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THE EFFECTS OF IN-SITU WATER COLUMN NUTRIENT ENRICHMENT ON THE SEAGRASS *THALASSIA HEMPRICHII* (EHRENB.) ASCHERS.: A PILOT STUDY AT ST. JOHN'S ISLAND, SINGAPORE

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ABSTRACT. — Nutrient enrichment of nearshore waters has been extensively implicated in the global decline of seagrass populations. Ex-situ manipulative experiments are invaluable in studying mechanisms and direct effects of elevated nutrients on seagrasses, but may underestimate the importance of indirect consequences of enrichment and need to be corroborated in the field. However, conducting in-situ experiments are notoriously difficult with real-world variability in conditions easily confounding attempted treatments. This paper presents the results of an in-situ pilot study conducted at St John's Island (also known as Pulau Sakijang Bendera), Singapore, to investigate experimental conditions that can properly examine the impacts of elevated water column nutrients on intertidal populations of a seagrass *Thalassia hemprichii* (Ehrenb. ex Solms) Asch. (Hydrocharitaceae). Plants were experimentally exposed to two sources of nitrogen (NH_4 and NO_3) and phosphate (PO_4) added into the water column over a three-month period. In addition, an assessment to evaluate suitable physiological and morphological response variables of *T. hemprichii* were made, and the effects of potential competitors such as epibionts (biomass) and phytoplankton (measured by chlorophyll a) on their well-being. Nutrient levels over the study period were highly variable within treatments, with measured concentrations in control plots exceeding intended dosing regimes. Increases in nitrate and nitrite concentrations were evident at enriched plots, but these were inconsistent within and among treatments, suggesting large variability in hydraulics. Some evidence of enrichment effects on plants exposed to nutrient treatments were observed, from reduced leaf C:P, enhanced leaf total chlorophyll concentrations, lowered leaf chlorophyll a/b ratios, and increased leaf length and area. However, these responses were mostly not statistically significant as the high variation observed potentially reduced the power of statistical tests. The results are discussed in the context of improvements to the experimental setup. This study highlighted the importance of pilot studies in planning manipulative in-situ studies to minimise high natural variation. A more robust design would provide clearer quantitative information on the impact of anthropogenic nutrient enrichment on local seagrasses.

KEYWORDS. — water column, canopy water, nutrient enrichment, seagrass, response variables, *Thalassia hemprichii*

INTRODUCTION

The major plant nutrients nitrogen and phosphorus are essential for the maintenance of primary productivity, but loadings from anthropogenic sources currently outweigh natural occurrences such that these now constitute a potential cause of impact to coastal habitats (de Jonge et al., 2002). Global declines and losses of many seagrass habitats have

been primarily attributed to the deterioration of coastal waters due to nutrient enrichment, although these conclusions are based on correlative rather than causal evidence (Tomasko et al., 1996; Cardoso et al., 2004; Lapointe et al., 2004). Environmental correlates provide a useful tool to assess potential causes of declines in seagrass health over broader spatial scales, but these studies were often limited in their ability to directly pinpoint causality of seagrass decline under enriched conditions (Cambridge & McComb, 1984). Manipulative experiments, on the other hand, can provide better understanding of responses to stressors, and give clearer quantitative data which would be useful for ecosystem understanding, modelling and management.

More manipulative studies have focused on nutrient enrichment of bed sediments (see review by Leoni et al., 2008), with the assumption that the primary route for nutrient uptake in seagrasses is through the roots (Zimmerman et al., 1987). Sediment enrichment often has positive effects on seagrasses, as plants tend to experience nutrient limitation (Lee & Dunton, 2000). However, enrichment of the water column may be a more critical issue for many coastal species (Rabalais et al., 2009), firstly because anthropogenically originated nutrients are directly received by near-shore water column (Jickells, 1998) and secondly, these nutrients are not immediately stored in the sediments until eventual adsorption, settlement and entrainment processes (Udy & Dennison, 1997; Valiela & Cole, 2002). Hence, sediment nutrient concentrations in the immediate vicinity of point sources of loadings often under-represent the true concentrations in receiving habitats (Szmant & Forrester, 1996). In addition, concentrations of water column nutrients may be further elevated by interactive effects of water physico-chemistry and sediment nutrients (Simon, 1989), especially under high temperatures and low levels of dissolved oxygen (Cerco, 1989).

The impacts of water column enrichment on seagrass performance and health have been widely assessed in ex-situ and in-situ experiments by loading external nutrients as a source of enrichment. Many recent ex-situ mesocosm experiments have devoted considerable effort in replicating field conditions (Table 1), but it is this ability that constitutes a major shortfall in being able to translate the results to the natural system. As a case in point, indirect effects of nutrients are thought to be more prevalent than direct negative effects on seagrasses, of which reduced light availability through algal proliferation is the most common mechanism (Romero et al., 2006). In-situ studies, in contrast, are realistic and these results can potentially provide substantive evidence for direct and indirect enrichment impacts on coastal habitats (Table 1).

In this study, the results of a pilot experiment to examine the effects of water column nutrient enrichment on seagrasses are presented. Pilot studies are useful to identify potential issues and problems with research prior to committing resources to the execution of a full sampling or experimental scheme (Legendre et al., 2004; Balata et al., 2010). Considerations to the design of pilot experiments are therefore necessary to pre-emptively address the limitations of in-situ experiments so as to reduce problems with the results. These would include treatment conditions, choice of nutrient source (e.g. suitable for autotrophic assimilation, dissolution rate), frequency of loading and nutrient deployment techniques (see review by Worm et al., 2000). The aims of this current study were to (1) set up a trial of an in-situ experiment exploring the responses of local seagrasses to nutrient enrichment of the water column, and (2) recommend methods for the improvements for a field study on the effects of water column nutrient enrichment on seagrass ecosystems.

MATERIAL AND METHODS

Study site. — The study was conducted in an intertidal seagrass bed (1°12.86'N, 103°51.00'E) located south of St. John's Island (officiallly known as Pulau Sakijang Bendera), along the Sisters Fairway (Fig. 1). This site consisted primarily of *Thalassia hemprichii* (Ehrenb. ex Solms) Asch. (Hydrocharitaceae) (Ascherson, 1871), covering an area of 38 by 33.5 m with an estimated percent cover of 65.4 ± 2.3%, and to a lesser extent, *Halophila ovalis* (R.Br.) Hook.f. (Hydrocharitaceae) (Hooker, 1858), found only at the upper

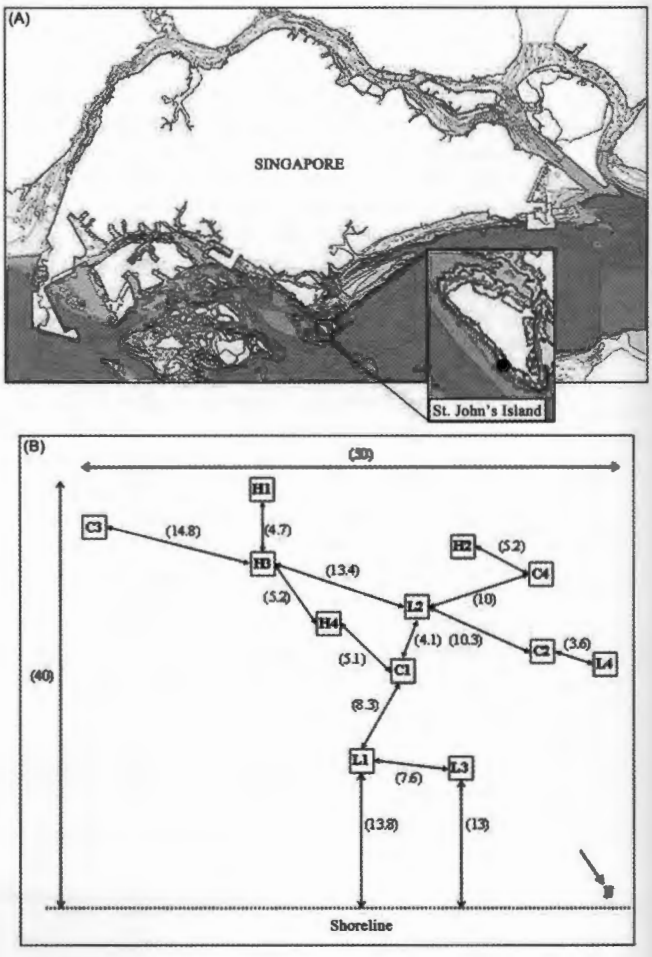

Fig. 1. Location and layout of the study site. (A): Map showing the seagrass bed on St. John's Island represented by solid circle (B): Layout of 12 experimental plots at the study site. C: Control plots, L: Low-nutrient treatment plots, H: High-nutrient treatment plots. Values in parentheses refer to distances measured in metres.

Table 1. Comparison of the advantages and disadvantages of in-situ and ex-situ manipulative studies conducted to assess impacts of water column nutrient enrichment.

Manipulative enrichment design	References
In-situ studies	
Advantages	
1. Offer a natural setting, accounting for ecosystem dynamics and interactive effects (e.g. seasons, hydrodynamics, biotic competition)	Harlin & Thorne-Miller, 1981; Heck et al., 2000; Frankovich et al., 2009
2. Provide a realistic quantitative measurement, with the ability to have sufficient replication	
Disadvantages	
1. High risk of loss of added nutrients due to rapid dissolution of the nutrient source and diffusion into surrounding medium	Hatcher & Larkum, 1983; Nielsen, 2001
2. Small-scale vagaries in receiving concentrations within experimental plots	Hatcher & Larkum, 1983; Williams & Ruckelshaus, 1993
Ex-situ studies	
Advantages	
1. Able to manipulate and control environmental (e.g. temperature, light) and biological (e.g. grazing communities) factors	Neckles et al., 1993; Short et al., 1995; Moore & Wetzel, 2000
2. Examine direct, monotonic responses close to limit and threshold concentrations	Brun et al., 2002
Disadvantages	
1. Effects observed in ex-situ studies may contradict field results, e.g. discrepancies in severity of algal growth responding to nitrogenous and phosphorus nutrients inputs in ex-situ and in-situ experiments	Harlin & Thorne-Miller, 1981; Taylor et al., 1995; Valiela et al., 1997
2. Are costly	
3. Reduced statistical power as experiments tend to have reduced replication capability and are shorter in length for cost-effectiveness	Underwood, 1994
4. Effects observed ex-situ must be correlated and validated with field studies to have any realistic significance	Tomasko & Lapointe, 1991; Skelly, 2002; Stachowicz et al., 2008

intertidal area (pers. obs.). *Thalassia hemprichii* was selected as the model species for this study because being a smaller late-successional species, they tend to be less resilient to disturbances (Duarte et al., 1997), and require longer recovery periods following disturbances (Olesen et al., 2004).

Nutrient enrichment. — Four replicate plots (0.5 m by 0.5 m) were randomly assigned to two experimental treatments (low and high) and ambient controls (Fig. 1). All plots were spaced at least 3 m apart (Fig. 1) for independence, a distance within the ranges described in nutrient enrichment designs by other authors (Agawin et al., 1996; Frankovich et al., 2009). Nutrients were introduced into the water column of experimental plots via nutrient dispensing tubes (4 cm diameter by 15 cm length). All nutrient tubes had four equally spaced vertical rows, and each row comprised of four equidistant 6 mm holes. Nutrient-loaded tubes were prepared by filling with approximately 200 g dry weight of the slow-release fertilizer Osmocote® Plus (8% NH_4-N, 7% NO_3-N, 9% P_2O_5, 12% K_2O). The ends of all tubes were capped with PVC caps. To remove potentially confounding hydraulic effects, all plots were allocated two dispensing tubes, anchored above the substratum in the centre via a PVC pipe driven into the sediment. Nutrient loading among treatments was manipulated by a combination of loaded and empty tubes. In experimental plots designated for high nutrient addition, labelled as 'high treatment', the two nutrient dispensing tubes contained fertilizer. Plots assigned to receive lower nutrient loads, labelled as 'low treatment', had one tube containing fertilizer and the other tube was empty. Both nutrient tubes placed within control plots were empty.

Sampling regime. — The experiment was conducted over three months (March to May 2010). Nutrients were introduced at low and high nutrient treatment plots at the start of the experiment. All experimental plots were sampled at the start of the experiments, to represent pre-enrichment conditions, and monthly thereafter during the spring low tide periods.

Methods. — During each sampling, in-situ measurements of water column chlorophyll a concentrations (as a proxy of phytoplankton biomass) were conducted with a chlorophyll a probe, YSI 6025, attached to an optical monitoring system, YSI 600 OMS (YSI Incorporated, Yellow Springs, USA). Seawater samples were collected whenever there was approximately 10 cm of water over the experimental plots, filtered with Whatman GF/C filters and frozen for nutrient analysis. Concentrations of ammonium (NH_4) and the sum of nitrate and nitrite (NOx) were analysed colorimetrically (QuikChem® 8000 Series IC + FIA, Lachat Instruments, USA). Phosphate (PO_4) concentrations were determined by spectrophotometric methods described by Grasshoff et al. (1999).

Seagrass parameters were determined from the oldest leaves excised above the leaf sheath, excluding senescent and brown leaves. Older leaves were sampled as they represent tissues that have steady growth, have greater development of epibiont cover (Sand-Jensen, 1977), and provide adequate biomass for analysis. Detached leaves were kept in seawater, placed on ice and immediately transported to the laboratory for processing. Leaves that were not analysed immediately were properly stored and kept within reasonable time. Leaves first cleaned of

Table 2. Results of univariate three-factor ANOVA testing the effects of nutrient enrichment Treatment, Plot(Treatment) and Time on the concentrations of water column nutrients. Significant differences are denoted by $^{\#}p < 0.05$, $^{\wedge}p<0.01$, $^{+}p < 0.001$.

		NH_4		NOx		PO_4	
Source	df	MS	*F*	MS	*F*	MS	*F*
Treatment	2	0.026	0.151	0.585	2.973	0.011	1.172
Plot(Treatment)	9	0.410	2.385$^{\#}$	0.518	2.634$^{\#}$	0.056	5.778^{+}
Time	2	3.704	21.52^{+}	0.795	4.041$^{\#}$	0.249	25.78^{+}
Treatment*Time	4	0.173	1.007	0.754	3.832$^{\wedge}$	0.033	3.411$^{\#}$
Plot(Treatment)*Time	18	0.314	1.827$^{\#}$	0.429	2.181$^{\#}$	0.018	1.856$^{\#}$
Error	72	0.172		0.197		0.010	

attached epibionts using a razor blade were used to determine the photochemical efficiency of photosystem II (F_v/F_m ratio), pigment contents (chlorophylls a and b, total carotenoids) and leaf size (length and area). F_v/F_m ratio was measured with a Plant Efficiency Analyser (Hansatech Instruments Ltd, King Lynn, England) on dark-adapted leaves. Pigment contents were extracted with N,N-dimethylformamide (Ralph et al., 2005), and analysed spectrophotometrically (Wellburn, 1994). Epibiont-free leaves were dried at 60°C to constant weight to determine leaf dry weight and elemental (carbon, nitrogen, phosphorus) content. Carbon (C) content was measured using a Total Organic Carbon Analyzer (TOC-V_{CSH}, Shimadzu, Japan) equipped with a Solid Sample Module (SSM-5000A, Shimadzu, Japan). Total Nitrogen (N) was extracted using Kjeldahl digestion (Tecator™ Digester, FOSS, Höganäs, Sweden) and concentrations were determined by steam distillation (*Kjeltec™ 2300* Analyzer Unit, FOSS, Höganäs, Sweden). Phosphorus (P) content was determined by dry ashing (Fourqurean & Zieman, 1992) and colorimetric analysis (Grasshoff et al., 1999). Dry weight of individual leaves was measured to determine the leaf mass area, calculated by normalising dry weight with one-sided leaf area (Lee et al., 2004). Scraped epibionts were collected to determine the dry biomass per unit leaf area. Samples were dried to constant weight at 60°C to determine the dry biomass and ashed at 550°C for 2 h to quantify the ash-free dry weight.

At the end of the experiment, the fertilizer in nutrient-loaded tubes were collected and dried to constant weight at 60°C to estimate daily nutrient loading rates. Daily fertilizer loss rates were estimated from the difference in fertilizer weight from the point of deploying to recovery of these tubes (Morris et al., 2007). Daily nitrogen and phosphorus release rates and loading rates were determined from the amount of nutrients lost (Heck et al., 2006).

Data analysis. — Data were statistically analysed using STATISTICA v10 (Statsoft 2010). A three-factor multivariate analysis of variance (MANOVA) design was performed separately to examine the effects of Treatment, Plot (Treatment) and Time on 1) the concentrations of nutrients in the water column, 2) physiological and morphological responses of *T. hemprichii* and 3) changes in epibiont biomass and water column productivity. All variables were square-root transformed to normalise the data prior to MANOVA analyses. Post-hoc comparisons were conducted using Tukey's HSD (Honestly Significant Differences) test to examine significance of treatment levels with critical values set at $p < 0.05$ (Zar, 2010).

RESULTS

Water column nutrient concentrations. — The concentrations of nutrients in the water column were significantly influenced by interactions between Treatment and Time (Pillai's $Trace_{12,216} = 0.35$, $p < 0.01$) and Plot(Treatment) and Time (Pillai's $Trace_{54,216} = 0.98$, $p < 0.001$). Subsequent univariate analyses demonstrated that Treatment*Time was significant only for the concentrations of NOx and PO_4, but the Plot (Treatment)*Time interaction had significant influences on the concentrations of all nutrient species (Table 2). This reflected the high variation in water column nutrients among plots (Fig. 2), and is likely to have decreased the power of the Treatment*Time test. There were no appreciable gains in mean concentrations of NOx at low nutrient loadings (Fig. 2B) and no increases in mean PO_4 at any treatment loadings, although the upper ranges for the latter appeared greater than before loading (Fig. 2C). There was a large, but statistically non-significant increase in mean NOx concentrations in the high treatment (Fig. 2B). Inconsistent loadings of nutrients within and between high and low treatments were not a function of variable Osmocote™ fertilizer dissolution as loss rates in high treatments were approximately twice that in low treatments (Table 3). Variation within treatments was all consistently less than 3% of mean loss and difference between mean treatment was roughly double between high and low treatments (Table 3).

Seagrass responses. — *Thalassia hemprichii* responses to experimental enrichment of the water column were also significantly influenced by interactions between Treatment and Time (Pillai's $Trace_{32,272} = 0.64$, $p < 0.05$) as well as between Plot(Treatment) and Time (Pillai's $Trace_{144,576} = 2.67$, $p < 0.001$). The results of the univariate analyses are presented in Table 4. High intra-treatment variation was also observed in seagrass responses (Figs. 3 to 6), with Plot(Treatment)*Time interactions significant for five of the eight parameters examined (Table 4). Although significant Treatment*Time interactions were detected on some parameters, the direction and magnitude of change in these were often inconsistent

Table 3. Daily loss, release and loading rates of Osmocote™ fertilizer at low and high nutrient treatment plots. Conversion to release and loading rates of nitrogenous and phosphorus nutrients was based on nutrient product information for Osmocote® Plus. N: Nitrogen, P: Phosphorus.

Daily rates	Nutrients	Low Treatment	High Treatment
Loss rate (g tube^{-1} d^{-1})		0.74 ± 0.02	1.48 ± 0.03
Release rate (mmol tube^{-1} d^{-1})	N	7.93 ± 0.19	15.82 ± 0.29
	P	2.15 ± 0.05	4.29 ± 0.08
Loading rate (mmol m^{-2} d^{-1})	N	31.71 ± 0.75	63.27 ± 1.14
	P	8.60 ± 0.20	17.17 ± 0.31

with treatment and contrary to expectation (Figs. 3 to 6). While plant negative responses, such as lowered leaf C:P ratio (Fig. 3B), increased total chlorophyll content (Fig. 4A), reduced chlorophyll a/b ratios (Fig. 4B), increased leaf length (Fig. 6A) and lowered leaf mass area (Fig. 6B) were observed, differences among treatment levels were either non-significant or inconsistent with the expected H>L>C effect size. There was also no indication that enrichment led to poor photosynthetic condition in receiving seagrasses; in fact both total chlorophyll:total carotenoids ratio and F_v/F_m ratios indicated that these plants were less healthy prior to the onset of enrichment (Figs. 4C, 5).

Community responses. — MANOVA results indicated that both epibiont biomass and water column chlorophyll *a* showed significant interactions between combinations of Treatment*Time (Pillai's $Trace_{12,216} = 0.33$, $p < 0.05$) and Plot(Treatment)*Time (Pillai's $Trace_{54,216} = 0.93$, $p < 0.01$), with the former significant only for epibiont dry weight and the latter only for epibiont ash-free dry weight (Table 5). As with seagrass responses, epibiont biomass response to enrichment treatment was contrary to expectation, with decreasing gradient from control to high treatments over the study period (Fig. 7). Only Plot (Treatment) and Time effects were significant on water column chlorophyll *a* concentrations (Table 5). The direction of change in water column primary productivity after experimental enrichment was also unexpected. Water column chlorophyll *a* concentrations were high even before treatment, but declined across all treatments after one month of loading (Fig. 8).

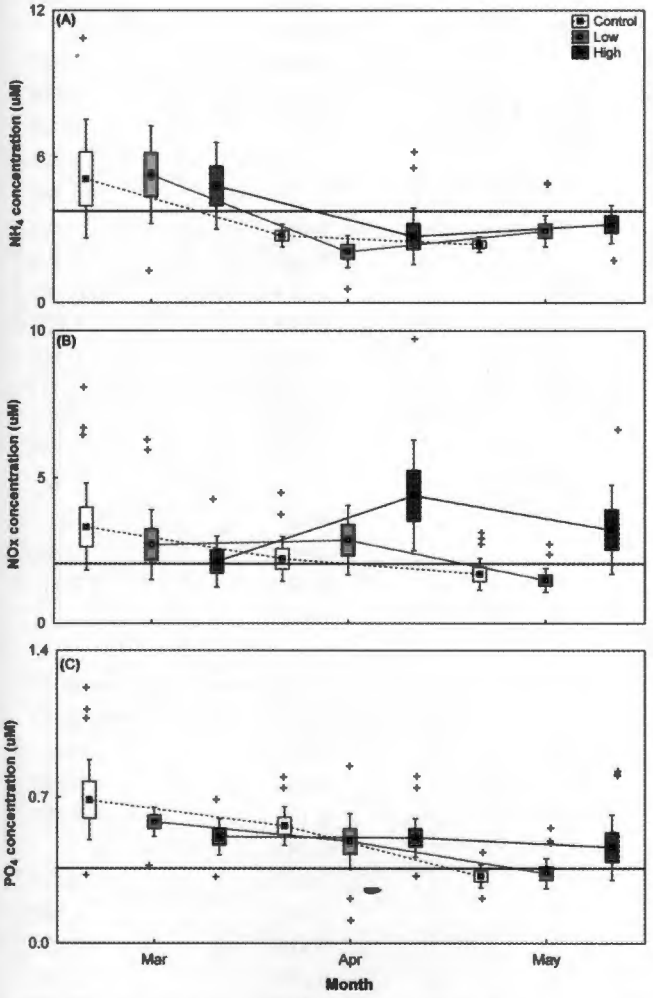

Fig. 2. Box (1 standard error) and whisker (95% CI) plot showing the temporal changes and variability in mean water column nutrient concentrations; (A) NH_4, (B) NO_x and (C) PO_4, among experimental treatments. The straight line represents the mean ambient nutrient concentration derived from a one-year study conducted at the study site (Mohamed Ali, 2011). Outliers (+) are defined as values that are either more or less 1.5 x 1 standard error.

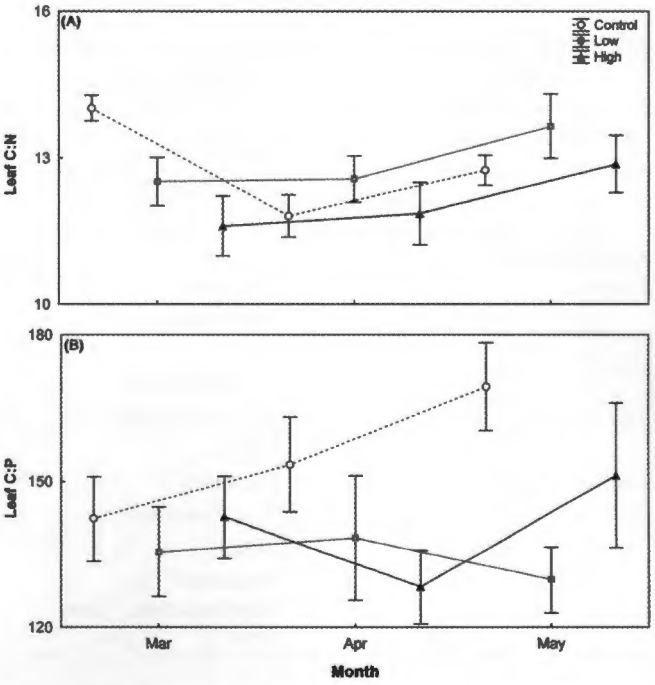

Fig. 3. Temporal changes in *Thalassia hemprichii* leaf tissue nutrient content among experimental treatments. Each data point represents the mean ± 1 standard error, n = 12 at each time point. (A): C:N ratio, (B): C:P ratio.

Table 4. Results of univariate three-factor ANOVA testing the effects of enrichment Treatment, Plot(Treatment) and Time on the measured physiological and morphological responses of *Thalassia hemprichii*. Significant differences are denoted by # $p < 0.05$, ^ $p < 0.01$, + $p < 0.001$. C: Carbon, N: Nitrogen, P: Phosphorus, Chl: Chlorophyll.

Parameters	Source	df	MS	F
Physiological				
C:N	Treatment	2	0.160	3.628#
	Plot(Treatment)	9	0.150	3.403^
	Time	2	0.192	4.355#
	Treatment*Time	4	0.156	3.539#
	Plot(Treatment)*Time	18	0.101	2.297^
	Error	72	0.044	
C:P	Treatment	2	7.208	4.287#
	Plot(Treatment)	9	4.032	2.398#
	Time	2	1.858	1.105
	Treatment*Time	4	2.220	1.320
	Plot(Treatment)*Time	18	2.457	1.461
	Error	72	1.682	
Total ch	Treatment	2	0.023	0.801
	Plot(Treatment)	9	0.144	5.017+
	Time	2	1.386	48.29+
	Treatment*Time	4	0.020	0.697
	Plot(Treatment)*Time	18	0.076	2.646^
	Error	72	0.029	
Chl a/b	Treatment	2	0.009	0.193
	Plot(Treatment)	9	0.071	1.504
	Time	2	0.631	13.30+
	Treatment*Time	4	0.132	2.777#
	Plot(Treatment)*Time	18	0.073	1.546
	Error	72	0.047	
Total chl:carotenoids	Treatment	2	0.141	2.929
	Plot(Treatment)	9	0.324	6.741+
	Time	2	0.649	13.51+
	Treatment*Time	4	0.044	0.907
	Plot(Treatment)*Time	18	0.082	1.713
	Error	72	0.048	
F_v/F_m ratio	Treatment	2	0.002	1.079
	Plot(Treatment)	9	0.007	3.482^
	Time	2	0.025	12.87+
	Treatment*Time	4	0.001	0.646
	Plot(Treatment)*Time	18	0.005	2.541#
	Error	72	0.002	
Morphological				
Length	Treatment	2	0.997	23.10+
	Plot(Treatment)	9	0.343	7.942+
	Time	2	2.785	64.55+
	Treatment*Time	4	0.194	4.509^
	Plot(Treatment)*Time	18	0.146	3.388+
	Error	72	0.043	
Leaf mass area	Treatment	2	0.052	0.471
	Plot(Treatment)	9	0.417	3.749+
	Time	2	0.816	7.326+
	Treatment*Time	4	0.105	0.942
	Plot(Treatment)*Time	18	0.319	2.863+
	Error	72	0.111	

Table 5. Results of univariate three-factor ANOVA testing the effects of enrichment Treatment, Plot(Treatment) and Time on the responses of epibionts and water column productivity. Significant differences are denoted by $^{\#}$ $p < 0.05$, $^{\wedge}$ $p < 0.01$, $^{+}$ $p < 0.001$.

Parameters	Source	df	MS	*F*
Epibiont				
Dry weight	Treatment	2	0.047	2.110
	Plot(Treatment)	9	0.054	2.424$^{\#}$
	Time	2	0.405	18.03^{+}
	Treatment*Time	4	0.030	1.343
	Plot(Treatment)*Time	18	0.069	3.083^{+}
	Error	72	0.022	
Ash-free dry weight	Treatment	2	0.011	1.887
	Plot(Treatment)	9	0.016	2.762$^{\wedge}$
	Time	2	0.272	45.49^{+}
	Treatment*Time	4	0.021	3.506$^{\#}$
	Plot(Treatment)*Time	18	0.009	1.489
	Error	72	0.006	
Water column productivity				
Chlorophyll *a*	Treatment	2	0.084	1.702
	Plot(Treatment)	9	0.154	3.120$^{\#}$
	Time	2	2.266	45.81^{+}
	Treatment*Time	4	0.049	0.997
	Plot(Treatment)*Time	18	0.056	1.135
	Error	72	0.049	

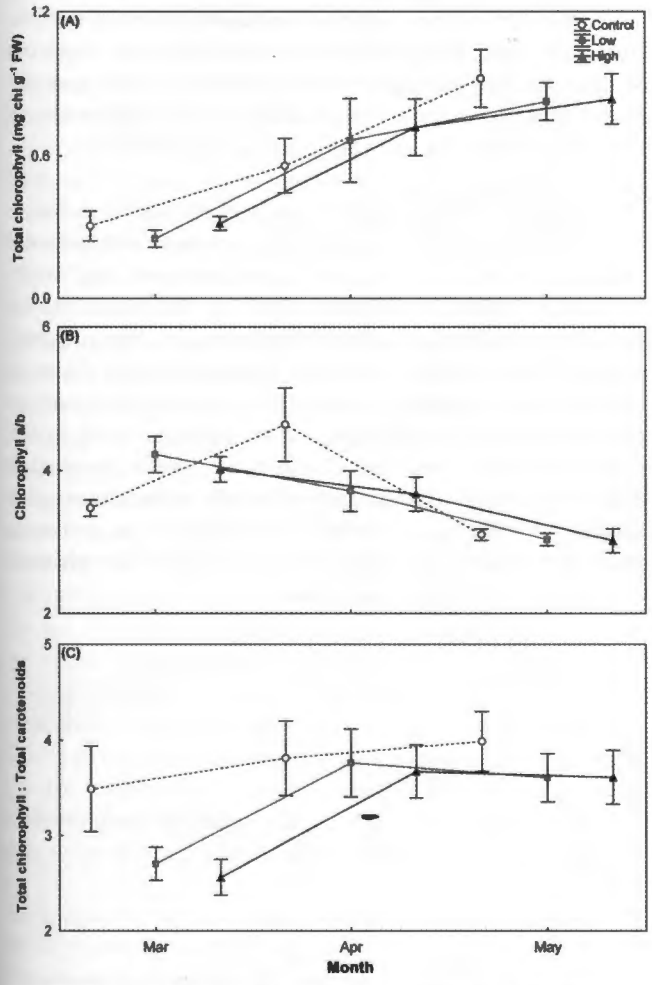

Fig. 4. Temporal changes in *Thalassia hemprichii* leaf pigment content among experimental treatments. Each data point represents the mean ± 1 standard error, n = 12 at each time point. (A): Total chlorophyll content (chlorophyll a + b), (B): Chlorophyll a/b ratio, (C): Total chlorophyll:total carotenoids ratio.

DISCUSSION

One of the main findings of this current pilot study was that the intra-treatment variation in measured water column and seagrass parameters was very high, with standard errors approaching 11.9 ± 0.8% of the mean. The high variation reduced the power of statistical tests thereby contributing to the lack of significance in many of the parameters examined. High variation in water column nutrients may be due to localised hydrodynamics at commensurate scales (Smith, 1986), potentially leading to the observation that current levels of nutrient enrichment could not be reliably detected in the water column. However, there was also the possibility of efficient nutrient cycling to meet the high nutrient demand of seagrasses and other autotrophs (Erftemeijer & Middleburg, 1995). Rapid nutrient uptake by autotrophs could have also contributed to the apparent difference in NH_4 and NOx concentrations after one month of loading, which suggested preference for NH_4 as a nitrogen source for seagrasses

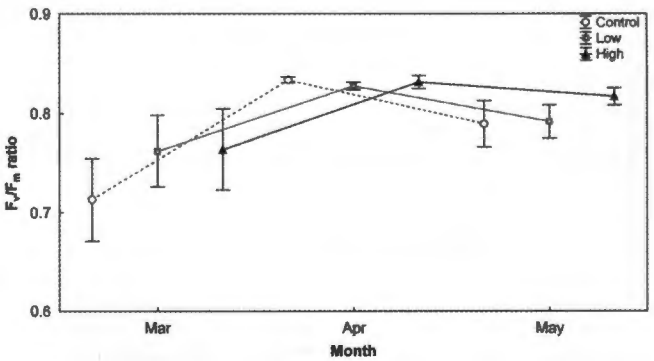

Fig. 5. Temporal changes in *Thalassia hemprichii* photochemical efficiency of photosystem II, F_v/F_m ratio, among experimental treatments. Each data point represents the mean ± 1 standard error, n = 12 at each time point.

(Stapel et al., 1996) and other primary producers (Turpin, 1991). These observations suggested that water column nutrient levels are not an effective indicator for monitoring loads (Tomasko et al., 1996), necessitating perhaps the use of plants' responses to indicate enrichment (Duarte, 1990; Lee et al., 2004).

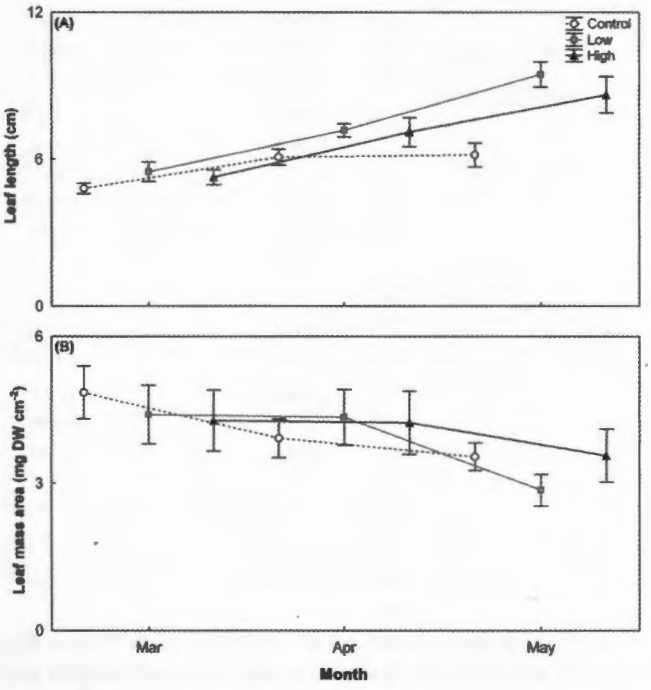

Fig. 6. *Thalassia hemprichii* leaf growth responses to experimental treatments over time. Each data point represents the mean ± 1 standard error, n = 12 at each time point. (A): Length, (B): Mass area.

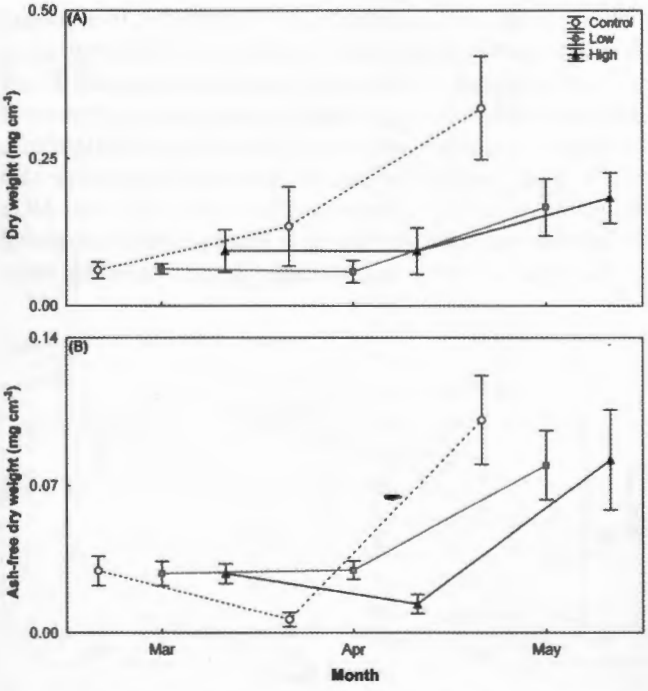

Fig. 7. Temporal changes in epibiont biomass across experimental treatments. Each data point represents the mean ± 1 standard error, n = 12 at each time point. (A): Dry weight, (B): Ash-free dry weight.

There was some supporting evidence of enrichment on *Thalassia hemprichii*, where lowered leaf C:P ratios in plants exposed to high nutrient treatments signalled internalisation of nutrients. This observation agrees with seagrasses subjected to experimental enrichment (Touchette et al., 2003; Invers et al., 2004) and those found in naturally high nutrient conditions (Pérez et al., 2008). In contrast, there were no observable decline in leaf C:N ratios in treated plants, a result contrary to expectation as C:N ratio in seagrasses has been established as a sensitive indicator of nutrient availability (Touchette et al., 2003; Lee *at al.*, 2004). In this study, leaves from enriched plants also had higher total chlorophyll concentrations and lower chlorophyll a/b ratios. This is likely to be a response to higher photosynthetic performance, required under enriched condition for nutrient assimilation, rather than a response to low light conditions (Invers et al., 2004). Increases in *Thalassia* leaf length and declines in leaf mass area noted in this study did not correspond with observations of enriched seagrasses, where leaves were smaller due to light limitation and excessive grazing of nutrient-rich leaves (Tomasko & Lapointe, 1991; Short et al., 1995). Instead, morphological changes observed in this study appeared to be characteristic of plants that are nutrient limited (Udy & Dennison, 1997; Fourqurean et al., 2010), although this was perhaps refuted by leaf concentrations of N and P above the values of 1.8% N and 0.2% P proposed by Duarte (1990) as indicators of nutrient limitation. This may be because local seagrasses exposure to nutrients may not have reached threshold levels, and that plants may have been opportunistic in utilising increased nutrients to improve growth (Perez et al., 1994).

The findings from this pilot study indicated that future field design for water column enrichment experiments need to consider the high spatial and temporal variability in environmental conditions. This can be overcome by employing multiple control sites and time, e.g., Before-After-Control-Impact (BACI) repeated designs (Stewart-Oaten et al., 1986), to examine any variation prior to experimentation and validate if a significant change have occurred in the course of experimentation (Long et al., 1996). Increasing experimental plot distance can reduce issues with cross-plot transfer of introduced nutrients, although too far distances may not be useful especially when there are microhabitat differences (Penagos et al., 2008).

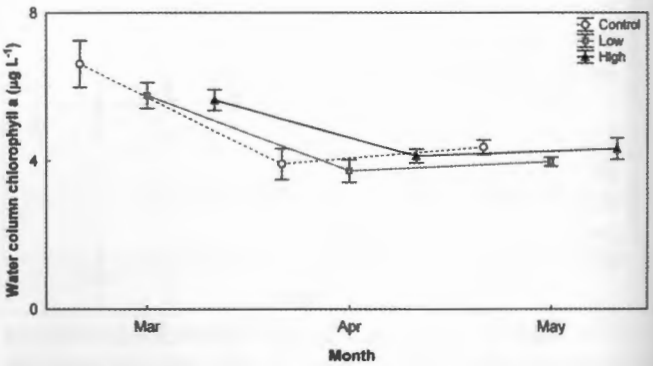

Fig. 8. Temporal changes in water column chlorophyll a concentrations among experimental treatments. Each data point represents the mean ± 1 standard error, n = 12 at each time point.

Nutrient load signals could be further enhanced by manipulating nutrient concentration, e.g. by increasing load dosage and applying nutrients evenly within treatment plots (Morris et al., 2007). Although nutrient application frequency in this study was within those described in other similar studies (Tomasko & Lapointe, 1991; Wear et al., 1999; Leoni et al., 2006, 2007), increasing frequency can also enhance loading concentrations. However, an examination of the decay rates of nutrient sources, accounting for flow conditions and temperature effects, should be conducted to determine their longevity and provide a better indication on the suitable application frequency to maintain a higher dose of enrichment within treatment plots (Heck et al., 2000). Detection of nutrient loading can be improved by reducing the sampling intervals (Leoni et al., 2007; Morris et al., 2007) as observations from this study have provided some evidence of rapid nutrient cycling within local seagrass meadows and high nutrient loss rates from either biotic process or environmental conditions. A shorter sampling frequency can also capture early biotic responses to elevated nutrient concentrations, e.g. physiological responses (Leoni et al., 2007).

Other design considerations can focus towards improving measurements of biotic responses to enrichment. Observations of the F_v/F_m ratios of plants in this study showed that plants were under slight photosynthetic stress prior to enrichment but there was no indication of photosynthetic decline after treatment. F_v/F_m ratios of plants are highly influenced by prevailing environmental conditions (Krause & Weis, 1984), but these measurements provide a prompt and simple method to assess photosynthetic health in plants. Future experiments should instead consider pre-dawn chlorophyll fluorescence measurements since they better represent plants' photosynthetic state (Maxwell & Johnson, 2000). Determination of belowground processes, e.g. biomass, should also be factored in future studies to complement aboveground responses, as this comparison could not be conducted in this study due to destructive sampling and limited plant samples. This would provide a better insight on seagrass resource allocation upon nutrient availability since plants either increase their aboveground biomass if they are nutrient limited (Udy & Dennison, 1997), or reduce biomass due to decline in growth (Leoni et al., 2006). Current measurements of epibiont response should also be revised, e.g. assess re-growth of epibionts after experimental treatment, as epibiont increase is a pertinent indirect effect of water column enrichment (Wear et al., 1999; Leoni et al., 2006). Future studies can also consider testing enrichment effects at varying treatment levels, e.g. one that ranges from low (or levels close to nearby nutrient loads) to ultra high concentrations, which would provide important quantitative data to understand threshold levels in seagrasses.

In conclusion, the results from the present study highlighted the value of pilot studies, especially for in-situ manipulative experiments. Presence of very high variability across all treatment plots provided insights on the underlying natural variation often associated with near-shore seagrass habitats. In addition, the findings also provided new insights on the types of processes occurring at within-habitat scales that would be imperative for the planning and execution of field experiments.

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