 23.33, 17.77, 17.22, 15.77, 14.22 and 12.77 times the initial value. From the results, it is known that the allelopathic substance has little effect on the inhibition of C. vulgaris, but it seems to prolong the exponential phase of algae at the highest concentration. At the concentration of 0.1 mg/L allelochemicals, the growth promotion of C. vulgaris was observed. According to the change of antioxidant system in the inhibition experiment of C. vulgaris and C. pyrenoidosa by Li[14] using plant-derived EMA. Low concentrations of EMA can stimulate the production of antioxidant enzymes in algae, while high concentrations had an inhibitory effect on them. The experiment confirmed that EMA could effectively stimulate the secretion of enzymes in the antioxidant system, but when the concentration of EMA continued to increase, the increase of enzyme activity slowed down. Under the influence of low concentration EMA, the algae antioxidant system could effectively guarantee the growth of algae. Similar results have been found in other allelopathy experiments on Chlorella. In the studies of extraction from Salix babylonica leaf allelopathy on C.Pyrenoidosa proved this extract can stimulate algae growth at its low concentration. The research inferred that algae may be able to use low concentrations of allelochemicals as nutrients[15]. This may explain why the C. vulgaris growth is promoted under the effect of low concentration allelochemicals. In contrast, the inhibitory effect of EMA on C. pyrenoidosa is very obvious. Li[16] found that EMA can effectively change the permeability of the cell membrane of C.
pyrenoidosa. EMA can significantly increase the K\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\) exudation to the environment. Changes in cell membrane permeability result in destruction of algal cellular homeostasis and inhibition of algae growth. The similar inhibitory mechanism of linoleic acid on \textit{M. aeruginosa} was investigated. The generation of malondialdehyde (MDA) and superoxide anion radical (O\(_2\)\(^-\)) of the algal cells increased. But after adding vitamin C, the production of O\(_2\)\(^-\) decreased and the number of algae increased, which showed that allelochemicals did change the permeability of cell membrane and increased the content of MDA, which led to the lipid peroxidation of cell membrane and cell death \[17\].

The algae will transform the nitrogen forms while the algae absorb and use the nitrogen. Cell membrane system was the most important place for the transformation process. Nitrate, and nitrite was reduced to ammonium. Ammonia nitrogen is the most direct nitrogen source in the culture media. Even if there are different forms of nitrogen in water, algae will preferentially use ammonia nitrogen or oxidised nitrogen \[18\]. Algae convert all forms of nitrogen into ammonia nitrogen and synthesizes the amino acids in the chloroplast \[13, 19\]. During the experiment, the changing trend of the absorption of ammonia nitrogen and total nitrogen by two algae was shown in Figure 2. The absorption rates of ammonia nitrogen by \textit{C. vulgaris} were 47.92\%, 53.21\%, 54.72\%, 64.53\%, 67.17\%, 70.57\%, 71.89\%. The absorption rates of ammonia nitrogen by \textit{C. pyrenoidosa} were 54.15\%, 60.94\%, 65.09\%, 68.68\%, 71.51\%, 74.15\% and 79.06\%. Whether allelochemical affect the growth of algae or not, it can be seen that allelochemical promote the absorption of ammonia nitrogen by algae.

![Figure 1](image-url). Comparison of growth curve and specific growth rate of \textit{C. vulgaris} (left) and \textit{C. pyrenoidosa} (right) under different concentrations of allelochemicals.
The experimental results showed that the allelochemical is not specific to the effects of nitrogen absorption on the two species of Chlorella. In fact, the use of ammonia nitrogen by algae indicates that algae are synthesizing a large amount of amino acids, whether for reproduction or self-repair. These amino acids are used to synthesize protein. The ammonia nitrogen used by algae cells prefer to self-healing for *C. pyrenoidosa*. But for the *C. vulgaris* which is not inhibited but stimulated by allelochemicals. Presumably, the reason was that large amounts of antioxidant enzymes need to resist harmful free radicals. This process required a lot of protein. Studies have shown that the allelochemicals secreted by the *Nymphoides peltatum* can effectively inhibit the synthesis of chlorophyll and related proteins in the *Microcystis Aeruginosa*[20]. However, chloroplasts are the main organelles that consume NH$_4^+$-N. The conclusion that the absorption of NH$_4^+$-N under high concentration EMA is higher than that of low concentration can be inferred that EMA has no significant effect on the chloroplast of Chlorella. Instead, it promotes the consumption of photosynthesis.

Total nitrogen (TN) is the main indicator for measuring the pollution of water environment. With the self-destruction of algae and metagenesis, algae will release nitrogen and phosphorus elements that have not been fixed in the cells. As these nutrients are released into the environment, they will increase the total nitrogen in the environment. The content of TN in algae liquid during the experiment was shown in Figure 3. The absorption rates of TN by *C. vulgaris* were 41.45%, 45.44%, 25.59%, 24.72%, 24.22%, 17.85% and 5.99%. The absorption rates of TN by *C. pyrenoidosa* were 46.19%, 58.80%, 50.69%, 50.06%, 49.56%, 45.69% and 40.32%.

![Figure 2](image1.jpg)

**Figure 2.** Changes of NH$_4^+$-N in *C. vulgaris* (left) and *C. pyrenoidosa* (right) culture media.

![Figure 3](image2.jpg)

**Figure 3.** Changes of TN in *C. vulgaris* (left) and *C. pyrenoidosa* (right) culture media.
According to the analysis of TN results, the high concentration of allelochemicals would significantly promote the TN content in the culture mediums. However, combined with the analysis of ammonia nitrogen content, nitrogen is released again into the water after being utilized by algae in the form of ammonia nitrogen. According to the related research on algae residues, the residues of algae decomposition can produce a large number of granular, dissolved and colloidal nutrients, resulting in the release of nutrients [21,22]. In summary, high concentrations of allelochemicals have a significant effect on the uptake of ammonia by algae. These nitrogen can be used for self-repair or reproduction, while high concentrations of allelochemicals cause the promotion of TN content in the environment by metabolism or release of cell disruption. Compared with high concentrations of allelochemicals, EMA can promote the algae to absorb the nitrogen source in the water at the low concentration (0.1mg/L) without generating excessive nitrogen metabolites, so that the TN in the water is minimized. It plays a role to promoting the purification of water by algae.

As an important element for the growth of algae, phosphorus is often a major factor limiting algae growth. Algae can directly use phosphorus in water (generally phosphate, orthophosphate is most easily absorbed by algae), or store excess phosphorus in polyphosphate granules in case lacking of phosphorus [12]. The phosphorus absorption curve of algae in the experiment was shown in Figure 4. The absorption rates of TP by *C. vulgaris* were 95.06%, 93.83%, 88.89%, 88.89%, 71.60%, 60.49% and 39.51%. The absorption rates of TP by *C. pyrenoidosa* were 53.09%, 95.06%, 99.70%, 98.77%, 92.59%, 90.12% and 54.32%.

Allelochemicals have obvious effect on the absorption of phosphorus by algae. EMA significantly inhibits the absorption of phosphorus by *C. vulgaris* (even if the amount of algae is not inhibited). However, for *C. pyrenoidosa*, the absorption of total phosphorus increases first and then decreases with increasing EMA concentration. The absorption of phosphorus by *C. pyrenoidosa* was promoted under the condition of low concentration EMA, and was inhibited under the high concentration EMA. The reason for this phenomenon may be that *C. pyrenoidosa* absorbs phosphorus from the environment at low concentrations for the algae self-repair. Due to the inhibition of EMA on the growth of *C. pyrenoidosa*, the number of algae was significantly inhibited under high concentration of EMA, which led to a significant reduction in phosphorus utilization. The number of *C. vulgaris* cells was not limited by the concentration of EMA. However, with the increase of EMA concentration, the antioxidant capacity and phosphorus absorption were significantly inhibited. It can be inferred that EMA has an effect on the growth strategy of *C. vulgaris*. At the beginning of the experiment at 1.1 mg/L EMA concentration, higher than the initial value of phosphorus in the environment was observed, indicating that high concentration of EMA could induce *C. vulgaris* to release themselves stored phosphorus under certain condition.

![Figure 4](image_url). Changes of TP in *C.vulgaris* (left) and *C.Pyrenoidosa* (right) culture media.
Above view were verified by calculating the rate of nitrogen and phosphorus absorption by unitized algae. The calculation results were shown in Figure 5. The exponential phase growth rate and the total average absorption rate of algae were represented by the value in the experimental five days and the value in experimental nine days respectively. It can be seen from the figure that the allelochemical promote the ammonia nitrogen absorption capacity of the unit algae in both kind, but the differences in the absorption capacity of the phosphorus element by two algae are significant.

The ratio of nitrogen and phosphorus in water is also considered by researchers to be an important factor affecting algae growth. The earliest investigation proposed Red Field law which reveals nitrogen and phosphorus for algae growth required need to be taken in a certain proportion[23]. Later with the deepening research, it was found that the different algae have significant differences in absorption and utilization of nitrogen and phosphorus[24]. In this experiment, we also calculate the absorption ratio of nitrogen and phosphorus in the two types of chlorella under the influence of allelochemical. The results were shown in Table 2.

![Figure 5. Comparison of absorption rates of ammonia nitrogen and total phosphorus between C. vulgaris (left) and C. pyrenoidosa (right).](image)

It can be seen from the data in the table that the increase of EMA concentration leads to the gradual increase the ratio of nitrogen and phosphorus absorption of C. vulgaris. The ratio of C. pyrenoidosa first decreased and then increased with the increase of EMA concentration, indicating that different concentrations of EMA have different effects on the preference of algae for nutrients. Allelochemical change the preference of the two kinds of algae to nitrogen and phosphorus, so the ratio of nitrogen and phosphorus to algae treatment in water containing allelochemical should be different from the normal situation.
Table 2. Absorption ratio of N/P in C. vulgaris (top) and C. pyrenoidosa (bottom), (the ratio retains two decimal places).

| Concentration | 0    | 0.1  | 0.3  | 0.5  | 0.7  | 0.9  | 1.1  |
|---------------|------|------|------|------|------|------|------|
| 0th day       | 30   | 30   | 30   | 30   | 30   | 30   | 30   |
| 1st day       | 18.05| 21.69| 26.45| 29.63| 47.75| 97.51| -80.69|
| 3rd day       | 15.89| 19.05| 22.42| 22.52| 41.51| 52.12| -563.94|
| 5th day       | 13.20| 15.90| 18.34| 25.04| 37.64| 43.56| 98.58 |
| 7th day       | 14.98| 16.45| 18.34| 22.66| 39.70| -563.94| 69.32 |
| 9th day       | 15.12| 17.01| 18.47| 21.78| 28.14| 35.00| 54.59 |

| Concentration | 0    | 0.1  | 0.3  | 0.5  | 0.7  | 0.9  | 1.1  |
|---------------|------|------|------|------|------|------|------|
| 0th day       | 30   | 30   | 30   | 30   | 30   | 30   | 30   |
| 1st day       | 38.09| 18.34| 18.70| 26.09| 27.43| 30.97| 194.09|
| 3rd day       | 34.90| 18.41| 16.49| 19.56| 25.66| 24.81| 81.07 |
| 5th day       | 33.40| 20.43| 18.28| 19.99| 25.36| 25.28| 47.66 |
| 7th day       | 32.33| 19.66| 18.63| 19.77| 23.71| 25.28| 44.84 |
| 9th day       | 30.60| 19.23| 19.53| 20.86| 23.17| 24.68| 43.66 |

5. Conclusions
Based on the different inhibitory effects of allelochemical EMA on C. vulgaris and C. pyrenoidosa, the changes of nitrogen and phosphorus absorbed by the above two algae were investigated. Studies have found that low concentrations (0.1 mg/L) of EMA can promote the growth of C. vulgaris. EMA can effectively promote the absorption of ammonia nitrogen by algae and promote cell metabolism to release other forms of nitrogen to water. The highest ammonia nitrogen removal efficiency occurred in the 1.1 mg/L concentration of EMA, C. vulgaris, and C. Pyrenoidosa removal efficiency were 71.89% and 79.06% respectively. But the best removal efficiency of TN were 45.44% (0.1mg/L EMA) and 58.80% (0.1mg/L EMA) respectively. EMA also change the demand for phosphorus by algae. High concentration of EMA inhibited the utilization of phosphorus by two algae, while the utilization of phosphorus by C. pyrenoidosa was promoted at low EMA concentrations. The best removal efficiency of TP were 95.06% (0 mg/L EMA) and 99.70% (0.3mg/L EMA) respectively. EMA also changed the ratio of demand for nitrogen and phosphorus in the growth of two species algae. These conclusions could help to explore the relationship between plant allelopathy and algae growth, such as inhibiting algae growth, using the synergistic purification of plants and algae to purify wastewater or obtain algal by-products.

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