

Tolerance of four Malaysian chlorophytes to nitrate and ammonium pollution

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ABSTRACT Microalgae tolerant to high levels of NaNO₃ and NH₄Cl have the potential for use in bioremediation of nitrogen-rich agro-industrial wastewaters and as bioindicators for nitrogen enrichment in tropical freshwater environments. Four indigenous chlorophytes from Malaysia, namely *Chlorella vulgaris* UMACC 001, *Scenedesmus quadricauda* UMACC 039, *S. quadricauda* UMACC 041 and *Ankistrodesmus convolutus* UMACC 101 were grown at NaNO₃ and NH₄Cl levels ranging from 2.9 (control), 50, 75, 120, 170 to 250 mM. The control contained the level of nitrogenous compound found in Bold's Basal Medium. Of the four chlorophytes, *C. vulgaris* UMACC 001 was most tolerant to NaNO₃ and NH₄Cl as the cell number and final biomass were much higher when grown at increased levels (> 2.9 mM) of these nitrogen sources. In contrast, *S. quadricauda* UMACC 041 and *A. convolutus* UMACC 001 were sensitive to high levels of NaNO₃ and NH₄Cl as indicated by the very low cell number at levels above 75 mM as compared to the control. Within the range of NaNO₃ levels tested, the four chlorophytes grew best at 50 mM. For the range of NH₄Cl levels tested, *C. vulgaris* UMACC 001 grew best at 75 mM whereas the other chlorophytes grew best at 50 mM. Based on the growth responses to the varying levels of nitrogen, *C. vulgaris* UMACC 001 may be a potential species for treatment of agro-industrial wastewaters and as bioindicator for nitrogen pollution.

ABSTRAK Mikroalga yang boleh tumbuh pada aras NaNO₃ dan NH₄Cl yang tinggi mempunyai potensi untuk digunakan dalam bioremediasi air buangan agro-industri yang mengandungi aras nitrogen yang tinggi. Di samping itu, mikroalga tersebut juga boleh digunakan sebagai bioindikator untuk pencemaran nitrogen di perairan tropika. Empat alga hijau tempatan, *Chlorella vulgaris* UMACC 001, *Scenedesmus quadricauda* UMACC 039, *S. quadricauda* UMACC 041 dan *Ankistrodesmus convolutus* UMACC 101 ditumbuh dalam Bold's Basal Medium (BBM) mengandungi NaNO₃ dan NH₄Cl sebanyak 2.9 (kawalan), 50, 75, 120, 170 dan 250 mM. *Chlorella vulgaris* UMACC 001 mempunyai toleransi yang tertinggi terhadap NaNO₃ dan NH₄Cl berasaskan kepada bilangan sel dan biomas yang lebih tinggi apabila dikulturkan pada aras nitrogen lebih daripada 2.9 mM. Sebaliknya, bilangan sel *S. quadricauda* UMACC 041 dan *A. convolutus* UMACC 101 adalah lebih rendah daripada kawalan, apabila dikulturkan pada kepekatan NaNO₃ dan NH₄Cl melebihi 75 mM. Dalam julat NaNO₃ yang digunakan, keempat-empat klorofit tersebut tumbuh paling bagus pada 50 mM. Untuk NH₄Cl, *C. vulgaris* UMACC 001 tumbuh paling bagus pada 75 mM manakala ketiga-tiga klorofit yang lain tumbuh paling bagus pada 50 mM. Berdasarkan pada sifat pertumbuhan mikroalga-mikroalga tersebut pada aras nitrogen yang berbeza, *C. vulgaris* UMACC 001 mempunyai potensi untuk digunakan dalam pengolahan air buangan agro-industri dan sebagai bioindikator pencemaran nitrogen.

(chlorophytes, bioindicators, bioremediation, nitrate, ammonium, *Chlorella vulgaris*, *Scenedesmus quadricauda*, *Ankistrodesmus convolutus*)

INTRODUCTION

Enrichment of nitrogen, especially nitrate and ammonium in water bodies is one of the major

causes of eutrophication, which may also lead to harmful algal blooms [1,2]. Drinking water contaminated with excess nitrate poses a threat to health, as it can cause methaemoglobinaemia.

The Maximum Contamination Level of nitrate in drinking water allowed by the Environmental Protection Agency (EPA) of the United States is 0.71 mM [3]. Thus, there have been attempts to develop microalgal-based systems to remove excess nitrate from groundwater [4]. Recently, both ammoniacal-nitrogen ($\text{NH}_3\text{-N}$) and nitrate-nitrogen ($\text{NO}_3\text{-N}$) have been proposed for inclusion in the Environmental Quality Regulation of Malaysia as additional parameters for effluent discharge. The proposed standards for discharge of effluents are 0.36 mM $\text{NH}_3\text{-N}$ and 1.43 mM $\text{NO}_3\text{-N}$ (Yeoh, pers. comm.).

The level of nitrogen in unpolluted freshwater environments is about 0.07 mM [5]. In Malaysia, polluting wastewaters that contain high levels of nitrogen include palm oil mill effluent (2 – 12 mM $\text{NH}_3\text{-N}$), rubber effluent (21 mM $\text{NH}_3\text{-N}$, 0.21 mM $\text{NO}_3\text{-N}$) and textile finishing wastewater (0.34-1.20 mM $\text{NO}_3\text{-N}$) as reported by Phang and Ong [6], Geetha *et al.* [7] and Rakmi [8] respectively. In addition, the ranges of $\text{NH}_3\text{-N}$ and $\text{NO}_3\text{-N}$ in sewage are 1 - 3 mM and 0.05 - 0.27 mM respectively [9]. Efficient treatment systems are needed to reduce the nitrogen level of these wastewaters in order to meet the standards for discharge. Wastewater treatment using microalgae is attractive as it generates useful biomass besides reducing the pollutant load [6]. Thus, species that are tolerant to high nitrogen levels will be useful for treating such nitrogen-rich wastewaters.

Microalgae can serve as suitable biological indicators for nitrogen enrichment as they directly utilise the nutrient for growth. Use of a bioindicator provides information on the bioavailability of the pollutant, which cannot be derived from chemical analysis [10]. There are limited studies on using tropical species as bioindicators and so far, the focus has been mainly on heavy metal pollution [11]. Species that are useful bioindicators for nitrogen pollution will be those that are tolerant to high nitrogen levels.

The objective of this study was to compare the tolerance of four Malaysian chlorophytes to high levels of nitrate and ammonium. It is part of our on-going studies to screen for microalgae that can be used for bioremediation of wastewaters and as bioindicators for nitrate and ammonium enrichment in freshwater environments.

MATERIALS AND METHOD

Test organisms

Four chlorophytes from the University Malaya Algae Culture Collection were chosen for this study: *Chlorella vulgaris* UMACC 001, *Scenedesmus quadricauda* UMACC 039, *S. quadricauda* UMACC 041 and *Ankistrodesmus convolutus* UMACC 101 [12]. *Chlorella vulgaris* UMACC 001 and *A. convolutus* UMACC 101 were isolated from freshwater lakes while both isolates of *S. quadricauda* were from fish tanks. The cultures were maintained in Bold's Basal Medium (BBM) [12].

Culture conditions

All cultures were grown in BBM buffered with 10 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid) or HEPES, containing varying levels of NaNO_3 or NH_4Cl . The inocula (10%) from exponential phase cultures with an optical density at 620 nm (OD_{620}) of 0.2 were used for all the tests. In the range finding test, the chlorophytes were grown in NaNO_3 and NH_4Cl ranging from 2.9, 250, 500, 750 to 1000 mM using Nunclon multi-well plates. The control level was taken as 2.9 mM, which is the concentration of nitrogenous compound in BBM. Growth was assessed by cell count on day 4 and 8.

Based on results from the range finding test, a definitive test was conducted using flask cultures (100 mL) with NaNO_3 and NH_4Cl levels ranging from 2.9 (control), 50, 75, 120, 170 and 250 mM. Triplicate cultures were used for each level. The inoculum was centrifuged (3,000 rpm, 10 min) and the cells were resuspended in nitrogen-free medium. The cultures were grown in an orbital shaker (150 rpm) at 28°C with an irradiance of 42 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (12:12 h light-dark cycle). Growth was monitored every two days by cell count and dry weight was determined at the end of the study on day 8 [13].

RESULTS

The range finding test showed that growth inhibition of the four chlorophytes occurred from 250 mM NaNO_3 and NH_4Cl upwards. Thus, the definitive tests were conducted at NaNO_3 and NH_4Cl levels ranging from 2.9 to 250 mM.

Table 1. Final biomass (mg L⁻¹) of the four chlorophyte cultures grown at different levels of NaNO₃.

NaNO ₃ mM	Biomass (mg dry weight L ⁻¹)			
	<i>Chlorella vulgaris</i> UMACC 001	<i>Scenedesmus quadricauda</i> UMACC 039	<i>Scenedesmus quadricauda</i> UMACC 041	<i>Ankistrodesmus convolutus</i> UMACC 001
2.9 (control)	40.0	81.7	46.7	20.8
50	293.3	70.8	214.2	205.0
75	336.1	50.8	138.3	164.2
120	273.3	59.2	95.0	89.2
170	297.5	110.8	69.2	89.2
250	223.3	101.7	52.5	64.2

Effect of NaNO₃ levels

The growth curves of the four chlorophytes based on cell number are shown in Figure 1. All the chlorophytes except *S. quadricauda* UMACC 039 grew best at 50 mM NaNO₃ as the cell numbers attained at this level were generally highest. At increased NaNO₃ levels (> 2.9 mM), the cell number of *C. vulgaris* UMACC 001 was generally higher than the control, contrasting with the trend shown by *S. quadricauda* UMACC 039 (Figures 1a and 1b). There was no marked increase in the cell number of *C. vulgaris* UMACC 001 at 2.9 mM NaNO₃. The cultures of *C. vulgaris* UMACC 001 grown at 250 mM NaNO₃ showed a lag phase lasting two days (Figure 1a). The cell number of *S. quadricauda* UMACC 039 on day 8 for the cultures grown at 50 mM NaNO₃ (3.31 X 10⁴ cells mL⁻¹) was slightly lower than the control (3.72 X 10⁴ cells mL⁻¹).

For *S. quadricauda* UMACC 041 and *A. convolutus* UMACC 101, cell numbers of cultures grown at 50 – 75 mM NaNO₃ were generally higher than the control while at 120 – 250 mM, they were lower (Figures 1c and 1d). At 250 mM NaNO₃, the cell number of *S. quadricauda* UMACC 041 decreased markedly after day 4 (Figure 1c).

Maximum growth rates calculated based on the steepest slopes of the growth curves of *S. quadricauda* UMACC 039 was lower (0.19 – 0.24 day⁻¹) at NaNO₃ levels above the control (0.36 day⁻¹). The reverse was shown by *C. vulgaris* UMACC 001, with values ranging from

0.46 to 0.73 day⁻¹ at NaNO₃ levels higher than 2.9 mM (0.25 day⁻¹).

Of the four chlorophytes, *C. vulgaris* UMACC 001 attained the highest final biomass, which was 6 - 8 times higher when grown at NaNO₃ levels above the control (Table 1). Although the cell number was lower, the final biomass of *S. quadricauda* UMACC 039 grown at 170 and 250 mM NaNO₃ was higher than the control. Cells of this chlorophyte grown at such high NaNO₃ levels were mainly ovoid and unicellular, much larger than those from the control cultures, which consisted mainly of four-celled coenobia. The final biomass of both *S. quadricauda* UMACC 041 and *A. convolutus* UMACC 101 was higher when grown at increased NaNO₃ levels above 2.9 mM. The cells were also larger under such conditions.

Effect of NH₄Cl levels

Growth curves of the chlorophytes grown at different levels of NH₄Cl are shown in Figure 2. The cell number of *C. vulgaris* UMACC 001 was generally higher when grown at NH₄Cl levels above the control (Figure 2a). There was a lag phase of two days when this species was grown at 250 mM NH₄Cl. For *S. quadricauda* UMACC 039, the final cell number attained at 50 mM NH₄Cl (18.90 X 10⁵ cells mL⁻¹) was significantly higher than the control (3.37 X 10⁵ cells mL⁻¹) (Figure 2b). In contrast, the final cell number attained at 75 mM NH₄Cl (4.11 X 10⁵ cells mL⁻¹) was only slightly higher than the control. At NH₄Cl levels above 75 mM, there was only slight increase in cell number throughout the growth cycle.

Table 2. Final biomass (mg L⁻¹) of the four chlorophyte cultures grown at different levels of NH₄Cl.

NH ₄ Cl mM	Biomass (mg dry weight L ⁻¹)			
	<i>Chlorella vulgaris</i> UMACC 001	<i>Scenedesmus</i> <i>quadricauda</i> UMACC 039	<i>Scenedesmus</i> <i>quadricauda</i> UMACC 041	<i>Ankistrodesmus</i> <i>convolutus</i> UMACC 001
2.9 (control)	33.1	89.2	48.3	24.2
50	282.9	225.8	172.5	222.5
75	337.1	113.3	110.0	135.0
120	315.7	92.5	77.5	65.0
170	268.6	84.2	65.0	79.2
250	250.0	74.2	56.7	40.8

For both *S. quadricauda* UMACC 041 and *A. convolutus* UMACC 101, the cell numbers attained at 50 – 75 mM NH₄Cl were generally higher than the control while at 120 – 250 mM the reverse was observed (Figures 3c and 3d). Based on cell number, *A. convolutus* UMACC 101 did not grow in 250 mM NH₄Cl (Figure 3d).

The maximum growth rate of *C. vulgaris* UMACC 001 at above 2.9 mM NH₄Cl (0.42 day⁻¹) was higher than the control (0.49 – 0.70 day⁻¹). This trend contrasted with that of *S. quadricauda*

UMACC 039 which showed maximum growth rates decreased (0.18 – 0.30 day⁻¹) at NH₄Cl levels higher than the control (0.46 day⁻¹).

Final biomass attained by *C. vulgaris* UMACC 001 was highest at 75 mM NH₄Cl whereas for the other chlorophytes, it was attained at 50 mM (Table 2). At 250 mM NH₄Cl, the final biomass of *A. convolutus* UMACC 101 was doubled although its cell number was very much lower compared to the control. Enlarged unicells instead of clusters of 4 – 8 cells were observed.

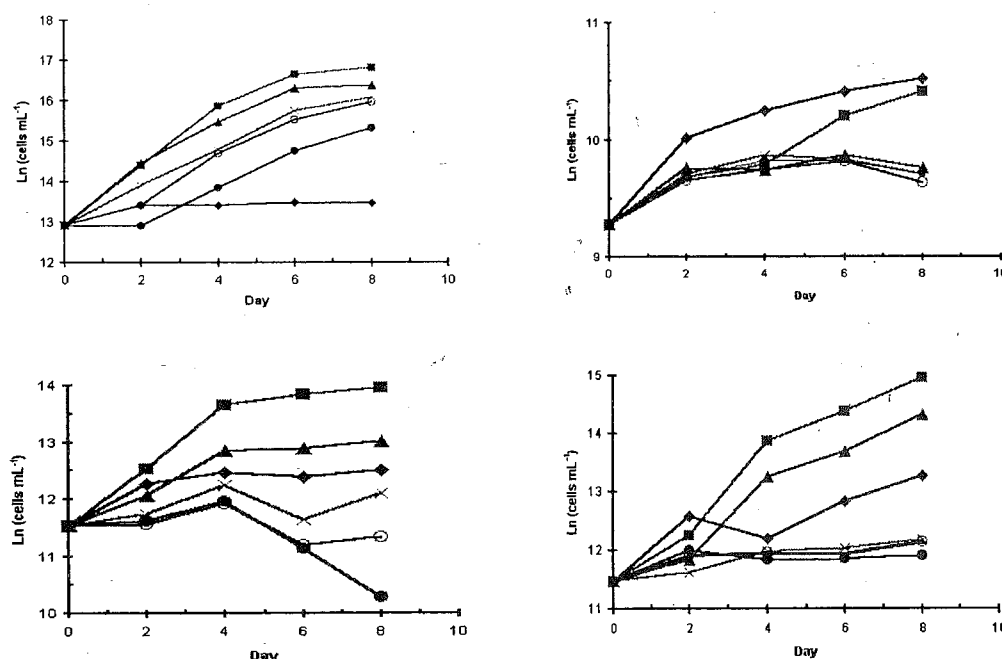


Figure 1. Growth curves of the four chlorophytes cultured at different levels of NaNO₃ (mM): ♦ 2.9 (control), ■ 50, ▲ 75, × 120, ○ 170, ● 250. *Chlorella vulgaris* UMACC 001 (top left); b) *Scenedesmus quadricauda* UMACC 039 (top right); c) *Scenedesmus quadricauda* UMACC 041 (bottom left); d) *Ankistrodesmus convolutus* UMACC 101 (bottom right)

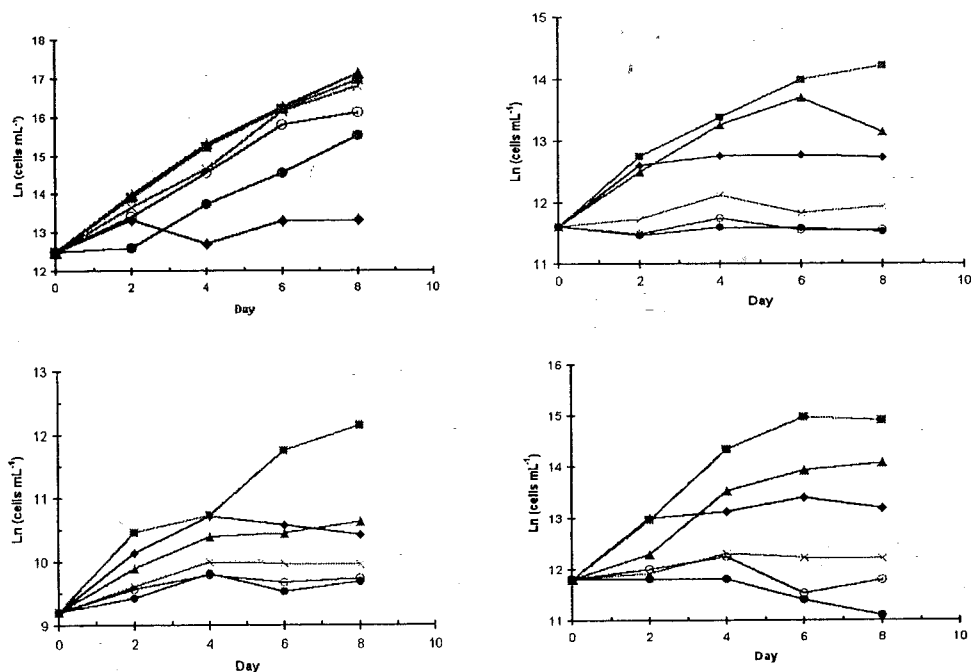


Figure 2. Growth curves of the four chlorophytes cultured at different levels of NH₄Cl (mM): ♦ 2.9 (control), ■ 50, ▲ 75, × 120, ○ 170, • 250. a) *Chlorella vulgaris* UMACC 001 (top left); b) *Scenedesmus quadricauda* UMACC 039 (top right); c) *Scenedesmus quadricauda* UMACC 041 (bottom left); d) *Ankistrodesmus convolutus* UMACC 101 (bottom right)

DISCUSSION

Of the four chlorophytes tested, *C. vulgaris* UMACC 001 was most tolerant to high levels of nitrogen, being able to grow at 250 mM NaNO₃ and NH₄Cl. This species did not grow well in BBM, which contained only 2.9 mM NaNO₃ or NH₄Cl. Both *S. quadricauda* UMACC 041 and *A. convolutus* UMACC were sensitive to high NaNO₃ and NH₄Cl levels, with growth markedly inhibited at above 75 mM. In comparison, the "high" levels of nitrogen used to grow chlorophytes reported by other workers are much lower than the levels tested in this study. Jeanfils *et al.* [14] reported another isolate of *C. vulgaris* that is able to grow at 97 mM KNO₃ while Tan *et al.* [15] demonstrated that *Trentepohlia odorata* grows well at 35 mM NH₄Cl. The optimum level of NaNO₃ for the growth of the four chlorophytes was 50 mM, which was much higher than that reported for other chlorophytes such as *Chlorella vulgaris* (6 – 12 mM) and *Ankistrodesmus*

convolutus (3.0 mM) reported by Jeanfils *et al.* [14] and Chu *et al.* [16] respectively.

Although the cell number of *S. quadricauda* UMACC 039 did not increase significantly at high NaNO₃ levels, there was increase of biomass, which resulted from the increased cell size. Other workers have reported changes in morphology of *Scenedesmus* in response to different culture conditions. For instance, the cell size of *S. microspina* increased two-fold, with marked ultra-structural changes, when grown in diesel fuel oil [17]. Colony formation in *S. obliquus* also changes when subjected to grazing pressure of *Daphnia* [18]. Enlarged cells of *A. convolutus* UMACC 101 grown at high nitrogen levels were also observed; probably this was a stress response. Chu *et al.* [13,16] observed similar changes in morphology and cell size of this chlorophyte grown under other stressed conditions such as high NaCl and glucose levels.

With the ability to grow at high nitrogen levels, *C. vulgaris* UMACC 001 is a potential species for bioremediation of nitrogen-rich agro-industrial wastes such as palm oil mill effluent and rubber effluent. In fact, Geetha *et al.* [17] demonstrated that *C. vulgaris* UMACC 001 grows well in rubber effluent in high-rate algal ponds, generating high biomass and achieving percentage NH₃-N reduction of more than 90%. It may also be a good bioindicator for nitrogen enrichment in freshwater environment.

There is interrelation between nitrogen with other nutrients, especially phosphorus and carbon in controlling algal growth. Thus, it is worthwhile to investigate the effect of N:P and C:N ratios on the growth of *C. vulgaris* UMACC 001. In addition, lower levels of nitrogen should be tested to determine the lower limits of tolerance (less than 2.9 mM) of these chlorophytes in order to further assess the suitability of these species as bioindicator organism for nitrogen enrichment.

CONCLUSION

Of the four chlorophytes tested, *C. vulgaris* UMACC 001 was most tolerant to increased levels (> 2.9 mM) of NaNO₃ and NH₄Cl while *S. quadricauda* UMACC 041 and *A. convolutus* UMACC 101 were sensitive to high levels of these nitrogen sources. Thus, *C. vulgaris* UMACC 001 is a potential species to be used in the treatment of agro-industrial wastes and as bioindicator for nitrogen enrichment.

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