DISCUSSION

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The ultimate objective of the present study was to evaluate the potential e of tropical microalgae as bioassay organisms for nitrogen and phosphorus richment in freshwater ecosystems. The approach used was to investigate the fects of minimal levels of nitrogen and phosphorus required to stimulate the owth of three chlorophytes within short growth duration (96 h). This approach fered from the previous study conducted by Foo *et al.* (2001) on the three ecies. In the previous study, the test organisms were subjected to high levels $NaNO_3$ and NH_4Cl, ranging from 2.9 to 250 mM, and the growth duration was days. In comparison, the levels of nitrogen tested in the present study were in e range of 0.03 to 18.75 mM. The levels tested were close to that encountered natural waters. For example, clean freshwater contains about 0.07 mM $NaNO_3$ rainor, 1984) while agro-industrial wastewaters such as rubber effluent may ntain 21 mM NH_3-N and 0.21 mM NO_3-N (Phang and Ong, 1988).

1. Selection of minimal culture medium for testing

Of the different strengths of BBM tested, 1% BBM was found to be the inimal medium that stimulated growth of *Chlorella vulgaris* UMACC 001. A mparison of nitrogen levels in various culture media and test media is shown in able 66. The nitrogen level in the minimal medium was close to that found in tural freshwater. Thus, this medium was chosen as the minimal medium for rther growth experiments. The 1% BBM was spiked with nitrogen and

osphorus levels at an increasing factor of five-fold. The overall growth trends d cell numbers attained after 12 and 96 h, of the test organisms were then mpared with that in Dilution Medium, which was without nitrogen and osphorus.

ble 66: Nitrogen levels in various culture media and test media.

Medium	Nitrogen (mM)
miya *et al.*	3.54 mM
ger and Granick	7.5 mM
iura and Iwasa	3.0 mM
stol	2.9 mM
u	0.5 mM
AP (Provisional Algal Assay Procedure)	0.3 mM
ture	0.07 mM
% BBM (Used in the present study)	0.03 mM

eference: Trainor (1984).

2. Growth responses of the three chlorophytes to variations in nitrogen d phosphorus levels

In the previous study (Foo *et al.*, 2001), the growth responses, both mulatory and inhibitory, of the chlorophytes to high nitrogen levels (up to 250 M) were investigated. In comparison, the present study aimed to compare the

imulatory growth response of the chlorophytes to low levels of nitrogen and hophorus. It was found that *Chlorella vulgaris* UMACC 001 was the most lerant to high nitrogen levels of the four chlorophytes tested by Foo *et al.* 001). However, tests using low levels of nitrogen failed to detect distinct fference in growth response towards the range of nitrogen used. Summary of ndings on the growth response of the three chlorophytes to different nitrogen nd phosphorus levels is shown in Table 67.

There was no growth inhibition of the three chlorophytes when grown at H_4Cl level as high as 18.75 mM. This finding contrasted with other studies hich showed that high levels of ammonium, especially in the form of ammonia, re toxic to microalgae. For example, Kallqvist and Svenson (2003) reported that oncentrations of NH_4^+ and NH_3 above 30 μM inhibit growth of *Nephroselmis yriformia*. Phytoplankton which are sensitive to high ammonia concentrations re usually those that inhabit environments with constant low levels of ammonia. he species used by Kallqvist and Svenson (2003) is a marine chlorophyte, and ost probably isolated from marine waters with low ammonium level. And thus, is could probably account for the difference in sensitivity to ammonia. In ontrast, the three chlorophytes tested in the present study were isolated from itrogen-rich habitats. For example, *Chlorella vulgaris* UMACC 001 was isolated rom an eutrophic lake at an experimental farm (Phang and Chu, 1996). This pecies was shown to be highly tolerant to high levels of nitrate and ammonium, growing well even at 250 mM $NaNO_3$ or NH_4Cl (Foo *et al.*, 2001).

Expt	N & P levels	Growth response	Comments
1	$NaNO_3$: 0.03, 0.3 & 3.0 mM	• 0.03 mM (1% BBM) : minimal level of $NaNO_3$ that enhanced growth compared to Dilution Water, at least for the initial 4 days of growth	• 1 % BBM was chosen as a 'minimal' medium for short-term (96 h) growth study
2	$NaNO_3$: 0.03, 0.15, 0.75, 3.75 & 18.75 Mm PO_4^{3-} :0.02 mM	• Growth enhanced after 48 h for the three chlorophytes • PGE-96 based on both cell numbers and OD_{620} increased with increasing $NaNO_3$ levels for the three chlorophytes	• Similar growth response of the three chlorophytes tested to the levels of $NaNO_3$ tested
3	NH_4Cl : 0.03, 0.15, 0.75, 3.75 & 18.75 mM PO_4^{3-} :0.02 mM	• *C. vulgaris* UMACC 001 & *S. quadricauda* UMACC 041: Similar growth trends for $NaNO_3$ levels between 0 – 18.75 mM • *A. Convolutus* UMACC 101 : growth was enhanced at $NaNO_3$ ≥0.03 mM $NaNO_3$; no marked difference in trends between 0.03 – 18.75 mM $NaNO_3$ • PGE-96 increased with increasing $NaNO_3$ levels for all species tested	• PGE-96 was a better parameter for bioassays of $NaNO_3$ levels compared to growth trends
4	$NaNO_3$: 0.03 mM PO_4^{3-} :0.02, 0.1, 0.5, 2.5 & 12.5 mM	• *C. vulgaris* UMACC 001: No marked difference in growth trends between 0 – 12.5 mM PO_4^{3-} • *S. quadricauda* UMACC 041: growth enhanced after 24 h, with respect to Dilution Water • *A. Convolutus* UMACC 101: cell numbers attained increased with increasing PO_4^{3-} level • Both PGE-12 and PGE-96 increased with increasing PO_4^{3-}levels	• The levels of PO_4^{3-} tested did not enhance growth of *C. vulgaris* UMACC 001 when $NaNO_3$ level used was low (0.03 mM) • Low $NaNO_3$ level sufficient for growth to use the excess PO_4^{3-}

Table 67: A summary of growth responses of the three chlorophytes to nitrogen and phosphorus levels in the medium.

Expt	N & P levels	Growth response	Comments
5	NH_4Cl : 0.03 mM PO_4^{3-} :0.02, 0.1, 0.5, 2.5, & 12.5 mM	• *C. vulgaris* UMACC 001: no growth enhancement between 0 – 12.5 mM PO_4^{3-} • *S. quadricauda* UMACC 041& *A. Convolutus* UMACC 101: growth enhanced in PO_4^{3-} containing medium after 48 h, no marked difference in growth trends between 0.02 – 12.5 mM • PGE-96 increased with increasing PO_4^{3-} levels, PGE-12 not affected by PO_4^{3-}levels	• PGE-96 was a good parameter for assessing growth enhancement due to increasing phosphate levels
6	$NaNO_3$: 18.75 Mm PO_4^{3-} : 0.02, 0.1, 0.5, 2.5, & 12.5 mM	• Growth enhance after 24 h in PO_4^{3-} containing medium for all species tested • PGE-96 increased with increasing PO_4^{3-} levels	• PGE-96 was a good parameter for assessing growth enhancement due to increasing phosphate levels
7	NH_4Cl : 18.75 mM PO_4^{3-} : 0.02, 0.1, 0.5, 2.5, & 12.5 mM	• Growth enhancement throughout the growth duration in PO_4^{3-} containing medium compared to Dilution Water for all species tested • PGE-96 increased with increasing PO_4^{3-} levels but not PGE-12	• PGE-96 was a good parameter for assessing growth enhancement due to increasing phosphate levels

This species has also been successfully used to treat rubber effluent and alm oil mill effluent, as high percentages removal of nitrogen and phosphorus ere achieved (Phang and Ong, 1988; Geetha *et al.*, 1994; Phang *et al.*, 2001). hile species sensitive to high ammonium levels are suitable for toxicity testing, lerant species apart from its use in treating wastewater, will also be useful as a oassay organism to detect the high levels of ammonium in agro-industrial astewater.

At low levels of $NaNO_3$ (0.03 mM, 1%BBM), the growth of *Chlorella ulgaris* UMACC 001 was not enhanced even at a phosphate level ten-fold of hat in BBM. However, at high $NaNO_3$ level (18.75 mM), growth of this species as markedly enhanced with increasing phosphate levels. Thus, the cultures rown at 0.03 mM $NaNO_3$ might be nitrogen-limited, and therefore, unable to use e excess phosphate efficiently.

Results showed that the cell number attained after 96 h was the most seful parameter for comparing the stimulatory effect of growth due to addition of w levels of nitrogen or phosphorus (Table 67). In comparison, cell number ttained after 12 h may not show a consistent trend with respect to the levels of trogen or phosphorus in the medium. Thus, Percentage Growth Enhancement 96 h (PGE-96) is a new parameter that we are introducing for use in bioassay nitrogen and phosphorus. The overall growth trends and specific growth rates d not seem to be good parameters for bioassay. One good example from the resent study was the response of *Ankistrodesmus convolutus* UMACC 101 to creasing phosphate levels. While μ did not vary markedly (6.04 – 9.51 d^{-1}), the

GE-96 increased from 111 to 216% with increasing phosphate levels from 0.02 12.5 mM.

3. Potential use of the chlorophytes for bioassay

Algal bioassays are used to study the biological responses to the changes water quality and to determine the levels of nutrients such as nitrogen and hosphorus, and pollutants such as heavy metals and pesticides in the water amples. One of the criteria in choosing an organism for assay purposes is that it hould be sensitive to low concentrations of the material (Pipe *et al.*, 1984). One f the common species used in bioassay is *Selenastrum capricornutum* because can be easily cultured, with minimum morphological changes during the opulation growth phase (Walsh *et al.*, 1984). This species is also highly ensitive and can be used to assay down to 0.01 mM nitrogen (Maestrini *et al.*, 984). There have been efforts to search for new, more sensitive test species at respond to both growth inhibitors and stimulators. For instance, De Vries and amphof (1984) explored the possible use of *Stigeoclonium* for bioassay of trogen in eutrophic waters. Recently, Kallqvist and Svenson (2003) used *ephroselmis pyriformis* in toxicity testing of ammonia in marine waters. Most of e species used in bioassays are temperate species, although there have been fforts to screen tropical phytoplankton for use in toxicity testing of heavy metals Melor *et al.*, 2002).

This present study was probably the first to assess the potential use of opical microalgae for bioassay of nitrogen and phosphorus enrichment. The

three chlorophytes seem to be useful as bioassay organisms based on their response of PGE-96 to the increasing levels of nitrogen and phosphorus. However, if *Chlorella vulgaris* UMMAC 001 was to be used as bioassay for phosphorus, high nitrogen should be added in the test medium. Other features that make these three species useful bioassay include homogeneity of the cultures and minimum changes in morphology during the growth test.

In general, testing of natural waters is usually based on IC_{50} and Algal Growth Potential (AGP). The first parameter is more useful for toxicity testing rather than assessing growth stimulation due to nutrient enrichment (e.g. Hallqvist and Svenson, 2003). The AGP test is based on the principle that the maximum cell yield or biomass attained by the test alga is proportional to amount of nutrient present in the water sample (Skulberg, 1995). For example, the AGP assay based on *Selenastrum capricornutum* (NIVA-CHL-1) was used to detect the phosphorus level in agricultural runoff (Skulberg, 1995). The water samples were first sterilised by gamma-radiation before being inoculated with the test organism and the algal assay was performed for 9 –12 days under standard growth conditions. Thus, longer time is required for AGP test compared to the bioassays of nitrogen and phosphorus enrichment demonstrated in the present study, which took only 96 h.

4. Areas for further studies

The blooming of microalgae in natural environments is usually due to the alterations of the ratio of nitrogen and phosphorus, and very unlikely to due solely

a single factor. For instance, Camacho and De Wit (2003) reported that adding nitrogen or phosphorus alone, or both nutrients at N: P ratio of 16 can cause ooming of different benthic microalgae in a hypersaline lake. Thus, one of the eas worth further studies is to investigate the interactive effects of both nitrogen nd phosphorus, using factorial experimental design, on the growth of the three hlorophytes. Thus, it is worthwhile to investigate the growth response of the icroalgae to variations in N: P ratios. The N: P ratios can be varied in batch ltures or by manipulating the growth rates of the test species in continuous lture.

The uptake of nitrogen and phosphorus is another area worthy for further udies. The uptake studies can be conducted using radiotracers such as N^{15} and ^{32}. Both bioassays and physiological tests should be conducted concomitantly lameed *et al.*, 1999). Apart from algal growth, the effects of nitrogen and hosphorus on physiological processes such as photosynthesis and ccumulation of cell materials such as lipids, proteins, carbohydrates, nucleic cids and ATP should be further characterised.

Investigations on the activities of enzymes involved in nitrogen and hosphorus utilisation by microalgae is another avenue of further studies. For xample, assaying of the alkaline phosphatase activity of symbiotic alga in coral an serve as a useful bioindicator for nitrogen status of the surrounding sea aters (Annis and Cook, 2002). Recently, Newman *et al.* (2002) reported that hosphatase activity is useful as an early warning indicator of wetland utrophication. The potential use of molecular markers as bioindicator for

hosphorus enrichment is worth exploring. For example, a luciferase gene that is ducible by alkaline phosphatase has been cloned in *Synechococcus harveyi* Schreiter *et al.*, 2001). Under phosphorus-limitation, the gene is induced and this auses bioluminescence.