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Nutrient limitation in Hong Kong waters inferred from comparison of nutrient ratios, bioassays and ^{33}P turnover times

Jie Xu[1,*], Kedong Yin[2,5], Alvin Y. T. Ho[1], Joseph H. W. Lee[3], Donald M. Anderson[4], Paul J. Harrison[1]

[1]Atmospheric, Marine and Coastal Environment (AMCE) Program, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR

[2]Key Laboratory of Tropical Marine Environmental Dynamics, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou

[3]Department of Civil Engineering, The University of Hong Kong, Pokfulam Road, Hong Kong SAR PR

[4]Biology Department, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

[5]*Present address:* Australian Rivers Institute, Griffith University, Nathan Campus, Brisbane, Queensland 4111, Australia

ABSTRACT: There is a need to determine the spatial and temporal dynamics of nutrient limitation to decide which nutrients should be removed during sewage treatment in Hong Kong. We compared 3 methods to assess potential or actual nutrient limitation. Ambient nutrient ratios were calculated, and nutrient enrichment bioassays were conducted, along with ^{33}P turnover times. Comparison of nutrient ratios and bioassays demonstrated that the ambient inorganic nutrient ratios, based on the Redfield Si:N:P ratio of 16:16:1, were a rapid and effective method that could be used to predict the potentially limiting nutrient of phytoplankton biomass, except in eastern waters in summer, since the DIN:PO_4 uptake ratio was occasionally below the Redfield ratio. The agreement between nutrient limitation indices of growth rate and biomass yield suggested that phytoplankton biomass and growth rate were P-limited in southern waters, with more stable conditions during summer. In contrast, a lack of agreement between these indicators showed that phytoplankton growth in potentially P-limited cases in western waters and Victoria Harbour was controlled by physical processes (e.g. strong hydrodynamic mixing and dilution). The limiting factor for phytoplankton growth varied spatially and temporally. In summer, there was a change from physical processes (e.g. the rapid dilution and possible light limitation due to strong turbulent mixing) in hydrodynamically active western waters and Victoria Harbour to P limitation, or N + P co-limitation, in southern and eastern waters with more stable conditions. In winter, phytoplankton growth was regulated by strong wind-induced vertical mixing. Hence, different seasonal sewage treatment strategies should be considered for nutrient removal.

KEY WORDS: Nutrient ratios · Nutrient enrichment bioassays · ^{33}P turnover times · Pearl River discharge · Sewage effluent · Hong Kong · Nutrient limitation · Light availability

INTRODUCTION

Eutrophication is a major global environmental problem in coastal waters, resulting from large anthropogenic inputs of nitrogen (N) and phosphorus (P) (Nixon 1995). Increased nutrient loading promotes primary production in coastal receiving waters with high stratification, resulting in bottom oxygen depletion due to the accumulation and decomposition of more organic matter at the bottom (Seliger et al. 1985, Cooper & Brush 1991). A typical case is the 'dead zone' in the northern Gulf of Mexico caused by the increased

*Email: xujie@ust.hk

nutrient loading from the Mississippi River watershed (Malakoff 1998).

The concept of nutrient limitation has been the keystone to understanding eutrophication impacts (Smith et al. 1999), and determining the limiting nutrient for phytoplankton growth was the central theme of eutrophication research in the 1970s (Jong 2006). Nutrient enrichment causes a change in phytoplankton biomass yield, as well as growth rate. Hence, the concept of nutrient limitation has 2 meanings: the limitation of biomass and/or of growth rate (Paasche & Erga 1988).

Nutrient availability in coastal waters can be strongly influenced by both freshwater inputs and oceanic and tidal exchange, with the latter typically diluting nutrient concentrations (Gobler et al. 2005). It has been debated which nutrient, N or P, limits primary production in coastal waters. Nitrogen has traditionally been viewed as the nutrient limiting productivity in coastal marine waters (Ryther & Dunstan 1971, Rudek et al. 1991, Oviatt et al. 1995). Recently, P limitation has often been observed in river-influenced coastal areas during periods of high river runoff with high N:P loading ratios (Harrison et al. 1990, Turner et al. 1990, Lohrenz et al. 1999, Labry et al. 2002, Fujiki et al. 2004), and N limitation, or N + P limitation, may occur during low river runoff (Fisher et al. 1992).

Hong Kong waters are influenced by exchange with the South China Sea, especially the southern and eastern waters. Hong Kong waters are influenced by 3 main nutrient inputs: the N-rich summer Pearl River discharge, relatively nutrient-poor coastal and oceanic waters from the South China Coastal Current, and year-round domestic sewage effluent that is discharged in the vicinity of Victoria Harbour. Nutrients from the domestic sewage effluent with high NH_4 and PO_4 and low Si are a major nutrient source, since Hong Kong discharges over 2×10^9 kg of sewage effluent daily (Broom et al. 2003). In 2001, the government of Hong Kong implemented the Harbour Area Treatment Scheme (HATs), which collects and transports ~70% of the sewage via a 26 km long tunneled sewage system to the Stonecutters Island (SCI) treatment plant. After receiving chemically enhanced primary treatment (CEPT) that removes PO_4 by precipitation with ferric chloride, the sewage effluent is discharged into shallow coastal waters near SCI through a short outfall. The enhanced degree of treatment and the relocation of sewage effluent discharge from a distributed mode through a number of sea outfalls in Victoria Harbour to one major point source discharge at the west end side of Victoria Harbour is having a significant effect on the dynamics of nutrients and phytoplankton biomass.

Previous observations of nutrient limitation have been made in Hong Kong waters in summer only (Yin et al. 2000, 2001). The assessment of seasonal variations in nutrient limitation was based on average ambient nutrient ratios (Yin 2002). As the optimum N:P ratio required by phytoplankton may vary from 10:1 to 30:1 for different algal species (Boynton et al. 1982, Atkinson & Smith 1983, Fong et al. 1993), more studies are necessary to evaluate the responses of phytoplankton growth to nutrient enrichment in Hong Kong waters in terms of nutrient limitation of biomass yield and/or of growth rate. The present study focused on a comparison of 3 methods, nutrient enrichment bioassays, ambient nutrient ratios and turnover times of phosphorus, which were used to assess which nutrient is the most limiting for phytoplankton growth and yield in Hong Kong waters on a seasonal basis. It is important to determine which nutrient is actually limiting, or has the potential to limit, algal biomass production so that a large increase in algal biomass, which may lead to hypoxia, can be alleviated or reduced by proper management.

MATERIALS AND METHODS

Study sites. Four stations were selected to represent different geographical regions and water quality zones: NM2 in the western waters, VM5 in Victoria Harbour, SM6 in the southern waters and PM7 in the Port Shelter — the same stations that have been sampled by the Hong Kong Environment Protection Department (EPD) (Fig. 1). They are representative of estuarine influence (NM2 and SM6), local sewage effluent impacts (VM5) and coastal/oceanic conditions (PM7). Seasonal sampling was conducted for all stations, except for PM7, in February, April, July and November 2006. At PM7, samples were collected in June, October and December 2005 for nutrient enrichment bioassays and in April and July 2006 for both nutrient enrichment bioassays and phosphate turnover time experiments. The 4 seasons were categorized as winter (December to February), spring (March to May), summer (June to August) and autumn (September and November).

Vertical profiles of salinity and temperature were measured with a YSI 6600. At PM7, salinity and temperature data were obtained from monthly average data from the 20 yr (1986 to 2005) monitoring data set obtained from the EPD. Photosynthetically active radiation (PAR) was measured with a Li-Cor underwater spherical quantum sensor (LI 193SA). In the present study, the mixed layer depth is defined as a depth at which $\Delta\sigma_t$ is >0.2 units m^{-1} (Therriault & Levasseur 1985). The stratification index (SI) was calculated as follows:

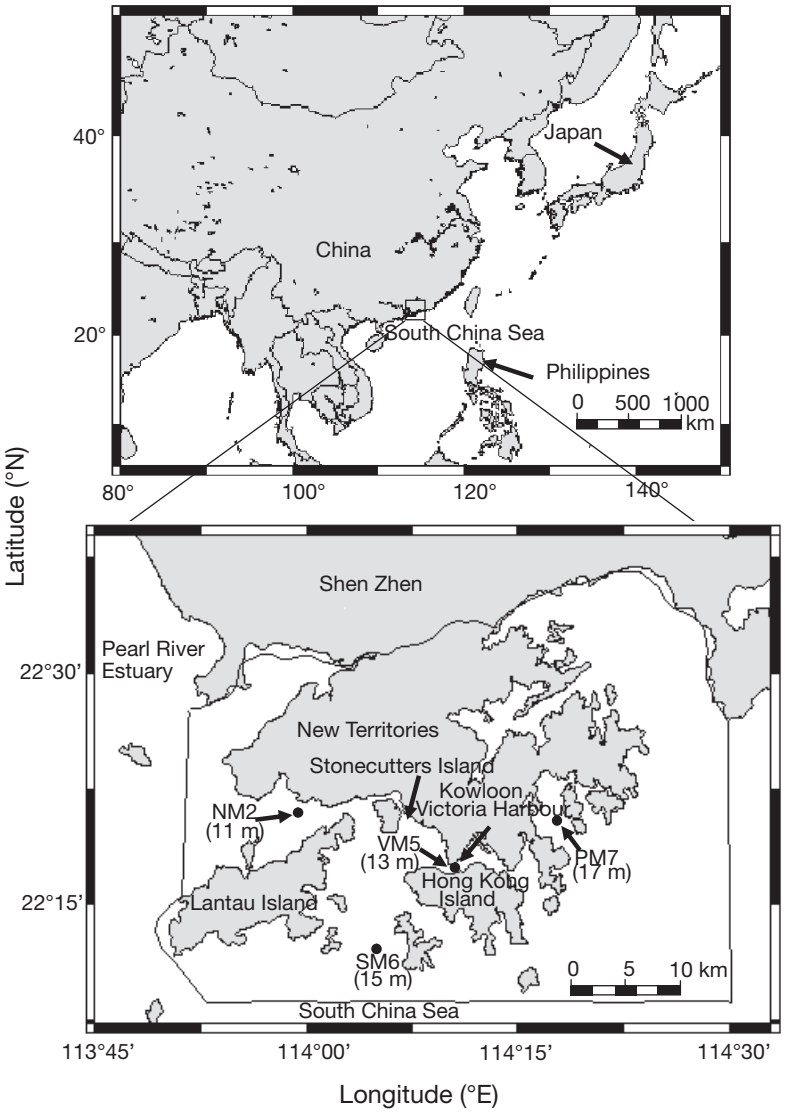

Fig. 1. Location of the 4 sampling stations in Hong Kong waters. The depths of the stations are shown in brackets. The 4 stations are the same as those monitored by the Environmental Protection Dept. (EPD), Hong Kong

$$SI = \frac{\delta\sigma_t}{h} \quad (1)$$

where $\delta\sigma_t$ (kg m^{-3}) is the difference in the seawater density (σ_t) between the surface and bottom and h is the depth (m) of the water column.

Water samples were collected at 1 m depth at all stations and transported in darkened polyethylene carboys to the laboratory. Nutrient samples were filtered through GF/F glass fibre filters and immediately frozen until analysed. Inorganic nutrient concentrations were determined colorimetrically with a SKALAR autoanalyser. NO_3, NO_2 and NH_4 were analysed by the Cu-Cd column reduction method (Strickland & Parsons 1968, Grasshoff et al. 1983) and the indophenol blue colour formation, respectively (Strickland & Parsons 1968). Soluble PO_4 (orthophosphate) was measured using the ascorbic acid method (Strickland & Parsons 1968), and SiO_4 was analysed using molybdate, oxalic acid and a reducing reagent (Strickland & Parsons 1968). Chlorophyll *a* (chl *a*) was filtered onto Whatman GF/F glass fibre filters, extracted with 90% acetone and analysed using a fluorometer (Knap et al. 1996).

Criteria for nutrient limitation based on inorganic nutrient ratios. The atomic Si:N:P ratio of marine diatoms is about 16:16:1 in a nutrient-replete ecosystem (Redfield 1958, Brzezinski 1985). In the present study, deviation from the Redfield ratio indicates the potential for N, P, or Si limitation of phytoplankton growth. We calculated 2 ambient nutrient ratios for each nutrient and applied the Redfield ratio to predict: (1) that N limitation occurs when DIN:PO_4 <16 and DIN:SiO_4 <1; (2) P limitation occurs when DIN:PO_4 >16 and SiO_4:PO_4 >16; and (3) Si limitation occurs when DIN:SiO_4 >1 and SiO_4:PO_4 <16. Dissolved inorganic nutrient (DIN) = NH_4 + NO_2 + NO_3.

Nutrient enrichment bioassays. Nutrient enrichment bioassays were conducted outdoors under natural sunlight. Water samples were screened through a 200 µm mesh nylon screen to remove large zooplankton and thus reduce grazing. Triplicate water samples were incubated in 2 l acid-washed polycarbonate bottles at 30 and 50% of natural sunlight during summer and winter, respectively. Running seawater was used to maintain the *in situ* temperature, and the bottles were stirred twice a day. Nutrients were added in 5 combinations: control (no addition), NO_3 + PO_4, NO_3 + SiO_4, PO_4 + SiO_4 and NO_3 + PO_4 + SiO_4, referred to as the control, N + P, N + Si, P + Si and N + P + Si treatments, respectively. The final concentrations that were added for samples were 20, 2 and 20 µM for NO_3, PO_4 and SiO_4, respectively. Since nutrient concentrations at PM7 were lower than at the other stations, the final nutrient concentrations that were added were 30, 3 and 30 µM for NO_3, PO_4 and SiO_4, respectively. The *in vivo* fluorescence was measured daily, and incubations were terminated 1 d after the daily fluorescence reached a maximum. Chl *a* samples were taken daily or every other day. However, chl *a* samples were always included at the time of

maximum fluorescence, when maximum chl *a* concentrations occurred. Comparisons of the maximal algal biomass were made between the control and other treatments and the control and the initial state.

Phosphate turnover times. Orthophosphate uptake kinetics were determined using carrier-free ^{33}P-orthophosphate. Water was screened through a 200 µm mesh nylon screen to remove large grazers. Incubations with ^{33}P were done in duplicate with 10 ml sub-samples in 20 ml polyethylene scintillation vials at approximately the *in situ* temperature and light when the samples were collected. Carrier-free ^{33}P-orthophosphate and K_2HPO_4 were added to a series of sub-samples to give a total count of approximately 1×10^6 counts per minute per 10 ml sample (max. 100 µl ml^{-1} sample), and addition of 0, 250, 500, 750 and 1000 nM cold orthophosphate provided a range of final concentrations. Incubation times varied from 15 min in summer to 3 h in other seasons, according to the ambient phosphate concentrations, except at PM7 where the incubation time was 15 min for all seasons. A cold chase of 0.1 mM K_2HPO_4 final concentration was added to all vials after incubation, and the contents were filtered immediately onto 25 mm polycarbonate filters of 0.2 µm pore size with a vacuum <0.5 bar. After filtration, the filters were rinsed twice with 3 ml of freshly prepared filter-sterilized seawater. The filters were saturated with 0.1 M cold K_2HPO_4 to reduce the adsorption of abiotic ^{33}P-orthophosphate. Formalin-killed controls were run in the same manner to determine background ^{33}P uptake rates, which were then subtracted from the overall uptake rates. The filters were transferred into scintillation vials. Then, 4 ml of cocktail (Optiface Hisafe 3) was added, and the radioactivity of the filters was measured using a liquid scintillation counter (Perkin Elmer, 1414) (Thingstad et al. 1998).

Estimation of turnover time was obtained using the method proposed by Thingstad et al. (1993). The consumed fraction $R(t)$ of the added label after incubation (t) was assumed to follow the theoretical expression:

$$R(t) = (1 - e^{-t/T_a}) \quad (2)$$

where t is the incubation time. For experiments with single incubation times, the turnover times T_a of the substrate in the incubation bottle were calculated from the rearranged Eq. (2):

$$T_a = \frac{t}{-\ln(1-R)} \quad (3)$$

Estimation of the turnover time was obtained following Wright & Hobbie (1966). The Michaelis-Menten kinetic equation can be rearranged as follows:

$$T_a = \frac{(K + S_n)}{V_m} + \frac{S_a}{V_m} \quad (4)$$

where T_a is the turnover time in the sample, S_n is the natural substrate concentration, S_a is the added substrate concentration, K is the half-saturation constant and V_m is the maximum reaction velocity. The turnover time, T, in the natural water sample can be estimated from the linear regression, and the y-axis intercept in the plots of the T_a value versus a series of added substrate concentrations. The uptake rate (v) is calculated by multiplying the consumed fraction $R(t)$ by the ambient phosphate concentration plus the final concentration of added phosphate. The half-saturation constant (K) is obtained by non-linear, least-squares regression of data fitted to the Michaelis-Menten equation. In the present study, the cut-off of 5 h for phosphate turnover time, as suggested by Nalewajko & Lee (1983), was used as the criterion to judge whether the *in situ* growth rate was limited by ambient PO_4 availability.

Phytoplankton species. One sample for each treatment was used to estimate the percentage of the most dominant phytoplankton genera/species in the phytoplankton community in the initial state and at the time of the maximum algal biomass. Phytoplankton samples were fixed with Lugol's solution and then identified, mainly to genus, by inverted light microscopy, using a local phytoplankton taxonomic guide book (AFCD 2008).

Growth rate. Growth rate was calculated using the following equation:

$$\mu = \ln(B_m/B_0)/\Delta t \quad (5)$$

where B_0 and B_m are the initial and maximum chl *a* concentrations, respectively. Δt is the time interval between the beginning of the experiment and the time when chl *a* reaches a maximum.

Statistical analyses. Statistical analysis was performed using SPSS software. An ANOVA analysis with an LSD (least-significant difference) multiple comparison technique was conducted to determine any significant difference between treatments ($\alpha = 0.05$) within each bioassay experiment. The error bars for the bioassay represent a pooled sample standard deviation of the mean.

RESULTS

Water column stratification

The water column was homogeneously mixed at all stations in winter, as well as at NM2, VM5 and SM6 in autumn and at VM5 in spring, accompanied by high salinity at the surface (>32) (Fig. 2, Table 1). The stratification index increased from 0.01–0.02 kg m^{-4} in winter to 0.38–1.49 kg m^{-4} in summer (Table 1). In spring, stratification occurred at NM2 and SM6, where the

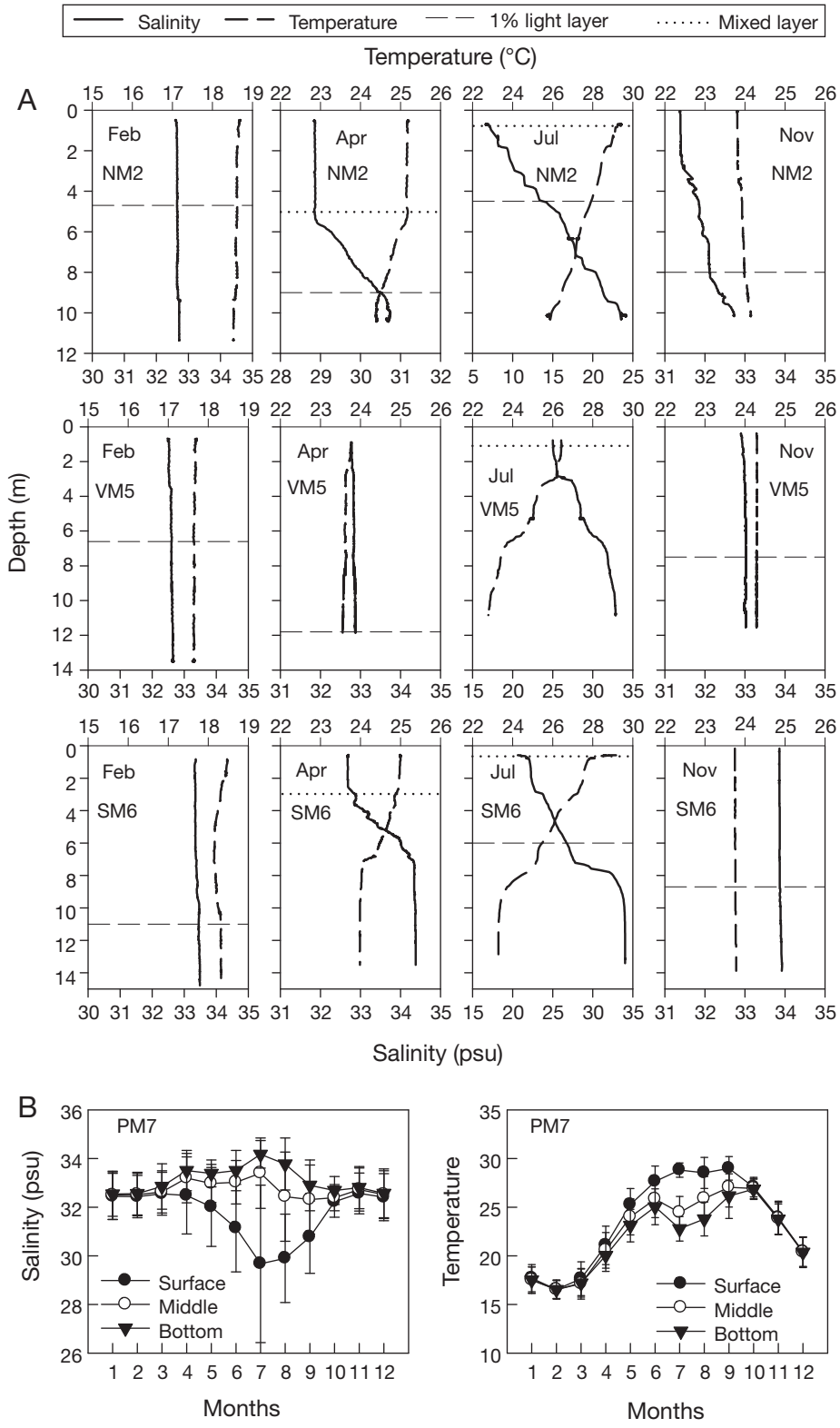

Fig. 2. (A) Vertical profiles of temperature and salinity at 3 stations (NM2, VM5, SM6) in February, April, July and November 2006. Horizontal dashed lines represent the 1% light depth. (B) Monthly average salinity (left) and temperature (right) at the surface, middle and bottom at PM7 from 1986 to 2005 (data from EPD, Hong Kong); vertical bars indicate ±1 SD, n = 20. Note: the mixed layer extended to the bottom at NM2 and SM6 in February and November 2006 and at VM5 in February, April and November 2006

Table 1. Mixed layer depth (MLD) and stratification index (SI) at Stns NM2, VM5 and SM6 in February (Winter), April (Spring), July (Summer) and November (Autumn) 2006 and the monthly average stratification index (SI) at PM7 from 1986 to 2005. See 'Results; Water column stratification' for details of MLD definition and SI calculation

Season or month	MLD (m)	SI (kg m^{-4})
NM2		
Winter	11	0.02
Spring	5	0.17
Summer	<1	1.49
Autumn	11	0.09
VM5		
Winter	13	0.02
Spring	13	0.02
Summer	1	0.70
Autumn	13	0.01
SM6		
Winter	15	0.01
Spring	3	0.13
Summer	<1	0.93
Autumn	15	0.01
PM7		
January	–	0.01
February	–	0.01
March	–	0.03
April	–	0.08
May	–	0.12
June	–	0.19
July	–	0.38
August	–	0.32
September	–	0.18
October	–	0.03
November	–	0.02
December	–	0.01

mixed layer depth was 5 and 3 m, respectively (Table 1). In contrast, the water column was strongly stratified at all stations in summer, when the mixed layer depth was ≤1 m in the river-influenced areas (e.g. NM2, VM5 and SM6) and surface salinity was a seasonal minimum due to the input of freshwater (i.e. Pearl River discharge and rainfall). Surface temperature exhibited clear seasonal changes and was high (26.4 to 29.4°C) in summer and relatively low (16.5 to 18.7°C) in winter (Fig. 2).

Nutrients and nutrient ratios

NO_3 and SiO_4 concentrations exhibited marked spatial and temporal variability. Their concentrations were highest (8 to 80 µM NO_3 and 17 to 108 µM SiO_4) in western waters (NM2), intermediate (mostly <7 µM NO_3 except for ~30 µM NO_3 in July and 9 to 35 µM SiO_4) at VM5 and SM6 and the lowest (mostly <2 µM NO_3 and 4 to 25 µM SiO_4) in eastern waters (PM7); seasonally, the highest values were in summer at all stations (Fig. 3).

There was clear spatial variation in NH_4 concentrations, with the highest (>12 µM) at NM2 most of the time, except in April 2006, intermediate (<5 µM) at SM6 and the lowest (mostly less than the detection limit of 0.2 µM) at PM7. At VM5, NH_4 concentrations varied temporally, with the highest (>11 µM) in April and November and the lowest (<2.5 µM) in February and July (Fig. 3).

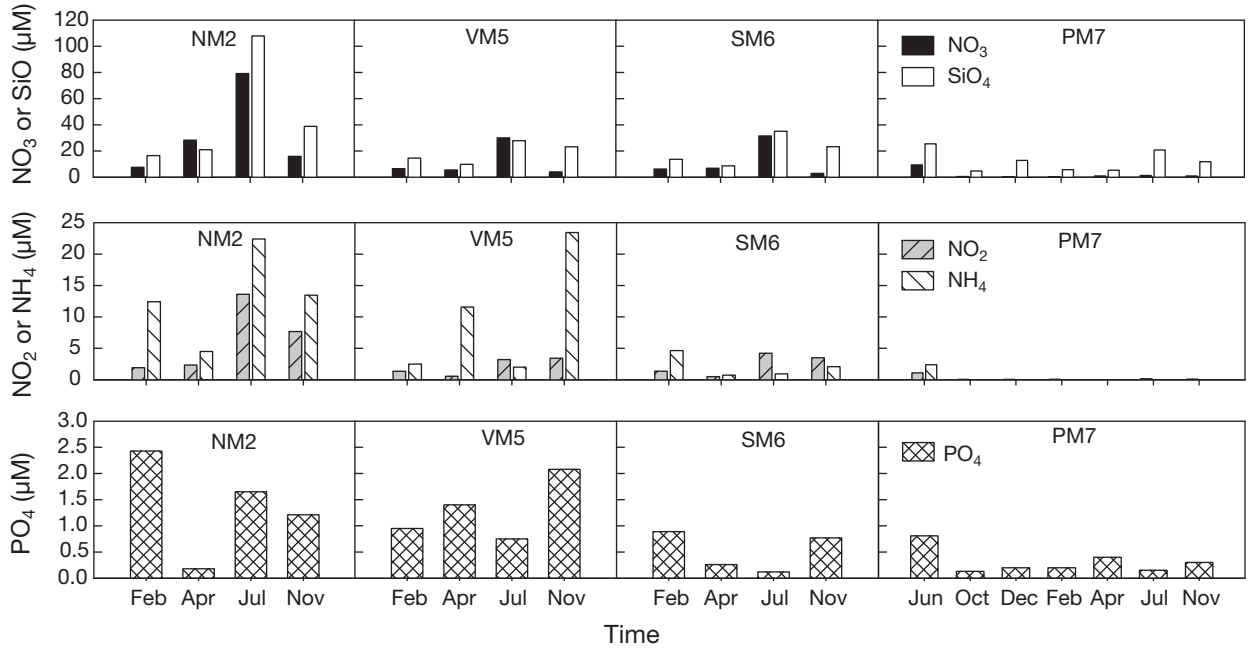

Fig. 3. Surface nutrients at 4 stations (NM2: estuarine water; VM5: Victoria Harbour; SM6: southern waters; PM7: coastal waters) in February, April, July and November 2006, as well as in June, October and December 2005 at PM7. Bars represent NO_3 and SiO_4

PO_4 concentrations varied spatially, with the highest (>0.75 µM, except for 0.18 µM at NM2, in April) at NM2 and VM5 and the lowest (<0.4 µM, except for 0.80 µM in June 2005) at PM7 (Fig. 3). However, at SM6, there was seasonal variation in PO_4 concentrations, with the highest (~0.8 µM) in the dry seasons (February and November) and the lowest (~0.2 µM) in the wet seasons (April and July).

The DIN:PO_4 and SiO_4:PO_4 ratios varied spatially and temporally, being greater than the Redfield ratio (16:1) in the freshwater-influenced areas in the wet season (i.e. NM2 during April to November, at SM6 in April and July and at VM5 in July; Fig. 4). At PM7, without the influence of the Pearl River discharge, the DIN:PO_4 ratios were less than the Redfield ratio (16:1) throughout the study, while the SiO_4:PO_4 ratios were >16:1 most of the time, except in April 2006. The DIN:SiO_4 ratios were greater than the Redfield ratio (1:1) at NM2 during February to July, and at VM5 during April to November, and <1:1 at PM7, during the whole study.

Chl *a*

Chl *a* concentrations demonstrated clear seasonality at VM5 and SM6, increasing from a minimum of <2 µg l^{-1} at the both stations in February when strong vertical mixing occurred and reaching a maximum of ~13 µg l^{-1} at VM5 and 73 µg l^{-1} at SM6 in July when stratification occurred (Fig. 5). At NM2 and PM7, chl *a* concentrations were relatively low (generally <5 µg l^{-1}) during the whole study, except for 6.3 µg l^{-1} at NM2 in April 2006 and 11 µg l^{-1} at PM7 in July 2006.

Phosphate turnover times

PO_4 turnover times varied seasonally and temporally. The shortest PO_4 turnover time at each station always occurred in the season with the highest chl *a* concentrations, especially in July when PO_4 turnover times (1.3 h) at SM6 and (0.7 h) at PM7 were less than

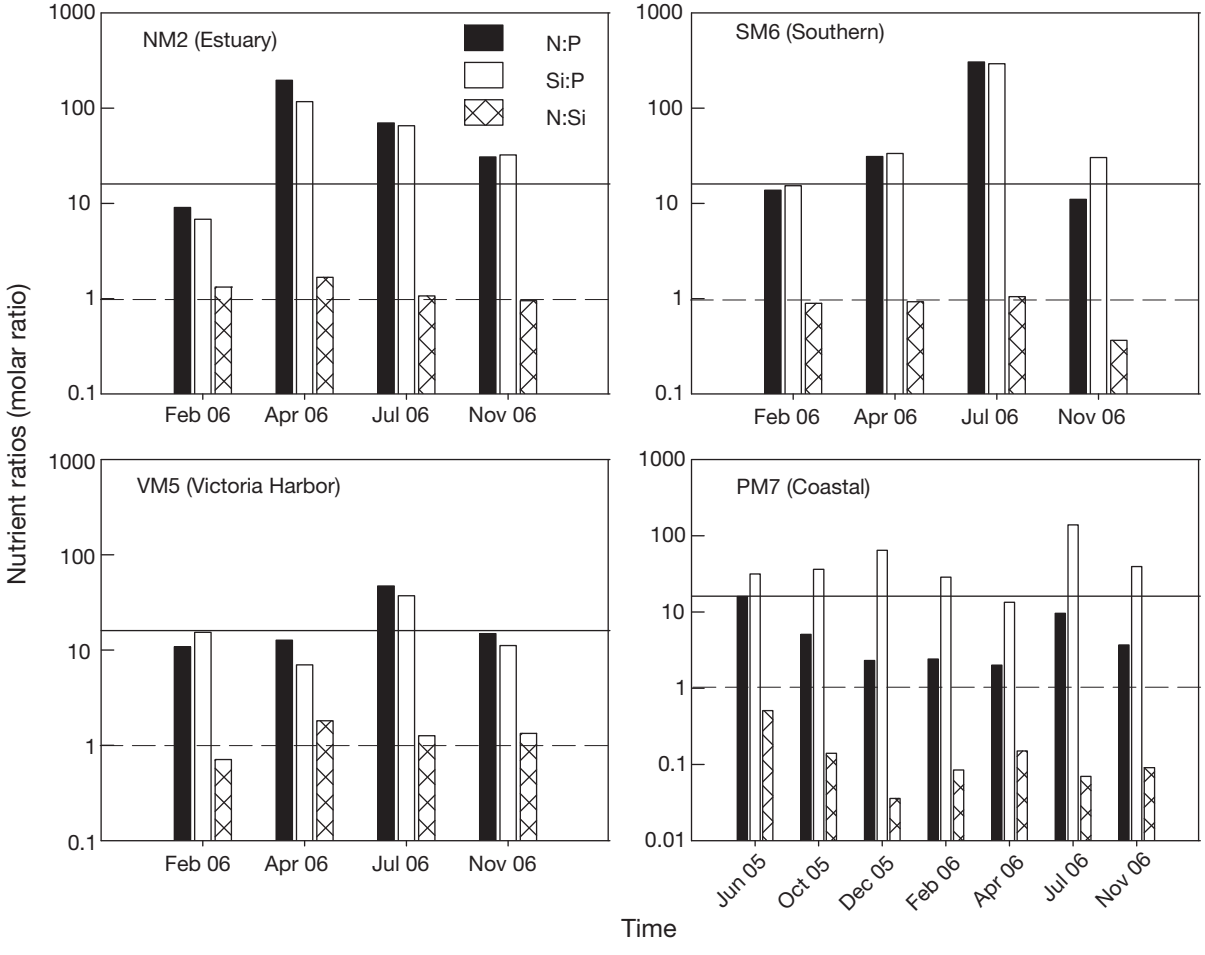

Fig. 4. Nutrient ratios depicted on a logarithmic scale at the surface at 4 stations in February, April, July and November 2006, as well as in June, October and December 2005 at PM7. Solid lines represent nutrient ratios of 16:1 (N:P or Si:P); dashed lines represent nutrient ratios of 1:1 (N:Si). N:P = DIN:PO_4; Si:P = SiO_4:PO_4; N:Si = NO_3:SiO_4. DIN: NH_4 + NO_2 + NO_3

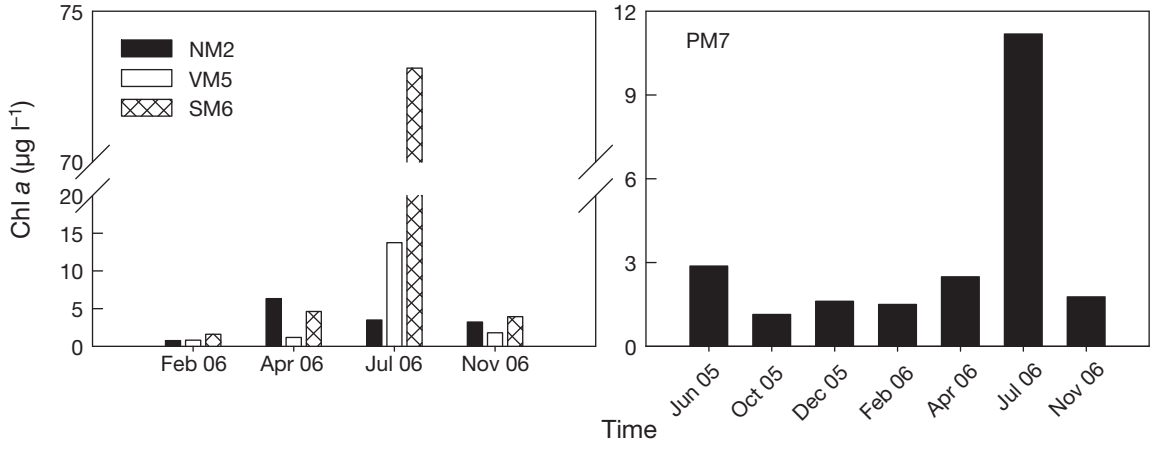

Fig. 5. Chlorophyll *a* concentrations at the surface of 4 stations in February, April, July and November 2006, as well as in June, October and December 2005 at PM7. Note the different scales for chl *a* and the break in the scale bar for the left figure

the threshold of 5 h (Fig. 6). In contrast, PO_4 turnover times (11 to 467 h) were much higher than the 5 h threshold in other cases (Fig. 6).

Nutrient enrichment bioassays

Nutrient enrichment bioassays clearly showed potential limitation and actual limitation in a few cases. Phytoplankton grew in the control treatments nearly as well as in the nutrient addition treatments until the achievement of the maximum chl *a* in the control in 14 of 17 cases, except at SM6 in July and at PM7 in April and July (Figs. 7 to 10). Moreover, the maximal chl *a* concentrations in the control were significantly higher than the initial chl *a* in these 14 cases, suggesting phytoplankton biomass production was limited by light or flushing. Given sufficient light and a long residence time of the water mass, nutrients would limit phytoplankton growth in these cases. There were significant spatial and temporal variations regarding which nutrient would potentially limit phytoplankton growth using enrichment bioassays. For the NM2 sample, the maximum chl *a* concentration was significantly higher in both the NO_3 and SiO_4 addition treatments (N + Si and N + P + Si) relative to in the control treatment in February (Fig. 7). In contrast, the maximum chl *a* concentration was significantly higher in the PO_4 addition treatments (N + P, P + Si, N + P + Si) during April to November (Fig. 7). For the VM5 sample, biomass was stimulated significantly by the NO_3 addition (N + P, N + Si, N + P + Si) relative to the control in February and April, by the PO_4 addition in July, and by the SiO_4 addition (N + Si, P + Si, N + P + Si) in November (Fig. 8). For the SM6 sample, there was a significant response to the NO_3 addition in February and November, both the NO_3 and PO_4 addition (N + P, N + P + Si) in April, and the PO_4 addition in July (Fig. 9). At PM7, compared to the control, the maximum chl *a* concentration was significantly higher in the NO_3 addition treatments during October 2005 to April 2006, in the PO_4 addition treatments in June 2005 and in both NO_3 and PO_4 addition treatments in July 2006 (Fig. 10).

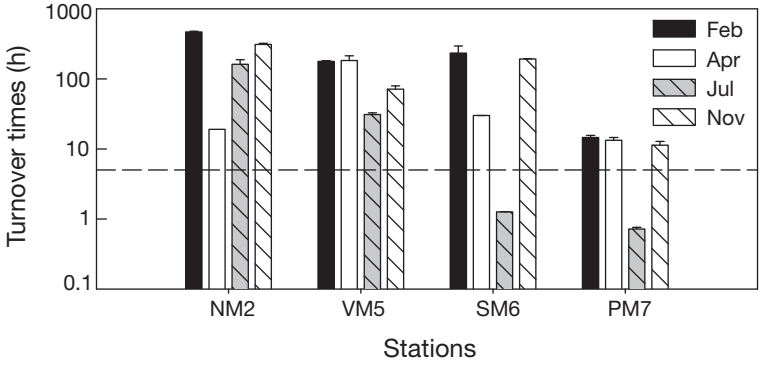

Fig. 6. Turnover times of orthophosphate on a logarithmic scale at 4 stations in February, April, July and November 2006. Vertical bars indicate ±1 SD and n = 2. Horizontal dashed line indicates the threshold value of 5 h, below which a short turnover time of <5 h indicates P-limitation

Phytoplankton species composition

Due to their fast growth rate, diatoms dominated at the end of the bioassays. For the initial samples at NM2, the diatom *Skeletonema costatum* dominated the phytoplankton community all year, followed by *Thalassiosira* spp. and *Chaetoceros* spp. These 3 species accounted for 34% of total cell density in April, 50 to 60% in February and November and 89% in July. For the ini-

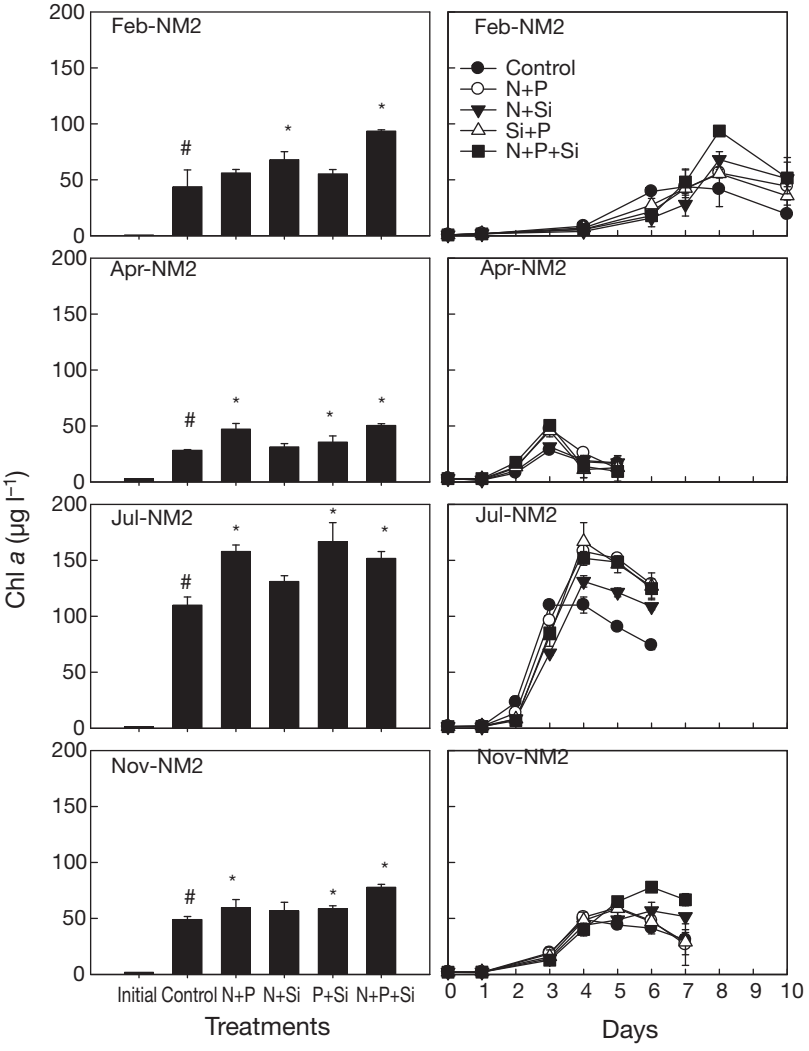

Fig. 7. Nutrient enrichment bioassays run in triplicate at NM2: maximum chlorophyll *a* concentrations (left) and the daily response of phytoplankton biomass to 5 treatments (right) in February, April, July and November 2006. Vertical bars indicate ±1 SD and n = 3. #: control was significantly different ($p < 0.05$) from initial value; *: treatment was significantly different ($p < 0.05$) from control, based on 1-way ANOVA

tial samples at VM5, the diatoms *Thalassiosira* spp. and *S. costatum* were dominant in February (47%) and July (82%). In April, the dinoflagellate *Scrippsiella* sp. was dominant (29%), followed by *S. costatum* (23%). In November, *Thalassiosira* spp. and *Nitzschia* spp. were dominant, accounting for 32 and 24%, respectively. For the initial samples at SM6, there was a seasonal shift in the dominant species from *Thalassiosira* spp. (~30%) in February and November to *Chaetoceros* spp. (23%) in April and *Nitzschia* spp. (81%) in July. For the initial samples at PM7, *Nitzschia* spp. dominated in June (47%) and December (26%) 2005, *S. costatum* (~70%), in October 2005 and April 2006, and *Rhizosolenia* sp. (64%), in July 2006 (Table 2).

The dominant species exhibited no obvious succession in any treatment during the incubation, except at VM5 and PM7 during April. In April at VM5, the dominant species shifted from the dinoflagellate *Scrippsiella* sp. in the initial sample to *Skeletonema costatum* in all N + Si addition treatments. However, at PM7, the dominant species shifted from *S. costatum* in the initial sample to *Pseudo-nitzschia* spp. in all N-addition treatments and to *Leptocylindrus* sp. in the P + Si addition treatments (Table 2).

DISCUSSION

Nutrient limitation versus assessment methods

The question of nutrient limitation of phytoplankton growth in marine ecosystems has been the subject of much controversy and debate (Hecky & Kilham 1988, Howarth 1988, Malone et al. 1996). To some extent, these disagreements are caused by different definitions of nutrient limitation (e.g. limitation of algal biomass yield vs. limitation of growth rate) and by the different methods used to evaluate nutrient limitation (Howarth 1988).

A variety of methods have been used to assess nutrient limitation in aquatic ecosystems. The approaches that are used commonly include inferences from ambient concentrations and ratios of ambient dissolved inorganic nutrients, bioassays, isotope techniques and enzyme activities (Xu 2007). These methods have their merits and limitations.

Comparison of the 3 methods

Nutrient ratios and nutrient enrichment bioassays

Ambient nutrient ratios reflect the integrated sum of different processes (i.e. riverine, oceanic inflows, biological uptake, grazing, sedimentation, and so on). Ambient nutrient ratios only indicate a potential, not necessarily an actual *in situ* nutrient limitation, since the ambient nutrient concentrations may not be low enough to limit phytoplankton growth (Justic et al. 1995). Therefore, ambient nutrient ratios have been used to predict potential nutrient limitation for biomass

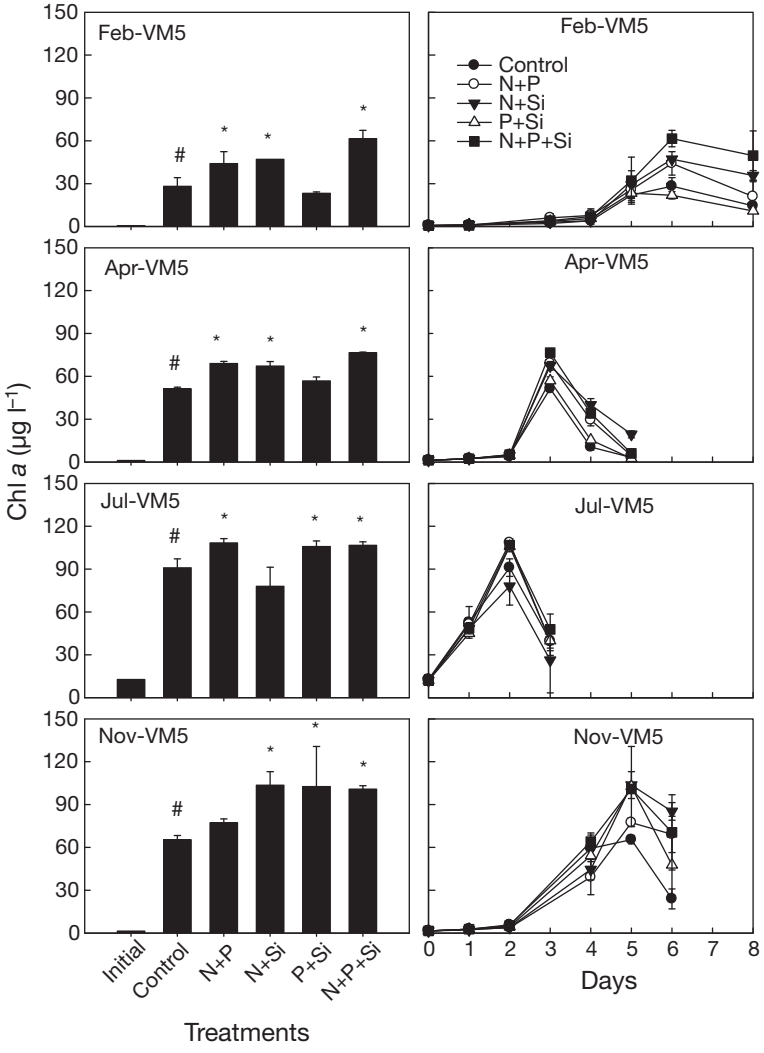

Fig. 8. Nutrient enrichment bioassays run in triplicate at VM5. All further details as in Fig. 7

yield in nutrient-enriched waters for several decades, because they can be easily obtained from available nutrient concentrations, but they may not be directly applicable to the oligotrophic ocean with nearly undetectable nutrient concentrations and low phytoplankton biomass. Other factors such as light or temperature might also limit growth even when nutrients are abundant. Predictions of which nutrient potentially limits algal growth from ambient nutrient ratios need to be confirmed by other methods (e.g. nutrient enrichment bioassays), since the optimum nutrient ratio required by phytoplankton may vary by >2-fold for different algal species (Boynton et al. 1982, Atkinson & Smith 1983, Fong et al. 1993). In addition, phytoplankton may also be able to use some organic nutrients (Howarth 1988), and not all P measured as PO_4 in the chemical analyses is biologically available (Zohary & Robarts 1998).

Nutrient enrichment bioassays that indicate which nutrient would be the most likely to become potentially limiting for phytoplankton biomass yield, only indicate the potential for nutrient limitation, since the enclosure of water in bottle bioassays changes the hydrodynamics (mixing, dilution and light), nutrient dynamics and grazing pressure (Zohary & Robarts 1998). However, mesozooplankton are often removed before the incubation, which changes not only the grazing pressure on phytoplankton but also nutrient regeneration. Furthermore, nutrient addition changes the nutrient ratios in the ambient concentration and may affect the relative abundance of different algal groups (Littler & Littler 1980). The enhanced concentration after nutrient addition often results in a dominant species shift from small to larger sized, fast-growing species (Fong et al. 1993).

Nutrient ratios and enrichment bioassays are extensively used in nutrient-enriched waters to assess the potential for an increase in algal biomass yield due to nutrient enrichment (Harrison et al. 1990, Fisher et al. 1992, 1999, Bernhard & Peele 1997, Holmboe et al. 1999). In the present study, predictions from nutrient ratios were always in agreement with the results from the nutrient enrichment bioassays, except in Victoria Harbour during spring and eastern waters during summer (Table 3), suggesting that the nutrient ratio of 16:16:1 (N:Si:P) as a criterion was always able to predict potential nutrient limitation in Hong Kong waters. In spring, in Victoria Harbour, the discrepancy between both methods in detecting the potentially limiting nutrient, as indicated by Si inferred from inorganic nutrient ratios and N from nutrient enrichment bioassays, may be due to the dominance of dinoflagellates (e.g. *Scrippsiella* sp.) whose growth does not require SiO_4. As a result, the phytoplankton community required less SiO_4 relative to nitrogen and phosphorus. In contrast, based only on the DIN:PO_4 ratio, the ratio indicated potential N deficiency relative to P and Si in spring, which is in agreement with nutrient enrichment bioassay. In eastern waters, DIN:PO_4 ratios were 16:1 and 10:1 in June 2005 and July 2006, respectively, while nutrient enrichment bioassays indicated potential P limitation in June 2005 and N + P co-limitation in July 2006. This result implied that the optimum DIN:PO_4 uptake ratio of the phytoplankton community might occur in a range of ratios of 10:1 to 16:1 in eastern waters during summer. Our results indicate that nutrient ratios are a relatively rapid and effective

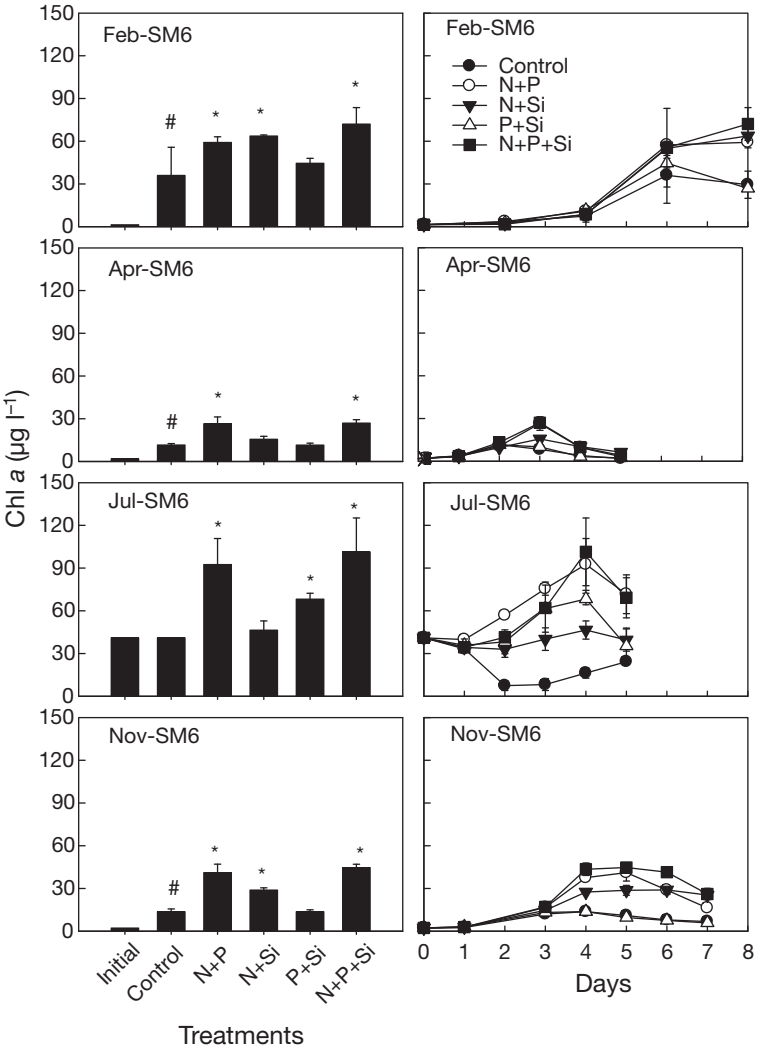

Fig. 9. Nutrient enrichment bioassays run in triplicate at SM6. All further details as in Fig. 7

method to predict the potentially limiting nutrient compared to nutrient enrichment bioassays, which are time consuming.

^{33}P turnover times and nutrient concentrations

Isotope tracer techniques that are used to determine uptake and turnover times of a nutrient are an estimate of the real-time response of bacterio- and phytoplankton to ambient nutrient concentrations. An isotope dilution approach has been used to estimate nutrient regeneration, based on the assumption that the incorporated label is not recycled over the course of the incubation (Harrison 1983). In freshwaters, a cut-off of 10 min for the phosphate turnover time is very commonly used (Lean et al. 1987). However, a threshold of 5 h appears to be more frequently applied in marine environments. Nalewajko & Lee (1983) reported that the phosphate turnover time of 5 h indicated phosphate limitation in the Sargasso Sea. Similarly, the orthophosphate turnover times were observed to be between 1 and 5 h in the P-limited surface water in Villefranche Bay (France) (Tanaka & Rassoulzadegan 2003). The proposed cut-off of 5 h for the phosphate turnover time has been used to determine whether the *in situ* growth rate of bacterio- and phytoplankton is limited by ambient PO_4 availability in marine environments (Van Den Broeck et al. 2004).

Turnover times of orthophosphate in seawater are a function of *in situ* concentrations and fluxes of orthophosphate (uptake by bacterio- and phytoplankton) (Van Den Broeck et al. 2004), which integrate the effects of ambient concentrations, internal storage and biomass (Lean et al. 1987, 1989, Fisher et al. 1992). Differences in turnover times between environments, in time and space, are most likely related to variations in both biological assimilation and PO_4 concentrations. The long turnover times of PO_4 are due to high PO_4 concentrations and/or very low rates of biological assimilation, while short turnover times are due to low PO_4 concentrations and/or moderate to rapid fluxes (Zohary & Robarts 1998). Therefore, the sole use of nutrient concentration as an indicator of nutrient limitation for growth rate is biased, since turnover times might be greatly different because of biological uptake, even if ambient PO_4 concentrations were the same between environments. For example, phosphate concentrations were similar in southern waters during summer and in western waters during spring, while turnover times of phosphate were very different in both waters. This discrepancy is possibly explained by differences in chl *a* concentrations, as indicated by the significant correlation between phosphate turnover times and chl *a* concentrations (Fig. 11).

In southern waters during summer, short PO_4 turnover times, associated with high chl *a* concentrations (73 µg l^{-1}), suggested that the phytoplankton growth rate was P-limited, which was in agreement with the results from nutrient ratios and bioassays. Fisher et al. (1992) concluded that the agreement between nutrient concentrations, ratios, bioassays and nutrient turnover times indicates that the phytoplankton growth rate was the dominant process controlling biomass accumulation, as the growth rate possibly reflected the state of

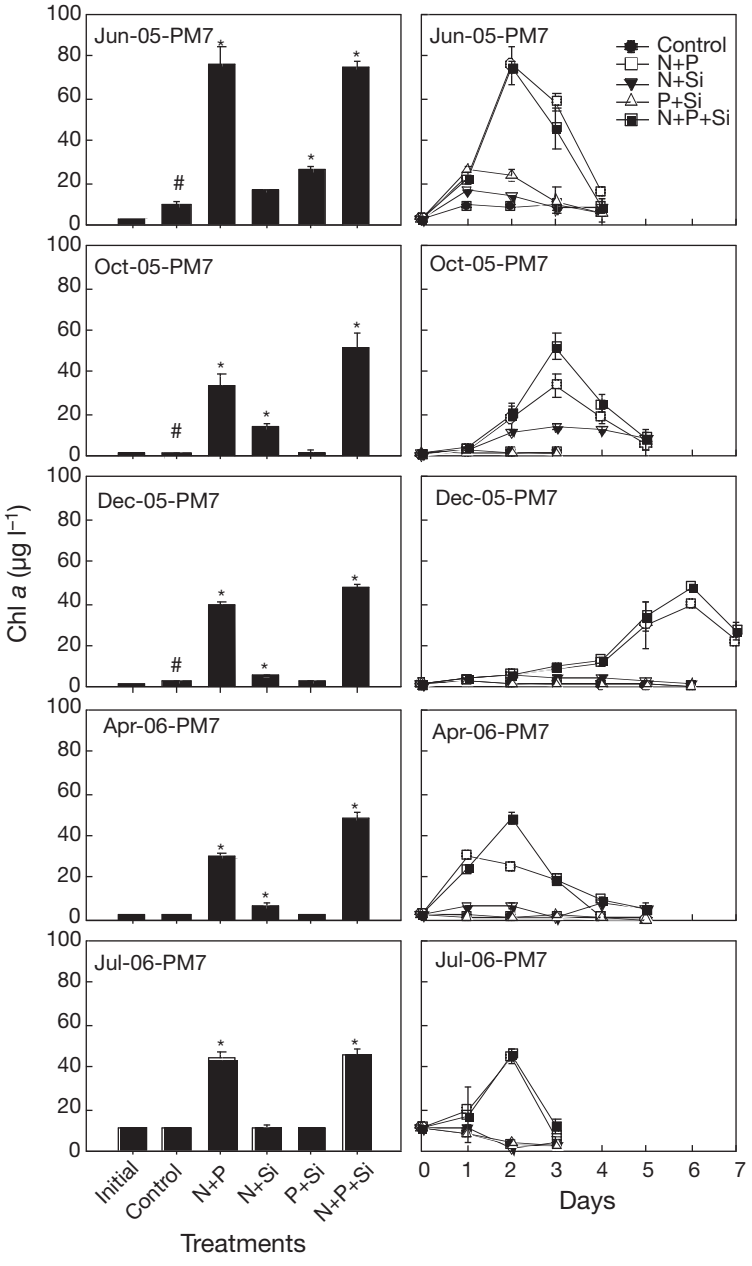

Fig. 10. Nutrient enrichment bioassays run in triplicate at PM7. All further details as in Fig. 7

the ecosystem. This is supported by our observation that the development of algal blooms was regulated by the phytoplankton growth rate, due to PO_4 depletion under strongly stratified conditions in this region. The observed phosphate concentration at SM6 is much below the half-saturation constant (Figs. 3 & 12). Hence, the removal of PO_4 in the treatment of sewage will help control the magnitude of algal blooms in southern waters during summer.

In contrast, in western waters during spring, long PO_4 turnover times were a result of high PO_4 supply from Pearl River discharge (Fig. 1) and low biological uptake (6.3 µg chl *a* l^{-1}), suggesting that the phytoplankton growth rate was not limited by PO_4 availability. This indicates that the growth rate is not the dominant process regulating biomass accumulation in western waters. However, bioassays indicated that potential PO_4 limitation of biomass yield would occur within a few days with significantly reduced physical processes (tidal mixing and flushing). These results implied that the processes (i.e. tidal mixing and flushing) that were excluded in the bioassays dominated the ecosystem responses in the field. This suggestion was supported by our observation that the maximum chl *a* concentration obtained in the bioassays was significantly higher (up to 5 times) than the ambient chl *a* concentration (Fig. 7). As a result, the *in situ* growth rate of phytoplankton was not limited by P availability in this region, since physical processes (e.g. dilution and flushing) supplied P faster than P uptake. The same explanation was given in other potentially P-limited cases (e.g. western waters during summer and autumn and in Victoria Harbour during summer).

Currently, an important challenge for interpreting assays of nutrient limitation is to extrapolate the laboratory studies to an understanding of nutrient limitation on a large scale (Beardall et al. 2001). Hence, the best approach is to use a suite of techniques and parameters to demonstrate nutrient limitation in terms of biomass yield and the growth rate of phytoplankton.

Seasonal and spatial variations in the factors regulating phytoplankton biomass

Eastern waters

The eastern waters are relatively far away from the Pearl River discharge (Fig. 1), and hence they have more oceanic characteristics such as low nutrients (generally < 2 µM DIN) and relatively high surface salinity during most of the year (Lee et al. 2006). Hence, N was the potentially limiting nutrient during most of the year, except in the summer. Limitation by N at high salinities has been documented in the Gulf of Mexico (Lohrenz et al. 1999). By comparison, in summer, during the period of highest rainfall, P limitation, or N + P co-limitation, was observed, possibly due to the contribution of rainfall with a high DIN:PO_4 ratio (50:1, N:P) (Yin 2002). The magnitude of the algal bloom was regulated by N and P availability.

Table 2. The most dominant phytoplankton species and the percentage of total cell density at NM2, VM5 and SM6 in the periods given. Numbers in brackets represent percentage of total cell density. For details on phytoplankton enumeration see 'Materials and methods'. A: *Skeletonema costatum*; B: *Thalassiosira* spp.; C: *Chaetoceros* spp.; D: *Scrippsiella* sp.; E: *Nitzschia* spp.; F: *Pseudo-nitzschia* spp.; G: *Prorocentrum* spp.; H: *Pleurosigma* spp.; I: *Leptocylindrus* sp.; J: *Rhizosolenia* sp.

Stns	Period	Treatments					
		Initial	Control	N + P	N + Si	P + Si	N + P + Si
NM2 (Western waters)	Feb 2006	A(34), B(18)	A(43), B(32)	A(47), B(39)	A(49), B(38)	A(59), B(33)	A(44), B(41)
	Apr 2006	A(21), C(13)	A(39), C(33)	A(47), C(28)	A(46), C(23)	A(54), C(34)	A(41), C(39)
	Jul 2006	A(51), B(28)	A(47), B(35)	A(54), B(31)	A(61), B(33)	A(70), B(26)	A(60), B(37)
	Nov 2006	A(38), C(19)	A(47), C(24)	A(37), C(31)	A(53), C(21)	A(61), C(25)	A(58), C(31)
VM5 (Victoria Harbour)	Feb 2006	B(37), A(10)	B(63), A(11)	B(57), A(19)	B(67), A(9)	B(74), A(10)	B(66), A(21)
	Apr 2006	D(29), A(23)	D(49), A(37)	D(52), A(39)	A(43), D(38), E(13)	D(47), A(35), E(9)	A(45), D(41), E(7)
	Jul 2006	A(58), B(24)	A(51), B(41)	A(60), B(37)	A(55), B(43)	A(70), B(28)	A(64), B(34)
	Nov 2006	B(32), E(24)	B(60), E(19)	B(51), E(23)	B(65), E(31)	B(57), E(29)	B(69), E(20)
SM6 (Southern waters)	Feb 2006	B(32), A&E(18)	B(58), A(29)	B(47), A(43)	B(50), A(27), E(9)	B(61), A(34)	B(49), A(48)
	Apr 2006	C(23), F(14), G(11)	C(23), F(14)	C(21), F(40), G(10)	C(49), F(44)	C(63), F(24)	C(71), F(15)
	Jul 2006	E(81)	E(70)	E(69), F(15)	E(75)	E(79)	E(74), F(21)
	Nov 2006	B(34), H(10)	B(70), H(14)	B(62), H(17)	B(66), H(11)	B(70), H(10)	B(73), H(9)
PM7 (Eastern waters)	Jun 2005	E(47), F(21)	E(60), F(20)	E(39), F(24)	E(77), F(14)	E(64), F(15)	E(57), F(29)
	Oct 2005	A(71), E(15)	A(64), E(14)	A(61), E(22)	A(48), E(40)	A(83), E(9)	A(80), E(10)
	Dec 2005	E(26), B(23)	E(41), B(35)	E(36), B(38)	E(60), B(18)	E(46), B(42)	E(54), B(31)
	Apr 2006	A(74), F&I(19)	A(50), F(38)	F(40), A(37)	F(45), I(38)	I(51), A(30)	F(54), A(31)
	Jul 2006	J(64), I(16)	J(54), I(31)	J(67), I(21)	J(60), I(19)	J(69), I(22)	J(73), I(10)

Table 3. Ambient chlorophyll *a* (chl *a*) concentrations (con.), potential maximum biomass in non-enriched samples (±1 SD, n = 3) and comparison of the limitation nutrient (N, P, Si, or co-limitation) derived from 3 methods (ambient inorganic nutrient ratios, phosphate turnover times and nutrient enrichment bioassays) at 4 stations during 4 seasons

Stns	Seasons	Chl *a* (µg l^{-1})		Nutrient ratios	^{33}P turnover times	Bioassays
		Ambient chl *a* conc.	Potential maximum biomass			
NM2	Spring	6.33	28.3 ± 0.55	P		P
	Summer	3.48	110 ± 7.24	P		P
	Autumn	3.21	49.1 ± 2.66	P		P
	Winter	0.75	44.0 ± 14.9	Si		N + Si
SM6	Spring	4.61	11.6 ± 0.90	P		N + P
	Summer	73.0	73.0	P	P	P
	Autumn	3.92	13.7 ± 1.89	N		N
	Winter	1.60	36.1 ± 19.6	N		N
VM5	Spring	1.16	51.3 ± 1.04	Si		N
	Summer	13.7	91.0 ± 6.15	P		P
	Autumn	1.78	65.4 ± 2.93	Si		Si
	Winter	0.81	28.2 ± 5.97	N		N
PM7	Spring	2.50	2.50	N		N
	Summer	11.2	11.2	N	P	P, N + P
	Autumn	1.14	1.81± 0.53	N		N
	Winter	1.61	3.27 ± 0.35	N		N

For example, in July 2006, the addition of 30 µM N and 30 µM Si, or 3 µM P and 30 µM Si, never stimulated algal growth, while algal biomass increased by 30 µg chl l^{-1} after the 30 µM N and 3 µM P addition.

Western waters

Western waters neighbouring the Pearl River estuary (Fig. 1) are typically influenced by river discharge in the wet season, as indicated by the low surface salinity and the occurrence of stratification in the water column (Fig. 2). The DIN:PO_4 and SiO_4:PO_4 ratios often dramatically deviated from the Redfield ratio, due to the input of freshwater with high N and Si concentrations (Yin 2002, Xu et al. 2008). P was deficient relative to N and Si during the river-influenced period. By contrast, in winter, when the Pearl River discharge has little influence on western waters due to low flow rate, the high NH_4 and PO_4 concentrations were possibly attributable to the discharge of sewage effluent at Stonecutters Island, which moves up towards the Pearl River estuary during flood tides (Lee et al. 2006). The intrusion of sewage effluent, which is P rich and Si poor, resulted in N and Si deficiency relative to P, as N is deficient relative to P in both sewage effluent (Xu et al. 2008) and oceanic water from the South China Sea (Chen 2005).

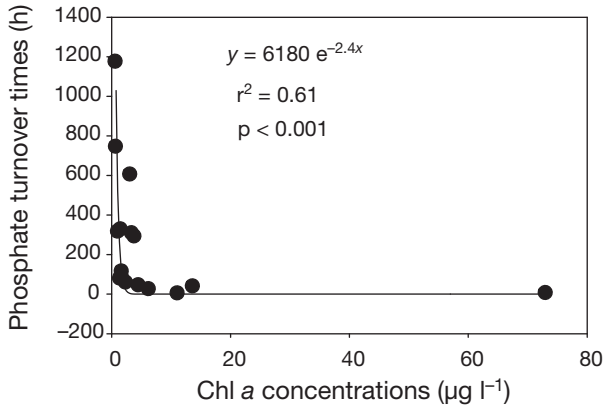

Fig. 11. A significant correlation between orthophosphate turnover times and chlorophyll *a* concentrations at the surface of 4 stations (NM2, VM5, SM6 and PM7) during 4 seasons in 2006

Nutrient limitation of the growth rate did not occur at any time, as the production of phytoplankton biomass was primarily restricted by physical processes (e.g. high flushing rate and mixing) in this area (Yin 2002, Xu et al. 2008). The evidence for this suggestion is that the high maximum chl *a* level of 28 to 110 µg l^{-1} was observed in the control bioassay treatment that eliminated the effects of vertical mixing and the flushing rate. During the wet season, flushing more likely regulated the phytoplankton biomass production than light, since stratification increased the light availability and the euphotic zone was deeper than the mixed layer. During summer, the turbulent mixing and suspended solids carried by the Pearl River discharge reduced the light availability, resulting in a shallow euphotic zone (~4.5 m). The high flushing, caused by the Pearl River discharge in the wet season, diluted the phytoplankton biomass and resulted in more rapid P supply than P uptake. In contrast, in the dry season, the strong turbulent mixing reduces the light availability and distributes the phytoplankton cells evenly in the water column. As a result, phytoplankton was often mixed down and out of the euphotic zone. Likewise, light could also be important as a limiting factor for turbid and highly flushed estuaries, as has been reported for Chesapeake Bay (Fisher et al. 1999).

Southern waters

During summer, high light intensity and temperature favoured algal growth, and strong stratification sustained the phytoplankton cells in the euphotic zone, in the relatively weakly flushed southern waters. As a result, algal blooms occurred, with a peak chl *a* value of 73 µg l^{-1}. The frontal regions of plumes have also been found to have high chl *a* concentrations relative to inshore and offshore waters in other coastal waters such as the Strait of Georgia (Yin et al. 1997), Chesapeake Bay (Breitburg 1990, Harding 1994) and the Mississippi River plume (Grimes & Finucane 1991). The relatively high chl *a* level in intermediate salinity areas may be associated with waters that have a longer residence time in the coastal plume due to increased stratification. The algal bloom resulted in PO_4 depletion (~0.12 µM), which is below the approximate half-saturation constant for P uptake (PO_4 = 0.96 µM; Fig. 12), where the magnitude of algal blooms was regulated by PO_4 availability. The importance of P as a limiting nutrient has been documented in the coastal systems of other major rivers, such as the Yellow River (Turner el al. 1990), Yangtze River (Hu et al. 1990) and Mississippi River (Lohrenz et al. 1999).

In spring, during the transition between the wet and dry seasons, the absence of PO_4 limitation in the growth rate and low biomass may be related to the weak and short-lived stratification in the water column and zooplankton grazing. In the winter dry season, N was deficient relative to P and Si, due to the invasion of N-deficient coastal/oceanic water. The low chl *a* concentration was likely caused by light limitation, due to strong vertical mixing.

Victoria Harbour

Victoria Harbour is subject to the impact of Pearl River discharge in summer, year-round sewage effluent inputs and coastal/oceanic water intrusion in winter. In summer, during the greatest amounts of Pearl River discharge, high light intensity and deeper light penetration (e.g. euphotic zone >11 m) favoured the development of algal blooms (~13 µg chl *a* l^{-1}) in the stratified waters under usually low wind conditions and high temperature (~30°C). Nevertheless, the potentially limiting nutrient (PO_4) concentration was

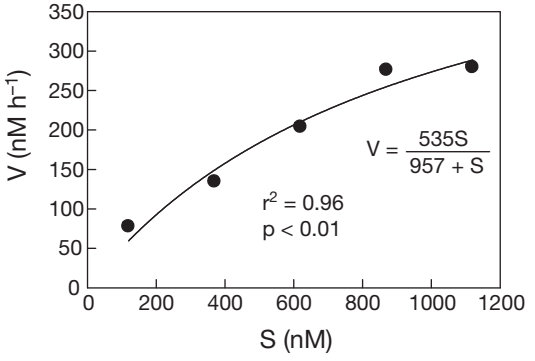

Fig. 12. Phosphate uptake rate as a function of phosphate concentrations for SM6 in July in 2006. Line denotes the fitted Michaelis and Menten equation

still relatively high (~0.75 µM) and was enough to support phytoplankton growth for 2 d in the control treatment (Fig. 8). The absence of a severe algal bloom and PO_4 limitation of the growth rate may be associated with a high flushing rate relative to the growth rate. The dilution rate due to flushing is estimated to be between 0.4 and 0.67 d^{-1}, based on a flushing time of 1.5 to 2.5 d in the wet season (Lee et al. 2006), accounting for approximately 50% of the growth rate (0.98 d^{-1}) (Table 4). In other words, the tidal flushing may lead to a 50% loss in biomass production. Furthermore, it is difficult to maintain the stratification for a long time due to strong tidal mixing in Victoria Harbour. Therefore, physical processes (e.g. vertical turbulent mixing and tidal flushing) are likely responsible for the rare occurrence of algal blooms and the absence of PO_4 limitation of the growth rate in Victoria Harbour (Wong et al. 2007).

In other seasons, Victoria Harbour is characterized by homogenous mixing and high surface salinity (>32). The potentially limiting nutrient shifted from N in spring and winter to Si in the autumn. In winter, NH_4 concentrations were lower (~2.5 µM) than the normal NH_4 concentration of >10 µM, possibly due to less input of sewage effluent. However, low light availability and tidal flushing, rather than nutrients, were responsible for the low algal biomass during these periods.

SUMMARY

The application of 3 methods for assessing nutrient limitation has provided new insights in terms of how nutrients control the biomass and growth rate of phytoplankton in Hong Kong waters. Our comparison of bioassays and nutrient ratios demonstrated that ambient nutrient ratios are an effective method to predict the potentially limiting nutrient of the phytoplankton biomass in Hong Kong waters, except in Victoria Harbour in spring and eastern waters in summer. The agreement between indicators of the nutrient limitation of the growth rate (e.g. ^{33}P turnover time) and biomass yield (nutrient ratios and enrichment bioassays) suggested that the growth rate was the dominant process regulating biomass accumulation. In contrast, when these indicators of the nutrient limitation of the growth rate and biomass yield do not agree, this indicates that physical processes (e.g. mixing and freshwater flushing) dominated the water mass in that region.

Table 4. Initial and maximum chlorophyll *a* (chl *a*), Δt (days to reach maximum chl *a*), growth rate (calculated from the initial to the maximum chl *a*) and temperature in nutrient bioassays conducted at VM5 (Victoria Harbour) in February, April, July and November 2006 (± 1 SD and n = 3)

Months	— Chl *a* (µg l^{-1}) — Initial	Maximum	Δt (d)	Growth rate (d^{-1})	Temp. (°C)
Feb	0.63	28.2 ± 5.97	6	0.59 ± 0.03	17 ± 2
Apr	1.16	51.3 ± 1.04	3	1.26 ± 0.01	27 ± 2
Jul	13.0	91.0 ± 6.15	2	0.98 ± 0.03	28 ± 2
Nov	1.46	65.4 ± 2.93	5	0.74 ± 0.04	23 ± 2

Biological processes are coupled with hydrodynamics in Hong Kong waters (Harrison et al. 2008). The seasonal alteration of Pearl River discharge and coastal/oceanic water intrusion induced by monsoons, sewage effluents and physical properties (vertical mixing) play important roles in the spatial and temporal variations in nutrients and phytoplankton biomass. Hence, the factors regulating phytoplankton biomass are complex in Hong Kong waters. In the winter dry season, the northeast monsoon wind and the Coriolis force move the estuarine water to the west side of the estuary. Thus, Pearl River discharge has little influence on Hong Kong waters, which are dominated by coastal/oceanic water. During this period, low chl *a* concentrations were primarily attributed to strong vertical mixing, which can transport phytoplankton cells out of the euphotic zone (1% light depth) and reduce light availability for growth. This effect is possibly enhanced by strong winds and heavy navigation traffic in Victoria Harbour (Yin 2002). The resistance of a coastal ecosystem to increasing eutrophic conditions has also been observed in the Bay of Brest due to the high hydrodynamic mixing there (Le Pape et al. 1996).

In summer, during the period of highest rainfall and Pearl River discharge, there is clear spatial variability in the limiting factor for phytoplankton growth. In the western waters and Victoria Harbour, phytoplankton growth was primarily limited by a combination of rapid tidal flushing and possible light limitation, due to strong turbulent mixing, reducing the eutrophication impacts. By comparison, southern waters are frequently at the edge of the coastal plume during the period when algal blooms often occur, due to the stable stratification and the input of nutrients from the Pearl River estuary. As a result, phytoplankton growth is limited by P availability. At Port Shelter (eastern waters), where there is little influence from Pearl River discharge in summer, P or N + P becomes the primary limiting nutrient instead of N, due to the input of high rainfall with a high N:P ratio.

Our results provide information on phytoplankton resource limitation in the coastal waters of Hong Kong that can assist in the development of coastal sewage management strategies. Seasonal variations in the factors that regulate phytoplankton biomass represent an opportunity for temporary cost savings by using different seasonal management strategies for nutrient re-

moval. In summer, the removal of P should be the primary consideration in sewage treatment. The cost of P removal is much lower than the removal of N. In contrast, the Hong Kong coastal ecosystem is more resistant to eutrophication impacts in winter, due to the strong vertical turbulent mixing, and, hence, nutrient removal is less important during the winter period.

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