Malaysian Journal of Science **25** (2): 55 – 66 (2006)

Factors Affecting Variability of Heterotrophic and Phototrophic Microorganisms at High Water in a Mangrove Forest at Cape Rachado, Malaysia

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Received 9th June 2005, accepted in revised form 6th July 2006.

ABSTRACT We sampled hourly, surface water in a mangrove forest at Cape Rachado, Port Dickson (in the Straits of Malacca) on four occasions (4 January, 18 April, 25 July and 29 December, 2003). Water level in this intertidal zone was between 0.3 and 1.6 m. Seawater temperature ranged 28 – 33°C whereas salinity ranged 25 – 33. Dissolved oxygen (DO) concentration was low ranging between 110 and 270 μM, and reflected a reducing environment (as shown by the negative redox potential, –40 to –70 mV). Chlorophyll *a* (Chl *a*) varied between 0.80 – 7.0 μg L^{-1} whereas ammonium, nitrite, nitrate, phosphate and silicate ranged 0 – 6.50 μM, 0.10 – 0.80 μM, 0.50 – 4.40 μM, 0 – 6.70 μM and 8.79 – 27.27 μM, respectively. For biotic variables, phototrophic picoplankton abundance was relatively stable, ranging between 1.8 – 14.6×10^5 cells mL^{-1} whereas bacterial abundance was about one order higher, ranging 0.5 – 28.4×10^6 cells mL^{-1}. Using principal component analysis, we found that the physical condition of the water could account for both Chl *a* and phototrophic picoplankton variation whereas inorganic nitrogen was important for phototrophic picoplankton. We also showed through nutrient limitation study that phytoplankton was limited by both P and Si. Bacterial abundance correlated significantly with Chl *a* (R^2=0.288, n=29, p<0.01) indicating bacteria–phytoplankton coupling.

ABSTRAK Kami menyampel air setiap jam, dari permukaan air paya bakau di Teluk Rachado, Port Dickson (sepanjang Selat Melaka) sebanyak empat kali (4 Januari, 18 April, 25 Julai dan 29 Disember, 2003). Paras air di zon inter-tidal ini adalah antara 0.3 dan 1.6 m. Suhu air laut adalah antara 28 – 33°C manakala saliniti adalah antara 25 – 33. Kepekatan oksigen terlarut adalah rendah,dengan julat antara 110 dan 270 μM, dan menggambarkan keadaan penurunan (seperti yang ditunjukkan oleh potensi redoks negatif, antara –40 dan –70 mV). Klorofil *a* (Chl *a*) adalah antara 0.80 – 7.0 μg L^{-1} manakala ammonia, nitrit, nitrat, fosfat dan silikat adalah antara 0 – 6.50 μM, 0.10 – 0.80 μM, 0.50 – 4.40 μM, 0 – 6.70 μM dan 8.79 – 27.27 μM, masing-masing. Bagi variabel biotik, kelimpahan pikoplankton fototrofik adalah agak stabil, antara 1.8 – 14.6×10^5 sel mL^{-1} manakala kelimpahan bakteria adalah lebih kurang satu order lebih tinggi, antara 0.5 – 28.4×10^6 sel mL^{-1}. Menggunakan analisa komponen prinsipal, kami mendapati bahawa keadaan fizikal air boleh menjelaskan variasi pada kedua-dua Chl *a* dan pikoplankton fototrofik. Nitrogen inorganik pula adalah penting untuk pikoplankton fototrofik. Kami juga menunjukkan melalui kajian penghad nutrien bahawa fitoplankton dihadkan oleh kedua-dua P dan Si. Kajian kami juga mendapati ada korelasi signifikan di antara kelimpahan bakteria dengan Chl *a* (R^2=0.288, n=29, p<0.01), menunjukkan kaitan antara bakteria–fitoplankton.

(Nutrient limitation for phytoplankton; bacteria–phytoplankton coupling; principal component analysis; Straits of Malacca)

INTRODUCTION

Microbial ecological research has shown the importance of bacteria in nutrient cycling and energy flow in various aquatic ecosystems [1, 2, 3]. Evidence suggests that the microbial food web is a key component in both coastal and estuarine ecosystems [4]. Microbial food web is characterized by organisms with high growth potential (i.e. >1 d^{-1}), and relatively short reaction time to environmental change. Therefore, a short-timescale sampling strategy should be able to detect temporal variation in biotic or abiotic variables that affect microbial activity. These short-term changes can drive long-term patterns that we often observe [5]. However, most studies only consider long-term patterns [e.g. 6, 7], and there are few short-timescale studies, in particular the daily scale.

Microbial ecology, especially in tropical mangrove waterways is not well studied. This present study was an attempt to fill this void, and was part of a research project to investigate the microbial food web in a mangrove ecosystem. Cape Rachado is located south of Port Dickson which is a popular resort town located along the Straits of Malacca. The sampling area was near a small fringe mangrove forest populated with both *Sonneratia* and *Rhizophora* trees. Several seaside resorts are located near this site. We measured the short-timescale variation of both biotic and abiotic variables on four separate sampling periods. However this study did not show any diel patterns for the variables measured but there were differences among each sampling period. Using principal component analysis (PCA), we found that variation in the abiotic factors can account for the variation in both phytoplankton (chlorophyll *a*) and phototrophic picoplankton. For bacterial abundance, chlorophyll *a* (Chl *a*) concentration had the most effect.

METHODS

The sampling station was located in an inter-tidal zone at Cape Rachado (2°24.8'N, 101°51.5'E; Figure 1). We sampled hourly during flood tide periods on four occasions (4 January, 18 April, 25 July and 29 December 2003). *In-situ* parameters measured included seawater temperature, pH, redox potential (Jenway 3071, UK) and salinity (Atago S/Mill-E, Japan). Water samples were taken at about one foot depth with acid-cleaned polyethylene bottle. Sub-samples for the determination of both bacteria and phototrophic picoplankton abundance were preserved with filtered (0.2 µm pore size) 1% glutaraldehyde (final concentration). Samples for dissolved oxygen (DO) analysis were obtained using 50 mL DO bottles, and fixed with a divalent manganese solution together with a strong alkali [8].

Seawater samples were filtered through pre-combusted (450°C for 3 hrs; CEM MAS7000, US) Whatman GF/F filter (nominal pore size 0.7 µm). Both the filtrate and filter were stored at –20°C until nutrient analysis, and extraction for chlorophyll *a* (Chl *a*), respectively. Chl *a* was extracted with 90% acetone, and measured with a spectrophotometer (Beckmann DU7500i, US) [9]. On one occasion, we determined the limiting nutrient for phytoplankton. Seawater sample was diluted five times with 20 µm filtrate to reduce grazing pressure, and then incubated under light condition with different nutrient addition, at 28°C for 3 d. For nitrogen (N), a final concentration of 32 µM NH_4Cl was added, whereas for phosphorus (P), 2 µM K_2HPO_4 was added. For silicon (Si), a silicate standard solution (Merck, Germany) was added at 47 µM final concentration. The nutrients were added in the following combinations: N, P, Si, N+P, N+Si, P+Si, N+P+Si, including a control (C) without any nutrient addition. Chl *a* analysis was then carried out.

For nutrient analyses, dissolved inorganic nutrients e.g. ammonium (NH_4), nitrite (NO_2), nitrate (NO_3), phosphate (PO_4) and silicate (SiO_4) were measured [9]. On several occasions, replicates were measured, and the coefficient of variation (*CV*) were regularly <5% for NH_4 (n=5), PO_4 (n=21) and SiO_4 (n=5) analyses, and <10% for NO_2 (n=5) and NO_3 (n=6) analyses. For DO measurements, the *CV* was <6% (n=19).

For the determination of bacterial abundance, samples were stained with 4'6-diamidino-2-phenylindole (DAPI) (0.1 µg L^{-1} final concentration) for 7 min [10]. Prepared filters were examined under an epifluorescence microscope (Olympus BX60, Japan), and >300 cells were counted for each slide. Unstained samples were also prepared, and examined for autofluorescence from the phototrophic picoplankton (both prokaryotic and eukaryotic). A minimum of 15 fields were counted.

Statistical analysis was carried out according to [11]. Level of significance was accepted at $p<0.05$, and mean values presented in both the text and figures were reported as mean ± standard deviation (SD). Count data (bacteria and phototrophic picoplankton) were natural log-transformed to stabilize the variance. For the nutrient limitation experiment, the Tukey test was used to carry out multiple comparison tests. We also used the principal component analysis (PCA) to identify the combination of variables that best explained the variation in our multivariate data set. We selected all the principal components that explained more than the average proportion of information [12]. The selected principal components were then used in multiple regression analyses to understand the variation in our biotic variables.

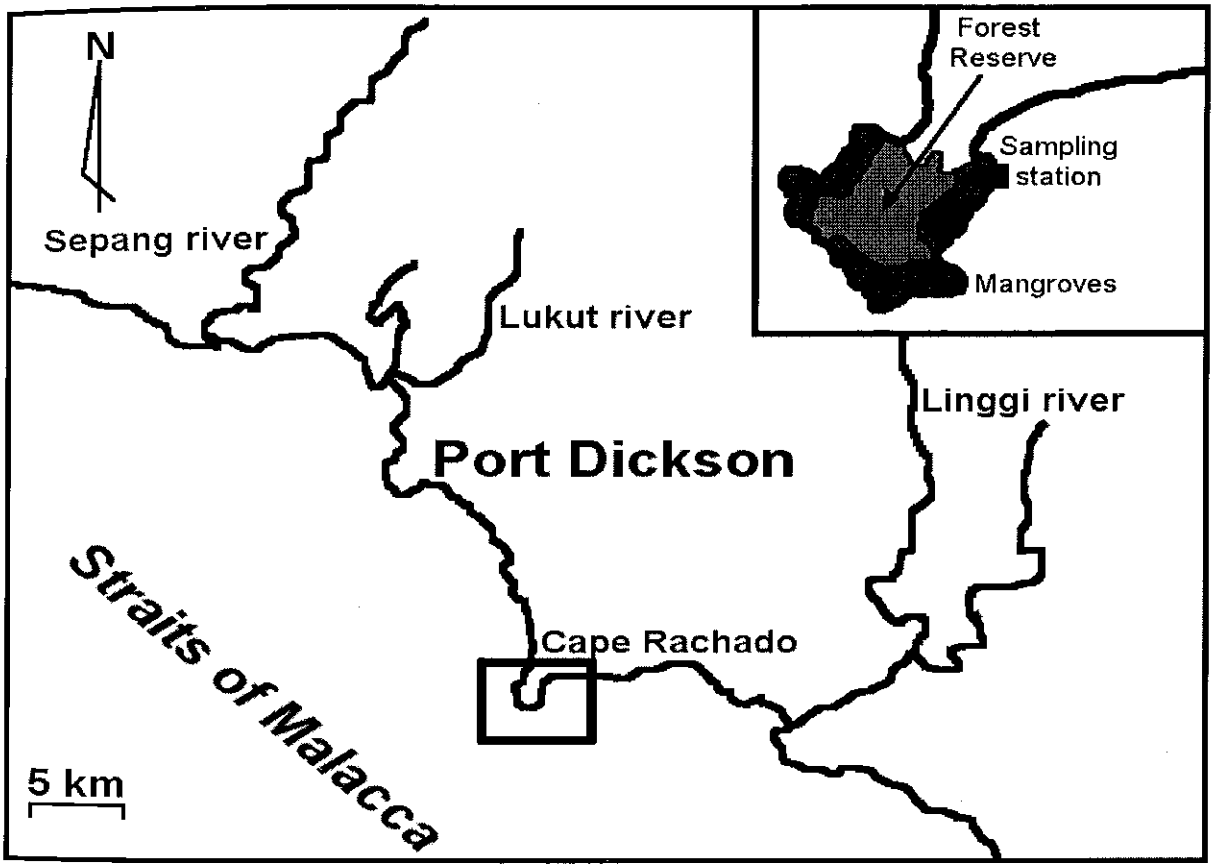

Figure 1. Location of the sampling station (2°24.5'N, 101°51.6'E) at Cape Rachado, Port Dickson. Inset: The boundary for the forest reserve and the mangrove forests are shown.

RESULTS

In this study, water level was between 0.3 and 1.6 m. Seawater temperature was relatively stable (CV = 2.9 – 6.3%), ranging between 28 to 33°C whereas salinity ranged 25 – 33 (CV = 1.6–7.1%) (Table 1). Figure 2 shows that both DO and Chl *a* concentrations were generally in synchrony, usually increasing with daylight, and decreasing as night lengthened. DO concentration was relatively low, and ranged 110 – 270 µM. Chl *a* concentration as an indicator of phytoplankton biomass varied between 0.8 and 7.0 µg L^{-1}.

Nutrient concentration varied throughout sampling (Figure 3) where NH_4 ranged 0 – 6.50 µM whereas NO_2, NO_3 and PO_4 ranged 0.10 – 0.80 µM, 0.50 – 4.40 µM and 0 – 6.70 µM, respectively. SiO_4 was measured only in December, and ranged 8.80 – 27.30 µM (Table 1). Figure 4 shows the microbial abundance measured in each sampling period. The abundance for both bacteria and phototrophic picoplankton were relatively stable. Bacterial abundance ranged 0.5 – 28.4×10^6 cells mL^{-1} whereas phototrophic picoplankton abundance was about one order lower, ranging from 1.8 to 14.6×10^5 cells mL^{-1}.

For nutrient limitation experiment (Figure 5), single nutrient addition (N, P or Si) did not significantly stimulate Chl *a* increase relative to the control. However multiple nutrient addition (N+P, N+Si, P+Si and N+P+Si) increased Chl *a* significantly to three ($p<0.001$), one ($p<0.05$), 17 ($p<0.001$) and 20 ($p<0.001$) times, respectively. From our results, we concluded that both P and Si were important in the bottom-up control of primary production.

Table 1. Range, mean (± standard deviation) and coefficient of variation (CV, %) for each abiotic and biotic variable measured.

PARAMETERS	04-JAN-2003	18-APR-2003	25-JUL-2003	29-DEC-2003
Depth (m)	0.3 – 1.2 (0.9 ± 0.3) (CV = 37%)	0.4 – 1.6 (1.0 ± 0.4) (CV = 41%)	0.5 – 0.7 (0.6 ± 0.1) (CV = 11%)	–
Temperature (°C)	28.0 – 30.5 (29.5 ± 0.9) (2.9%)	28.6 – 32.6 (30.2 ± 1.2) (3.9%)	27.6 – 31.6 (29.9 ± 1.9) (6.3%)	–
Salinity	29 – 30 (30 ± 0) (1.6%)	25 – 33 (30 ± 2) (7.1%)	25 – 27 (26 ± 1) (2.2%)	–
Dissolved oxygen (μM)	129 – 165 (152 ± 14) (8.9%)	136 – 270 (214 ± 37) (17.3%)	206 – 216 (212 ± 4) (1.9%)	113 – 153 (135 ± 16) (11.8%)
NH_4 (μM)	0.36 – 1.45 (0.91 ± 0.43) (47.1%)	0.02 – 2.89 (1.28 ± 0.97) (75.3%)	–	1.51 – 6.46 (3.71 ± 1.88) (50.0%)
NO_2 (μM)	0.10 – 0.84 (0.39 ± 0.25) (63.3%)	0.14 – 0.50 (0.33 ± 0.11) (33.1%)	0.32 – 0.69 (0.54 ± 0.15) (28.8%)	0.08 – 0.16 (0.12 ± 0.03) (28.3%)
NO_3 (μM)	0.48 – 3.27 (1.95 ± 0.98) (50.5%)	0.85 – 2.73 (1.68 ± 0.57) (34.3%)	0.46 – 4.23 (1.39 ± 1.40) (100.3%)	2.46 – 4.37 (3.65 ± 0.72) (19.8%)
PO_4 (μM)	0.40 – 1.48 (0.73 ± 0.32) (44.2%)	0 – 1.20 (0.46 ± 0.47) (104.1%)	0.61 – 4.02 (2.11 ± 1.25) (59.2%)	2.42 – 6.67 (4.63 ± 1.60) (34.5%)
SiO_4 (μM)	–	–	–	8.79 – 27.27 (18.30 ± 6.94) (37.9%)
Chlorophyll *a* (μg L^{-1})	0.8 – 4.2 (2.0 ± 0.9) (47%)	2.0 – 7.0 (3.6 ± 1.4) (38%)	2.9 – 5.4 (4.0 ± 1.1) (28%)	2.9 – 5.0 (3.7 ± 0.9) (24%)
Bacteria (cells mL^{-1})	$4.9 – 10.9\times10^5$ $(7.6 \pm 1.6\times10^5)$ (22%)	$2.7 – 28.4\times10^6$ $(11.7 \pm 8.8\times10^6)$ (76%)	$3.1 – 5.3\times10^6$ $(3.9 \pm 6.8\times10^6)$ (17%)	–
Phototrophic picoplankton (cells mL^{-1})	$1.8 – 12.7\times10^5$ $(5.8 \pm 4.2\times10^5)$ (73%)	$2.1 – 14.6\times10^5$ $(6.1 \pm 4.4\times10^5)$ (72%)	$4.6 – 7.2\times10^5$ $(5.5 \pm 1.0\times10^5)$ (18%)	–

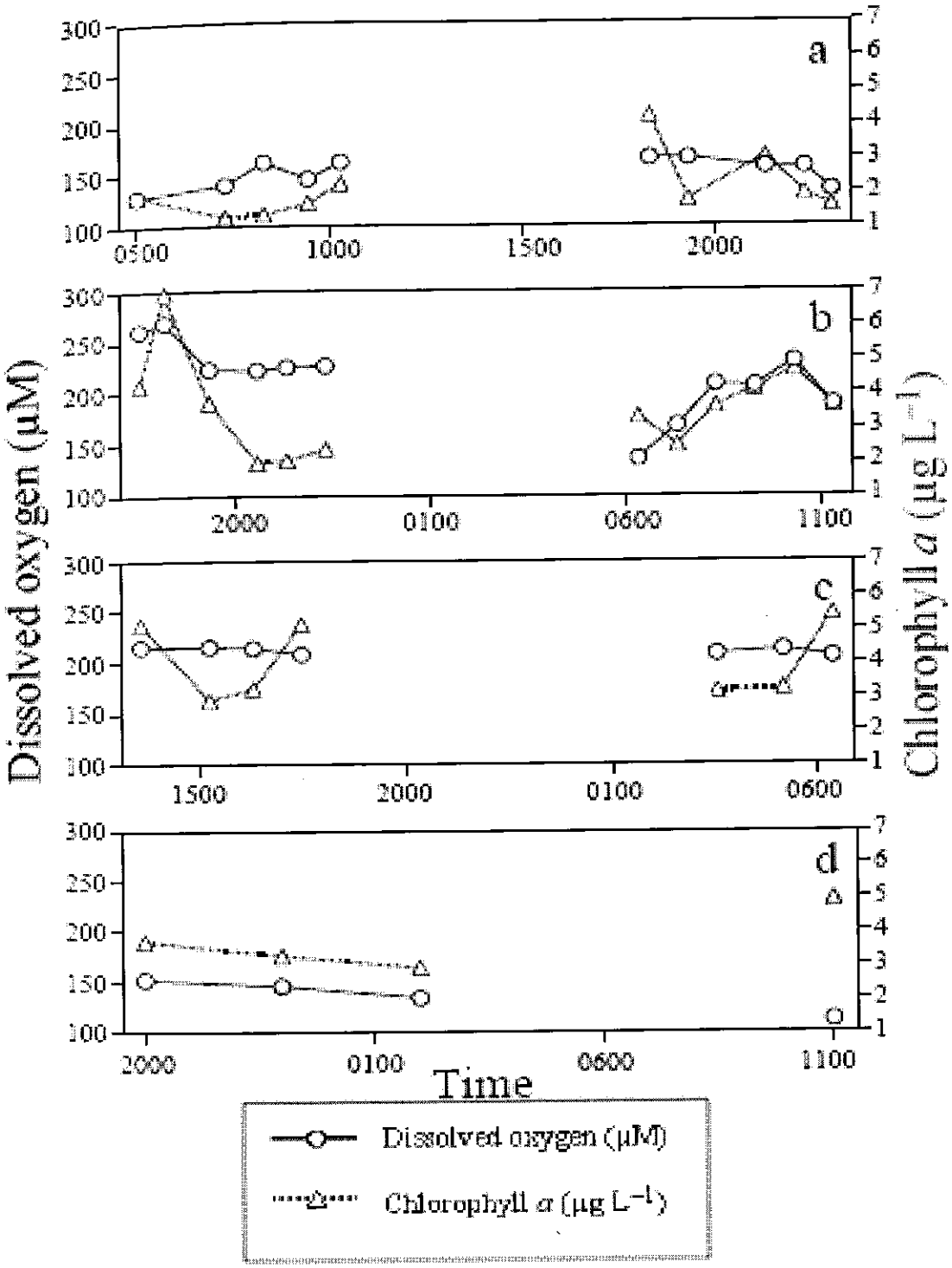

Figure 2. Variation in dissolved oxygen (μM) and chlorophyll *a* (μg L^{-1}) in the (a) January, (b) April, (c) July and (d) December sampling periods.

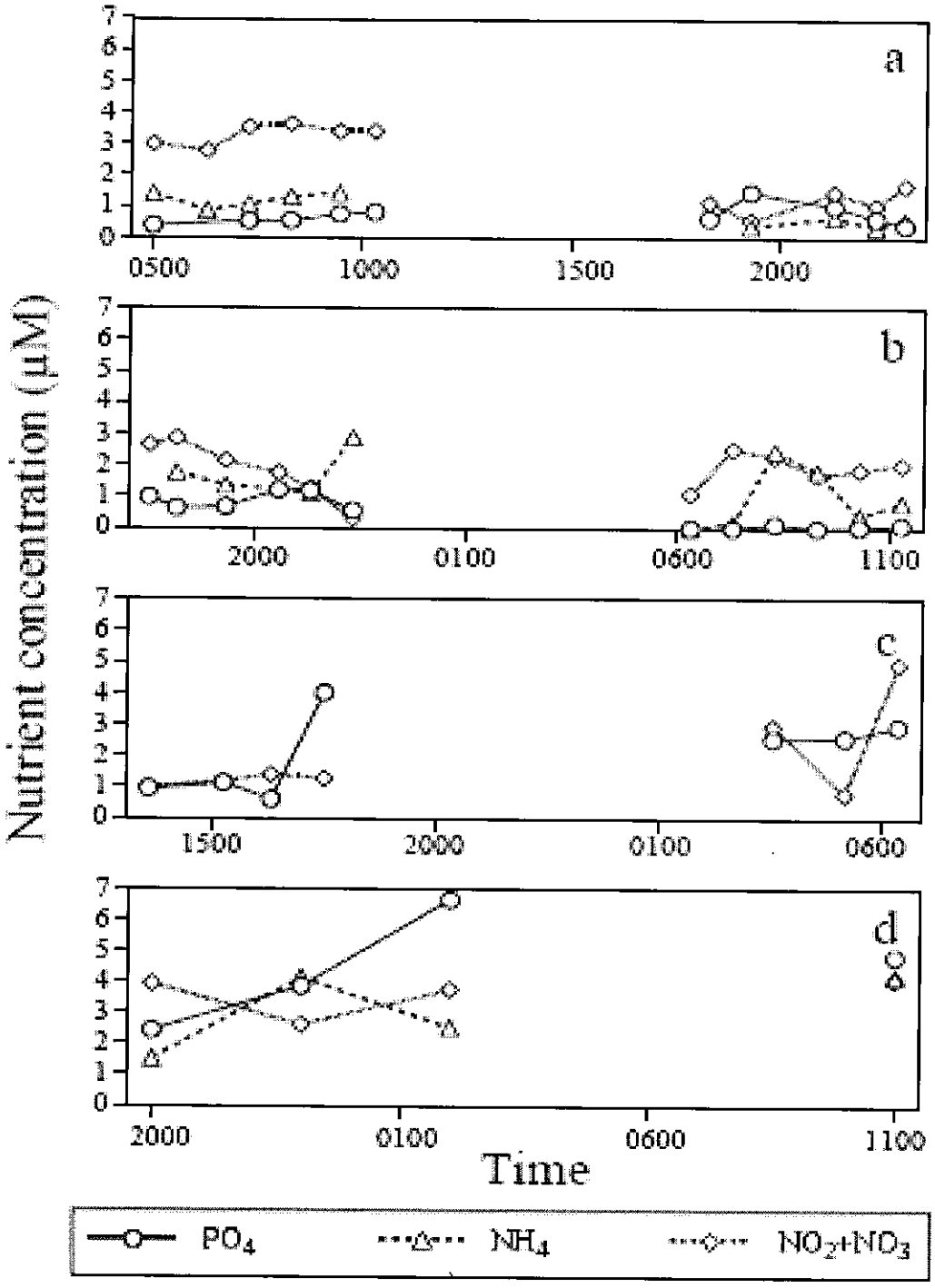

Figure 3. Variation in phosphate (PO_4, µM), ammonium (NH_4, µM) and nitrite+nitrate (NO_2+NO_3, µM) in the (a) January, (b) April, (c) July and (d) December sampling periods.

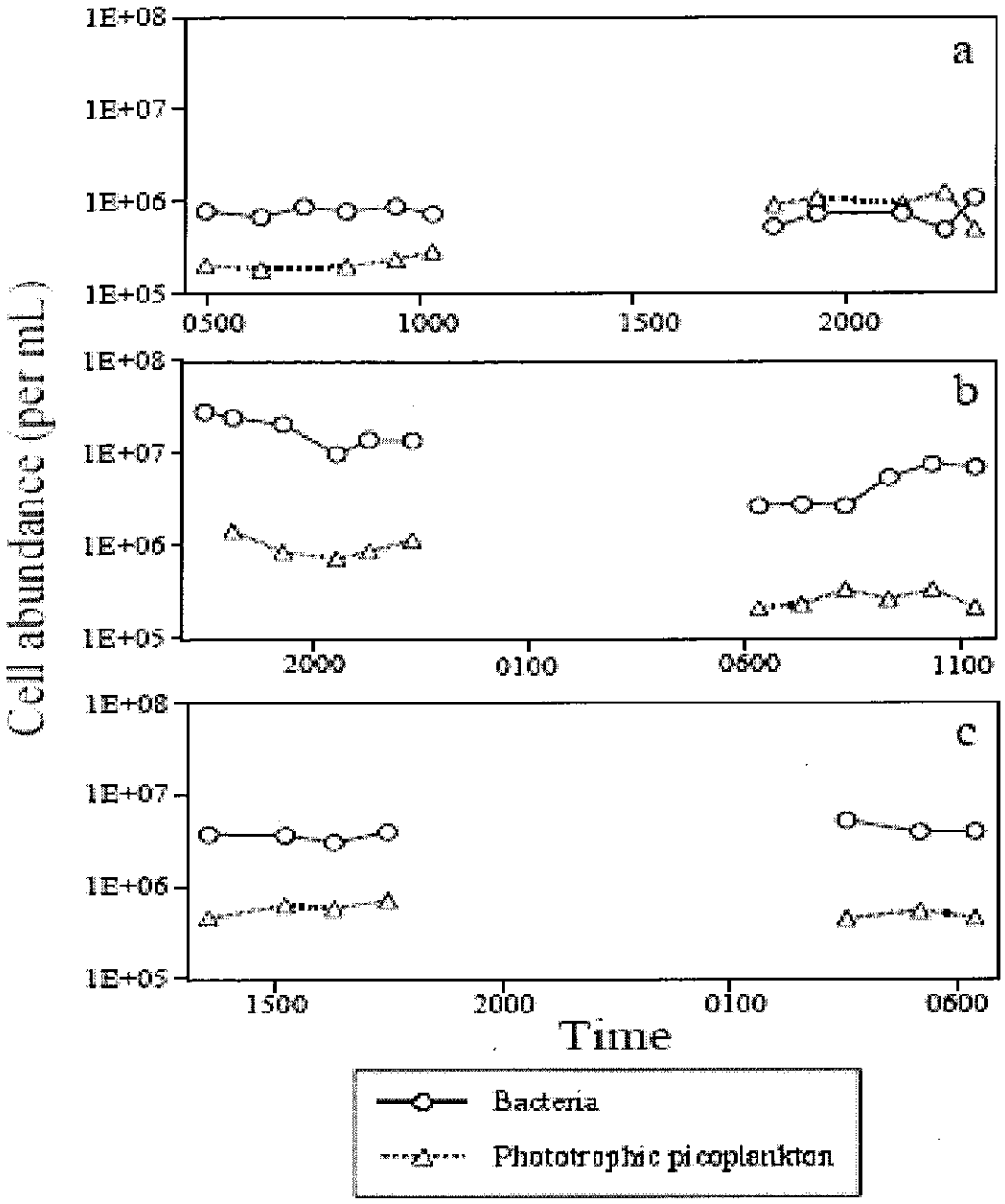

Figure 4. Variation in bacterial and phototrophic picoplankton abundance (cells mL^{-1}) in the (a) January, (b) April and (c) July sampling periods.

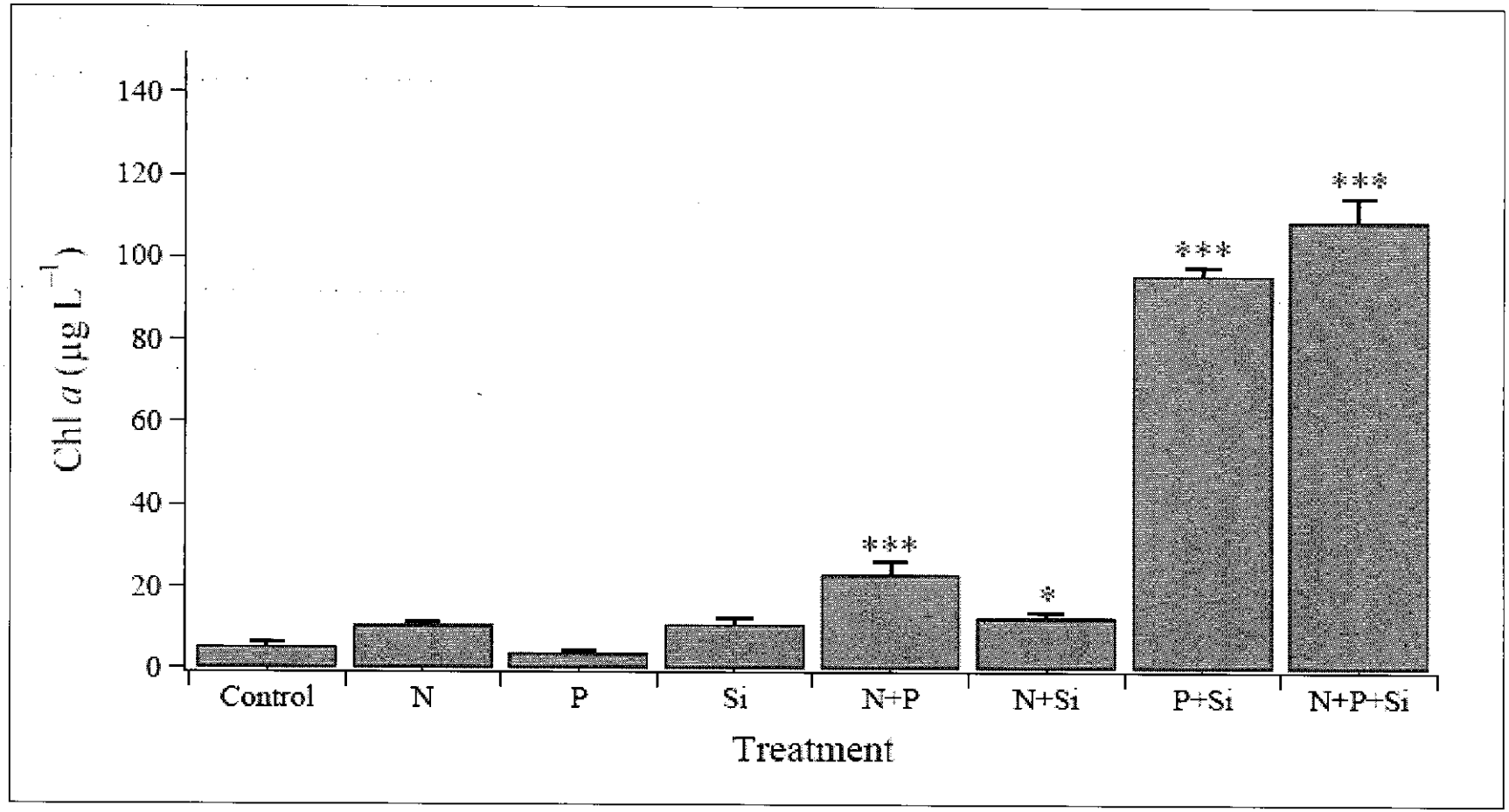

Figure 5. The chlorophyll *a* concentration (μg L^{-1}) after incubation with different combinations of nutrient addition . (Control, N, P, Si, N+P, N+Si, P+Si and N+P+Si). * – treatment was significantly different from control at $p<0.05$, *** – treatment was significantly different from control at $p<0.001$

DISCUSSION

DO concentration was relatively low, and were <150 μM on several occasions. Adequate DO concentration is important as low DO concentration allows anaerobic processes that usually produce an unpleasant odor [13]. This is detrimental especially to the nearby resorts. DO is consumed by respiration and replenished through photosynthesis and the physical processes of gas exchange [8]. In this study, photosynthesis is important as DO concentration correlated significantly with Chl *a* (R^2=0.401, n=29, p<0.001). The data on both inorganic nutrient and Chl *a* concentrations demonstrated mesotrophic condition, as opposed to eutrophic (>60 μg L^{-1} Chl *a*) [14] or oligotrophic systems [15]. In this study, NH_4, NO_2, NO_3 and PO_4 concentrations were within the range previously reported for estuarine mangroves in Malaysia [16].

Chl *a* concentration varied within a small range, similar to other mangrove ecosystems e.g. in Australia [17]. Chl *a* concentration includes contribution from phytoplankton, nanophytoplankton and phototrophic picoplankton [18, 19]. In this study, phototrophic picoplankton was not a significant contributor to the total phototrophic plankton biomass as Chl *a* concentration did not correlate with phototrophic picoplankton abundance (p>0.05). This is common as the major constituent of primary producers in mangrove waters is usually nanophytoplankton or net phytoplankton (up to 80%) [18]. The phototrophic picoplankton observed here was within the range previously reported [4, 20], and included both cyanobacteria and eukaryotic phototrophic picoplankton.

Bacterial abundance was within the range reported for estuaries and marshes (0.1 – 35.4×10^6 mL^{-1}) [1]. Bacterial abundance fluctuated within a small range, and the short-timescale sampling strategy employed did not detect any diel pattern within each sampling period. However our results showed marked differences of about one order among the three sampling periods (average bacterial abundance: 0.8, 11.7 and 3.9×10^6 cells mL^{-1} for 4 January, 18 April, and 25 July 2003, respectively). We used principal component analysis (PCA) to identify the combinations of abiotic variables that best explained the variation for our biotic variables. This approach also overcame problems linked to the collinear nature of abiotic data.

In this study, PCA analysis generated eight components, of which the first three explicated a total of 74% variance (Table 2). Component loadings for PC1, PC2 and PC3 were also given. For PC1, the positive component loadings were dominated by physical variables (depth and temperature) and to a lesser extent, DO concentration. Inorganic nutrients (N based: NO_3, NO_2 and NH_4) dominated PC2 whereas for PC3, salinity was the most influential. We then proceeded with multiple regression analyses to determine whether these principal components were sufficient to explain the variation in bacteria, phototrophic picoplankton and Chl *a* (representing total phototrophs).

These principal components (PC1, PC2 and PC3) were insufficient to account for the variation in bacterial abundance (Table 3A) (df = 16, F = 2.19, p = 0.14). However, if Chl *a* was added to the analysis, the result was highly significant (Table 3B) (df=16, F = 9.01, p = 0.001). Bacteria was strongly coupled to phytoplankton, and Chl *a* alone was sufficient to explain the variation in bacterial abundance (R^2 = 0.288, n = 29, p<0.01). We know that causality cannot be inferred from correlation analysis, but the correlation between bacteria and Chl *a* indicated a form of bacteria–phytoplankton coupling that is well documented for various aquatic ecosystems [15, 21]. Although our study suggested that bacteria was dependent on phytoplankton production, other researchers [22, 23, 24] have reported the importance of other carbon sources (e.g. mangrove litter) in mangrove waters.

PC1, PC2 and PC3 were, however, sufficient to account for the variability of both phototrophic picoplankton (df = 15, F = 14.7, p<0.001) and Chl *a* concentration (df= 16, F = 7.57, p = 0.004) (Table 3C and 3D). For phototrophic picoplankton, only the partial regression coefficients for PC1 and PC2 were significant (p = 0.001 and p<0.001, respectively). By referring to the component loadings of these principal components, phototrophic picoplankton abundance was affected by both the physical condition (temperature and depth) and nitrogen concentration (NO_3, NO_2 and NH_4) in the water. Chl *a* concentration was also affected by the physical conditions of the water (p = 0.05). However nitrogen concentration was not

important for its variation. This was confirmed by our nutrient limitation experiment that showed both P and Si were limiting primary production. Although limitation by P has also been shown [25], the role of Si remains largely unknown. Our results showed that although Si was widely available, conditions where it becomes limiting can exist.

This study presents new data on the microbial ecology of mangrove waters in the Straits of Malacca. Although we employed short-timescale sampling strategy, we were unable to detect diel variation in both biotic and abiotic variables. However using PCA, we found that the physical condition of the water could account for both Chl *a* and phototrophic picoplankton variations whereas inorganic nitrogen was important for phototrophic picoplankton alone. We also showed that phytoplankton was limited by both P and Si. Evidence of bacteria-phytoplankton coupling was also shown.

Table 2. Percentage of explication and eigenvalues for the principal components, and the loadings of abiotic variables on the first three principal components (PC1, PC2, and PC3). Values in bold indicate the influential abiotic variables for the principal components.

COMPONENTS	EIGEN VALUES	% OF EXPLICATION	CUMULATIVE % OF EXPLICATION
1	2.672	33.41	33.41
2	1.911	23.89	57.30
3	1.329	16.61	73.91
4	0.976	12.19	86.10
5	0.520	6.50	92.60
6	0.377	4.72	97.32
7	0.117	1.46	98.78
8	0.098	1.22	100.00

	PRINCIPLE COMPONENT LOADING		
ABIOTIC VARIABLES	PC1	PC2	PC3
Temperature	**0.535**	–0.175	0.232
Salinity	–0.070	0.173	**0.685**
Depth	**0.551**	–0.014	–0.191
DO	**0.440**	–0.056	0.322
NH_4	0.344	**0.445**	0.038
PO_4	0.281	–0.352	–0.263
NO_2	0.115	**0.502**	–0.500
NO_3	0.043	**0.602**	0.130

Table 3.
A. Results of multiple regression analysis of bacterial abundance against PC1, PC2 and PC3. Whole model test using the standard least squares method was insignificant ($df = 16, F = 2.19, p = 0.14$).
B. Results of multiple regression analysis of bacterial abundance against PC1, PC2, PC3 and Chl *a*. Whole model test using the standard least squares method was very highly significant ($df = 16, F = 9.01, p = 0.001$).
C. Results of multiple regression analysis of phototrophic picoplankton abundance against PC1, PC2 and PC3. Whole model test using the standard least squares method was very highly significant ($df = 15, F = 14.7, p<0.001$).
D. Results of multiple regression analysis of Chl *a* concentration against PC1, PC2 and PC3. Whole model test using the standard least squares method was highly significant ($df = 16, F = 7.57, p = 0.004$).

A	**COEFFICIENTS**	**STANDARD ERROR OF COEFFICIENTS**	***t***	***p***
Intercept	14.73	0.28	51.9	<0.001
PC1*	0.41	0.18	2.3	0.04
PC2	–0.05	0.21	–0.2	0.83
PC3	0.27	0.25	1.1	0.31

B	**COEFFICIENTS**	**STANDARD ERROR OF COEFFICIENTS**	***t***	***p***
Intercept	12.06	0.63	19.2	<0.001
PC1	0.11	0.13	0.8	0.44
PC2	–0.05	0.13	–0.4	0.70
PC3*	–0.58	0.25	–2.3	0.04
Chl *a**	0.93	0.21	4.5	<0.001

C	**COEFFICIENTS**	**STANDARD ERROR OF COEFFICIENTS**	***t***	***p***
Intercept	12.93	0.10	132.6	<0.001
PC1*	0.25	0.06	4.3	0.001
PC2*	–0.39	0.08	–5.0	<0.001
PC3	0.17	0.09	1.9	0.08

D	**COEFFICIENTS**	**STANDARD ERROR OF COEFFICIENTS**	***t***	***p***
Intercept	2.89	0.24	11.9	<0.001
PC1*	0.33	0.15	2.2	0.05
PC2	0.01	0.18	0.1	0.97
PC3*	0.92	0.22	4.2	0.001

* indicates significant partial regression coefficients

Acknowledgements This work was supported in part by grants from the National Oceanographic Directorate of Malaysia (NOD/R&D/P7/0005/03) and University of Malaya (F0377/2002A and PPF/FP003/2002D). We would also like to thank Chin Yao Hua, Mohamed Azroie, Kahlil Khidzir and Angie Ng Yee Fang for their help in both sampling and analysis. Bong is sponsored by the National Science Foundation (Malaysia) post-graduate scholarship.

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