Growth and Physiological Responses of the Singapore Strain of *Heterosigma* to Various Nitrogen Sources and Light Conditions

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Abstract— ***Nutrient pollution of coastal environments particularly by nitrogen (N) sources can lead to harmful algal blooms. Ameliorating the incidence of such events requires an understanding of how harmful taxa respond to different N sources. In particular, the blooms of toxic Heterosigma have been known to cause costly and highly detrimental effects to surrounding ecosystem function. The threat is especially dire in the case of Singapore where growth dynamics of Heterosigma species under tropical environmental conditions are poorly understood. The present study examined how the growth rates of Heterosigma species isolated from the Johor Straits of Singapore differ under various N sources as well as varying light intensity. Heterosigma was found to grow well under both nitrogen sources, but grew at a faster rate when exposed to nitrate compared to ammonium. In addition, the study also observed tolerance for high- and low-light conditions. These results indicate that present modifications to the coastal environment through high sedimentation and nutrient pollution rates might be creating conditions conducive to the growth of Heterosigma.***

Keywords - Heterosigma; Singapore; coastal management; pollution; harmful; bloom

I. INTRODUCTION

HABs of the marine raphidophyte *Heterosigma* have been known for massive fish- and shellfish-kills throughout the world [1-3]. *Heterosigma* can cause detrimental effects through neurotoxins [4-7] even without attaining high cell concentration and dominance of the water column [8-9]. In recent years, raphidophyte *Heterosigma* species found in water samples collected around Singapore indicated that they could be dominant at times [10-11]. However, there is at present little knowledge about tropical strains [12], making it difficult to mitigate growth and productivity levels.

Current measures to address the occurrence of *Heterosigma* must seek to ameliorate the formation of HABs which would severely damage the coastal environment. Information on their growth and physiological responses to varying environmental conditions are therefore essential for implementing measures that can protect commercial aquaculture as well as public health.

It is widely recognised that marine environments are N-limiting for marine algae growth [13]. In general, increased agricultural development as well as sewage and industrial output have led to increased inorganic-N levels in coastal environments [14] which are commonly in the forms of ammonium and nitrate. In Singapore, its waters are similarly susceptible to N-pollution due to nearby coastal and urban development [15]. N-pollution can enter marine environments through surface run-off, atmospheric deposition or groundwater discharge [16], entering coastal waters at different depths and resulting in vertical inhomogeneity of inorganic-N levels.

In the present study, we therefore exposed *Heterosigma* species to different light exposure and different form of N-sources and concentrations to examine their growth responses to such varying conditions.

II. MATERIALS AND METHODS

Heterosigma strains used in the experiment were isolated from the East Johor Straits by Dr. Sandric Leong. Cultures grown in 1-litre sterilised screw-top polycarbonate bottles under semi-continuous conditions followed the protocol of Leong et al (2010) [17]. All cultures were maintained at 25 ^{o}C and at irradiance of 350 µmol m^{-2} s^{-1} on a 12:12 hour light-dark cycle using cool white fluorescent tubes. Throughout the study, F/2 modified media [18] without silicate and containing varying concentrations of either nitrate or ammonium was used.

Prior to experimentation, cells were acclimated to modified media containing 3 µM-N of nitrate and exposed to a light intensity of 350 µmol m^{-2} s^{-1}. Acclimation took place over a period of 18 days with regular inoculation into fresh media every two days. *In vivo* chlorophyll-*a* levels were measured every two days to monitor growth rates.

Fluorescence readings were measured in triplicate using the *Aqua*fluor™ fluorometer. All measurements were carried out in raw fluorescence units (RFU). Chlorophyll-*a* density

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was subsequently derived by comparison against a calibration chart (Fig. 1). The linear relationship is represented by y = 2.94x + 3.356 where y represents chlorophyll-*a* density value and x represents RFU values.

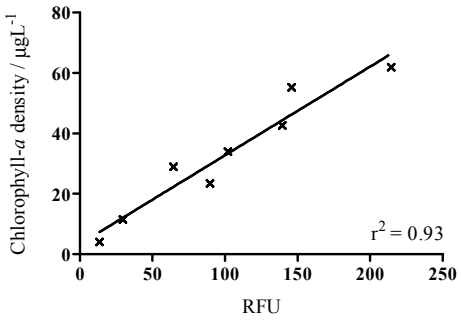

Fig. 1: Calibration chart to determine chlorophyll-*a* density (y-axis) from RFU values (x-axis). RFU values were obtained by triplicate measurements using the Aquafluor™ fluorometer and chlorophyll-*a* values are duplicate measurements following EPA Method 446.0 [19].

Light experiments were conducted by growing cells at a nitrate concentration level of 3 µM-N. Low-light was simulated by using neutral light density filter which attenuated incubator light by about half. High-light conditions were achieved by directly exposing the culture bottles to the incubator light settings.

Nutrient experiments were conducted by inoculating exponentially-growing cultures into modified media containing 3, 6 and 9 µM-N of nitrate or ammonium.

For each variable, three sets of cultures were grown over a period of five days. Specific growth rates (µ day^{-1}) were calculated from fluorescence measurements taken at regular twenty-four intervals on each of the five days according to the equation:

$$\mu = \frac{Ln(N_t) - LN(N_0)}{t_1 - t_0} \quad (1)$$

where N_t and N_0 are the chlorophyll-*a* density in μgL^{-1} on the day of measurement (t_1) and at the start of the culture (t_0). For each experimental culture, the initial chlorophyll-*a* density was kept below 6.3 μgL^{-1} (<10 RFU).

III. Results

A. Ammonium vs. nitrate experiments

Heterosigma cells that were cultured under nitrate-enriched (Fig. 2) and ammonium-enriched (Fig. 3) conditions were observed to enter stationary growth phase by Day 3. There was no evident lag phase when nitrate-acclimated cells were inoculated into ammonium-enriched media. Exponential growth rates were found, using a two-way ANOVA test, to be significantly affected by both factors of nitrogen concentration levels ($p < 0.0001$; 83.27% influence on total variation) and nitrogen type ($p < 0.01$; 7.21% influence on total variation). There was insignificant interaction between the two factors. The values obtained are presented in Table 1.

TABLE I. Exponential Growth Rates of Experimental Cultures

Conc. (µM)	NO_3-enriched medium		NH_4-enriched medium	
	Mean (day^{-1})	*S.D.*	*Mean (day^{-1})*	*S.D.*
3	0.70	0.1	0.66	0.04
6	1.08	0.06	0.89	0.1
9	1.25	0.07	1.10	0.05

Cultures inoculated in nitrate-enriched media yielded higher growth rates than ammonium-enriched media. Using Michaelis-Menton kinetics (Fig. 4), nitrate-enriched cultures attained maximum growth rate (V_{max}) of 2.06 ± 0.2 day^{-1} with half-saturation constant (K_m) of 5.71 ± 0.9 µM-NO_3. Ammonium-enriched cultures attained a V_{max} of 1.65 ± 0.2 day^{-1} with K_m of 4.71 ± 1.3 µM-NH_4.

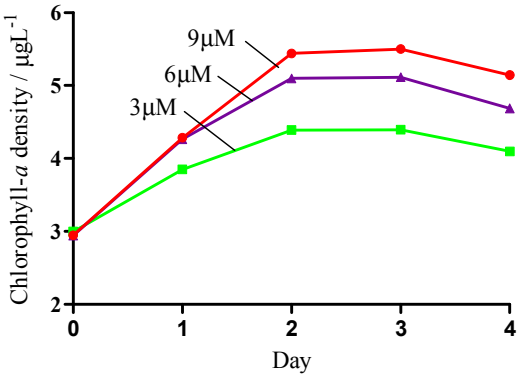

Fig. 2: Growth curves for *Heterosigma* at 3 µM-NO_3 (green closed squares), 6 µM-NO_3 (purple closed triangles) and 9 µM-NO_3 (red closed circles). Chlorophyll-*a* density values were obtained by conversion of RFU readings using the calibration chart. Values are plotted against a logarithmic vertical axis. The points shown depict mean values.

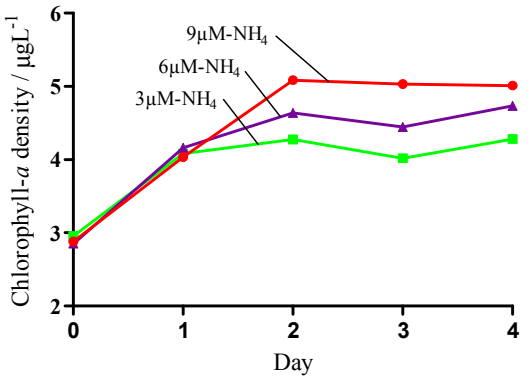

Fig. 3: Growth curves for *Heterosigma* at 3µM-NH_4 (green closed squares), 6µM-NH_4 (purple closed triangles) and 9µM-NH_4 (red closed circles). Values are plotted against a logarithmic vertical axis. Points presented are derived from mean values.

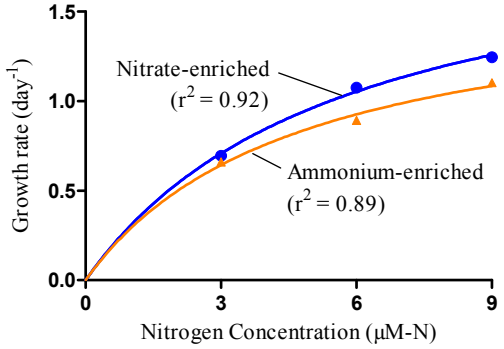

Fig. 4: Growth rates of *Heterosigma* in relation to increasing nitrate (blue closed circles) and ammonium (orange closed triangles) concentration levels. The line is derived according to Michaelis-Menten kinetics and points depict mean values.

B. High-light vs. low-light experiments

Exponential growth rates were found to be statistically similar for cultures grown under low-light and high-light conditions. The obtained results are presented in Table 2.

TABLE II. EFFECT OF LIGHT ON EXPONENTIAL GROWTH RATES (DAY^{-1})

Light levels	Exponential growth (day^{-1})		
	Mean	***S.D.***	***p-value***
High	0.70	0.1	0.5
Low	0.76	0.1	

a. p-values are calculated using single-factor ANOVA

In general, the differences in the sampling parameters are statistically-insignificant, demonstrating the tolerance of *Heterosigma* cultures over the experimental range of light levels. Cultures grown under high-light (Fig. 5A) were also observed to have a sharper exit from the exponential growth phase. Under low-light conditions (Fig. 5B), cultures exhibited a more prolonged exponential growth phase.

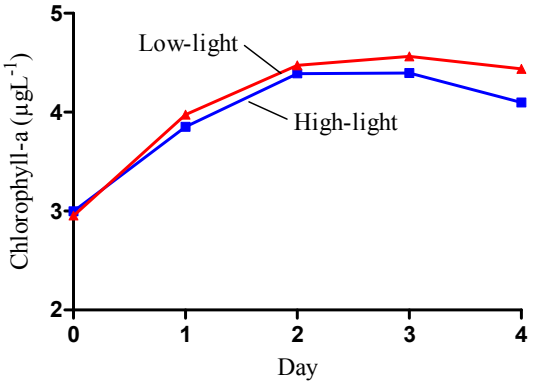

Fig. 5: Growth curves for *Heterosigma* under high-light (blue closed squares) and low-light (red closed triangles) conditions. Chlorophyll-*a* density values are plotted against a logarithmic vertical axis. Points depict mean values.

Discussion

For the experiment between ammonium and nitrate sources of nitrogen, the present study has demonstrated that *Heterosigma* exhibited a preference for nitrate compared to ammonium. Nevertheless, both sources were suitable for the growth of *Heterosigma* cultures. Nitrate-uptake behaviour is understood to be thermodynamically more costly than ammonium uptake [20]. *Heterosigma* cells used in this study were therefore able to cope with the higher energy demand associated with nitrate-uptake. Although separate studies have indicated similar trends for several phytoplankton species [21], other studies have observed *Heterosigma* exhibiting a stronger preference for ammonium sources [22-23]. Such contrasting observations may indicate strain-specific biological mechanisms that may not yet be well-understood.

In addition, the absence of a lag phase when nitrate-acclimated *Heterosigma* cells were inoculated into ammonium-enriched media indicates its ability to rapidly switch from nitrate-uptake to ammonium-uptake behaviour. Conversely, inoculation of ammonium-acclimated cells into nitrate-enriched media has been found to require a lag phase associated with the physiological development of nitrate-uptake carrier proteins [24]. The uptake of nitrate and ammonium is therefore likely to require different physiological features.

In addition, this study also observed that *Heterosigma* cells cultured under low light intensity had growth rates that did not significantly differ from cells cultured under high light intensity, indicating the ability of *Heterosigma* cells to tolerate shaded conditions.

At present, the coastal waters of Singapore are dominated by non-toxic phytoplankton species [11]. The high diversity of local species assemblage may therefore serve to alleviate HAB-formation by decreasing the availability of open niches [25-27]. However, new niches can be created by human-driven changes to the coastal environment. For instance, low light conditions typically unfavourable to most marine algae [28] are being enhanced by sedimentation brought about through activities like reclamation, dredging and ship movement. The effect is compounded by N-pollution particularly through underground discharge which can provide suitable environments at depth for *Heterosigma* growth.

In addition, studies have also observed a relationship between industrialisation activities and increased nitrate pollution. Increased NO_x emissions have led to nitrate-spiking of hydrological systems which discharge into coastal waters [e.g. 29-31]. If nitrate pollution is similarly present in Singapore, it could cause rapid growth of *Heterosigma* cells, increasing the opportunities for toxic algal bloom events.

The Singapore strains of *Heterosigma* are observed to be tolerant of a wide range of physical conditions and are hence able to rapidly colonise a wide range of niches. In order to ameliorate *Heterosigma* bloom events, it is important that favourable conditions are not provided and that open niches do not become available. Understanding the physiological responses of *Heterosigma* strains to various N sources as well as light intensity is an important step towards the mitigation of

bloom behaviour. Policy measures are therefore crucial to regulating the human-modification of coastal environments and to promoting the survival of the prevalent species assemblage.

Acknowledgements

We thanked the staff and interns of TMSI; Ms SM Chew, Ms P Chen and Mr A Lee for assisting and overseeing the set-up and execution of the various experiments.

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