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EFFECT OF AMMONIA CONCENTRATIONS ON GROWTH OF *CHLORELLA VULGARIS* AND NITROGEN REMOVAL FROM MEDIA

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Abstract

The effect of ammonia concentration on growth and physiology of a unicellular green alga, Chlorella vulgaris, *was investigated. Growth occurred in all ammonia concentrations examined (10–1000 mg N l^{-1}) although less growth was found in cultures containing either very low (10 mg N l^{-1}) or very high (750 and 1000 mg N l^{-1}) ammonia concentrations. At NH_3-N concentrations between 20 and 250 mg N l^{-1}, there were no significant differences in specific growth rates and maximal cell densities attained. Growth in these media was comparable to growth in the commercial Bristol medium which contains nitrate as the nitrogen source. Higher chlorophyll and protein contents were found in cell cultures with higher ammonia concentrations. The algal growth was accompanied by a decrease in nitrogen content in the medium, indicating that nitrogen removal was due to algal uptake and assimilation. In cultures containing nitrogen lower than 40 mg N l^{-1}, nitrogen was completely removed at the end of the cultivation period. Over 95% ammonium removal was found in cultures containing 40–80 mg N l^{-1}. The percentage N reduction decreased with the initial N concentrations in cultures containing more than 80 mg N l^{-1}. Copyright © 1996 Elsevier Science Ltd.*

Key words: Microalgae, *Chlorella*, ammonium, growth, physiology, nitrogen removal.

INTRODUCTION

Wastewater generated from agriculture and domestic sources contains high concentrations of organic matter, nitrogen and phosphorus, and causes eutrophication in receiving water. Many algal species, especially *Chlorella*, are rather tolerant to organic pollution and will rapidly colonize any milieu rich in N, P and organic compounds. Microalgae have traditionally been used as a tertiary treatment process to remove inorganic N and P from wastewater after its organic matter (BOD and COD) has been reduced by conventional secondary treatments (Lavoie & de la Noue, 1985; Martin *et al.*, 1985; Oswald, 1988). Recent studies have reported that many algal species, particularly *Chlamydomonas*, *Scenedesmus* and *Chlorella*, can assimilate partially degraded organic compounds under light condition (mixotrophy) and they are also capable of heterotrophic growth on simple molecules, such as acetate, glucose and organic acids in the dark (Laliberte & de la Noue, 1993; Mayo & Noike, 1994). It has been suggested that a microalgal system can be employed as an alternative secondary treatment process for simultaneous removal of nutrients and organic matter from wastewater (Przytocka-Jusiak *et al.*, 1984; Tam & Wong, 1990).

One limitation in employing an algal system as the secondary treatment process is the presence of high concentrations of ammonia and urea in raw wastes, especially those discharged from livestock and food industries, which inhibit algal growth and physiological activity (Przytocka-Jusiak, 1976). However, previous research has mainly been focused on the effects of N-deficiency and competitive interaction between nitrate and ammonia uptake at low N level. Relatively little information is available on the problems of growing algae in extremely high concentrations of ammonia. Moreover, the concentration at which ammonia toxicity becomes effective varies greatly with individual algal species and culture conditions. Przytocka-Jusiak (1976) reported a 50% inhibition on cell growth of *C. vulgaris* at 330 mg l^{-1} NH_3-N, while 700 mg l^{-1} NH_3-N at pH 8–9 caused a complete inhibition of cell division for the same algal species (Matusiak *et al.*, 1976). Azov and Goldman (1982) showed a 50% inhibition of photosynthesis in *S. obliquus* at 16.8 mg l^{-1} NH_3-N, but Konig *et al.* (1987) demonstrated that both *Chlorella* and *Euglena* exhibited no ammonia toxicity at 560 mg l^{-1} NH_3-N. Ammonia toxicity, although of

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concern, has not been investigated in detail and the information available is mostly fragmentary. The present study, therefore, aimed to examine the effects of ammonia concentrations on growth and physiology of a unicellular green alga, *Chlorella vulgaris*. The degree of ammonia removed by the algal culture was also estimated.

METHODS

Stock culture of *Chlorella vulgaris* (Carolina Biological Supply) was kept aseptically in a commercial Bristol medium (also called Bold Basal medium containing 40 mg N l^{-1} in the form of KNO_3 and 53 mg P l^{-1} in the form of KH_2PO_4). After 14 days of growth, stock algal suspension was transferred to 250 ml conical flasks containing 150 ml sterilized culture media of different ammonia concentrations. The culture media were prepared in the same method as that of the Bristol medium except the nitrate component was replaced by different amounts of ammonium sulphate. A total of 12 ammonia concentrations: 0, 10, 20, 40, 50, 60, 80, 125, 250, 500, 750 and 1000 mg N l^{-1}, were prepared. The commercial Bristol medium was used as the control. The initial cell density was 1×10^6 cells ml^{-1}. The pH values of all culture media were adjusted to 7.0 ± 0.2 before algal inoculation. The algae were axenically grown at 20 ± 2°C, with an illumination of 4300 ± 300 lux from cool white fluorescent tubes and light–dark cycles of 16–8 h for 21 days. Each flask was aerated with filtered air which provided atmospheric CO_2 and a mixing process. All treatments were in duplicate.

At 3 or 4 day intervals, algal cell number was determined by using the improved Neubauer haemacytometer and two counts were performed for each flask. The specific growth rate constant (k, day^{-1}) of *C. vulgaris* in each culture was determined by a simple linear regression analysis on $\ln(N_t/N_0)$ and t, where N_t, N_0 denoted the final and initial cell numbers, respectively. The k value was the slope of the regression line. The pH values of the cultures were measured and maintained at neutral pH by the addition of either sterilized and diluted NaOH or HCl. The chlorophyll content was determined at 7-day intervals by methanol–chloroform extraction. At the same time intervals, algal suspensions were centrifuged at 3000 *g* for 10 min and the amounts of ammonia and nitrate ions remaining in the culture medium were respectively examined by Nesslerization method and ultraviolet spectrophotometry (APHA, 1989). All samplings and measurements were carried out at the same time of the day and during the light period. At the end of the cultivation period, the percentages of NH_3-N removal and the specific NH_3-N uptake rates were calculated. The algal proteins were extracted in 0.5 N NaOH and assayed by modified Folin–Lowry method (Lowry *et al.*, 1951). The algal growth (cell number) and chlorophyll content were treated by two-way analysis of variance to determine any significant difference between ammonia concentration and incubation time.

RESULTS AND DISCUSSION

Growth of *Chlorella vulgaris* under different concentrations of ammonia nitrogen

The growth pattern of *C. vulgaris*, expressed as the increase in cell number during 21 days of cultivation, was similar in all media containing either ammonia or nitrate nitrogen, with an initial lag phase, followed by log, stationary and declining phases (Fig. 1). Most cells appeared to reach their stationary phase after 17 days of cultivation and started to decline thereafter. The drop in cell number towards the latter part of the experiment was most obvious in the control (containing nitrate as the nitrogen source) and its cells disintegrated from day 17 onwards. In culture containing 10 mg N l^{-1}, growth became steady from day 10 onwards with a lower stationary phase cell yield (Fig. 1). Above this concentration, from 20 to 250 mg N l^{-1}, there was no significant difference in growth. The maximum cell density attained (around 5×10^7 cells ml^{-1}) and the constant values of specific growth rate (about 0.22 day^{-1}) were similar to those of the control (Table 1). When ammonia concentration increased up to 500 mg N l^{-1}, the specific growth rate still remained comparable to the control, although the maximal cell density achieved was significantly lower (Table 1). Growth was possible even in cultures containing very high ammonia contents (750 and 1000 mg N l^{-1}), although the maximum cell numbers observed were significantly lower than those found in control and other ammonia concentrations. Similar results were reported by Przytocka-Jusiak *et al.* (1977). They observed that cell division of *C. vulgaris* was inhibited at ammonia concentrations greater than 750 mg l^{-1}, although such concentrations did not cause any visible lethal effect. The tolerance of *Chlorella vulgaris* to high concentrations of ammonia suggests that this algal species can be used to treat wastewater rich in NH_3-N.

Chlorophyll and protein content

Total chlorophyll contents (μg ml^{-1}) increased gradually with the incubation time in all cultures, with the highest chlorophyll content recorded at day 21. The chlorophyll content generally increased with the initial ammonia concentrations ranging from 0 to 50 mg N l^{-1}. At ammonia concentrations higher than 50 mg N l^{-1}, there was no significant difference ($P < 0.05$) in chlorophyll content. This suggests that at low N concentrations, chlorophyll formation was limited by N supply while excessive N did not cause

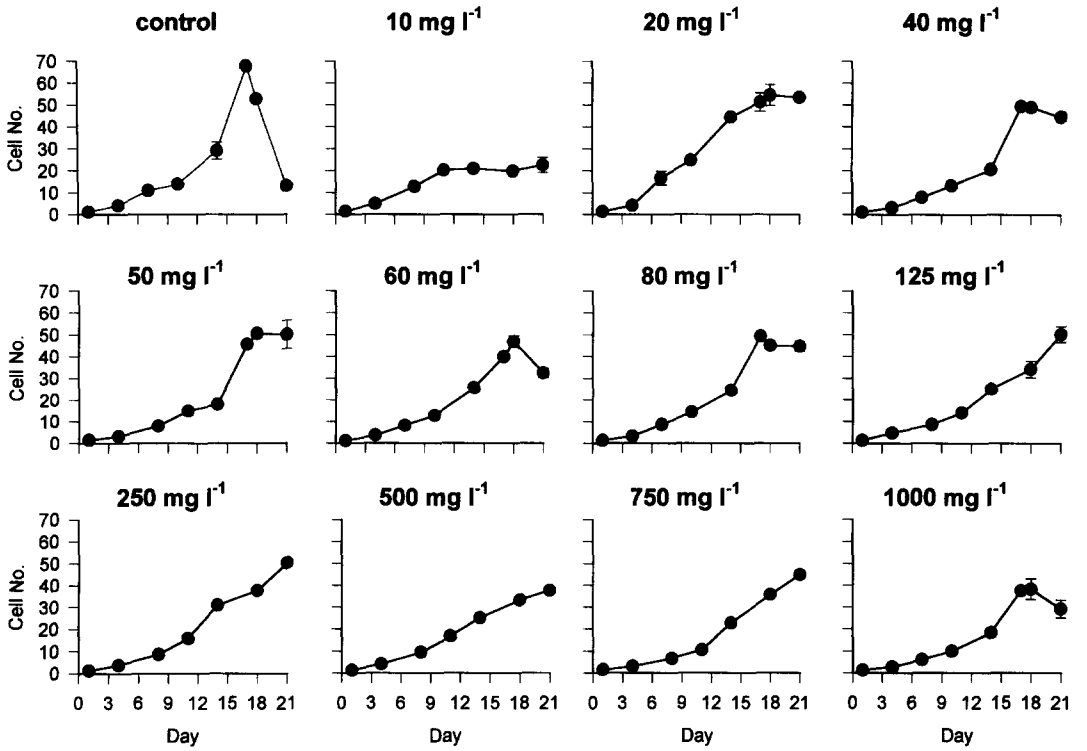

Fig. 1. Cell number ($\times 10^6$cells ml^{-1}) of *Chlorella vulgaris* cultivated at different ammonia concentrations (0–1000 mg N l^{-1}). Mean and standard deviation values of four replicates are shown.

additional synthesis of chlorophyll. When chlorophyll content was expressed on a per cell basis, its values (pg $cell^{-1}$) also increased with the initial ammonia concentrations up to 50 mg l^{-1}, followed by levelling-off afterwards (Table 2). The chlorophyll content per cell basis (pg $cell^{-1}$) had less fluctuation with incubation time than those measured in terms of μg ml^{-1}. These results indicate that the physiological activity of *C. vulgaris* was not reduced by high ammonia concentrations.

When the ammonia concentration in the medium increased, the final protein content (μg ml^{-1}) and the protein value (per cell basis) generally increased (Table 2). Maximum protein concentrations (expressed as μg ml^{-1} culture) were found at 60 and 125 mg N l^{-1}, whereas maximum contents of protein (based on pg $cell^{-1}$) were obtained at 60 and 1000 mg N l^{-1}. It is generally recognized that the protein content of microalgae depends on the amount of nitrogen available in the culture medium, an increase in nitrogen concentration in the medium is generally parallel to an increase in cellular protein content (Przytocka-Jusiak *et al.*, 1977; Fabregas *et al.*, 1989). The high protein values (pg $cell^{-1}$) were

Table 1. The growth properties of *Chlorella vulgaris* under different nitrogen concentrations

NH_3-N conc. (mg l^{-1})	Specific growth rate (day^{-1})[a]	R^2	Maximum count ($\times 10^6$ cells ml^{-1})[b]	Final count ($\times 10^6$ cells ml^{-1})[c]
0	0.054a	0.87	3.16 (day 14)	2.64
10	0.204b	0.90	22.59 (day 21)	22.59
20	0.228c	0.89	54.48 (day 18)	53.46
40	0.214c	0.97	49.55 (day 17)	44.70
50	0.212c	0.98	50.69 (day 18)	50.18
60	0.211c	0.96	46.85 (day 18)	32.50
80	0.219c	0.97	49.30 (day 17)	44.70
125	0.213c	0.95	50.30 (day 21)	50.30
250	0.236c	0.99	50.39 (day 21)	50.39
500	0.221c	0.97	37.75 (day 21)	37.75
750	0.204b	0.99	44.89 (day 21)	44.89
1000	0.199b	0.99	38.30 (day 18)	29.18
Bristol medium	0.229c	0.96	67.85 (day 17)	13.19

R^2: Coefficient of determination.
[a]Values followed by different letters indicate that they were significantly different at a probability level of 0.05 according to ANOVA test.
[b]Values in bracket are the dates of cultivation showing the maximum cell count.
[c]Initial cell density was 1×10^6cells ml^{-1}.

Table 2. The chlorophyll and protein contents of *Chlorella vulgaris* at the end of 21 days cultivation under different nitrogen concentrations

NH_3-N conc. (mg l^{-1})	Chlorophyll content ($\mu g\ ml^{-1}$)	Chlorophyll conc. (pg $cell^{-1}$)	Protein content ($\mu g\ ml^{-1}$)	Protein conc. (pg $cell^{-1}$)	NH_3 conversion efficiency (%)[a]
0	0.44	0.11	0.02	0.01	—
10	3.22	0.10	33.20	1.27	53.12
20	35.51	0.67	67.37	1.26	53.89
40	39.56	0.89	117.64	2.50	47.06
50	71.01	1.42	115.41	2.30	36.93
60	55.07	1.69	133.35	4.12	35.56
80	72.10	1.61	113.32	2.54	22.66
125	81.16	1.60	139.78	2.72	17.89
250	77.54	1.54	116.18	2.31	7.44
500	69.57	1.84	113.94	3.03	3.65
750	72.46	1.63	110.57	2.50	2.36
1000	64.50	2.12	109.39	3.47	1.75
Bristol medium	ND	ND	29.42	2.23	NC

[a]The NH_3-N conversion efficiency is calculated as follows: (i) transform the final protein content to total cellular N by dividing the protein content by 6.25; and (ii) divide the total cellular N by the amount of ammonia N added to the culture medium, then times by 100%.
NC: not calculated, as Bristol medium did not contain any ammonia N, also because a lot of cells died on day 21 in Bristol medium.
ND: not determined, as a lot of cells died on day 21 in Bristol medium.

not only affected by the initial ammonia concentrations, they were also related to the low cell density recorded at the end of the experiment. In contrast, the efficiency of converting ammonia-N to protein-N (calculated as the ratio between the amount of nitrogen added to the medium and the protein nitrogen produced per culture) decreased with an increase in initial ammonia concentration (Table 2). The highest conversion efficiency was found at 10 and 20 mg N l^{-1}, with a value around 53%, and very low conversion efficiency (<10%) was observed in media containing more than 250 mg N l^{-1}. These findings reveal that in media containing high initial ammonia content, nitrogen supply was in excess and algal growth was probably limited by phosphorus.

Removal of ammonia by algal culture

At initial ammonia concentrations of 10 and 20 mg N l^{-1}, ammonia N was found to be completely depleted by day 7. The continual growth of algae in these two cultures suggests that cells can utilize their reserve N, lipids and carbohydrates to maintain their growth. This is reflected by their low protein content (less than 1.3 pg $cell^{-1}$) at the end of the experiment (Table 2). In cultures containing initial N of more than 40 mg N l^{-1}, the residual NH_3-N in the culture media decreased gradually with time. The residual ammonia N in cultures containing 40–80 mg l^{-1} initial N was less than 3 mg l^{-1} at the end of the experiment, i.e. more than 95% N was removed by algal uptake (Fig. 2). In cultures with more than 80 mg l^{-1} initial ammonia, more than 50% of initial N still remained in the culture at day 21, indicating that excessive N was not taken up by algae. The percentage N removal decreased with initial ammonia concentrations, i.e. the higher the initial N, the lower the removal efficiency (Fig. 2). At 1000 mg N l^{-1}, the percentage N reduction was the lowest (<20%), about 180 mg l^{-1} NH_3-N was removed from the culture medium. This amount of ammonia reduction was comparable to that reported in the literature. Matusiak *et al.* (1976) stated that 160 mg l^{-1} NH_4^+-N out of 1005 mg l^{-1} was removed by stationary *C. vulgaris*. On the contrary, the specific NH_3-N uptake in 21 days (mg N uptake per million cells) increased with initial ammonia concentrations, and the highest uptake rate was recorded in algae grown in medium containing 1000 mg N l^{-1}.

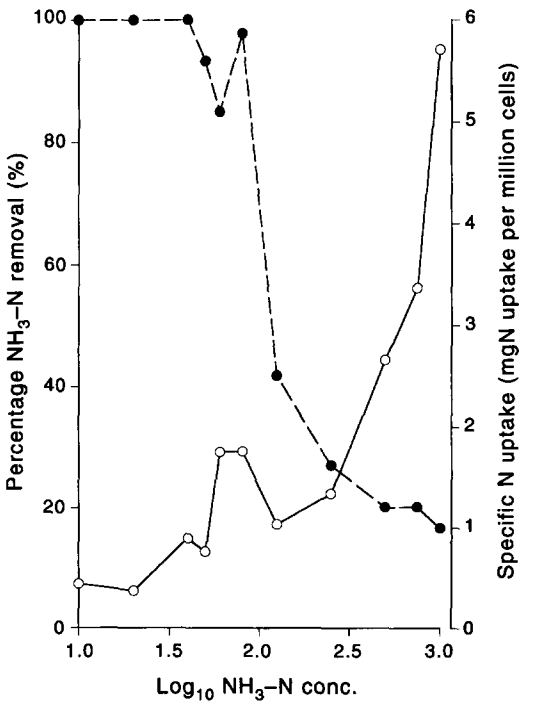

Fig. 2. Mean percentage removal and specific uptake of NH_3-N at the end of 21 days cultivation of *C. vulgaris* (○: specific N uptake rate; ●: percentage NH_3-N removal).

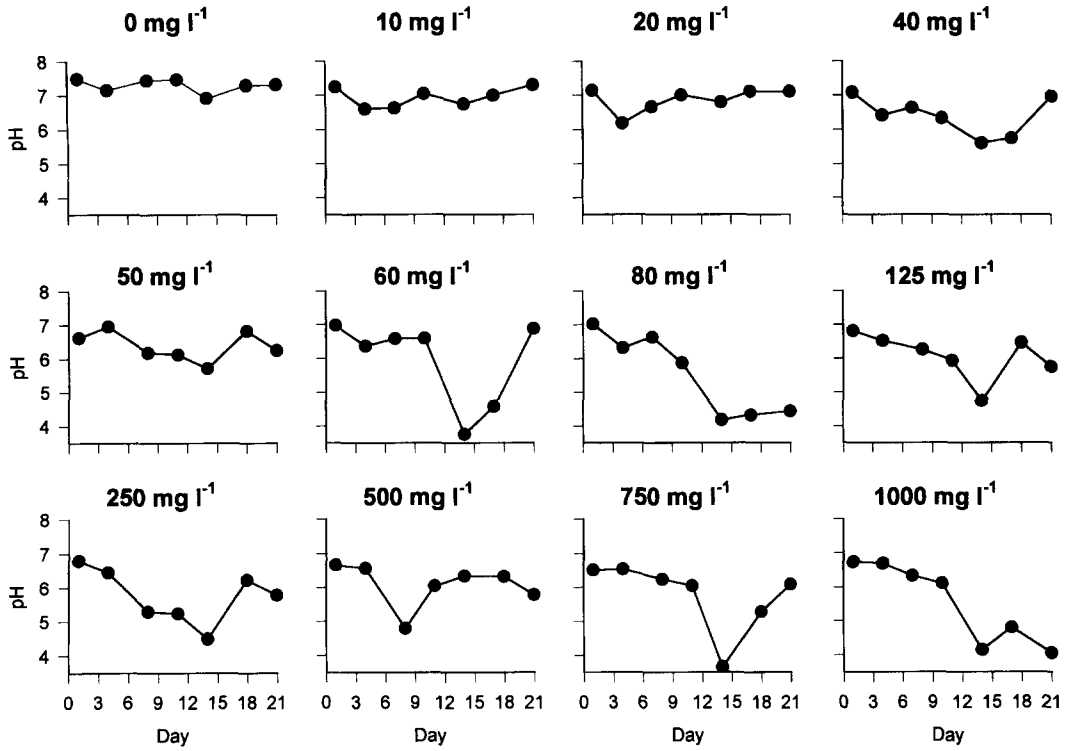

Fig. 3. Changes in pH values of the culture media during cultivation of *C. vulgaris* at different ammonia concentrations. Acidic pH was readjusted to neutral pH immediately after each measurement.

The algal growth and their removal of ammonia N from media coincided with a decrease in pH values. Figure 3 shows that the pH of the culture medium containing more than 50 mg N l^{-1} decreased significantly throughout the cultivation period, despite the fact that acidic pH was readjusted to neutral pH immediately after each measurement. This suggests that a large amount of H^+ ions was generated in media containing high ammonia concentrations. In many cultures, pH dropped to the minimum value between days 12 and 15, then rose towards the end of the cultivation period. Przytocka-Jusiak *et al.* (1977) found that the growth of *C. vulgaris* in cultures containing ammonia nitrogen was usually accompanied with the change of pH; the medium became acidic in the logarithmic phase of growth then changed to alkaline when the cells were in lag or stationary phases and in the absence of growth.

The low pH reduces the formation of ammonia gas and most N will exist in ammonium ion form, which is less toxic to algae. It has been reported that algal photosynthesis was inhibited at ammonia concentrations above 28 mg l^{-1} if the culture pH exceeded 8.0, when non-toxic NH_4^+ dissociates to toxic NH_3 (Azov & Goldman, 1982). This explains why *Chlorella* still maintained certain growth at 1000 mg N l^{-1}. The low pH also suggests that the reduction of ammonia via ammonia volatilization and stripping was unlikely in this study as ammonia stripping is significant only under high alkaline and high temperature conditions. Moreover, nitrite was not detected and nitrate was found at very low levels in all cultures (<0.1 mg l^{-1}), indicating that the removal of ammonia from the culture media was not due to nitrification. These results demonstrate that the loss of N from the media was mainly due to algal assimilation. Przytocka-Jusiak *et al.* (1984) also showed that dense cultures of *C. vulgaris* were adapted to high NH_3-N concentration and were capable of removing N from highly loaded nitrogenous industrial wastewater by incorporation into their biomass.

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