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亞熱帶水庫系統鹼性磷酸酶活性降尺度研究

Downscaling Alkaline Phosphatase Activity in a Subtropical Reservoir

曾于芳

Yu-Fang Tseng

指導教授：夏復國 博士

Advisor: Fuh-Kwo Shiah, Ph.D.

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本論文係**曾于芳**君（學號 **D93241005**）在國立臺灣大學海洋研究所完成之博士學位論文，於民國 100 年 7 月 14 日承下列考試委員審查通過及口試及格，特此證明。

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所 長：＿＿＿＿＿＿＿＿（簽名）

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摘 要

本論文首先藉由降尺度法 (群聚-族群-細胞-生理層次) 於 2006 至 2009 年進行亞熱帶翡翠水庫系統浮游生物缺磷指標 - 鹼性磷酸酶活性之系統性分析，以探討水體中浮游生物之缺磷狀態。野外調查結果顯示，水庫上層水體 (0~20 m) 有高的鹼性磷酸酶活性 (1.6~95.2 nM h^{-1}) ，且顯示明顯的季節性變化，推測此系統浮游生物呈現季節性缺磷狀態。多元迴歸分析結果顯示，混合層深度 (即水體磷酸鹽可利用度指標) 為主要影響水體鹼性磷酸酶活性季節性變動的調控因子。利用分層過濾探討不同浮游生物階層對水體鹼性磷酸酶活性貢獻度發現，磷酸酶主要來源為超微型浮游生物。進一步利用螢光標定酵素分析法輔以螢光顯微鏡觀察發現，細菌為主要鹼性磷酸酶的供應者，推測在亞熱帶磷缺乏系統中，異營性細菌對於整體磷循環扮演相當重要的角色。光強度佐以營養鹽操控實驗結果顯示，此系統中超微浮游生物的生長機制受不同環境因子調控，其中細菌生長主要受到磷酸鹽調控，而超微藍綠藻生長則主要受到光的調控。另外，操控實驗結果亦證實在磷缺乏系統中，高的光照強度會刺激超微藍綠藻的生長，甚而勝過細菌的生長，推測光強度為影響超微藍綠藻與細菌競爭磷酸鹽的主要決定因子。此外本論文首先觀察到夏季強烈颱風事件為影響水體鹼性磷酸酶活性年間變異的主要調控因子，證實強烈擾動事件 (颱風與強烈降雨) 對水體所造成的物理化學因子變動，將直接影響水體浮游生物生理缺磷狀態。未來研究將著重於探討亞熱帶磷缺乏系統中，颱風事件的強度與頻度對水體鹼性磷酸酶活性表現的調控機制，以探討強烈水體擾動伴隨營養鹽注入對於浮游生物缺磷程度的影響。

關鍵字: 鹼性磷酸酶、磷缺乏、超微型浮游生物、颱風事件、翡翠水庫。

Abstract

This dissertation was conducted by downscaling study to understand phosphorus (P)-deficient status of different plankton and the role of alkaline phosphatase activity (APA) in subtropical Feitsui Reservoir. Results from field survey showed that bulk APA (1.6~95.2 nM h^{-1}) was widely observed in the epilimnion (0~20 m) with an apparent seasonal variations, suggesting that plankton in the system were subjected to P-deficient seasonally. Mixed layer depth (an index of phosphate availability) is the major factor influencing the variation of bulk APA and specific APA (124~1,253 nmol mg C^{-1} h^{-1}), based on multiple linear regression analysis. Size-fractionated APA assays showed that picoplankton (size 0.2~3 μm) contributed most of the bulk APA in the system. In addition, single-cell APA detected by enzyme-labeled fluorescence (ELF) assay indicated that heterotrophic bacteria are the major contributors of APA. Thus, we can infer that bacteria play an important role in accelerating P-cycle within P-deficient systems. Light/nutrient manipulation bioassays showed that bacterial growth was directly controlled by phosphate, while picocyanobacterial growth is controlled by light and can out-compete bacteria under P-limited condition with the aid of light. Further analysis revealed that the strength of summer typhoon is a factor responsible for the inter-annual variability of bulk and specific APA. APA study demonstrated the episodic events (e.g. strong typhoon and extreme precipitation) had significant influence on APA variability in sub-tropical to tropical aquatic ecosystems. Hence, the results herein will allow future studies on monitoring typhoon disturbance (intensity and frequency) as well as the APA of plankton during summer-to-autumn in subtropical systems.

Key words: alkaline phosphatase activity, phosphorus deficiency, picoplankton, typhoon event, Feitsui Reservoir.

Table of Contents

Table List

Figure List

Chapter 1

Introduction

Phosphorus (P) is an essential element for all living organisms (for a review, see Karl 2000). A growing amount of research has indicated that P plays an important role in controlling plankton growth and production in aquatic environments, especially in freshwater systems (Schindler 1977, Coveney & Wetzel 1992, Hudson *et al.* 2000, Guildford *et al.* 2005). Among various forms of P in water, orthophosphate (*i.e.* phosphate) is the preferred form for microbial growth (Cotner & Wetzel 1992). However, ambient phosphate concentrations in freshwater systems are often low and insufficient to satisfy plankton demand (Hudson *et al.* 2000, Karl 2000). This perceptible lack of phosphate has encouraged research to focus on the role of dissolved organic phosphorus (DOP) playing in the biogeochemical cycles of aquatic ecosystems.

In oligotrophic freshwater systems, DOP concentrations generally exceed phosphate concentrations and comprise a significant proportion of total phosphorus (TP). For instance, Karl & Yanagi (1997) observed that phosphate constituted <25% of the TP in an oligotrophic system, and the remaining 75% occurred in the forms of DOP and inorganic polyphosphate compounds. Consequently, the cycling of DOP could potentially control P-availability, and affect biomass and production of natural plankton communities (Sebastian *et al.* 2004). This has led to an emphasis on the study of extracellular phosphatase activity due to its important roles in regulating P regeneration from DOP and in increasing the concentration of bio-available P.

Among extracellular phosphatase groups, alkaline phosphatase (APase) is considered as the most important enzyme in controlling DOP degradation (Cotner & Wetzel 1992, Huang & Hong 1999, Labry *et al.* 2005, Dyhrman & Ruttenberg 2006, Ivancic *et al.* 2009). A brief diagram of the ecological importance of APase in aquatic ecosystems is shown in Fig. 1.1. Numerous literatures indicated that when ambient

phosphate is sparse, microorganisms can produce extracellular APase to hydrolyze DOP in compensating for their P-deficiency (Berman 1970, Jansson 1976, Pettersson 1980, Hashimoto *et al.* 1985, Chrost & Overbeck 1987, Istvanovics *et al.* 1992, Nausch 1998, Strojsova *et al.* 2003, Sebastian *et al.* 2004, Labry *et al.* 2005, Gao *et al.* 2006). Further, APase activity (APA) was found repressed when phosphate became available (Perry 1972, Elser & Kimmel 1985, Jamet *et al.* 1997, Dignum *et al.* 2004, Labry *et al.* 2005, Cao *et al.* 2010). In addition, the performance of APase was inversely correlated to extracellular and intracellular phosphate concentrations (Jansson *et al.* 1988, Dyhrman & Palenik 1999). Therefore, APA has been used in many aquatic studies as an indicator of P-deficiency of natural plankton populations (Jansson *et al.* 1988, Istvanovics *et al.* 1992, Kalinowska 1997, Rose & Axler 1998, Ammerman & Glover 2000, Hoppe 2003, Cao *et al.* 2005).

Feitsui Reservoir is an artificially build reservoir with a surface area of 10.24 km^2 and a mean depth of 39.6 m. The dam-site is the deepest place with a depth of ~100 m. As a major source of drinking water for mega Taipei City, Feitsui Reservoir has been less influenced by anthropogenic impact since 1976. According to the long-term records of Carlson's TSI (range from 38 to 46; Chou *et al.*, 2007), Feitsui Reservoir has been considered as a mesotrophic system. However, many investigations have indicated that this system has had high (>200) molar ratios of dissolved inorganic nitrogen to phosphorus (N/P ratios) all year round (Chang & Wen 1997). The unbalanced N/P ratios for microbial requirements (N/P ratio = 16 for phytoplankton and 9 for bacteria) (Redfield 1958, Chrzanowski *et al.* 1996, Vrede *et al.* 2002) and low concentrations of phosphate lead to an assumption that P is the limiting element for microbial growth in Feitsui Reservoir.

However, these cannot be used to explain the real nutrient status of plankton in Feitsui Reservoir for the following reasons: Firstly, the analytical determination of phosphate concentrations does not account for the organic form, which could be utilized by living plankton (Karl & Yanagi 1997, Baldwin 1998, Benitez-Nelson 2000). Secondly, low phosphate concentrations do not necessarily represent deficiency under fast regeneration and efficient utilization (Hudson *et al.* 2000, Karl 2000). APA method is one of direct determinations of the P-status of plankton community since it is a physiological phenomenon induced or repressed by extracellular or intracellular phosphate pools (Chrost & Overbeck 1987). In another word, APA is the method portraying more details about the bio-cycling of DOP in aquatic ecosystems.

Many APA methods have been used (Perry 1972, Hoppe 1983, Scanlan & Wilson 1999, Ammerman & Glover 2000, Sebastian & Niell 2004). Among them, the fluorometric technique (Perry 1972, Gonzalez-Gil *et al.* 1998) of using artificial soluble organic phosphate substrates (3-0-methylfluorescein phosphate; 3-0-MFP) has been the most popular one.

Bulk APA assay reveals the total amount of enzyme being produced by microorganisms in a sample (Istvanovics *et al.* 1992, Newman & Reddy 1993, Gao *et al.* 2006), which provides general information about the P-status at the community level. However, bulk APA assay is not able to distinguish the activities of APase between particulate and dissolved fractions (Solorzano & Sharp 1980). Moreover, the assay is incapable to differentiate the sources of APA from different plankton, including phytoplankton, bacterioplankton, and zooplankton. Measuring APA by size-fractionation method may allow one to differentiate “loosely” the signals come from particles of different size and dissolved fraction. However, the applicability of this method could be

doubtful for plankton groups (*i.e.* picophytoplankton and bacterioplankton) that are in the same size range.

To overcome the problems mentioned above, a single-cell Enzyme-Labeled Fluorescence (*i.e.* ELF) method has been developed to detect the P-status information at the individual taxon level. It involves an addition of a phosphomonoesters substrate (ELF-97 phosphatase substrate; Molecular Probes) to samples; however, instead of releasing soluble products to the medium, fluorescent precipitates only forms at the site where APase hydrolysis occurred, which gives this method a high cell-specific capacity. The ELF method has been applied widely to the studies of natural plankton communities (Rengefors *et al.* 2003, Strojsova *et al.* 2003, Lomas *et al.* 2004, Ranhofer *et al.* 2009, Cao *et al.* 2010), and P-status between classes or even between individual cells of the same species can be determined (Dyhrman & Palenik 1999, Dyhrman *et al.* 2002).

Based on the introduction mentioned above, the P-status of different plankton and their roles in P-cycling were “systematically” (see the last paragraph) studied in a subtropical reservoir. This dissertation entitled “Downscaling Alkaline Phosphatase Activity in a Subtropical Reservoir” has six chapters in it, and organized as follow:

Chapter 1: Introduction

Chapter 2: Temporal variations of alkaline phosphatase activity in a subtropical reservoir

Chapter 3: Temporal variations of alkaline phosphatase activity in four size fractions in a subtropical reservoir

Chapter 4: A comparison of alkaline phosphatase activity of osmotrophs by Enzyme-Labeled Fluorescence (ELF) Method

Chapter 5: Light/nutrient effects on the osmotrophs behaviors in a subtropical reservoir

Chapter 6: Conclusions

The term “downscaling” means to study a phenomenon first at larger or broader scales, then its results are used as a boundary condition for the continuing study at smaller or narrower scales, and so on. The major purposes form Chapters 2 to 5 were in a hierarchy of this logic. The main purposes of Chapter 2 were to define bulk APA behavior in Feitsui Reservoir and to find out the controlling mechanisms for its seasonal and inert-annual variations. Using the finding of bulk APA as a boundary, the main purpose of Chapter 3 was to identify which size fraction of plankton APA determined bulk APA variation in general. And it turned out to be the pico-fraction (0.2~3 μm) which was composed mainly by osmotrophs. The ELF method was adopted in Chapter 4 to make out the relative contribution of osmotrophs to the APA of the pico-fraction. In Chapter 5, a series of light/nutrient manipulation experiments were performed to examine their effects on osmotrophs’ growth.

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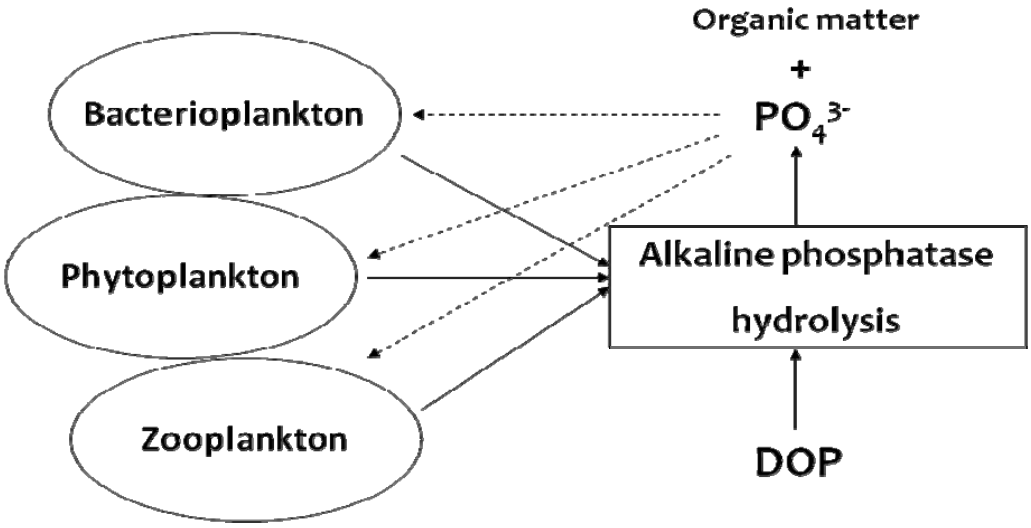

Fig. 1.1. Brief diagram shows the ecological importance of alkaline phosphatase (APase) in aquatic ecosystems. In phosphorus (P)-limited conditions, plankton (including bacterioplankton, phytoplankton, and zooplankton) could produce extracellular APase to hydrolyze dissolved organic phosphorus (DOP) and get additional phosphate (PO_4^{3-}) source in compensating for their P-deficiency. The figure was modified from the book "Enzymes in the Environment: Activity, Ecology, and Applications" (Chrost & Siuda 2002).

Chapter 2

Temporal Variations of Alkaline Phosphatase Activity in a Subtropical Reservoir

Abstract

Weekly to bi-weekly samplings of alkaline phosphate activity (APA) as well as related environmental variables were investigated in a deep (100 m depth) subtropical reservoir during the period of 2006~2009. Within the epilimnion (depth <20 m), integrated-averaged bulk APA (1.6~95.2 nM h^{-1}) and biomass normalized specific APA (124~1,253 nmol mgC^{-1} h^{-1}) varied obviously during the investigation period. Multiple linear regression analysis indicated that in the order of importance, mixed layer depth (MLD, 3~90 m, an index of phosphate availability), picocyanobacteria abundance ($0.3\sim3.7\times10^{11}$ cells m^{-3}), light intensity (0~102 mE m^{-2} d^{-1}), and soluble reactive phosphorus concentrations (<0.02~0.15 μM P) were the four major factors that accounted for 65% of the variation of bulk APA. As to specific APA, light intensity, MLD, and temperature (17.5~32.1℃) explained 66% of its variation. Further analysis depicted that the strength of summer typhoon was the factor responsible for the inter-annual variability of bulk and specific APA. The temperature responses of bulk and specific APA in the strong-typhoon-years (2007 & 2008) were significant, while those of the weak-typhoon-years (2006 & 2009) became either lower or insignificant. This highlighted the importance of episodic events (*e.g.* strong typhoon and extreme precipitation) in affecting the seasonal cycles of plankton APA in sub-tropical to tropical aquatic ecosystems.

2.1 Introduction

In phosphorus (P)-limited aquatic ecosystems, phosphate is usually in a concentration of nano molar, which is far lower than the detection limit of chemical (spectro-photometry) method (~20 nM; Parsons *et al.* 1984). Alternatively, extracellular enzyme activity has been adopted as an indicator for the responses of plankton communities to P-deficiency. In aquatic systems, plankton can utilize dissolved organic phosphate (DOP) as an alternative P-source to sustain their growth by producing extracellular alkaline phosphatase (*i.e.* APase) under P-limited conditions (Chapter 1, Fig. 1.1). Numerous researches have demonstrated that APase activity (*i.e.* APA) changed proportionally with the status of P-deficiency (Berman 1970, Jones 1972, Jansson 1976, Jansson *et al.* 1988b, Newman *et al.* 1994, Cao *et al.* 2010). This phenomenon has been named as the "induction-repression" mechanism (Jansson *et al.* 1988a). Accordingly, APA has been suggested as a good indicator for P-status in plankton communities (Healey & Hendzel 1980, Pettersson 1980, Istvanovics *et al.* 1992, Rose & Axler 1998, Ammerman & Glover 2000, Kahlert *et al.* 2002, Cao *et al.* 2005, Guildford *et al.* 2005, Gouvea *et al.* 2006, Strojsova & Vrba 2009).

The purposes of this study were: (1) to investigate seasonal variations of APA and its ecological relationships (controlling mechanisms) with other environmental factors, and (2) to examine summer typhoon impact on the inter-annual variations of APA, so that the regulation mechanisms of APA at seasonal and inter-annual scales could be explored. The physical measurements included temperature, light intensity, mixed layer depth; the chemical measurements included dissolved inorganic nitrogen and soluble reactive phosphorus concentrations; and the biological measurements included chlorophyll *a* concentrations and the abundances of picocyanobacteria and heterotrophic bacteria.

2.2 Materials and Methods

2.2.1 Study site and sampling

The study site (Feitsui Reservoir) locates in northern Taiwan (24°55'N, 121°35'E; Fig. 2.1) with an averaged surface area of 10.24 km^2 and a mean depth of 40 m. It is an artificially-build reservoir, and has served as the major source of drinking water for mega Taipei city. This reservoir (and its tributaries) has been well-protected from anthropogenic activities since 1976.

Weekly to biweekly sampling was conducted at the dam-site (depth ~100 m) from Jan 2006 to Dec 2009. The 5-Liter Go-Flo bottles were used for water sampling. The vertical sampling was conducted at 10 depths (0, 2, 5, 10, 15, 20, 30, 50, 70, and 90 m) from the surface to the near bottom manually. Conductivity-temperature-depth (CTD) and the sensors attached onto it were used to record the vertical structures of the measurements, which included temperature, photosynthetic available radiance (PAR), and chlorophyll fluorescence. Water samples stored in 20 L polycarbonate bottles were transported back to the laboratory within 2 hrs for the measurements listed below. Daily hydrographic data (reservoir water levels, water discharges, and precipitation data) were obtained from the web-site (www.feitsui.gov.tw) of Taipei Feitsui Reservoir Administration Bureau. The mixed layer depth (MLD) is defined as the depth at which its temperature is 0.5℃ lower than the surface (Levitus *et al.* 1982). Typhoon impact index was calculated as the product of daily maximum wind speed and daily maximum precipitation for each typhoon event.

2.2.2 Inorganic Nutrients

Water samples for nutrient analysis were filtered through 500℃ pre-combusted 47-mm GF/F filters under low (<100 mmHg) pressure. The filtrates were used for

nutrients analysis immediately. Nitrate, nitrite, and soluble reactive phosphorus (SRP) concentrations were determined following the methods of Parsons *et al* (1984) with a spectrophotometer (Shimadzu, UV-1201). Dissolved inorganic nitrogen (DIN) was the sum of nitrate and nitrite. In calculating dissolved N/P ratio, the SRP data below the detection limit (0.02 μM P) were not included. Total phosphorus (TP) concentrations of the surface water were obtained from Taipei Feitsui Reservoir Administration Bureau.

2.2.3 Chlorophyll *a* (Chl *a*)

Chl *a* concentrations were determined by the non-acidification fluorometric procedure of Welschmewer (1994). Water samples were filtered through 47-mm GF/F filters, the filters were extracted with 100% v/v acetone in the dark at -20℃ for 12~16 hrs. Fluorescence was measured using a fluorometer (Turner Designs, TD-700). Algal biomass in carbon (C) unit was determined with a C: Chl *a* factor of 50 gC gChl a^{-1} (Antia *et al.* 1963).

2.2.4 Abundance of picocyanobacteria (CYA) and heterotrophic bacteria (BA)

CYA and BA (<3 μm size fraction) were enumerated by flow cytometry (Partec CyFlow) quipped with a 15 mW, 488 nm argon laser, and the FloMax analysis software. CYA signals were identified by their signatures in a plot of red fluorescence versus orange fluorescence. For BA, samples were pre-diluted 10 times and then stained with SYBR Green (Molecular Probes; final concs., 2.5 μM) for 15 mins. BA signals were identified by their signatures in a plot of side scatter vs. green fluorescence. A solution of yellow-green 1 μm latex beads (~10^3 beads mL^{-1}; Polysciences) was used as the size indicator. Samples were run at speeds of 800~1200 particles s^{-1} until ~30000 counts were made. Bacteria biomass in C unit was converted by a conversion factor of 20 fg C $cell^{-1}$ (Lancelot & Billen 1984).

2.2.5 Alkaline phosphatase activity (APA)

Bulk APA was derived by a fluorometric assay using 3-0-methylfluorescein phosphate (3-0-MFP; Sigma) as the substrate (Perry 1972). Water samples were pre-filtered through a 100 μm nylon sieve to remove large zooplankton. Triplicate 6 mL subsamples were incubated with 750 μL of 3-0-MFP (final concs., 200 nM) in the dark at 25℃ for 1 hr. The fluorescence produced by the 3-0-methylfluorescein (3-0-MF; excitation, 435 nm; emission, 520 nm) was measured with a fluorometer (Turner Designs, TD-700). Calibration was performed with 3-0-MF standard solutions (Sigma) in the range 20~200 nM. Specific APA (nmol mg C^{-1} h^{-1}) was derived from the division of APA by the sum of the biomass (in C unit) of Chl *a* and bacteria. This was based on an assumption that phytoplankton (eukaryotic algae and cyanobacteria) and heterotrophic bacteria constituted the majority of plankton biomass.

2.2.6 Statistical analysis

The depth-integrated averages within epilimnion (upper 20 m) were acquired using trapezoidal method. This is because the signals of APA and many other measurements appeared mostly at a depth <20 m (see the Result section). Statistical analyses including linear correlation analysis, multiple linear regression analysis, one-way ANOVA, and ANCOVA were performed using the statistical software SPSS 12.0™.

2.3 Results

2.3.1 Physical environment

The depth contour of water temperature (15.9~32.1℃; Fig. 2.2A) revealed that the water column was well-mixed during winter (Dec~Feb of the next year). Stratification occurred during the period of early Apr to late Oct. Surface water temperature (17.5~32.1℃; Fig. 2.3A) varied seasonally with the coldest and warmest temperature recorded in Feb and Aug, respectively. Values of the mixing layer depth (MLD) ranged 3~90 m, with the shallowest and the deepest MLD in summer and winter, respectively (Fig. 2.3B). Weekly light intensity (0~102 mE m^{-2} d^{-1}; Fig. 2.3A) showed apparent seasonality but seemed varied more than that of surface temperature. Daily precipitation ranged from 0~406 mm (Fig. 2.3C) with lower values recorded in the dry seasons, which covered the period from Nov to Feb of the next year. Higher precipitations came from two sources, the summer evening thunder-showers and the rainfalls induced by typhoon (Fig. 2.3C).

2.3.2 Chemical variables

In the epiliminion, most of the individual SRP (<0.02~0.25 μM P; Fig. 2.2B) and depth-integrated averaged SRP (SRP_{DIA}; <0.02~0.15 μM P; Fig. 2.4B) concentrations were under detection limit (<0.02 μM P) during the stratified seasons except several spikes recorded during post-typhoon periods (Fig. 2.3C). Higher individual SRP and SRP_{DIA} concentrations occurred in the mixing seasons, especially in the winter. The temporal changes of the SRP_{DIA} concentrations in the hypolimnion (20~90 m) were higher than those recorded in the epilimnion (0~20 m) (Fig. 2.4B), but with similar trends (r = +0.60, p<0.01, n=126). Strong inter-annual variation of SRP_{DIA} in both stratified and mixing seasons was noted (Fig. 2.4B). For the stratified seasons, SRP_{DIA} concentrations in 2007 were very high, even higher than the winter-spring (Dec~May of

the next year) values recorded in 2008. SRP_{DIA} concentrations in the mixing season of 2007 were most undetectable.

During the investigation period, individual dissolved inorganic nitrogen (DIN, = nitrate + nitrite; 14~87 μM N; data not shown) concentrations were always detectable, even in the epilimnion during summer (Fig. 2.4A). In term of stoichiometry, DIN seemed to be much surplus to SRP. The molar ratios of N/P ranged from 230 to 2,995 molN $molP^{-1}$ with an average of 1,432±685 mol N $mol\ P^{-1}$ (Fig. 2.4C), which was about 100-fold greater than that of the Redfield ratio (N/P = 16; Redfield 1958). Total phosphorous (TP, = SRP + DOP) in the surface water ranged from 0.16 to 2.06 μM P with an average of 0.47±0.38 μM P (Fig. 2.5). SRP constituted ca. 8% of the TP.

2.3.3 Biological measurements

Vertical contours of Chl *a* (data not shown) indicated that phytoplankton biomass was restricted in the upper 20 m. Epilimnic depth-integrated averaged Chl *a* concentrations (Chl_{DIA}; Fig. 2.6A; range, 0.5~9.7 μg L^{-1}; mean, 2.4±1.2 μg L^{-1}) varied seasonally, and basically followed the trend of temperature (Table 2.1). In this system, the scale of spring bloom was less significant when compared with that of autumn, as evident by the very high Chl_{DIA} (>9 μg L^{-1}) recorded in Oct 2006. Vertical contour of picocyanobacteria (data not shown) indicated that they distributed mostly in the upper 20 m, and the abundance was higher in the surface water and then decreased with depth. Epilimnic depth-integrated average of picocyanobacteria abundance (CYA_{DIA}) ranged from 0.3 to 3.7×10^{11} cells m^{-3}, with an average of $1.5\pm0.6\times10^{11}$ cells m^{-3} (Fig. 2.6B). The values of CYA_{DIA} were positively correlated with Chl_{DIA} (Table 2.1), and higher CYA_{DIA} values were generally recorded in late autumn. CYA were the most abundant species of the phytoplankton community in Feitsui Reservoir. During 2006 and 2007, CYA on

average constituted 87±12% of the total algal cell counts (Fig. 2.7). Heterotrophic bacteria abundance (BA) was high in the upper 20 m, and then dwindled with depth (data not shown). In term of seasonal variation, depth-integrated average of BA (BA_{DIA}; Fig. 2.6B; range, 0.8~4.6×10^{12} cells m^{-3}; mean, 2.3±0.8×10^{12} cells m^{-3}) changed positively with temperature and CYA_{DIA} (Table 2.1). A negative correlation was observed for BA_{DIA} vs. MLD.

2.3.4 Seasonal and inter-annual analyses of bulk APA

Signals of bulk alkaline phosphatase activity (APA) were only observed in the upper 20 m, and then decreased significantly with depth (Fig. 2.2C). Epilimnic depth-averaged bulk APA (APA_{DIA}; Fig. 2.6C) varied ~100X with a range of 1.6~95.2 nM h^{-1}, and a mean of 40.4±21.5 nM h^{-1}. In the epilimnion, values of the biomass normalized bulk APA (*i.e.* specific APA_{DIA}; Fig. 2.6C) varied ~10X with a range of 124~1,253 nmol mgC^{-1} h^{-1}, and a mean of 391±207 nmol mgC^{-1} h^{-1}.

In term of seasonal variation, values of bulk APA_{DIA} were positively correlated with of the changes of temperature, light intensity, Chl_{DIA}, CYA_{DIA}, and BA_{DIA}; values of bulk APA_{DIA} were also negatively correlated with MLD, DIN_{DIA}, and SRP_{DIA} concentrations (Table 2.1). A closer examination indicated that the temperature response of bulk APA_{DIA} of each year were different (Fig. 2.8). The slope of bulk APA_{DIA} vs. temperature of 2009 was insignificant, while the slopes of the other 3 years were significant with values ranged 0.08~0.14. The slope of 2008 was significantly different from those of 2006 and 2007 (ANCOVA, $p<0.05$), while the latter two were not different from each other (ANCOVA, $p>0.05$). The relationships of specific APA_{DIA} to other variables were the same as those of bulk APA_{DIA} (Table 2.1).

Results of the multiple linear regression analysis indicated that 65% of the bulk

APA_{DIA} variability could be explained by the combination of light intensity, MLD, DIN_{DIA}, SRP_{DIA}, and CYA_{DIA} (Table 2.2). The relative importance (standardized regression coefficient, *i.e.* Beta weight) of these independent variables on bulk APA_{DIA} in order was -0.40 for MLD, 0.30 for CYA_{DIA}, -0.22 for DIN_{DIA}, 0.21 for light intensity, and -0.15 for SRP_{DIA}. The same procedure was performed on the data of each single year. The results showed apparent inter-annual variability of the independent variables in explaining the variation of bulk APA_{DIA}. In 2006, Chl_{DIA} and BA_{DIA} were the best factors for the changes of bulk APA_{DIA}. In 2007, the best combination switched to MLD only. In 2008, MLD and Chl_{DIA} were responsible for 72% of the variation. In 2009, light intensity and CYA_{DIA} explained 72% of the variation. Basically the analysis results of specific APA_{DIA} were the same as those of bulk APA_{DIA}. For the pooled data set, light (*Beta* = 0.45) and MLD (*Beta* = -0.41) were the two factors affecting the variability of specific APA_{DIA} most.

2.3.5 Typhoon impact

During the investigation period, a total of 18 typhoons had swept through the study site during summer periods (Fig. 2.3C). To test typhoon effects on the behaviors of bulk APA_{DIA} and specific APA_{DIA}, the data collected during the period of Jul~Sep were analyzed. Table 2.3 indicated that 2007 and 2008 could be categorized as the strong-typhoon years with typhoon impact indices of 12.2±9.2 and 13.1±6 $m^2\ S^{-1}$, respectively. On the other hand, typhoon impact indices of 2006 (4.3±2.4 $m^2\ S^{-1}$) and 2009 (2.8±3.3 $m^2\ S^{-1}$) were ~one-third of those in 2007 and 2008. Accordingly, 2006 and 2009 were considered as the weak-typhoon years. Physical parameters (*i.e.* temperature, light intensity, and MLD) showed no difference between strong- and weak-typhoon periods. SRP_{DIA} concentration (0.06±0.03 μM P) in 2007 was ~2-fold higher than those of the other years ($p < 0.01$). CYA_{DIA} value ($1.3±0.4 \times 10^{11}$ cells m^{-3})

recorded in 2008 (one of the strong-typhoon years) was significantly lower than the weakest typhoon year (2009; $1.9\pm0.3\times10^{11}$ cells m^{-3}). BA_{DIA} values of the strong-typhoon years ($3.2\sim3.4\times10^{12}$ cells m^{-3}) were higher than those of the weak-typhoon years ($2.2\sim2.7\times10^{12}$ cells m^{-3}). Bulk APA_{DIA} and specific APA_{DIA} showed no difference between the strong- and the weak-typhoon years. Correlation analysis of the pooled data set showed that bulk APA_{DIA} was positively correlated with Chl_{DIA} and CYA_{DIA}, and specific APA_{DIA} correlated positively with bulk APA_{DIA} (Table 2.4).

2.4 Discussion

In terms of physical structures, the study site is characterized with strong seasonality. The deepening of MLD (Fig. 2.3B) in the cold seasons apparently served as a regular source of inorganic nutrients for plankton growth in the epilimnion. The extremely high N/P ratios (1,432±685 molN $molP^{-1}$; Fig. 2.4C) recorded in the epilimnion indicted that many plankton (phytoplankton and bacterioplankton) were subjected to P-deficiency, especially during the warm and stratified seasons, as revealed by higher readings of bulk APA_{DIA} and specific APA_{DIA} (Fig. 2.6C). However, both enzymatic readings fluctuated greatly during the stratified seasons, implying that episodic event (*i.e.* typhoon) might also affect the SRP concentrations at the dam-site (Fig. 2B), and thus the behaviors of bulk APA_{DIA} and specific APA_{DIA} (more discussion below).

Bulk APA is an enzymatic reaction. Its expression in the field is subjected to physical, chemical (*i.e.* substrate availability), and biological regulations. Table 2.1 indicated that the values of bulk APA in this system as a whole could be affected by the changes of physical (temperature and light intensity), chemical (SRP concentrations and availability, *i.e.* MLD), and biological (Chl *a*, the abundances of picocyanobacteria and bacteria) parameters. Multiple linear regression analysis (Table 2.2) indicated that MLD (*Beta* = -0.41) seemed affected the total variations (seasonal and inter-annual) of bulk APA_{DIA} more than the biomass of CYA (*Beta* = +0.30). Overall, it suggested that the changes of phosphate availability (as inferred from MLD) and pico-phytoplankton biomass were the two important factors in determining the seasonal and inter-annual variability of bulk APA in the epilimnion. After biomass normalization, MLD still was one of the essential factors responsible for the variation of specific APA_{DIA}.

Based on the negative correlation between phosphate concentrations and APA, many field and enclosure studies have concluded that phosphate supply could be one of the most important factors regulating APA (Chrost & Overbeck 1987, Siuda & Chrost 1987, Istvanovics *et al.* 1992, Zhou & Zhou 1997, Nausch 1998, Labry *et al.* 2005). High phosphate concentrations often repressed the synthesis rate of APase (Perry & Eppley 1981, Jamet *et al.* 1997, Kruskopf & Du Plessis 2004, Labry *et al.* 2005, Kim *et al.* 2007, Cao *et al.* 2010). An "induction-repression" mechanism of phosphate availability on APA has been proposed by Jansson *et al* (1988a). This study verified the negative relationship of SRP_{DIA} (consider as phosphate) concentration on bulk APA_{DIA} and specific APA_{DIA} (Table 2.1). However, it is further identified that the "availability" of phosphate that is the changes of the mixed layer depth, is more appropriate and representative than "concentration" itself in explaining the variations of bulk and specific APA (Table 2.2). Several studies suggested that the stoichiometry of inorganic nutrient (*i.e.* N/P ratios) might affect the expression of APA in the field. For instance, Petterson (1985) found that specific APA in oligotrophic Lake Erken (with dissolved inorganic N/P ratio varied from >1,200:1 in Apr to 8:1 in Sep) increased 10 fold during P-limited period (May~Jun) but decreased to undetectable during N-limited season (Sep). A identical phenomenon was also be found in Chesapeake Bay (Fisher *et al.* 1992). However, the results of analyses of correlation (Table 2.1) and multiple linear regression analysis (Table 2.2) indicated that this is not the case for the study site. Potential reason might be that DIN concentrations (14~87 μMN) and N/P ratios (1,432±685 mol N mol P^{-1}) were too high in this system, so that N-limitation could never occurred.

In addition to physical mixing processes and limiting-mineral availability, light intensity, through its effects on autotrophs, could be also important in regulating bulk

and specific APA. In fact, surface light intensity ranked 4th among the five most suitable variables for bulk APA, and ranked 1st among the four most suitable variables for specific APA (Table 2.2). Intuitively, light may enhance autotrophs' C-fixation rate and results in a higher demand of non-carbon materials (*e.g.* phosphate) simultaneously. An elevation of APA under higher light intensities eventually would be expected. It is well known that the physiological responses of heterotrophs including bacteria, are light-independent. Light might still affect bacterial APA indirectly because of the mineral-competition between picocyanobacteria and heterotrophic bacteria (*i.e.* osmotrophs) in many mineral-limited environments (Thingstad *et al.* 1993). In another word, it is suspected that light might have an additive (or even multiplicative) effect on either bulk or specific APA. Light effect on osmotrophs' APA behaviors will be specifically examined in Chapter 5.

Typhoon is a summer-to-autumn episodic event in the northern Hemisphere. On average, more than 20 typhoons were formed in the tropical Pacific Ocean each year, and 6~7 of them passed through Taiwan (data source, Taiwan Central Weather Bureau, www.tcwb.gov.tw). During typhoon events, free phosphate and particle-attached phosphate would be transported from up-stream and the tributaries down to the study site by hyper-pycnal flow formed at the depths of 40~80 m (Chen *et al.* 2006). The magnitude of the sub-surface SRP maximum (Fig. 2.2B) formed in the mid-waters reflected the strength of typhoon, and served important phosphate source for plankton grown in the epilimnion (Tseng *et al.* 2010). The strength of summer typhoons and thus phosphate supply could affect the seasonal trend of bulk APA. This was justified by the results of Fig. 2.8 indicating that the temperature responses (*i.e.* the slopes) of bulk APA_{DIA} were quite different among the four sampling years. This implies that the potential impact of episodic events (typhoon and extreme precipitation) can't be ignored

especially for systems located at typhoon prevailing areas. Recent studies indicated that the intensity (and frequency) of strong typhoon (Chan & Liu 2004, Webster *et al.* 2005, Wu *et al.* 2005) and extreme precipitation (Alexander *et al.* 2006, Kwon *et al.* 2007) might be enhanced under warming climate. Based on this line of reasoning, many mineral-limiting freshwater ecosystems in sub-tropical to tropical areas might become less P-deficit for plankton growth in summer.

In this system, DOP contributed >90% of total phosphate (TP = SRP +DOP; Fig. 2.5). DOP could serve as an additional source of P for plankton growth. The turn-over times of TP (=TP inventories/APA) estimated to be in the range of 0.2~11.8 d^{-1}, which were within the range (12~24 d^{-1}) reported by Labry *et al* (2005). The reported values of bulk APA (1~95 nM h^{-1}) of this study were comparable to the oligotrophic ecosystems (Table 2.5), such as the Red Sea (40~150 nM h^{-1}) and the Baltic Sea (40~160 nM h^{-1}).

Since bulk APA is a function of living biomass and specific APA (Bulk APA = biomass x specific APA), specific APA has been recognized as a better indicator for P-deficiency of plankton. In this study, specific APA was derived from the normalization of bulk APA by the biomasses of phytoplankton and bacteria, despite of the fact that all plankton groups or cells respond equally to P-stress (Rengefors *et al.* 2003, Lomas *et al.* 2004). Healey & Hendzel (1979), Pettersson (1980, 1985) and Gage & Gorham (1985) defined that a system would be in a status of “critical” and “severe” P-deficiency when the observed specific APA were in the range of 40~250 nmol mgC^{-1} h^{-1} and >250 nmol mgC^{-1} h^{-1}, respectively (Table 2.6). In this system, average values of the specific APA during the cold-mixing seasons were 158±138 nmol mgC^{-1} h^{-1}, implied that plankton were critically P-deficient. In warm-stratified seasons, plankton were facing severe P-deficiency since the averaged specific APA reached 391±207 nmol mgC^{-1} h^{-1}.

2.5 Conclusion

The study system was an oligotrophic environment where plankton were subjected to conditions of “critically phosphate-deficiency” and “severely phosphate-deficiency” during cold-mixing and warm-stratified seasons, respectively. Seasonal variations of bulk APA and biomass normalized APA were mainly controlled by the changes of phosphate availability (*i.e.* mixed layer depth) and light intensity. Typhoon strength in the summers accounted for the inter-annual variations of bulk and specific APA. Picocyanobacteria and heterotrophic bacteria were the two most abundant plankton in this system, their relative contributions to bulk APA is one of the important issues to be identified.

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Table 2.1. Linear correlation matrix of surface temperature (T), weekly-averaged light intensity (L), and the epilimnic (0~20m) depth-integrated averages ($_{DIA}$)[@] collected from the study site during the period of 2006~2009. All are significant at p <0.01.

Parameters	**T**	**L**	**MLD**	**DIN_{DIA}**	**SRP_{DIA}**	**N/P_{DIA}**	**Chl_{DIA}**	**Ln-CYA_{DIA}**	**Ln-BA_{DIA}**	**APA_{DIA}**	**$SAPA_{DIA}$**
Units	℃	mE m^{-2} d^{-1}	m	μM N	μM P	mol N mol P^{-1}	mg Chl m^{-3}	10^{11} cells m^{-3}	10^{12} cells m^{-3}	nM h^{-1}	nmol mg C^{-1} h^{-1}
T	1										
L	0.36	1									
MLD	-0.65	-0.44	1								
DIN_{DIA}	-0.32	-0.24	0.30	1							
SRP_{DIA}	-	-	-	0.25	1						
N/P_{DIA}	-	-	-	0.44	-0.64	1					
Chl_{DIA}	0.23	-	-	-	-	-	1				
Ln-CYA_{DIA}	-	-	-0.28#	-	-	-	0.39#	1			
Ln-BA_{DIA}	0.58#	-	-0.53#	-	-	-	-	0.41#	1		
APA_{DIA}	0.48	0.47	-0.68	-0.42	-0.25	-	0.27	0.47#	0.44#	1	
$SAPA_{DIA}$	0.24	0.61	-0.55	-0.32	-0.22	-	na	0.28#	na	0.77	1

@, T, L, MLD, DIN_{DIA}, SRP_{DIA}, N/P_{DIA}, Chl_{DIA}, CYA_{DIA}, BA_{DIA}, APA_{DIA}, and $SAPA_{DIA}$ indicated surface temperature, weekly-averaged light intensity, and epilimnic depth-integrated averages of mixed layer depth, dissolved inorganic nitrogen (nitrate + nitrite), soluble reactive phosphorus, ratio of DIN to SRP, chlorophyll *a*, picocyanobacteria abundance, bacteria abundance, alkaline phosphatase activity, and specific phosphatase activity, respectively. Ln-, natural-log transformed; #, power function fit. -, zero correlation.

Table 2.2. Multiple linear regression analysis of year-to-year and pooled APA and specific APA (SAPA) over other environmental factors[@]. Numerical indicated the standardized regression coefficient (Beta weight). R^2, coefficient of determination. na, not analyzed.

Year	**T**	**L**	**MLD**	**DIN_{DIA}**	**SRP_{DIA}**	**Chl_{DIA}**	**CYA_{DIA}**	**BA_{DIA}**	**R^2**
units	℃	mE m^{-2} d^{-1}	m	μM N	μM P	mgChl m^{-3}	10^{11} cells m^{-3}	10^{12} cells m^{-3}	
2006 APA	-	-	-	-	-	0.44	-	0.44	0.49
2007 APA	-	-	-0.66	-	-	-	-	-	0.65
2008 APA	-	-	-0.49	-	-	0.51	-	-	0.72
2009 APA	-	0.62	-	-	-	-	0.57	-	0.72
Pooled APA		0.21	-0.41	-0.22	-0.15	-	0.30	-	0.65
2006 SAPA	-	-	-	-	-	na	-	na	0.30
2007SAPA	-	-	-0.69	-	-	na	-	na	0.72
2008 SAPA	-	-	-0.58	-	-	na	na	na	0.62
2009 SAPA	-	0.52	-1.50	-	-	na	-	na	0.80
Pooled SAPA	-0.27	0.45	-0.40	-0.22	-	na	-	na	0.66

@, the same as Table 2.1.

Table 2.3. A year-to-year comparison of the averages (±SE)[#] of the parameters[@] collected during the typhoon season (Jul ~ Sep).

Parameters	Units	2006 (a)	2007 (b)	2008 (c)	2009 (d)
Typhoon index	$m^2\ s^{-1}$	4.3±0.9 (0)[bc]	12.2±3.8 (2)[ad]	13.1±6.5 (2)	2.8±1.6 (0)
Precipitation©	mm	1276	1658	1819	673
T	℃	29.3±0.5	29.6±0.4	29.4±0.4	29.3±0.3
L	$mE\ m^{-2}\ d^{-1}$	63±5	67±5	67±4	81±8[abc]
MLD	m	7±0.7	7±0.4	9±1.0	8±1.5
DIN_{DIA}	μM N	17±0.6[cd]	23±0.9[cd]	46±1.2	41±3.9
SRP_{DIA}	μM P	0.03±0.005	0.06±0.007[acd]	0.02±0.001	0.03±0.005
N/P_{DIA}	$mol\ N\ mol\ P^{-1}$	827±29[cd]	518±101[cd]	2316±79[abd]	1617±332[abc]
Chl_{DIA}	$mg\ m^{-3}$	3.3±0.2[b]	2.2±0.2	2.8±0.2	2.4±0.4
CYA_{DIA}	10^{11} cells m^{-3}	1.5±0.15	1.4±0.10	1.3±0.11[d]	1.9±0.13
BA_{DIA}	10^{12} cells m^{-3}	2.2±0.12[bc]	3.2±0.19	3.4±0.23	2.7±0.25[bc]
APA_{DIA}	$nM\ h^{-1}$	50.2±3.6	47.7±4.5	47.5±5.4	53.3±6.4
$SAPA_{DIA}$	$nmol\ mg\ C^{-1}\ h^{-1}$	212±26	243±15	203±17	302±46

@, the same at Table 2.1. #, numeric with superscript a, b, c, and d indicate it is different from those values of year 2006, 2007, 2008, and 2009 (ANOVA). The numeral in parenthesis indicated strong typhoon number.©, the sum of precipitation during typhoon event.

Table 2.4. Linear correlation matrix of surface temperature (T), weekly-averaged light intensity (L), and the epilimnic (0~20m) depth-integrated averages ($_{DIA}$)[@] collected from typhoon seasons (Jul~Sep) during the period of 2006~2009. All are significant at p <0.01.

Parameters	**T**	**L**	**MLD**	**DIN_{DIA}**	**SRP_{DIA}**	**N/P_{DIA}**	**Chl_{DIA}**	**Ln-CYA_{DIA}**	**Ln-BA_{DIA}**	**APA_{DIA}**	**$SAPA_{DIA}$**
	℃	mE m^{-2} d^{-1}	m	μM N	μM P	mol N mol P^{-1}	mg Chl m^{-3}	10^{11} cells m^{-3}	10^{12} cells m^{-3}	nM h^{-1}	nmol mg C^{-1} h^{-1}
T	1										
L	0.40	1									
MLD	-0.53	-	1								
DIN_{DIA}	-	-	-	1							
SRP_{DIA}	-	-	-	-0.44	1						
N/P_{DIA}	-	-	-	0.89	-0.71	1					
Chl_{DIA}	-	-	-	-	-0.39	-	1				
Ln-CYA_{DIA}	0.46[#]	-	-	-	-	-	-	1			
Ln-BA_{DIA}	0.41[#]	-	-	-	-	-	-	-	1		
APA_{DIA}	-	-	-	-	-	-	0.51	0.55[#]	-	1	
$SAPA_{DIA}$	-	-	-	-	-	-	na	na	-	0.61	1

@, the same as Table 2.1. Ln-, natural-log transformed; #, power function fit. -, zero correlation.

Table 2.5. A comparison of bulk alkaline phosphatase activity (APA; nM h^{-1}) derived from this and other aquatic ecosystems.

Systems	Trophic status	APA	References
Mediterranean Reservoir (Spain)	Eutrophic	500~3400	Nedoma *et al.* (2006)
Rimov Reservoir (Czech)	Eutrophic	7~727	Vrba *et al.* (1993)
Baltic Sea, Kiel Fjord	Mesotrophic	4~160	Hoppe (1986)
Red Sea	Oligotrophic	40~150	Li *et al.* (1998)
East China Sea	Meso to oligotrophic	<1~74	Huang *et al.* (2007)
Central North Pacific	Oligotrophic	<1~8	Perry (1972)
Subtropical Feitsui Reservoir	Oligotrophic	1~95	This study

Table 2.6. The level of specific alkaline phosphatase activity (SAPA) as an indicator for P-starvation in this and other studies. The definition of "constitutive" here means the specific APA always exists and is independent to external or internal phosphate concentration.

References	**Data type**	**Levels of SAPA (nmol mgC^{-1} h^{-1})**		
		constitutive	**Critical P-starvation**	**Severe P-starvation**
Healey & Hendzel (1979)	Algal cultures	<40	40~200	>200
Pettersson (1980, 1985)	Lake plankton	6~50	50~140	>140
Gage & Gorham (1985)	Lake plankton	<50	50~250	>250
FT reservoir				
Mixed seasons	Lake plankton	158±138 (critical P-starvation)		
Stratified seasons	Lake plankton	391±207 (severe P-starvation)		

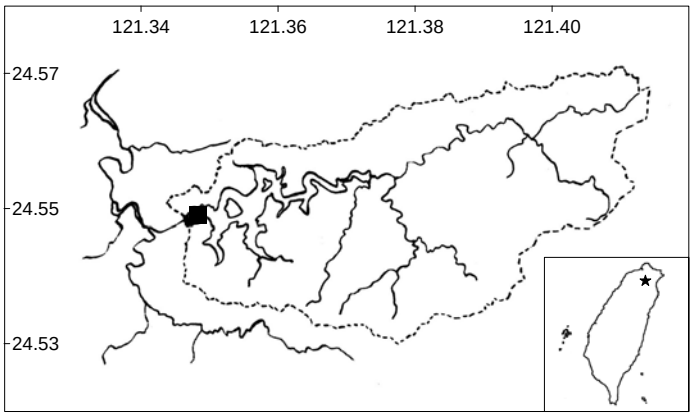

Fig. 2.1. Map of the Feitsui Reservoir showing the sampling site (the dam-site, ■).

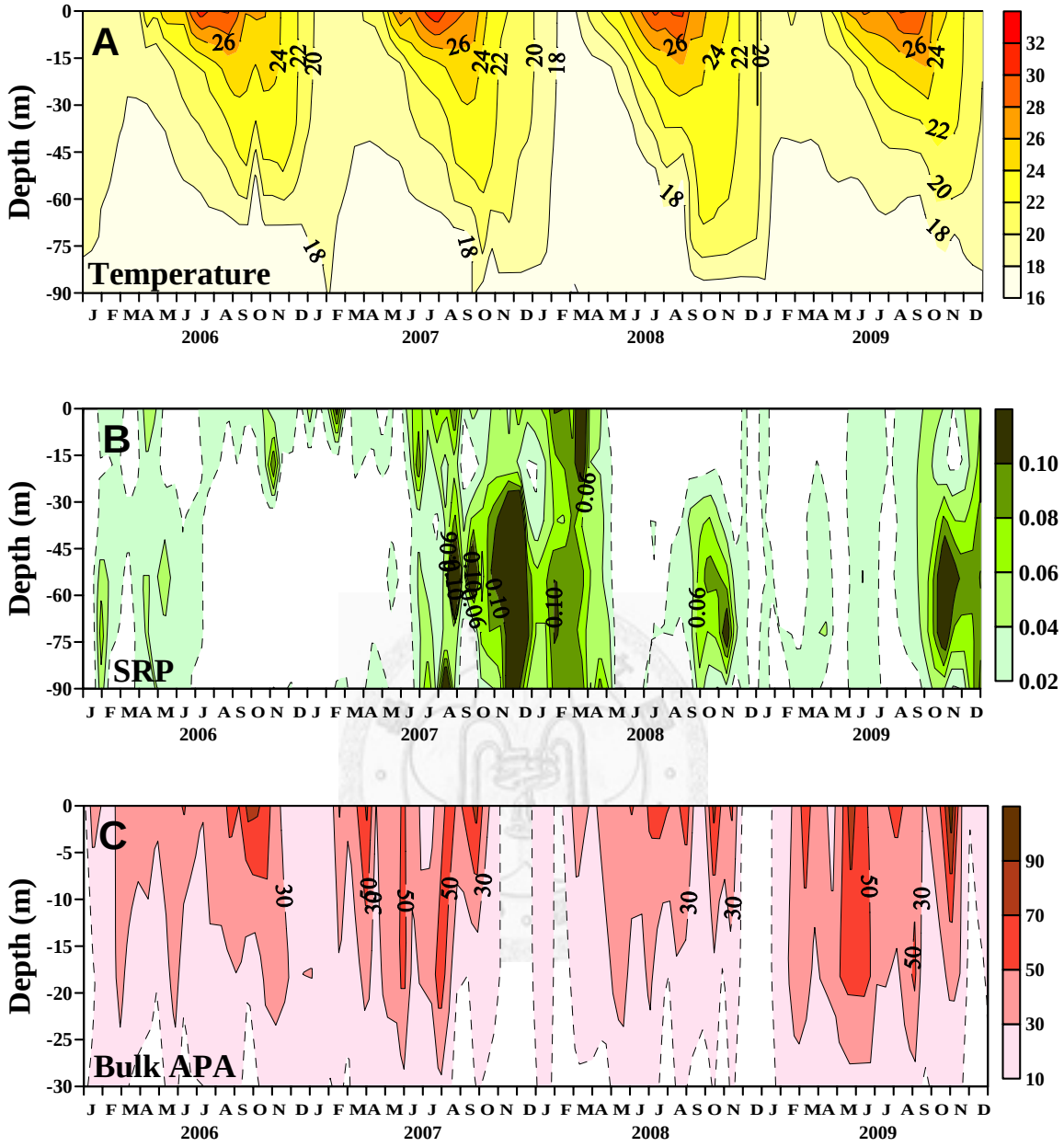

Fig. 2.2. Depth contours of (A) water temperature (℃), (B) soluble reactive phosphorus concentrations (SRP; μM P), and (C) bulk alkaline phosphatase activity (APA; nM h^{-1}).

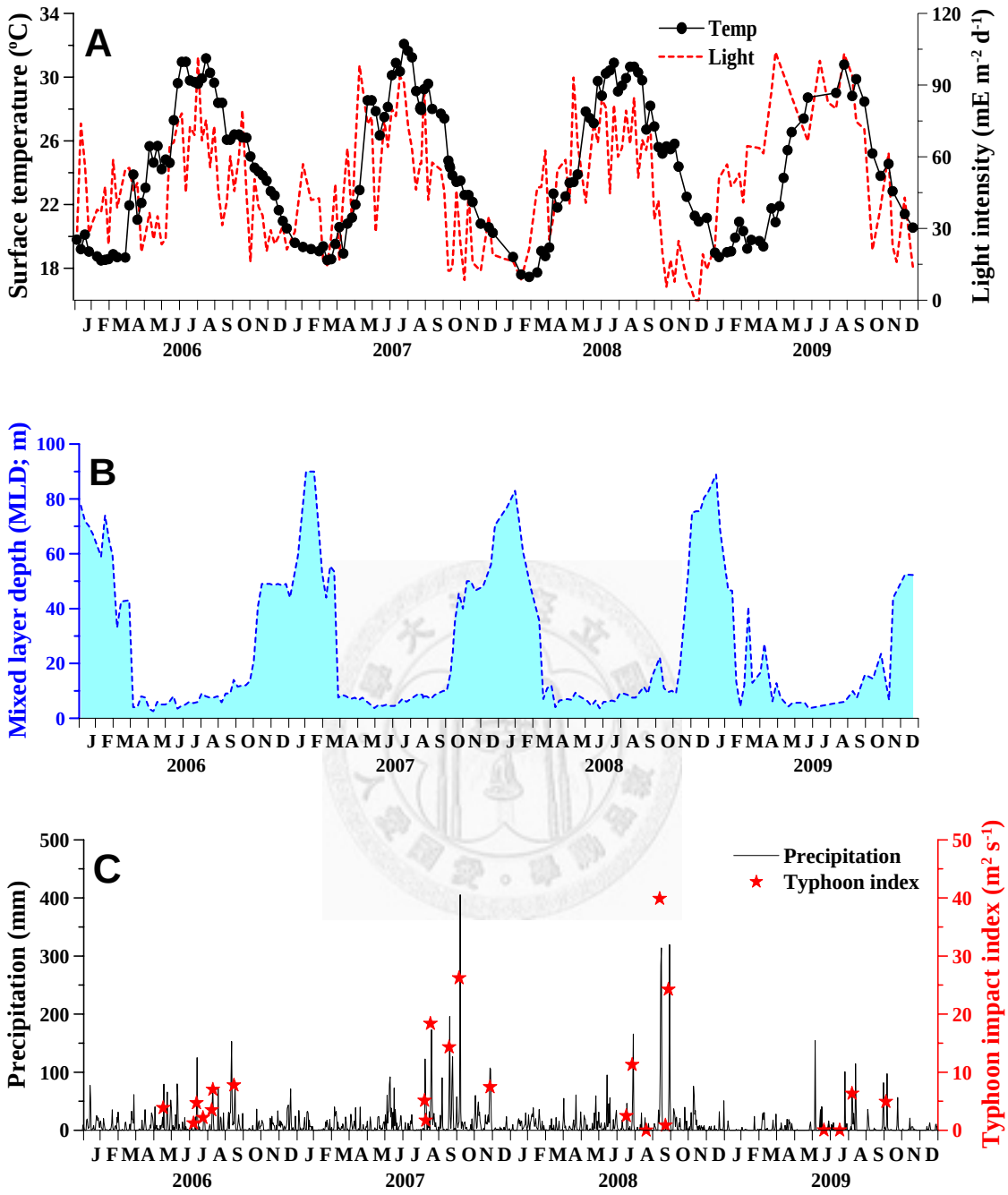

Fig. 2.3. Time series of (A) surface temperature and surface light intensity, (B) mixed layer depth, and (C) daily precipitation and typhoon impact index (★).

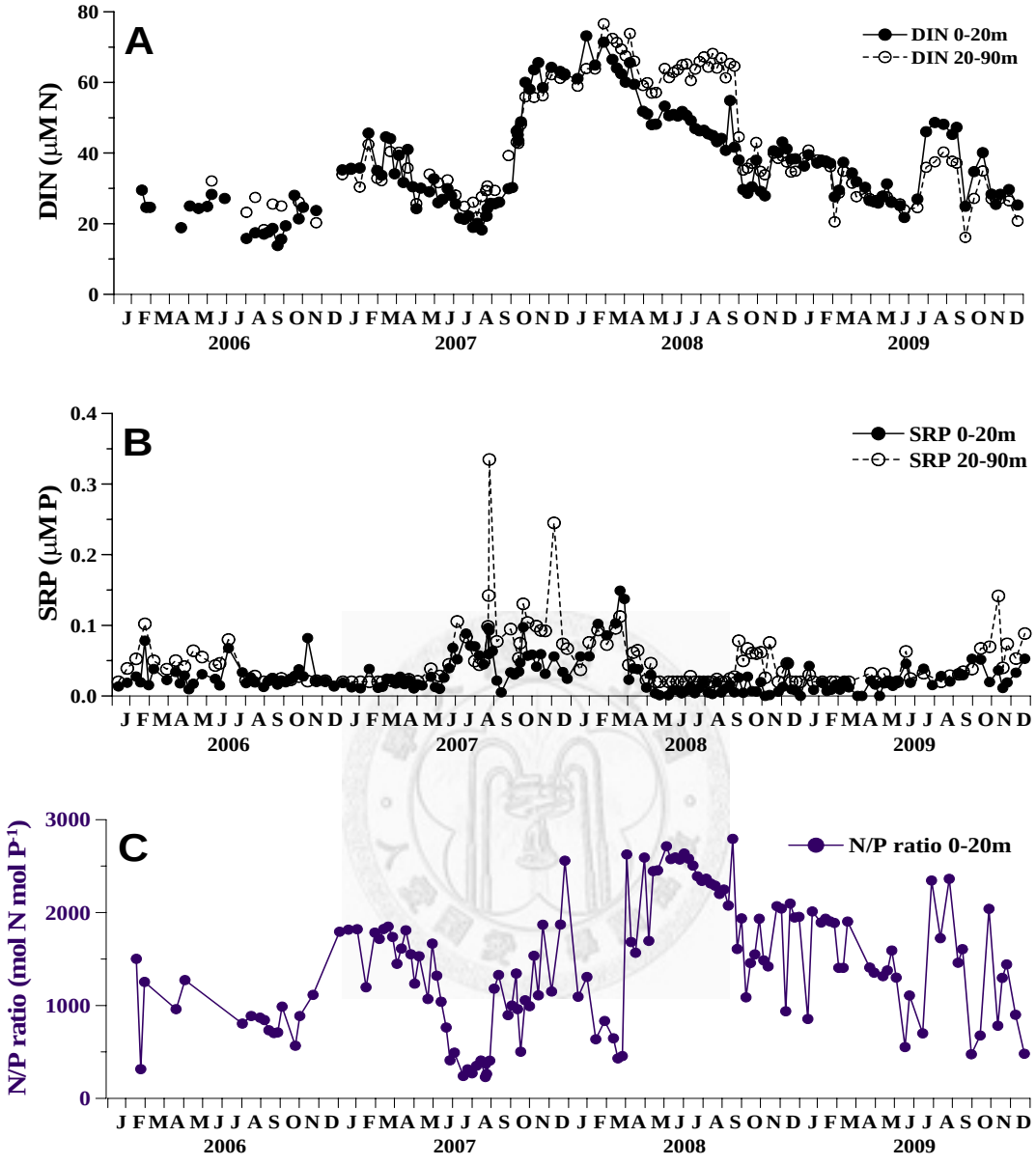

Fig. 2.4. Time series of epilimnic (0-20m) and hypolimnic (20-90m) depth-integrated averages of dissolved inorganic nitrogen (A; DIN), soluble reactive phosphorus concentrations (B; SRP), and epilimnion depth-integrated averages of the N/P ratios (C).

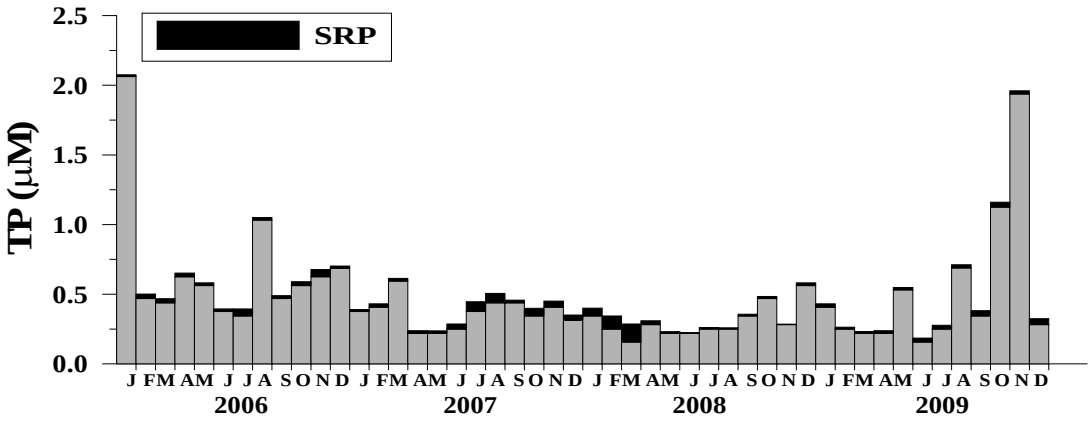

Fig. 2.5. Time series of total phosphate concentrations (TP) during 2006~2009. Data source from Taipei Feitsui Reservoir Administration Bureau.

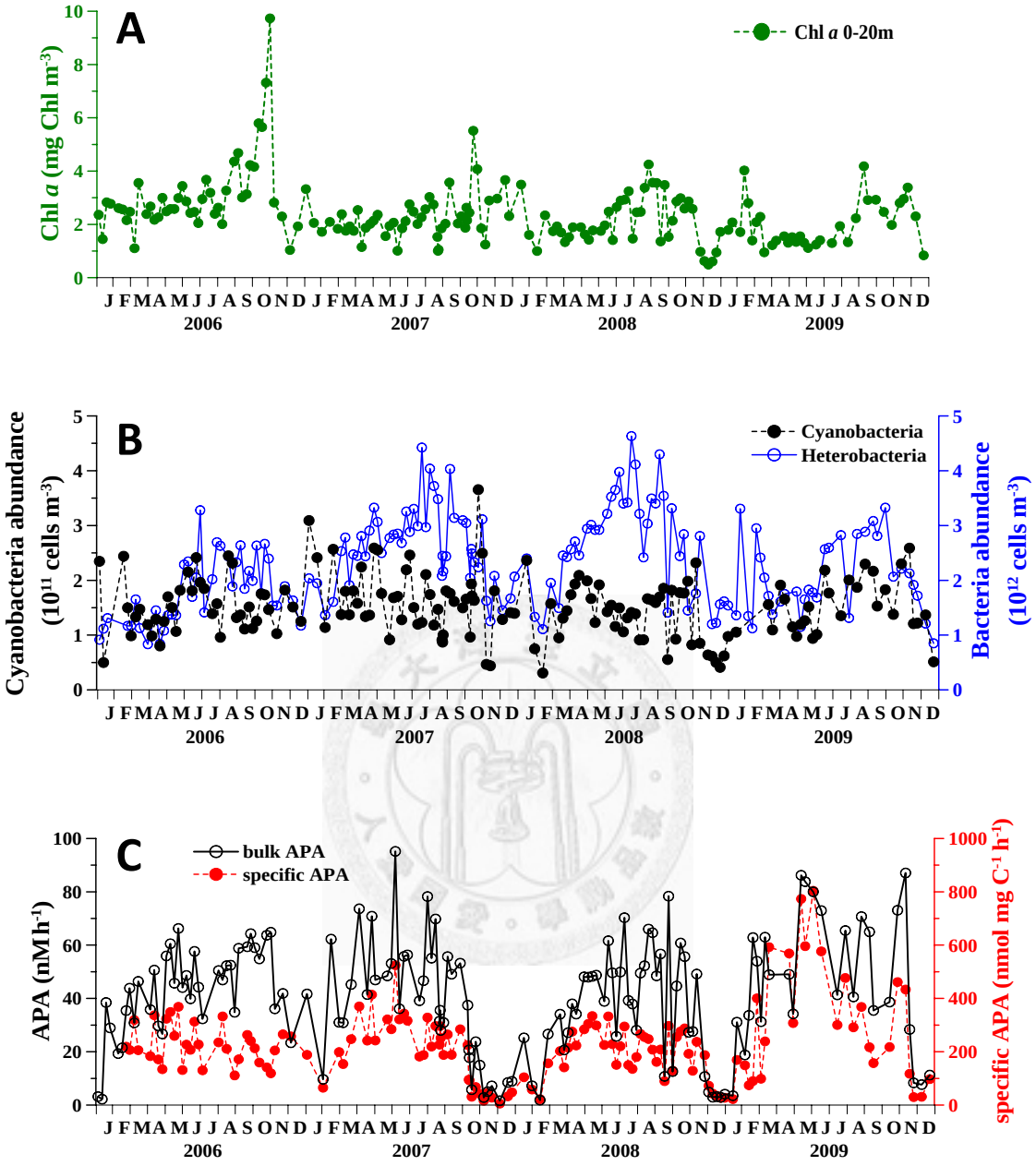

Fig. 2.6. The same as Fig. 2.5, but for epilimnic depth-integrated averages. (A) Chl *a* concentrations, (B) abundances of picocyanobacteria and bacteria, and (C) bulk APA and specific APA.

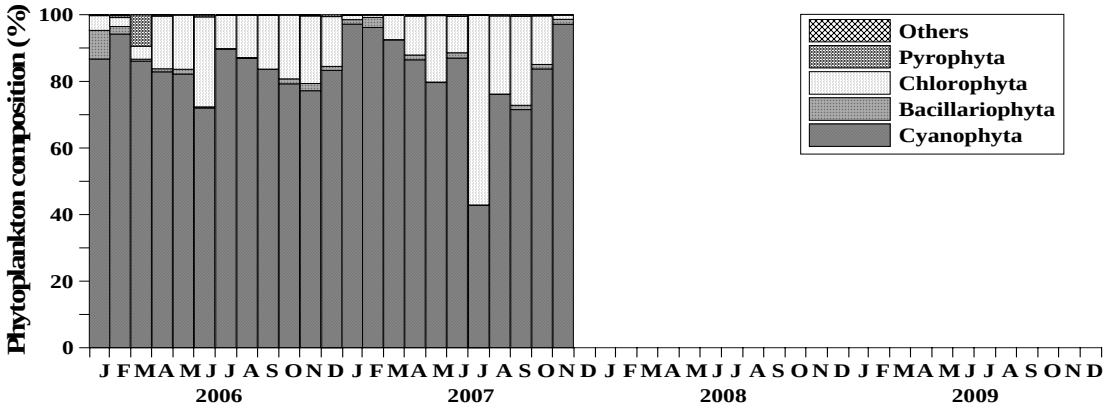

Fig. 2.7. Time series of phytoplankton composition percentage (%) during the period of Jan 2006~Nov 2007. Data source from the laboratory of Dr. Wo, Jiunn-Tzong, Biodiversity research center, Academia Sinica.

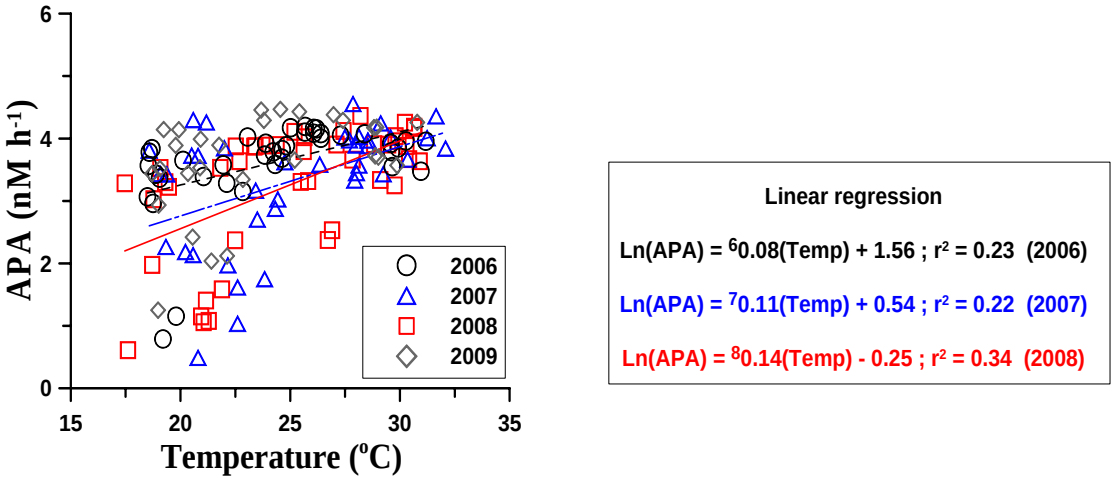

Fig. 2.8. Scatter plots of Ln transformed bulk alkaline phosphatase activity vs. temperature. Slopes were significant at p=0.05 level if shown. Superscripts 6~8 indicated slope of the given year was different from that of the other years by ANCOVA test (p<0.05).

Chapter 3

Temporal Variations of Alkaline Phosphatase Activity in Four Size Fractions in a Subtropical Reservoir

Abstract

Temporal variations of APA in three particulate fractions (0.2~3 μm, 3~10 μm, and 10~100 μm; named as APA_{pico}, APA_{nano}, and APA_{micro}, respectively) and dissolved fraction (<0.2 μm; APA_{dissol}) were studied in a subtropical reservoir from 2006 to 2009. The contribution of particulate fractions to bulk APA was high, varying 28~98% with a mean of 83±11%. Picoplankton APA (0.2~3 μm; APA_{pico}) was the major fraction (71±24%) of the particulate APA. 50% of the variation of bulk APA was determined by the changes of APA_{pico}. Multiple linear regression analysis indicated that APA (an index of P-deficient status of plankton) in different size fractions might be controlled by different mechanisms. APA_{pico} (an index of picoplankton P-deficiency) was related to the changes of mixed layer depth, light intensity, dissolved N/P ratio, and picocyanobacteria abundance. For APA_{nano} and APA_{micro} (indices of nanoplankton and microplankton P-deficiency), dissolved inorganic nitrogen was the major controlling factor. APA_{dissol} showed no relationship with any environmental factor.

3.1 Introduction

In Chapter 2, it was demonstrated that the plankton of the study-site were subjected to P-deficiency. Their seasonal growth was either "critically" or "severely" limited by P-availability. Because the plankton community is composed of living organisms with different sizes (picoplankton, nanoplankton, and microplankton…etc) and functions (autotrophy, heterotrophy, and mixotrophy…etc), it is of interest to know whether plankton in different size categories respond equally to P-deficiency. Studies indicated that APA of algal community were enhanced when extracellular or intracellular phosphate concentrations were low (Kruskopf & Du Plessis 2004, Ivancic *et al*. 2009), while repression of APA occurred under high phosphate concentrations (Robarts *et al*. 1998, Labry *et al*. 2005, Tanaka *et al*. 2006). In contrast, heterotrophic bacteria could maintain their high APA at high phosphate concentrations (Chrost & Overbeck 1987, Jamet *et al*. 1997). It is suspected that APA (an index of P-deficiency of plankton) in different size categories might respond differently at the same P-status.

Bulk APA consists of particulate and dissolved fractions. Phytoplankton, bacterioplankton, protozoans, and even zooplankton are the potential contributors of the former. The dissolved fraction may come from biogenic processes including release/excretion of cell surface phosphatases and zooplankton grazing …etc (Jansson *et al*. 1988). The origins of APA in aquatic ecosystems could be quite diverse, and have not been fully identified. For instance, the APA in mesotrophic Lake Erken of Sweden was mainly particulate (Pettersson 1980), while in oligotrophic Michigan lakes, >50% of the bulk APA was dissolved (Stewart & Wetzel 1982). Furthermore, it has been suggested that APA could be a function of plankton composition (Chrost & Overbeck 1987, Vrba *et al*. 1993, Dyhrman & Ruttenberg 2006, Cao *et al*. 2010), and/or related to the trophic status of lakes (Olsson 1990). Berman (1970) found that phytoplankton

(dinoflagellate) were the significant contributors to bulk APA during bloom events occurred in Lake Kinneret, Israel. Whereas Stewart and Wetzel (1982) attributed all APA to bacteria. Seasonality effects have been reported by Chrost & Overbeck (1987) showing that ~50% of bulk APA could be attributed to phytoplankton during the period of spring to autumn, while bacteria contributed 45% of bulk APA in winter.

In this study, temporal variations of APA in four size fractions namely dissolved (<0.2 μm), pico (0.2~3 μm), nano (3~10 μm), and micro (10~100 μm) were identified. The relationships of these four components with environmental factors and their relative contributions to bulk APA were analyzed.

3.2 Materials and methods

3.2.1 Study site and sampling

The geographical properties of the study site (Feitsui Reservoir), sampling frequency and period at the dam-site were described in Chapter 2.

3.2.2 Physical and chemical factors

The methods for physical (temperature, light intensity, and MLD), chemical (DIN and SRP), and biological (abundance of picocyanobacteria and bacteria) factors determination can be inferred from Chapter 2.

3.2.3 Size-fractionation of chlorophyll *a*

Water samples from 2 m depth of the dam-site were filtered sequentially through 10-μm, 3-μm, and 0.2-μm polycarbonate filters (dia., 47-mm). Duplicate filters of each size fraction were used for chlorophyll *a* determination using a fluorometer (Turner Designs, TD-700). See also Chapter 2 for details.

3.2.4 Size-fractionation of alkaline phosphatase activity (APA)

Water samples at the five depths (0, 2, 5, 10, 15, and 20 m) within the epilimnion were pre-filtered through a 100-μm mesh to remove larger organisms. The filtrates then were filtered sequentially through 10-μm, 3-μm, and 0.2-μm polycarbonate filters under low pressure (<100 mmHg). Alkaline phosphatase activity (APA) derived from the 10~100 μm, 3~10 μm, 0.2~3 μm, and <0.2 μm filtrate fractions were defined as microplankton APA (APA_{micro}), nanoplankton APA (APA_{nano}), picoplankton APA (APA_{pico}), and dissolved APA (APA_{dissol}), respectively. Bulk APA was the sum of the four above mentioned size fractions. Triplicate APA measurement was performed immediately after samples collection. See also Chapter 2 for the details of the APA method.

3.2.5 Statistical analysis

Statistical analyses including linear correlation analysis, multiple linear regression analysis, and one-way ANOVA were performed using the statistical software SPSS 12.0™.

3.3 Results

3.3.1 Temporal variations of size-fractionated Chl *a* and APA

Temporal variations of physical, chemical, and biological measurements were shown in Chapter 2. Bulk chlorophyll *a* (Chl *a*) concentrations (Fig. 3.1; range, 0.5~9.7 μg L^{-1}; mean, 2.4±1.2 μg L^{-1}) varied seasonally, and basically followed the trend of temperature (Table 2.1, r = 0.23, n = 171, p <0.01). Spring blooms hardly occurred while autumn blooms were significant as indicated by the high Chl *a* concentrations (>6 μg L^{-1}) recorded in Oct 2006 and Oct 2007. In term of relative contribution, pico-phytoplankton were the major contributor of the bulk Chl *a* in winter and spring (*i.e.* Chl_{pico}, ranged 30~68%, mean 55±14%), and then decreased in summer and autumn (ranged 22~46%, mean 33±11%). In autumn, bulk Chl *a* was dominated by nano- and micro-phytoplankton (*i.e.* Chl_{nano} and Chl_{micro}), together they contributed 55~77% of the bulk Chl *a*, with a mean of 67±10%. Overall, phytoplankton biomass in cold seasons (winter and spring) was dominated by pico-phytoplankton. The system turned to be nano- and micro-phytoplankton dominated during warm seasons (summer and autumn).

Seasonal patterns of APA values of the four different size fractions were not clear except APA_{pico}, which showed a positive and a negative correlation with light and mixed layer depth, respectively (Table 3.1). Noticeable inter-annual difference was observed between the first two years (2006 and 2007) and the last two years (2008 and 2009), with a clear cutoff occurring in Nov 2007 (Fig. 3.2, Table 3.3). During the first two years, APA_{micro} and APA_{nano} together contributed >40% to bulk APA, while APA_{pico} contributed 39±11% to bulk APA. During 2008 and 2009, three-fourth (74±11%) of bulk APA came from APA_{pico}, while APA_{micro} and APA_{nano} together constituted <5% of bulk APA. The contribution of APA_{dissol} to bulk APA seemed to be constant with an average of 19±8% (Fig. 3.2, Table 3.3). Further analysis (Table 3.2) indicated that APA values in

all fractions changed simultaneously with bulk APA. And that the changes of bulk APA was primarily driven by the variation of APA_{pico} values, which had the highest regression coefficient (*Beta* = 0.80) among the three effective fractions. More specifically, it said that ~45% [0.80 / (0.33+0.40+0.80+0.24)] of bulk APA variation was determined by the changes of APA_{pico}.

The results of multiple linear regression analysis (Table 3.1) indicated that APA_{dissol} was not related to any environmental factors. 26% of the APA_{micro} variability could be explained by the combination of total dissolved inorganic nitrogen (DIN) and total Chl *a*. The relative importance (Beta weight) of these two independent variables on APA_{micro} were -0.27 for DIN and 0.39 for Chl *a*. For APA_{nano}, the best-fit equation switched to DIN only, which explained 25% of the variation. The relative importance of environmental factors on APA_{pico} in order were -0.38 for mixed layer depth, 0.34 for light intensity, 0.23 for N/P ratio and 0.18 for CYA abundance. Together, these factors explained 51% of the variation. Bacterial abundance (and biomass) had no correlation with APA_{pico}. The variation of biomass normalized APA_{pico} ($SAPA_{pico}$) could be explained by a combination of light (*Beta* = 0.33), mixed layer depth (*Beta* = -0.31), and phosphate concentrations (*Beta* = -0.20).

3.4 Discussion

In aquatic systems, the origins of APA could be quite diverse, and the regulation mechanisms of APA could be different among plankton community with different size. Using the cascading filtration method, it was estimated that most APA in the study site was in particulate form (82±8%), and APA_{pico} accounted for the major portion (70±25%) of the particulate APA. The APA_{nano} and APA_{micro} to the bulk APA varied (0~87%) highly. The contribution of APA_{dissol} remained low (<20%). The proportions of size-fractionated APA in this study are comparable with ranges reported in the literatures. For instance, Pick (1987) found most particulate APA in Lake Ontario associated with <5 μm particles (Pico-fraction). Berman *et al*. (1970) also measured a wide range (<5~70%) of >20 μm (micro-fraction) particulate activity in oligotrophic Lake Kinneret of Israel. In addition, Newman and Reddy (1993) evaluated dissolved APA only contributed less than 10% of the bulk activity in a hypertrophic reservoir. Bulk APA co-varied with the four fractions except APA_{micro}, and its variation was majorly determined by APA_{pico} (Table 3.2). This indicated that plankton of smaller size could be more powerful than larger organisms in determining system's P-status. The same result was found in a subtropical system (Lil *et al*. 1998).

The results further indicated that the four APA size fractions might be regulated by different controlling mechanisms in this system (Table 3.1). Mixed layer depth (MLD), light intensity, N/P ratio, and picocyanobacteria abundance were the most four important factors in controlling the variation of APA_{pico}. However, for micro- and nano-fractions, DIN concentration was the most important factor controlling their variations. It is suggested that phosphate availability (as inferred from MLD) and light availability might alleviate and aggravate the P-stress of picoplankton, respectively. However, all three particulate fractions (APA_{micro}, APA_{nano}, and APA_{pico}) were more or

less independent of phosphate concentrations. This was in consistence with the finding by Jamet *et al*. (1997) showing that the APA was independent of phosphate concentrations in the oligomesotrophic Lake Pavin, France. According to the results, he suggested that in an extreme P-deficient system (phosphate concentrations were closed to detection limit), constitutive (always exist and active) and repressible APA could coexist and therefore a negative correlation between APA and phosphate concentrations might be diminish.

Nutrient status (*i.e.* the N/P ratio) caused dramatic shift of plankton community structure has been reported in many literatures (for review, see Wetzel, 2001). However, the relation between nutrient status and the composition of size-fractionated APA has rarely been discussed. This study demonstrated apparent inter-annual changes of APA in different size-fractions (Fig. 3.2 and Table 3.3). Before Nov 2007, APA_{pico} contributed ~50% to particulate APA, but such contribution increased up to 90% afterwards. This dramatically change was coincided with a shift of N/P ratio before and after Nov 2007 (Table 3.3). The dramatic increases of N/P ratios in 2008 and 2009 were due to higher DIN (Chapter 2, Fig. 2.4A) in these two years.

Several studies suggested that high DIN concentrations (and high N/P ratio) would make the system to be more P-deficit, which might enhance the activities of plankton APase (Feuillade *et al*. 1990, Ivancic *et al*. 2009, Liess *et al*. 2009). The inter-annual comparison (Table 3.3) indicated that the responses of different size fractions to N/P ratio seemed to be quite different. In the three particulate fractions, only APA_{pico} showed a positive trend with N/P ratio, while readings of APA_{nano} and APA_{micro} decreased under condition of higher DIN concentrations. The mechanism for such differential response is not clear.

3.5 Conclusion

This study demonstrated that picoplankton APA was the major portion and responsible for the variation of bulk APA. The controlling mechanisms of APA in different size fractions could be quite dissimilar. Light intensity and phosphate availability (inferred from MLD) were important for APA_{pico}, but had no effects on larger organisms. The negative reposes of APA in the micro- and nano-fractions were firstly reported, but the potential mechanisms were not clear.

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Table 3.1. The best-fit equations for alkaline phosphatase activity (APA) of the four size fractions and specific APA of pico-fraction ($SAPA_{pico}$) vs. environmental factors[@] by multiple linear regression analysis. Numerical represented Beta weight.

Parameters	**APA_{disso}** <0.2 μm	**APA_{micro}** 10~100 μm	**APA_{nano}** 3~10 μm	**APA_{pico}** 0.2~3 μm	**$SAPA_{pico}$** 0.2~3 μm
T	-	-	-	-	-
L	-	-	-	0.34**	0.33**
MLD	-	-	-	-0.38**	-0.31**
DIN	-	-0.27**	-0.50**	-	-
SRP	-	-	-	-	-0.20*
N:P ratio	-	-	-	0.23**	-
Chl *a*	-	0.39**	-	-	-
CYA	-	-	-	0.18**	-
BA	-	-	-	-	-
R^2	-	0.26	0.25	0.51	0.37

@, the same as Table 2.1. * and ** indicated p <0.05 and p <0.01 levels, respectively.

Table 3.2. Standardized regression coefficient (Beta weight) of alkaline phosphatase activity (APA) of the four size fractions vs. bulk APA with multiple linear regression analysis. All significant at p <0.01 level.

	APA_{micro} 10~100 μm	**APA_{nano}** 3~10 μm	**APA_{pico}** 0.2~3 μm	**APA_{dissol}** <0.2 μm	**R^2**
Beta weight	0.33	0.40	0.80	0.24	1

Table 3.3. A year-to-year comparison of the averages (±SE) [#] of the parameters[@] using one-way ANOVA analysis.

Parameters	**Units**	**2006 (a)**	**2007 (b)**	**2008 (c)**	**2009 (d)**
N:P ratio	molN $molP^{-1}$	948 ± 77^{cd}	1179 ± 89^{c}	1890 ± 105^{ab}	1450 ± 107^{a}
CYA	10^{11} cells m^{-3}	1.5±0.1	1.7±0.1	1.3±0.1	1.5±0.1
BA	10^{12} cells m^{-3}	1.8 ± 0.1^{bc}	2.6 ± 0.1^{ad}	2.7 ± 0.1^{ad}	2.0 ± 0.1^{bc}
Chl a_{micro}	%	40 ± 3^{d}	35 ± 2^{d}	38 ± 3^{d}	22 ± 2^{abc}
Chl a_{nano}	%	33 ± 2^{cd}	27 ± 2^{c}	17 ± 2^{ab}	21 ± 2^{a}
Chl a_{pico}	%	26 ± 2^{cd}	36 ± 3^{d}	45 ± 3^{a}	57 ± 3^{abc}
APA_{micro}	%	19 ± 3.6^{cd}	20 ± 2.8^{cd}	4 ± 0.4^{ab}	3 ± 0.5^{ab}
APA_{nano}	%	27 ± 2.1^{cd}	21 ± 2.0^{cd}	5 ± 0.5^{ab}	3 ± 0.5^{ab}
APA_{pico}	%	38 ± 2.3^{cd}	43 ± 2.4^{cd}	77 ± 1.3^{ab}	72 ± 3.1^{ab}
APA_{dissol}	%	16±1.6	17±1.4	14 ± 0.9^{d}	22 ± 3.3^{c}

#, superscripts a, b, c, and d indicated it is different from the value of year 2006, 2007, 2008, and 2009, respectively. @, the same as Table 2.1.

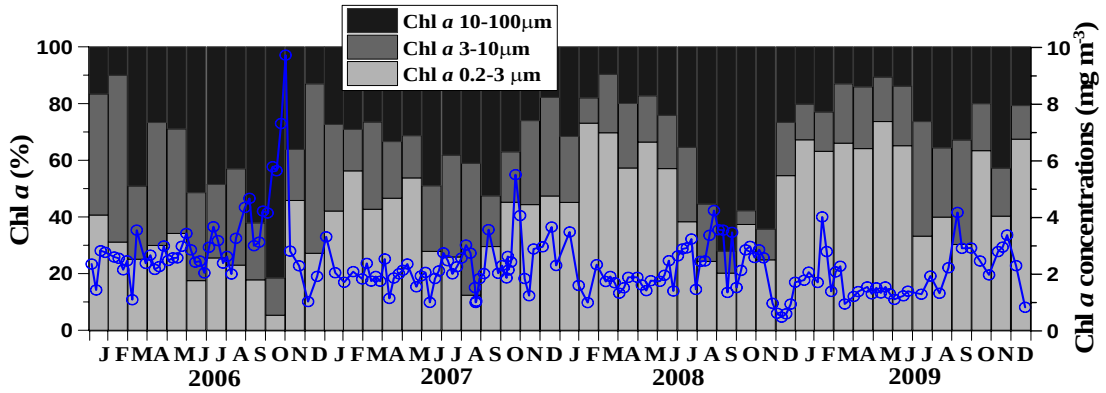


Fig.3.1. Seasonal variations of bulk chlorophyll *a* concentrations (blue open symbol) and the contribution percentage from the three size-fractions.

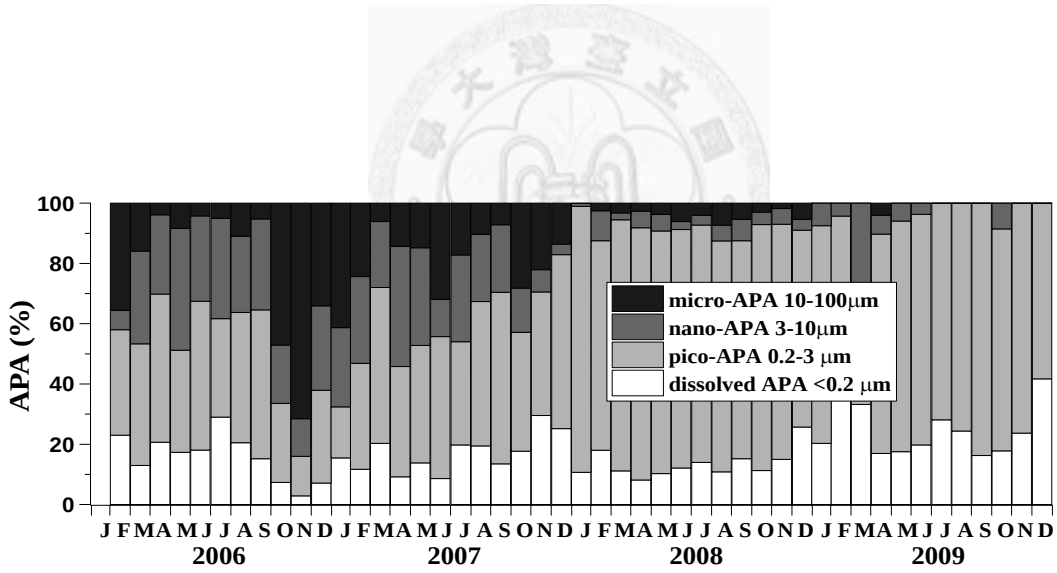
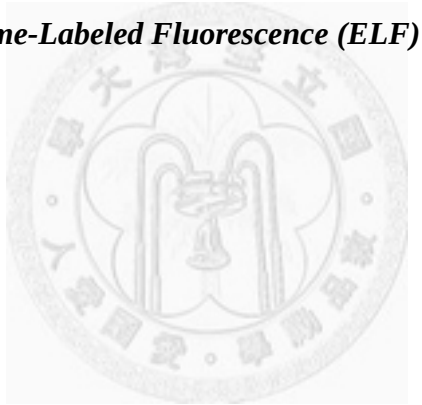


Fig. 3.2. Seasonal variations of the contribution percentage of bulk alkaline phosphatase activity (APA) from four different fractions.

Chapter 4

A comparison of Alkaline Phosphatase Activity of Osmotrophs by Enzyme-Labeled Fluorescence (ELF) Method



Abstract

Alkaline phosphatase activity (APA) of osmotrophs (*i.e.* picocyanobacteria and heterotrophic bacteria) in a sub-tropical reservoir were compared using enzyme-labeled fluorescence (ELF) method during the period of 2007~2008. Results showed that there was little or no (0~9%) ELF-labeling in picocyanobacteria during the investigation period, the rest of the ELF signals came from bacteria. In cold seasons, only 4~6% of the observed bacteria were ELF-labeled. However, 35~48% of the observed bacteria were ELF-labeled during warm and stratified periods. Correlation analysis suggested that the changes of APA in the pico (0.2~3μm) fraction was majorly determined by bacteria during the investigation. The variation of the percentage of ELF-labeled bacteria was controlled by phosphate availability as inferred from the negative relationship between the former and mixed layer depth. Meanwhile, the variation of ELF-labeled cyanobacteria was regulated by light availability as inferred from a negative relationship between the % of ELF-labeled picocyanobacteria and total suspended matters. Phosphate enrichment experiment showed significant reduction of ELF-labeled cell after the addition of phosphate.

4.1 Introduction

The results of Chapter 3 showed that picoplankton (0.2~3 μm) were the major contributors of bulk APA in the field. However, the differential filtration method revealed the APA associated with particles of different size. The method could not distinguish species-specific responses within plankton community (Dyhrman & Palenik 1999, Rengefors *et al*. 2001, Rengefors *et al*. 2003), nor could it discriminate the activities between picocyanobacteria and heterotrophic bacteria (*i.e.* bacteria) since they are within the same size fraction in many oligotrophic ecosystems.

Gonzalez-Gil *et al* (1998) developed a Enzyme-Labeled Fluorescence (*i.e.* ELF) method so that APA response at single cell level could be identified under microscope. Since then, this method had been adopted by many studies to determine the P-status of individual plankton species in aquatic systems (Dyhrman & Palenik 1999, Rengefors *et al*. 2001, Dyhrman *et al*. 2002, Strojsova *et al*. 2003, Lomas *et al*. 2004, Strojsova *et al*. 2005, Nicholson *et al*. 2006, Ou *et al*. 2006, Strojsova & Vrba 2006, Heil *et al*. 2007, Ranhofer *et al*. 2009). So far, the ELF technique has been successfully applied to phytoplankton (Rengefors *et al*. 2001, Nedoma *et al*. 2003, Rengefors *et al*. 2003, Cao *et al*. 2005, Nicholson *et al*. 2006, Cao *et al*. 2009) and bacteria (Labry *et al*. 2005, Nedoma & Vrba 2006, Van Wambeke *et al*. 2008) in both freshwater and marine samples.

The chemical principles and reactions for the ELF method are described below. Through phosphatase enzymatic hydrolysis, the water-soluble ELF-97 phosphate (ELFP) is converted to water-insoluble ELF-97 alcohol (ELFA; Appendix 4.1), which accumulates around the reaction sites of cell membrane and emits high yellow-green fluorescence under fluorescence microscope.

In this study, the single-cell ELF method was applied to study the P-status of picocyanobacteria and bacteria that are in the same size category. The major purpose was to identify the relative contribution of the osmotrophs to picoplankton APA in the field and the potential controlling mechanisms behind it.

4.2 Materials and Methods

4.2.1 Field survey

A total of 9 water samples were taken from the 5 m depth of the dam-site during the period of 2007~2008 (Table 4.1). Whole water samples were pre-filtrated through 3-μm GF/F filters to remove large-sized plankton. The 3-μm filtrates then were subjected to Enzyme-Labeled Fluorescence (ELF-97, see below) analysis.

4.2.2 Phosphate enrichment experiments

The experiment was carried out with the surface water (5 m depth) sample taken from the dam-site on June 11, 2008. The 3-μm filtrates were allocated to the control and phosphate-enriched (Final conc., ~100 nM P) treatments, and incubated for 2 hrs with the addition of ELF-97 phosphate (ELFP). Reactions were stopped by adding phosphate-buffered saline (PBS; pH 7.5; final conc., 10mM). Cell numbers of picoplankton and the signals of ELF-97 alcohol (ELFA) labeling were assessed.

4.2.3 ELF-97 method

The 3-μm filtrates were incubated in dark with pure ELFP substrate (E6588, Invitrogen; final conc., 20 μmol L^{-1}; (Duhamel *et al*. 2008)) at room temperature for 4 hrs. The reactions were stopped by the addition of PBS (pH 7.5; final concs., 10mM). The samples were filtered through 0.2-μm black polycarbonate filters and stained with DAPI (final conc., 2.5μg mL^{-1}) for 5 min in dark. Oil slides were made and subjected under an inverted fluorescence microscope (Zeiss, Axioskop) for microscopic examination. Three signals namely the autotrophic red fluorescence, the blue-fluorescent DAPI, and the yellow-green fluorescent ELFA precipitate could be seen under the microscope (Photo 4.1). A set of filters (Appendix 4.2) was used to separate these signals more accurately.

For image analysis, a Zeiss Axioskop inverted fluorescence microscopy equipped with a 200-W mercury arc lamp, a monochromatic digital integrating camera Axiocam MRm and a PC-based image analysis software was used. Picocyanobacteria (red signals), bacteria (blue signals), and ELF-labeling cell numbers (yellow-green signals) were identified. The cell was recorded as either negative (-ELF) or positive (+ELF) based on the absence or presence of the green fluorescent precipitate of each individual cell.

4.3 Results

4.3.1 Field study

In situ conditions of environmental factors during study period were listed in Table 4.1. Water temperatures were all >22℃. The periods of well-mixed (late Oct~Mar of the next year) and stratified (Apr~early Oct) could be identified by the changes of mixed layer depth (*i.e.* MLD, 4~40m). Surface photosynthetic available radiance (SPAR) varied 135~2314 μE m^{-2} s^{-1}, with the lowest and highest value recorded in Oct 2007 and Jun 2008, respectively. Total suspended matters (TSM) ranged 0.6~1.6 g m^{-3}, except two high readings (>4.3 g m^{-3}) appeared in Oct 2008. Total dissolved inorganic nitrogen (DIN, nitrate + nitrite) concentrations ranged from 30 to 66μM N, with lower values in Oct and higher values in early Apr. Soluble reactive phosphate (SRP) concentrations varied from undetectable in most of the stratified period to high values (0.06~0.1 μM P) in the well-mixed period.

Picoplankton chlorophyll *a* (Chl a_{pico}) concentrations were <1.2 mg Chl m^{-3} most of the time. A high value (2.5 mg Chl m^{-3}) was recorded in Oct 2007. Picoplankton alkaline phosphatase activity (APA_{pico}) showed apparent seasonal differences. Lower APA_{pico} values (1.4~9.8 nM h^{-1}) were recorded in the well-mixed period, while higher APA_{pico} values (22.5~52.8 nM h^{-1}) appeared in the stratified period. The ratios of APA_{pico} to bulk APA ($RAPA_{pico}$; 77~97%) indicated that on average, >80% of the bulk APA was contributed by APA_{pico}. Picocyanobacteria abundance (CYA) was in the range of 1.1~3.2 × 10^{11} cells m^{-3} with the highest value appeared in Oct 2007. Bacteria abundance (BA) ranged from 2.5 to 3.9 × 10^{12} cells ml^{-3} with the highest value appeared in Jun 2008.

4.3.2 ELF result

Within picocyanobacteria, the percentage of non ELF-labeled picocyanobacteria

(CYA_{-ELF}) was the major component, while those showing positive response (CYA_{+ELF}) constituted at most 9% of the total in the first 7 samples (Table 4.1). No signal of CYA_{+ELF} was observed in the last two samples in Oct 2008. For bacteria, the percentage of ELF-labeled bacteria (BA_{+ELF}) were quite low (0~9%) in the first 3 samples when the depth of mixing layer was >35m, and it increased up to ~40% in Apr 2008 and remained high afterwards during the stratified period (Table 4.1). Correlation analysis indicated that values of APA_{pico} were negatively correlated with SRP concentrations (Fig. 4.1A), but showed a positive response with BA_{+ELF} (Fig. 4.1B), which were also negatively correlated with MLD (Table 4.2). The readings of CYA_{+ELF} were negatively correlated with TSM.

4.3.3 Phosphate enrichment experiments

The percentage of ELF-labeled cells in the control and phosphate (Pi)-enriched treatments showed dramatic difference (Photo 4.2). After a 2hr-incubation, More than 30% of the cells grown in the control treatment were brightly ELF-labeled. A few (<1%) or no ELF-labeled cells were observed in the Pi-enriched treatments (Fig. 4.2).

4.4 Discussion

There are three major findings in this study. Firstly, the ratios of APA_{pico} to bulk APA ($RAPA_{pico}$ in Table 4.1) indicated that picoplankton (picocyanobacteria and bacteria) were the major contributor for bulk APA in this system. Secondly, ELF-positive bacteria were 6-fold of that of picocyanobacteria (Table 4.1), and might be the main factor driving the variation of APA_{pico} in the field (Table 4.2). And finally, the addition of phosphate inhibited the formation of ELF-positive cells.

Lomas *et al*. (2004) found that the expression of APA appeared to be more common in larger species (*i.e. Microcystis*) than in picocyanobacteria. In short-term enclosure experiments, Strojsova *et al*. (2008) documented that picocyanobacteria grew well in both Pi-depleted and Pi-enriched conditions, however cells in the latter treatment did not produce any APA. With the results derived from a 6-lakes survey, Cao *et al*. (2010) found that phytoplankton community was dominated by picocyanobacteria in the lakes with lower (soluble reactive) phosphate concentrations. However, none of them were ELF-positive. This and previous studies mentioned above all pointed out that picocyanobacteria APA was low or even none in low-P or P-deficient systems. The percentage of ELF-labeled picocyanobacteria showed a negative correlation with TSM (Table 4.2), implying that light availability but not intensity (SPAR) could be the major factor driving the expression of APA of picocyanobacteria in the field. We suggested that picocyanobacteria could produce more APA to obtain additional phosphate for satisfying their P-demand during growth phase.

Relative to picocyanobacteria, much fewer studies have paid attention to bacterial APA till recently. Bacteria could have higher P-demand than picocyanobacteria, but seemed to be easier suffered from P-limitation (Cotner & Wetzel 1992). It was clearly

demonstrated that bacterial P-demand was the major driving force of APA_{pico} in the field (Table 4.2). So a positive relationship between APA_{pico} and the abundance of ELF-labeled bacteria (Fig. 4.1B) was within expectation. The negative correlation of BA_{+ELF} vs. MLD (Table 4.2) further indicated that phosphate availability (as inferred from the changes of mixed layer depth) was the key factor in affecting bacterial APA behavior. It has been suggested that bacterial APA could be enhanced at condition when dissolved organic carbon (DOC) was limiting bacterial growth (Cotner & Wetzel 1992, Van Wambeke *et al*. 2008). This scenario was quite unlikely to be true in this system. Tseng *et al* (2010) confirmed that the DOC in the study-site was abundant, and most of it was labile. Therefore, DOC might never be a limiting factor for bacteria growth in this study. The reduction of ELF-labeled cells under Pi-enriched condition (Fig. 4.2) has been reported by Dyhrman & Palenik (1999), Dyhrman *et al* (2002), Labry *et al* (2005) and Dyhrman & Ruttenberb (2006).

The ELF-97 labeling method combined with microscopy seems to be convenient in identifying the P-deficient status at single-cell level. However, the observation would be interfered when the water samples were full of mucus secreted by phytoplankton, especially the *Microcystis* spp. which were abundant in summer in the study-site. It was difficult to separate the mucus by pre-filtration. To overcome the mucus problem, a new technique combing ELF-labeling with flow cytometry has been proposed by Telford *et al* (1999), Dignum *et al* (2004), Duhamel *et al* (2008), and Duhamel *et al* (2009). A higher resolution, a lower background and a much shorter operation time could be achieved. Unfortunately, this new method had not been aware during the study period.

4.5 Conclusion

During the investigation period, more than 80% of bulk APA could be ascribed to the osmotrophs. The amount of bacteria labeled by ELF was 6-fold of that of picocyanobacteria, indicating that the APA of the pico fraction was majorly determined by bacteria but not picocyanobacteria. The negative correlation between total suspended matters and percentage of ELF-labeled picocyanobacteria suggested higher light availability might induce picocyanobacteria APA expression, and thus their P-uptake capacity. Light effects on picocyanobacteria APA expression, rates of C-production and growth were explored in Chapter 5.

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Table 4.1. A list of the *in situ* ranges of environmental factors and Enzyme-Labeled Fluorescence (ELF) measurements[@] collected during the period of Oct 2007~Oct 2008.

Date	**T**	**SPAR**	**MLD**	**TSM**	**DIN**	**SRP**	**Chl a_{pico}**	**APA_{pico}**	**CYA**	**BA**	**$RAPA_{pico}$**	**CYA_{+ELF}**	**BA_{+ELF}**
Units	^{o}C	$\mu E\ m^{-2}\ s^{-1}$	m	$g\ m^{-3}$	μM N	μM P	mg Chl m^{-3}	$nM\ h^{-1}$	10^{11}cell m^{-3}	10^{12} cell m^{-3}	%	%	%
12-Oct-07	24.3	325	35	1.6	43	0.1	0.6	9.8	1.7	2.5	89	6	6
19-Oct-07	23.8	135	37	1.2	49	0.1	1.0	1.4	1.6	2.5	89	7	5
31-Oct-07	23.5	239	40	1.2	58	0.06	2.5	7.6	2.5	3.4	77	9	7
9-Apr-08	22.7	815	4	0.6	66	0.04	0.7	33.7	1.9	3	97	6	38
7-May-08	23.4	208	7	0.7	51	0.03	1.0	39.7	1.7	2.9	95	6	38
11-Jun-08	27.4	2314	5	0.6	51	<0.02	1.2	52.8	1.5	3.9	95	9	43
18-Jul-08	27.1	1645	7	1.0	51	<0.02	0.9	40.2	1.2	3.9	95	4	48
9-Oct-08	25.2	229	22	6.4	30	0.03	0.8	37.8	1.8	3.3	91	0	35
29-Oct-08	25.5	737	10	4.3	38	0.02	1.1	22.5	0.8	2.5	93	0	40
Average	24.8	739	19	2.0	49	0.05	1.1	27.3	1.6	3.1	91	5	29
std	1.7	761	15	2.0	11	0.03	0.6	17.7	0.5	0.6	6	3	18

@, T, SPAR, MLD, TSM, DIN, SRP, Chl a_{pico}, APA_{pico}, CYA, BA, $RAPA_{pico}$, CYA_{+ELF}, and BA_{+ELF} indicated surface temperature, surface photosynthetic available radiance, mixing layer depth, total suspending matters, dissolved inorganic nitrogen (nitrate + nitrite), soluble reactive phosphorus, chlorophyll *a* of 0.2~3 μm size fraction, alkaline phosphatase activity of 0.2~3 μm size fraction, cyanobacteria abundance, bacteria abundance, ratios of APA_{pico} to bulk APA, the percentage of ELF positive cyanobacteria to total cyanobacteria cell counts, and the percentage of ELF positive bacteria to total bacteria cell counts, respectively.

Table 4.2. Correlation matrix of the measurements[@] derived from field survey. * and ** indicated p <0.05 and p <0.01 levels, respectively.

Parameters	**T**	**SPAR**	**MLD**	**TSM**	**DIN**	**SRP**	**Chl_{pico}**	**APA_{pico}**	**CYA**	**BA**	**$RAPA_{pico}$**	**CYA_{+ELF}**	**BA_{+ELF}**
Units	°C	μE m^{-2} s^{-1}	m	g m^{-3}	μMN	μMP	mgChl m^{-3}	nM h^{-1}	10^{11}cell m^{-3}	10^{12} cell m^{-3}	%	%	%
T	1												
SPAR	0.79*	1											
MLD	-		1										
TSM	-		-	1									
DIN	-		-	-0.85**	1								
SRP	-		0.83**	-	-	1							
Chl_{pico}	-		-	-	-	-	1						
APA_{pico}	-	0.68*	-0.86**	-	-	-0.84**	-	1					
CYA	-	-	-	-	-	-	-	-	1				
BA	-	0.71**	-	-	-	-	-	-	-	1			
$RAPA_{pico}$	-	-	-0.85**	-	-	-	-0.76*	0.70*	-	-	1		
CYA_{+ELF}	-	-	-	-0.85**	0.73*	-	-	-	-	-	-	1	
BA_{+ELF}	-	-	-0.95**	-	-	-0.93**	-	0.90**	-	-	0.76*	-	1

@, the same as Table 4.1

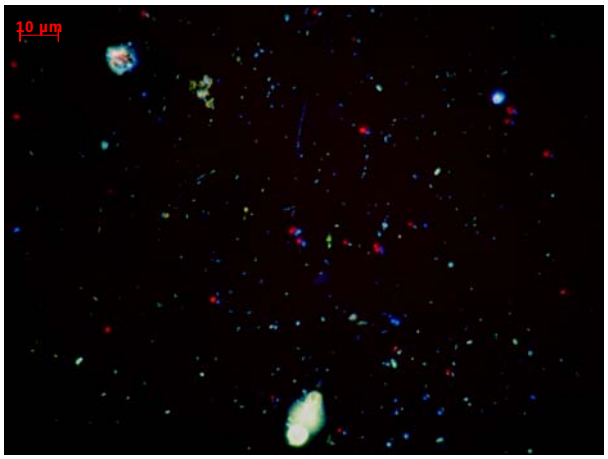

Photo 4.1. Image shows red-blue-green (RBG) overlay under an inverted fluorescence microscope. Autotrophic organisms (red fluorescence), DAPI labels (blue fluorescence) and ELFA precipitates (yellow-green fluorescence).

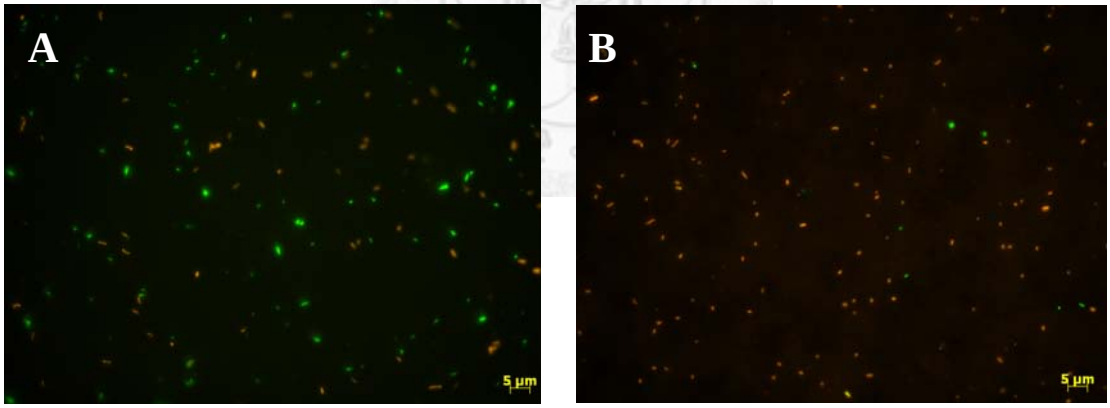

Photo 4.2. An image comparison of ELF-labeled (green fluorescence) cells in (A) the control and (B) phosphate-enriched treatments.

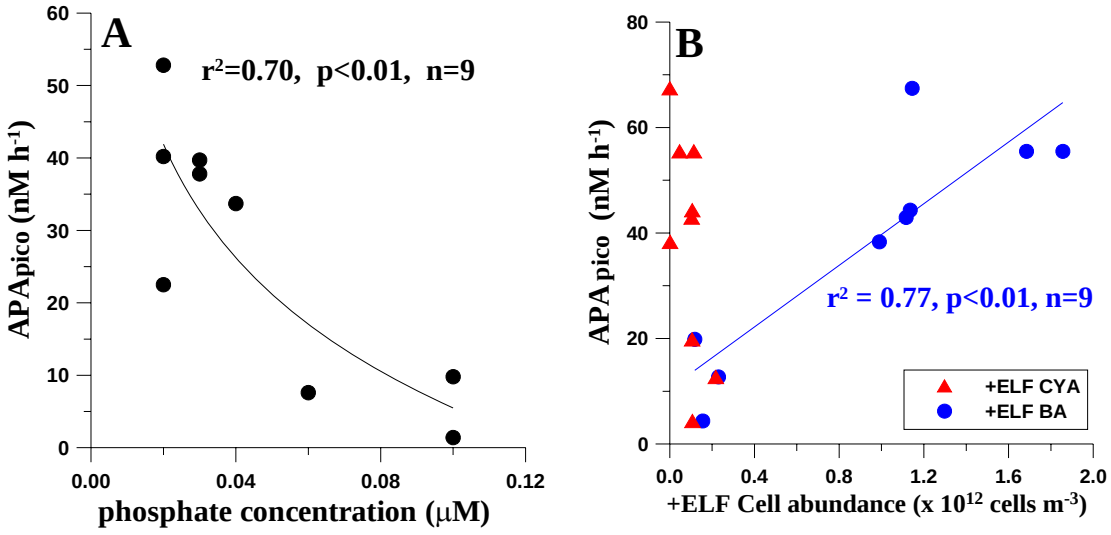

Fig. 4.1. Scatter plots of (A) picoplanktonic alkaline phosphatase activity (APA_{pico}) vs. phosphate concentrations and (B) APA_{pico} vs. abundance of ELF-positive cells (CYA_{+ELF} & BA_{+ELF}).

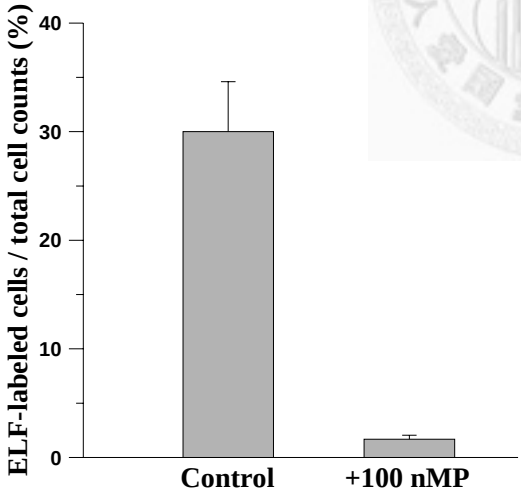

Fig. 4.2. The percentage (%) of ELF-labeled cells to total cell counts incubated in the control and phosphate-enriched treatments (final concs., 100 nMP). Error bars denoted standard deviations (n = 3).

Chapter 5

Light/Nutrient Effects on the Osmotrophs Behaviors in a Subtropical Reservoir

Abstract

Light/nutrient manipulation experiments were conducted with surface water samples taken from a subtropical oligotrophic reservoir. In the 8 monthly phosphate (Pi) enrichment experiments, the enhancement of pico-phytoplankton production (PP) occurred in the experiments conducted in summer (Jul~Sep) only, while that of heterotrophic bacterial production (BP) could be observed most of the year (May~Oct). BP could be enhanced at lower Pi-addition than PP. Three of the 7 positive cases showed co-limitation of phosphate and ammonia on bacterial production. These indicated that bacteria seemed to be more sensitive than algae to the changes of phosphate in the field. In the four light/nutrient manipulation experiments, bacterial turnover rates (BAμ) increased ca. 2~3-fold in treatments enriched with phosphate or dissolved organic phosphorus (DOP), and such elevation of BAμ seemed to be light-independent. The turnover rate of picocyanobacteria (CYAμ) on the other hand, was majorly controlled by light availability. Under illuminated condition, CYAμ in the control treatments were similar to, and sometimes higher than the BAμ in the counterpart treatments. These indicated that with the aid of light, picocyanobacteria could grow at rates similar to or higher than those of bacteria, and thus compete equally with or out-compete bacteria under low-Pi or Pi-deficient condition.

5.1 Introduction

In oligotrophic aquatic ecosystems, pico-phytoplankton (picocyanobacteria and picoeukaryotes…etc) are more important than lager algae in terms of primary production, abundance, and thus the roles in food-web dynamics and biogeochemical cycling (Stockner 1988, Raven 1998, Callieri & Stockner 2002, Callieri 2008). On the other hand, heterotrophic bacteria (termed bacteria below), the most abundant heterotrophs in the world, compete and even out-compete inorganic nutrient with phytoplankton in many mineral-limited environments. Many researchers have demonstrated that bacteria could take up limiting inorganic nutrient more efficiently than phytoplankton due to their smaller size, and thus higher surface area to volume (S/V) ratio (Currie & Kalff 1984, Thingstad *et al*. 1993).

To explain the coexistence of phytoplankton and bacteria, Thingstad *et al*. (1997) proposed a so-called "microbial-loop malfunction" theory based on a culture study and modeling results. This theory states that bacteria could out-compete phytoplankton in taking up limiting nutrient at the first place; however, bacteria then lost their competing advantage after running down dissolved organic carbon (DOC), which served as carbon or energy source for their growth. In another word, phytoplankton could dominate the uptake of limiting nutrient when bacteria are C-limited. However, this theory probably is not applicable for Feitsui Reservoir at which DOC concentrations are very high. Tseng *et al*. (2010) showed that DOC in this system seldom limited bacterial growth, and the degradation rate of DOC (by bacteria) was determined by the availability of phosphate.

The S/V ratio theory could be true for bacteria when against larger phytoplankton such as diatoms and autotrophic dinoflagellates; however, it might be inapplicable for

pico-phytoplankton which is at the size category of bacteria. Moutin *et al*. (2002) demonstrated that in the oligotrophic Mediterranean Sea, the affinity constants of picocyanobacteria and bacteria to limiting phosphate were in the same range. In some cases, the values of the former were lower than the latter, indicating that picocyanobacteria could compete equally and even out-compete bacteria for limiting phosphate. Light intensity could be one of the potential mechanisms for this, but was not proposed. Light intensity, an essential factors for picophytoplankton growth, could affect their capacity in taking up limiting nutrients (Jasser & Arvola 2003).

The results of Chapter 3 revealed that the APA of the size category of <3 μm (*i.e.* APA_{pico}) was the major contributor to bulk APA. On average, picoplankton accounted for ~60% of bulk APA. Based on the positive correlations of picocyanobacteria abundance vs. APA_{pico} and bacterial abundance vs. APA_{pico}, it was concluded that picocyanobacteria and bacteria (osmotrophs) were the major contributors to the enzyme activities in the study site. Further results of Chapter 4 indicated that most of APA_{pico} signals came from bacteria but not picocyanobacteria by ELF analysis.

During the period of 2006~2008, a series of light/nutrient manipulation experiments was performed with the water samples taken from the Feitsui Reservoir. Three scientific questions were addressed: (1) How did nutrient amendments affect osmotrophs productions? (2) Did added phosphate accelerate picocyanobacteria growth under well-illuminated condition? (3) How did APA respond in these light/nutrient manipulated experiments?

5.2 Materials and Methods

A series of monthly nutrient manipulation and four light-nutrient manipulation experiments were conducted in laboratory with the water samples taken from the dam-site during the period of 2006~2008. Water samples stored in 10L polycarbonate carboys were brought back to laboratory for bioassay experiments listed below within 2 hrs.

5.2.1 Bioassay 1: Nutrient manipulation experiments

A total of 8 experiments were performed in different months of 2006 (Table 5.1). Water samples taken from the 2 m depth of the dam-site were pre-screened through a 3-μm polycarbonate (PC) filter to remove larger organisms. The filtrates were filled into 250 mL acid-prewashed PC bottles. Six duplicate treatments were generated by adding different amount and types of inorganic nutrients. These treatments were (1) the control, (2) 0.1 μM Pi, (3) 0.5 μM Pi, (4) 1 μM Pi, (5) 5 μM NH_4 and (6) 0.5 μM Pi + 5 μM NH_4. Concentration units listed above were the final concentrations. All bottles were incubated under 10:00 AM daylight at room temperature (~25℃) for 2 hrs. Pico-phytoplankton production (PP), bacterial production (BP), APA, and phosphate concentrations were measured after incubation. Enrichment (of BP and PP) and inhibitory (for APA) effects were defined as the treatment reading was 10% higher than that of the control.

5.2.2 Bioassay 2: Light/nutrient manipulation bioassay experiments

Four light/nutrient manipulation experiments were conducted in 2008 (Table 5.2). The 3-μm filtrates were filled into 250 mL acid-prewashed PC bottles. *In situ* physical and chemical conditions as well as the treatments of the four experiments are shown in Table 5.2. Phosphate (Pi) and glucose-6-phosphate (G-6-P) were used as dissolved

inorganic phosphorus (DIP) and dissolved organic phosphorus (DOP) substrates, respectively. In brief, samples of experiment #1 were enriched with Pi, and then incubated under two light intensities (370 and 140 μE m^{-2} s^{-1}) in a 12h:12h light-dark cycle. In experiment #2, samples were incubated under two light intensities (450 and 250 μE m^{-2} s^{-1}) without adding any substrate. In experiment #3, samples were enriched with Pi and DOP, and then incubated under high-light condition (350 μE m^{-2} s^{-1}) in a 12h:12h light-dark cycle. Experiment #4 basically was a repeat of experiment #3. The incubation lasted for 24~60 hrs. Sub-sampling of picocyanobacteria abundance (CYA), bacterial abundance (BA), and APA was performed every 3~12 hrs.

5.2.3 Picophytoplankton production (PP)

PP was estimated by ^{14}C-incorporation method (Parsons *et al*. 1984). In short, radioisotope $NaH^{14}CO_3$ was inoculated into a PC bottle containing 100 mL of water sample. The final concentration was 10 μCi mL^{-1}. After one hour incubation under natural sun-light, subsamples were immediately filtered on 0.2-μm PC filters. Acidification of sample filters was performed by adding 0.5 mL of 0.5N HCl into a 25 mL scintillation vial. Then the filters were measured with a liquid scintillation analyzer (Packard, Tri-Carb 2700TR) after adding 10 mL scintillation cocktails (Ultima Gold, Perkin Elmer).

5.2.4 Bacterial production (BP)

BP was measured via the incorporation of ^3H-thymidine (Fuhrman & Azam 1982). Aliquot 1.7 mL water samples in 2.0 mL vials were spiked with ^3H-thymidine (S.A. = 6.7 Ci/mmol; final conc., 20 nM), and then incubated in a 25°C water bath. After 1 hr incubation, 40 μL of 37% formaldehyde was added to stop the reaction. Vials were centrifuged at 4℃ at a speed of 14,000 rpm for 7 mins. After the removal of upper clear

filtrate, retained pellets were rinsed with 1.7 mL ice-cold 5% trichloroacetic acid (TCA) and then went for centrifugation to collect the pellets. Subsequently, 1.7 mL ice-cold 80% ethanol was added for rinsing, and the third centrifuge was processed. After the removal of filtrate, vials were left dry in a hood. A 1.7 mL scintillation cocktail (Ultima Gold, Perkin Elmer) was added to the vial for radioactivity measurement in a liquid scintillation analyzer (Packard, Tri-Carb 2700TR).

5.2.5 Picoplankton abundances, phosphate concentrations, and APA

The methods for the abundance of picocyanobacteria (CYA) and bacteria (BA), phosphate concentrations, and APA were shown previously. See Chapter 2 for details.

5.3 Results

5.3.1 Bioassay 1

In situ conditions of physical, chemical, and biological variables of the 8 experiments were listed in Table 5.1. Ambient soluble reactive phosphorus (SRP) concentrations were either very low or under the detection limit (*i.e.* 0.02 μM P). On the other hand, concentrations of dissolved inorganic nitrogen (DIN = nitrate + nitrite), DOC, and Chl *a* were high, ranging from 16~28 μM N, 81~237 μM C, and 1.5~6.5 mg Chl m^{-3}, respectively.

Among the 40 (8 months x 5 treatments) combinations, eight of them showed that PP was enhanced by the addition of Pi (Table 5.3). One case (Sep) showed NH_4 addition effect. The higher the concentration of Pi added, the higher the percentage of PP enhanced. Note that the cases showing enrichment effects were samples taken from summer period (Jun~Sep). As to BP, among the 25 effective cases (Table 5.4), 18 of them were due to Pi addition, 3 could be ascribed to NH_4 effect and 3 showed interactive effects of Pi plus NH_4. Like that of PP, the higher the concentration of Pi added, the higher the percentage of BP enhanced. In the 0.1 μM Pi treatment, PP had no response except the data of Sep, while BP had two (Sep and Oct) positive cases. As the dosage increased, BP had more positive cases than PP. The inhibition of APA by Pi-addition was eminent even in the 0.1 μM Pi treatment (Table 5.5). Again, as the concentration of added Pi increased, the percentage of decrease elevated. In 3 cases (Sep~Dec), the addition of 5μM NH_4 did result in the reduction of APA, however, the decrease percentages were <18%.

5.3.2 Bioassay 2

The changes of CYA (Fig. 5.1A) and BA (Fig. 5.1B) in the six treatments of Exp. #

1 indicated that in the control group, the turnover rates of BA (BAμ; 0.17~0.19 d^{-1}; Table 5.6) in the dark-control, lowlight (LL)-control, and highlight (HL)-control treatments were similar to each other. On the other hand, CYA's turnover rates (CYAμ) in the illuminated-control treatments (0.17~0.18 d^{-1}) were significantly higher than that of the dark-control (0 d^{-1}). In the enriched group, the addition of Pi had no effect on CYAμ. On the other hand, BAμ in the dark-enriched and illuminated-enriched treatments were enhanced by ~50% when compared with their counterpart treatments. Overall, these suggested that the growth of CYA was light-dependent but not P-dependent. On the other hand, the growth of BA was controlled by the availability of phosphate instead of light.

The results of Exp. #2 (Fig. 5.2) verified the hypothesis of CYA's growth is light-dependent. When grew in an un-amended condition, CYAμ increased as light intensity increased, while BAμ remained the same irrespective of light's availability (Table 5.6). APA increased with the incubation time, indicating P-deficit was deteriorating (Fig. 5.2C). The change of APA in the dark treatment probably could be mainly ascribed to BA, while the increasing parts of APA in the illuminated treatments at any given time point might be contributed by CYA.

In Exp. #3, the abundance of CYA remained unchanged in the first 12hrs and then decreased slightly afterwards (Fig. 5.3A). Meanwhile, CYA in the HL-control treatment grew quickly with a rate of 0.78 d^{-1} (Table 5.6). The addition of Pi and DOP had no extra effects on CYAμ under light incubation. When compared with the results from the dark-control and HL-control treatments, enrichment effects of Pi and DOP on BAμ were noticeable at the first 12 hrs (Fig. 5.3B). Overall, it said that the change of BAμ was controlled by substrate. Pi and DOP enrichments had similar effects in enhancing BAμ (Table 5.6). CYA' growth was dominated by light availability.

In Exp. #4, CYA abundance decreased with time when incubated in the dark (Fig. 5.4A). In the dark, neither Pi nor DOP enrichment make their growth curves different from that of the dark-control treatment. When subjected to illuminated condition, CYA abundance in the HL-control treatment increased with a turn-over rate of 0.13 d^{-1} (Table 5.6). Values of CYAμ in the HL+Pi (0.23 d^{-1}) and HL+DOP (0.21 d^{-1}) treatments were ~1.7-fold of that of the light-control treatment. The changes of BA in the HL-control and dark-control treatments were similar, increasing during the first 24 hrs and then decreased afterwards (Fig. 5.4B). Values of BAμ (Table 5.6) derived from these two control treatments were 0.16 d^{-1} (dark-control) and 0.14 d^{-1} (HL-control). Values of BAμ (0.33~0.48 d^{-1}) of the enriched treatments were ~2.5-fold of those of the control treatment. Light intensity seemed had no effects on BAμ under amended conditions. DOP addition had similar effects to that of Pi addition in enhancing BAμ.

5.4 Discussion

Picocyanobacteria and heterotrophic bacteria (*i.e.* osmotrophs) are the two most abundant picoplankton in mineral-limited aquatic ecosystems. In oligotrophic environments, pico-phytoplankton may account for more than half of primary production (Raven 1998), while bacterial production plays a central role in the microbial-loop (Azam *et al*. 1983) and the mobilization of DOC, which is the largest organic carbon pool in aquatic ecosystems. Therefore, an understanding of the interactions between osmotrophs, particularly their competition on liming nutrient, is essential for the studies of tropho-dynamics and biogeochemical cycling.

The results of bioassay 1 indicated that phosphate (Pi) supply was the major, if not the only, controlling factor affecting the productions of picocyanobacteria (Table 5.3) and bacteria (Table 5.4). Several findings were noticeable in bioassay 1 (Table 5.3~5.5). BP was more sensitive than PP to Pi-addition at any given dosage and/or given sampling month. In the Sep experiment, the increase percentage of BP in the 0.1 μM Pi treatment was 28%, and it increased to 51% in the 1 μM Pi treatment, while the increase percentage of PP amplified >10-fold (*i.e.* △17% in the 0.1 μM Pi treatment and △191% in the 1 μM Pi treatment). Similar phenomenon could be found in the Aug experiment. These implied that the production (and growth, too) of picocyanobacteria could be enhanced to a greater magnitude than bacteria at higher phosphate concentrations with help from certain physical factor, and light intensity was the most possible one.

The four experiments of bioassay 2 were designed to have a direct observation on the changes of osmotrophs' abundance over longer period of time. The testing hypothesis was that picocyanobacteria could gain their competition advantage over

bacteria via the help of light. The results of Table 5.6 clearly indicated that CYA did not proliferate in the dark with or without Pi (or DOP) additions. Once incubated under light, CYA in the control-treatments could grow at rates no less and sometimes higher than those of BA. For example, in Exp. #2, values of CYAμ in the lowlight-control and highlight-control were 0.36 and 0.78 d^{-1}, respectively; while those of BAμ were in a similar range of 0.39~0.42 d^{-1}. These suggested that picocyanobacteria with their rapid adaptation to light intensity (Kana & Glibert 1987b, a, Fahnenstiel *et al*. 1991, Pick & Berube 1992) might give them an equal or even greater capacity than bacteria in competing limiting-P for growth.

Ikeya *et al*. (1997) found that the P-uptake of picophytoplankton grown in P-deprived medium (phosphate-limited cells) was markedly higher than the cells grown in the P-rich medium (phosphate-replete cells). The results shown in Table 5.6 seemed to contrast with their observations. They also found that ambient phosphate as low as 0.5 nM was enough to support the growth of cyanobacterium *Synechococcus* at a rate of 1.0 d^{-1}. These indicated that picocyanobacteria could still grow rapidly even at phosphate concentrations of nanomolar level. Moutin *et al*. (2002) reported that in P-limited systems, picocyanobacteria could occasionally prevail over bacteria in taking up limiting-P, although the potential mechanisms were not mentioned.

The enhancement of bacterial production (BP) by Pi-addition was obvious, although co-limitation of Pi and NH_4 occurred occasionally (Table 5.3). Several studies demonstrated that the addition of phosphate could stimulate BP (Coveney & Wetzel 1992) and bacterial specific growth rate (Toolan *et al*. 1991) in meso-eutrophic lakes. DOC limitation on BP was quite unlikely since its concentrations were quite high (Table 5.1; 81~237 μMC). Tseng *et al*. (2010) indicated that background (refractory) DOC in

this system was in a range of 30~40 μM C. The excessive DOC was bio-degradable, and its degradation rate was determined by phosphate concentration (availability). This indicated that the "microbial-loop malfunction theory" (Thingstad *et al*. 1997) is not applicable to the studied system.

Inhibition of APA by Pi-addition was eminent (Table 5.5). However, NH_4 amendment also depressed APA in the three cases (Sep, Oct, and Dec in Table 5.5) in bioassay 1 experiments. This contradicted to the results of Dyhrman & Ruttenberb (2006) suggesting that bulk APA of microbial community should increase under DIN enrichment. On the other hand, Rengefors *et al*. (2003) indicated that DIN-addition did not result in any increase of APA in Lake Erken, Sweden.

5.5 Conclusion

In oligotrophic systems, an understanding of the regulating mechanisms of the competition between picocyanobacteria and bacteria for limiting mineral is essential. This is because the former and the latter constitute the most abundant autotrophs and heterotrophs, respectively. In the monthly enrichment experiments, it was demonstrated that bacteria were more sensitive than picocyanobacteria to the Pi-addition. In the light/nutrient manipulation experiments, bacteria doubled its turnover rate when extra phosphate was added irrespective of light availability. Growth of picocyanobacteria on the other hand was light-dependent but not P-dependent. Several cases showed that picocyanobacteria could grow faster than bacteria under illuminated condition, indicating that light could be important for osmotrophs (picocyanobacteria and bacteria) behavior in competing limiting mineral in oligotrophic aquatic ecosystems.

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Table 5.1. *In situ* conditions (average ± standard deviation) of environmental factors[@] of bioassay 1 conducted in 2006.

Month	**T** (℃)	**L** (mE m^{-2} d^{-1})	**DIN_{DIA}** (μM N)	**SRP_{DIA}** (μM P)	**DOC_{DIA}** (μM C)	**Chl_{DIA}** (mg m^{-3})	**APA_{DIA}** (nM h^{-1})
Apr.	22.3±1.2	43±15	18±1	0.03±0.01	84±6	2.5±0.4	36±11
May	24.7±1.1	30±6	19±2	0.02±0.01	81±5	2.8±0.4	54±10
Jun.	26.6±2.4	58±22	25±1	0.02±0.01	97±29	2.5±0.3	48±8
Jul.	30.3±0.7	66±15	28±1	0.05±0.02	84±5	3.1±0.5	32±3
Aug.	30.1±0.7	74±17	17±1	0.02±0.01	173±9	3.1±1.0	47±7
Sep.	27.2±1.3	45±12	16±2	0.02±0.02	237±12	3.8±0.8	61±3
Oct.	26.0±0.6	49±23	23±4	0.03±0.01	133±29	6.5±2.5	61±5
Dec.	22.0±0.9	28±3	23±2	0.02±0.01	86±48	1.5±0.6	23±2

@, T, L, DIN_{DIA}, SRP_{DIA}, DOC_{DIA}, Chl_{DIA}, and APA_{DIA} indicated surface water temperature, weekly-averaged light intensity, and epilimnic depth-integrated averaged dissolved inorganic nitrogen (nitrate + nitrite), soluble reactive phosphorus, dissolved organic carbon, chlorophyll *a*, and alkaline phosphatase activity, respectively.

Table 5.2. *In situ* conditions of environmental factors[@] and the experimental setup[©] of bioassay 2 experiments conducted in 2008.

Exps.	**Date**	**T** (℃)	**SRP_{DIA}** (μM P)	**HL** (μE m^{-2} s^{-1})	**LL** (μE m^{-2} s^{-1})	**Pi** (μM P)	**DOP** (μM P)
Exp #1	Jan. 29	19	0.06	370	140	0.5	-
Exp #2	Jun. 04	28	<0.02	450	250	-	-
Exp #3	Sep. 03	30	<0.02	350	-	0.5	1
Exp #4	Oct. 22	26	<0.02	400	-	0.5	1

@, T and SRP are the same as Table 5.1. ©, HL, LL, Pi, and DOP represented treatments under high light intensity, low light intensity, phosphate enriched, and dissolved organic phosphorus (glucose-6-phosphate) enriched, respectively.

Table 5.3. The increase (in %) of pico-phytoplankton production (PP) in treatments enriched with phosphate (Pi), ammonia (NH_4), and their combination of bioassay 1. ns, the difference between treatment and control was <10%. Capitalized letters (N and P) indicated the limiting factor.

Month	**0.1 μM Pi**	**0.5 μM Pi**	**1 μM Pi**	**5 μM NH_4**	**0.5 μM Pi + 5 μM NH_4**	**Limiting factor**
Apr.	ns	ns	ns	ns	ns	-
May	ns	ns	ns	ns	ns	-
Jun.	ns	ns	ns	ns	ns	-
Jul.	ns	ns	21±12	ns	ns	P
Aug.	ns	13±7	30±16	ns	13±1	P
Sep.	17±3	83±6	191±11	20±10	76±213	P
Oct.	ns	ns	ns	ns	ns	-
Dec.	ns	ns	ns	ns	ns	-

Table 5.4. The increase (in %) of bacterial production (BP) in treatments enriched with phosphate (Pi), ammonia (NH_4), and their combination of bioassay 1. ns, the difference between treatment and control was <10%. Capitalized letters (N and P) indicated the limiting factor.

Month	**0.1 μM Pi**	**0.5 μM Pi**	**1 μM Pi**	**5 μM NH_4**	**0.5 μM Pi + 5 μM NH_4**	**Limiting factor**
Apr.	ns	ns	ns	12±6	32±16	N, P
May	ns	13±12	25±8	13±10	17±11	N, P
Jun.	ns	39±1	43±4	ns	44±3	P
July.	ns	ns	18±4	ns	ns	P
Aug.	ns	22±21	30±31	ns	31±31	P
Sep.	28±7	38±8	51±6	ns	37±5	P
Oct.	40±6	53±5	58±6	ns	52±3	P
Dec.	ns	18±3	32±5	35±5	53±6	N, P

Table 5.5. Initial picoplankton alkaline phosphatase activity (APA) and percentage of APA inhibition in treatments enriched with phosphate (Pi), ammonia (NH_4), and their combination of bioassay 1. ns, the difference between treatment and control was <10%.

Month	**Initial APA** ($nM\ h^{-1}$)	**0.1 μM Pi** (%)	**0.5 μM Pi** (%)	**1 μM Pi** (%)	**5 μM NH_4** (%)
Apr.	36±11	-48±11	-65±7	-83±3	ns
May	55±10	-45±10	-68±11	-89±10	ns
Jun.	49±8	-52±11	-76±10	-93±4	ns
July.	32±7	-29±19	-53±28	-73±22	ns
Aug.	47±7	-20±7	-40±16	-70±13	ns
Sep.	61±3	-25±6	-48±11	-66±10	-18±27
Oct.	61±5	ns	-14±19	-44±20	-18±33
Dec.	23±2	-60±2	-80±2	-89±2	-16±3

Table 5.6. A list of the turn-over rates (d^{-1})[@] of picocyanobacteria (CYAμ) and bacteria (BAμ) in the four experiments of bioassay 2.

	Exp #1		Exp #2		Exp #3		Exp #4	
Treatments[©]	**CYAμ**	**BAμ**	**CYAμ**	**BAμ**	**CYAμ**	**BAμ**	**CYAμ**	**BAμ**
Dark-control	0	0.18±0.02	0	0.39±0.04	0	0.21±0.04	0	0.16±0.01
Dark+Pi	0	0.28±0.03	-	-	-	-	0	0.33±0.01
Dark+DOP	-	-	-	-	-	-	0	0.48±0.02
LL-control	0.18±0.02	0.19±0.03	0.36±0.01	0.41±0.05	-	-	-	-
LL+Pi	0.18±0.01	0.28±0.03	-	-	-	-	-	-
LL+DOP	-	-	-	-	-	-	-	-
HL-control	0.17±0.02	0.17±0.01	0.78±0.04	0.42±0.04	1.01±0.02	0.20±0.06	0.13±0.03	0.14±0.01
HL+Pi	0.19±0.02	0.30±0.04	-	-	0.96±0.04	0.33±0.04	0.23±0.02	0.33±0.02
HL+DOP	-	-	-	-	1.00±0.05	0.35±0.06	0.21±0.04	0.42±0.02

@, derived from the slope of Ln transformed abundance (or APA) vs. time during the active growing phase. ©, Pi and DOP indicated phosphate and glucose-6-phosphate (G-6-P) enrichment. LL and HL indicated illumination with low and high light intensity. See also Table 5.2 for light treatments.

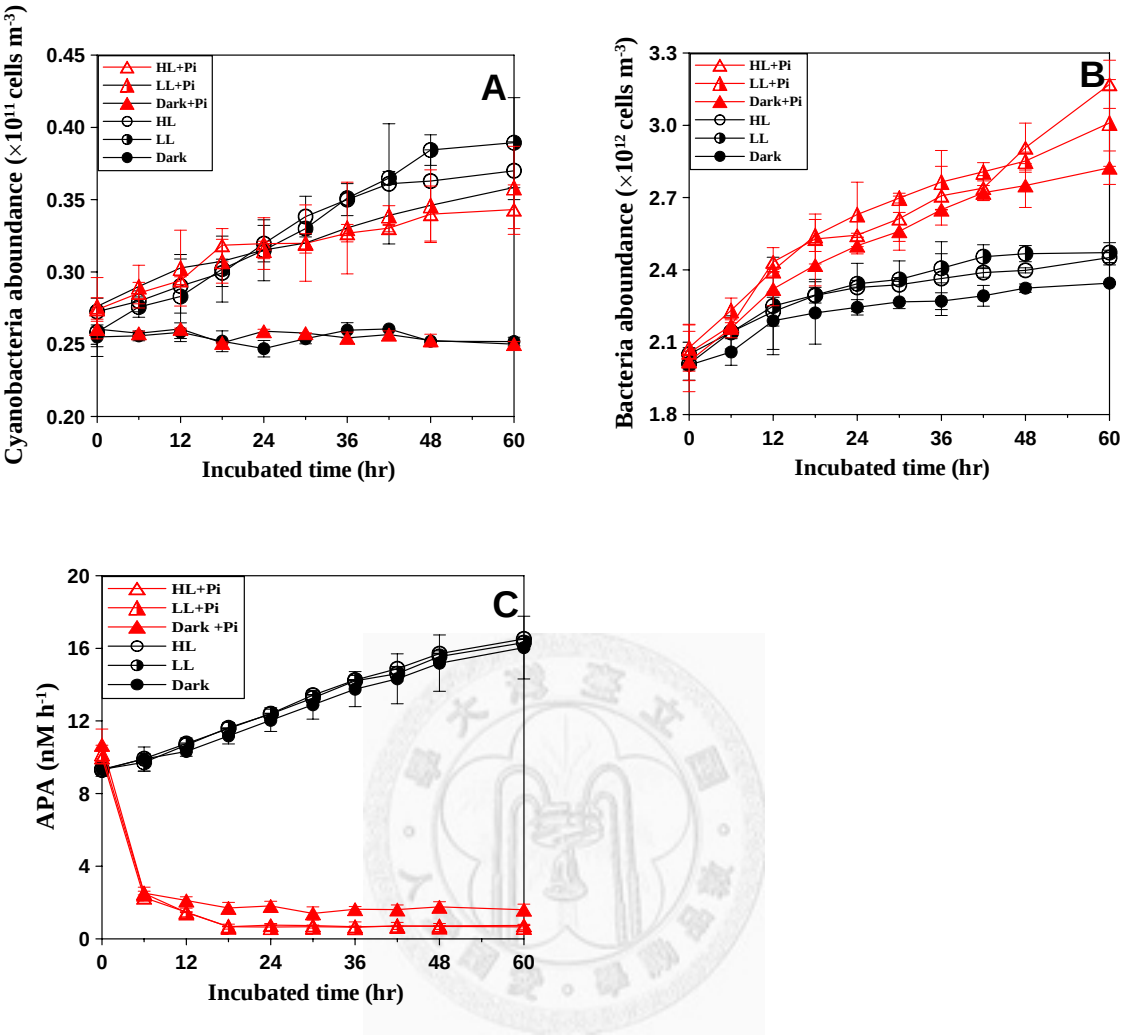


Fig. 5.1. The changes of (A) picocyanobacteria abundance, (B) bacteria abundance, and (C) picoplankton alkaline phosphatase activity (APA) of Exp. #1 conducted in Jan 2008. Incubation time 0 represented 8:00 AM. Refer Table 2 for the light and Pi levels used in this experiment. Vertical bars indicated standard deviations.

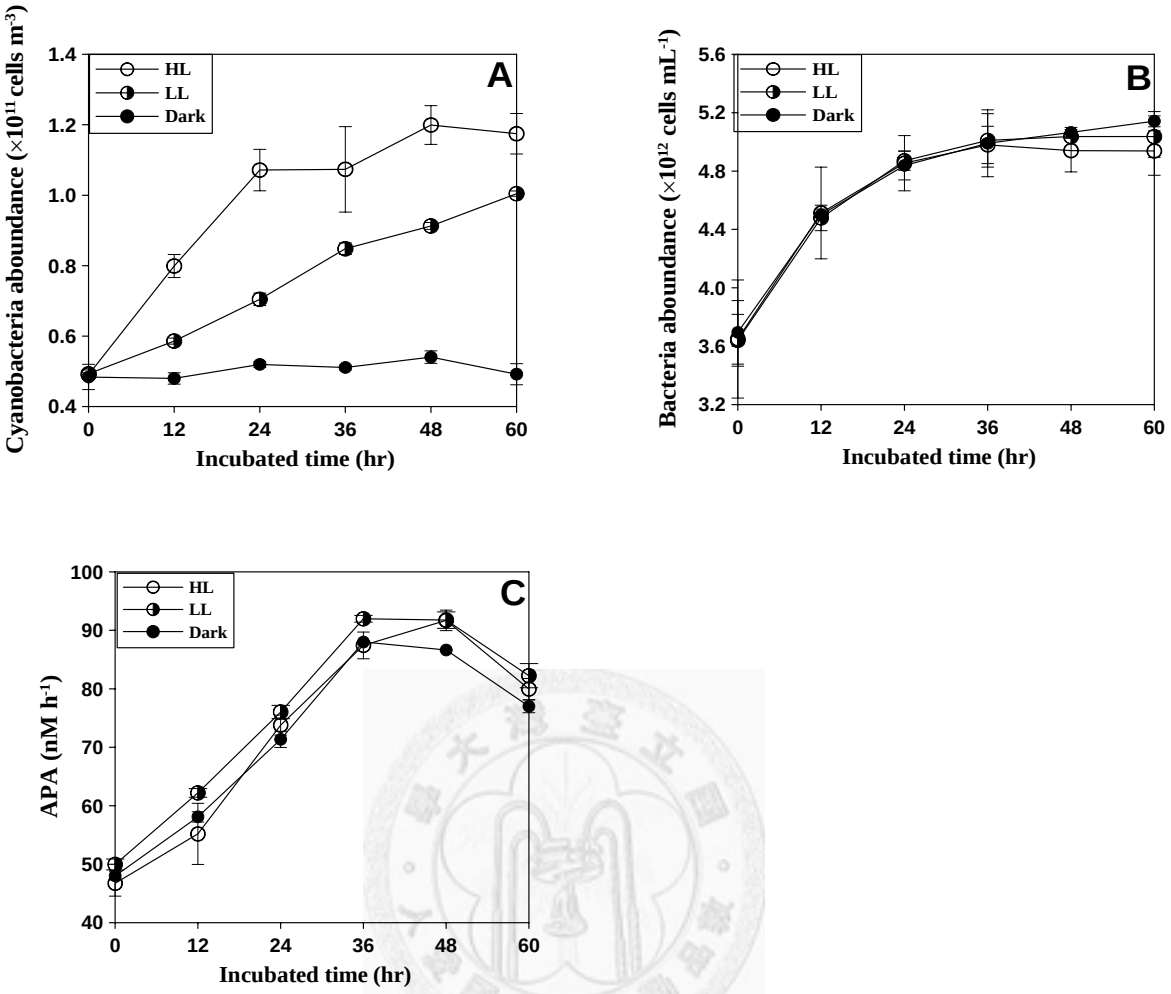


Fig. 5.2. The same as Fig. 5.1, but for Exp. #2 conducted in Jun 2008.

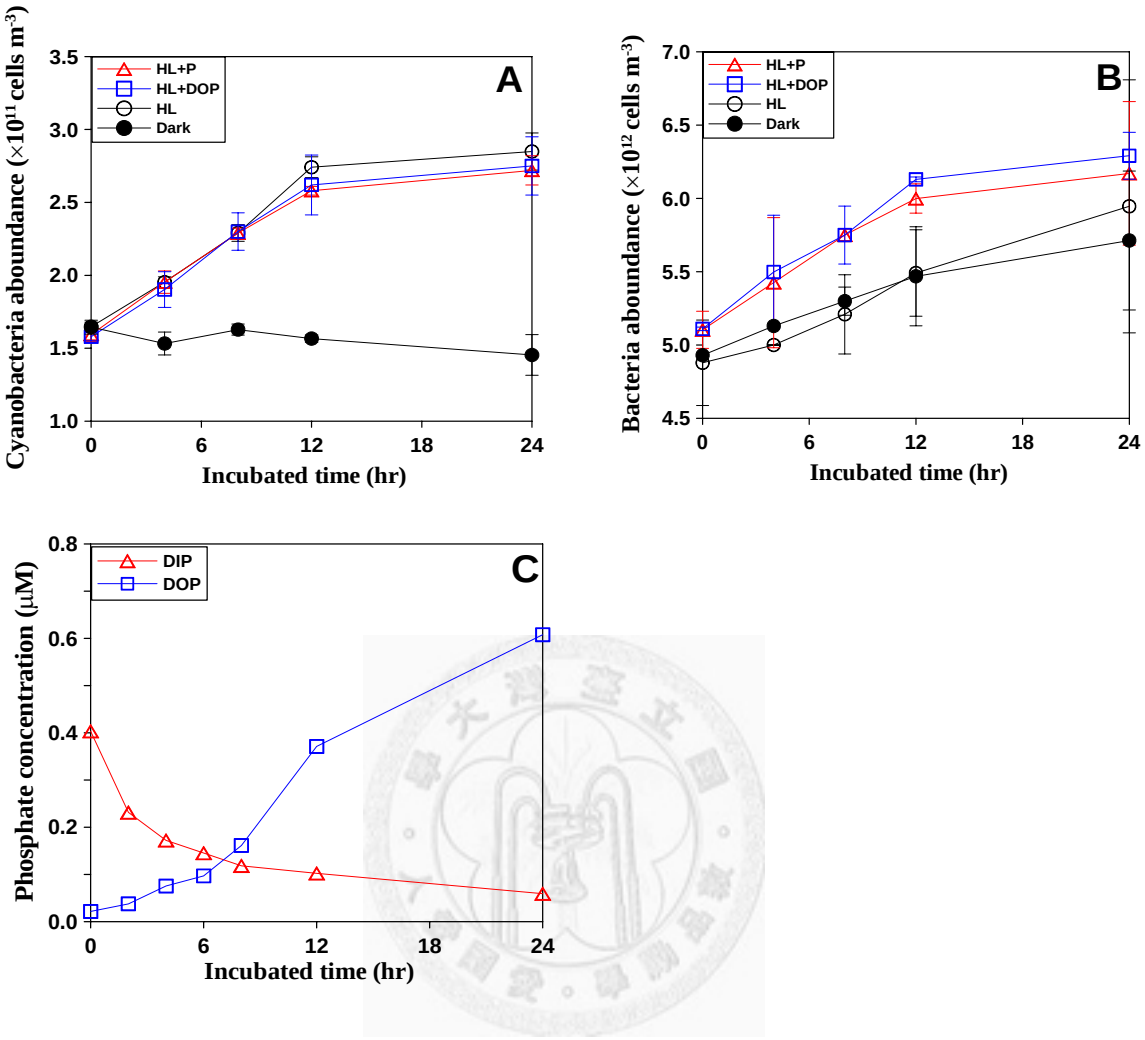


Fig. 5.3. (A) and (B) are the same as Fig. 5.1, but for Exp. #3 conducted in Sep 2008. (C) changes of the phosphate concentrations.

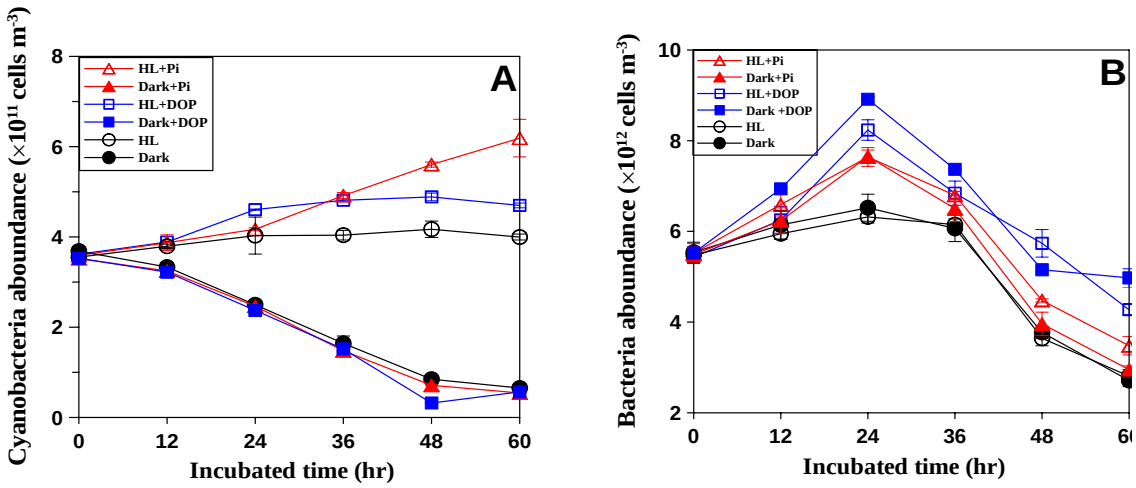


Fig. 5.4. The same as Fig. 5.1, but for Exp. #4 conducted in Oct 2008.

Chapter 6

Conclusion

6.1 Conclusion

Phosphorus (P) is an important element for all living organisms because it consists most of the cell structure. When water system is P-deficient, plankton could utilize dissolved organic phosphorus (DOP) by producing alkaline phosphatase (APase) for their P requirement. Therefore, bulk APase activity (APA) measurement, as a physical indicator to P-status of natural plankton population, has been wildly processed in many aquatic systems. However, there are two major factors which cause the measurement of bulk APA to be erroneous to represent the entire P-status in whole system: (1) the origins of APA in the system are diverse and are hardly to be identified; (2) the regulate mechanisms of APA in different organisms (*e.g.* picocyanobacteria and bacteria) are variable because of their discrepant competition strategies for mineral utilization. For the reasons, downscaling study of APA was conducted to understand the relationship between bulk APA and the entire P-status in an aqueous system. Results from this study can also be used for future studies as a boundary condition at narrow scales.

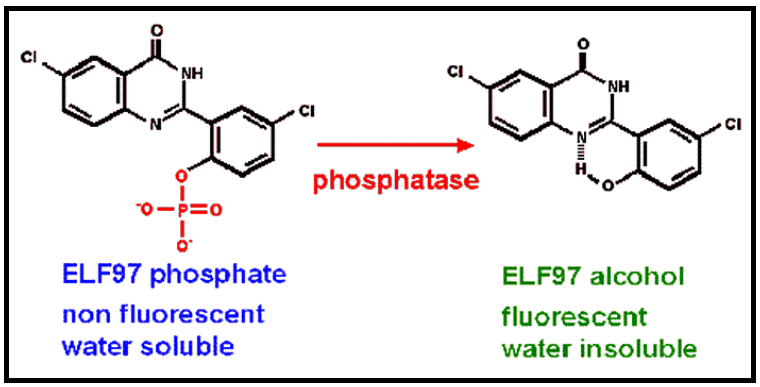
In this study, downscaling APA in a subtropical reservoir was examined systematically using bulk (Chapter 2), size-fractionated (Chapter 3), and single-cell APA assays (Chapter 4). Light-nutrient manipulation bioassay experiments (Chapter 5) was also conducted. The results from Chapter 2 showed that bulk APA and biomass normalized APA (specific APA) had apparent seasonal variations in the epilimnion of the reservoir with low activity in winter mixed seasons and high activity in well-stratified seasons. Hence, their seasonal variability was majorly controlled by the changes of phosphate availability (*i.e.* mixed layer depth) and light intensity. Furthermore, occurrence of episodic events (*e.g.* typhoon and extreme precipitation) revealed an important role in affecting the inter-annual variability of plankton APA in

sub-tropical to tropical aquatic ecosystems. Chapter 3 demonstrated that the picoplanktonic size fraction (0.2~3 μm) contributed most of the activities of APase in the system. In chapter 4, enzyme-labeled fluorescence (ELF) assays further illustrated that bacteria-mediate-APA is the major donator of APA. Results from the light/nutrient manipulation bioassay experiments in Chapter 5 showed three import findings: (1) APA increased in P-famine and inhibited in P-fertility, directly supported the hypothesis of "induction-repression" mechanism of APA; (2) bacterial growth in the system was phosphate-dependent but light-independent, while picocyanobacteria growth was only light-dependent; and (3) with the aid of light, picocyanobacteria could grow at rates similar to/or higher than those of bacteria, and thus, compete equally with or out-compete bacteria under low-P or P-deficit condition. Overall, P plays a significant role in the subtopic reservoir and is therefore likely to restrict the growth of plankton. In this P-deficient system, high APA supported the idea that biological processes (*i.e.* DOP degradation of APase) mainly accelerated P-cycle in resource-limited environments.

6.2 Further work

For further study of APA, estimation of appropriate ELF-labeling identification by flow cytometry will be conducted, since this assay can improve the resolution of ELF-signals and enhance in understanding the physiological response of auto- and hetero-picoplankton to P limitation. Also, the utilization of both the quantitative bulk APA (fluorometric measurement presented in Chapter 2 and size-fractionated APA measurement presented in Chapter 3) and the qualitative single-cell APA assays (ELF-labeling bioassays presented in Chapter 4) will be used together to provide comprehensive assessment of plankton P-stress from community level down to the individual taxon level in natural plankton communities in subtropical systems.

Appendix 1. Principle of enzyme-mediated formation of the fluorescent ELF-97 alcohol precipitate (ELFA) from the ELF-97 phosphatase substrate (ELFP). Source from J. Nedoma *et al* (2003).



Appendix 2. The filters setting for RGB (red-green-blue) monochromatic signals capturing in Zeiss inverted fluorescence microscope.

Filter sets	Fluorescences	Exciters	Emitters	Beam-splitters
DAPI	Blue	350±50 nm	460±50 nm	> 400 nm
ELF 97	Green	350±50 nm	>515 nm	> 400 nm
ET-DsRED	Red	545±30 nm	620±60 nm	> 570 nm