BIOPRODUCTIVITY AND BIODIVERSITY

IN SHALLOW FRESHWATER LAKES

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ABSTRACT

To address the lake eutrophication problem, a research framework integrating molecular biotechnology with environmental engineering was developed. Initially, the lake-like microcosms (Trophic State-Classified Algal Reactors, TSCARs) were designed and constructed for using scenario assessment. As the results, several patterns of algal growth were observed under many replication experiments performed.

By adjusting nutrient loading and hydraulic properties, TSCARs produced three classified trophic levels. The TSCARs' treatments, based on the Vollenweider model in conjunction with the practical works of environmental engineering, were conducted to investigate the relationships between lake biodiversity (LB) and algal bioproductivity (AB). The Chlorophyll-based estimation was developed for assessing the AB. Based on the estimate of AB, the time-varying algal populations were quantified.

The relationships between LB and AB were clearly demonstrated by DGGE (Denaturing Gradient Gel Electrophoresis) fingerprints. Data showed that the relationships were in agreement with previous studies. The Shannon index (H') indicated that the eukaryotic biodiversity of mestrophic level was higher than that of oligo and eutrotrophic levels. The prokaryotic biodiversity of mestrophic level was lower than that

of oligo and eutrotrophic levels. The similar trends were found in two sites of Lake Wilson under different trophic level.

The phase-oriented concept of the algal growth is firstly proposed to explain the varying relationships between LB and AB by examining DGGE under time-varying analysis. Two relationships: positive relation following a hump shape pattern (eukaryotic assemblage) and negative relation following a U shape pattern (prokaryotic assemblage) were found and exhibited clear correlations between LB and AB. Results from time-varying analysis provided exciting insight into the lake biodiversity. These results showed that LB was deeply affected by the history of algal growth. Moreover, critical timing points of algal growth history in terms of Pr(t) predicted that a shift in LB was imminent.

By conducting molecular cloning, four libraries were produced. The community structures sampled from the TSCARs were higher similarity in lakes. Finally, a minor finding is worthy of note in regard to the population dynamics of cryptophyta in lakes. It was found that the abundance of the cryptophyta was positively correlated with trophic levels in TSCARs.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AB: Algal Bioproductivity

BASINS: Better Assessment Science Integrating point & Non-point Sources

BBM: Bold's Basal Medium

BLAST: Basic Local Alignment Search Tool

CBD: Convention on Biological Diversity

Chl *a*: Chlorophyll *a*

CSTR: Completely Stirred Tank Reactor

CTAB: Cetyl Trimethyl Ammonium Bromide

DEM: Digital Elevation Model

DGGE: Denaturing Gradient Gel Electrophoresis

DNA: Deoxyribonucleic Acid

DO: Dissolved Oxygen

DSTP: Downstream of Sewage Treatment Plant

Ek4: Molecular Cloning Library of Eukaryote in Oligotrophic TSCAR

Ek5: Molecular Cloning Library of Eukaryote in Mesotrophic TSCAR

Ek6: Molecular Cloning Library of Eukaryote in Eutrophic TSCAR

EVM: Extreme Value Method

GIS: Geographic Information System

IC: Inorganic Carbon

LB: Lake Biodiversity

NFKS: North Fork Koukonahua Station

NMDS: Nonmetric Multidimensional Scaling

PCR: Polymerase Chain Reaction

Pk6: Molecular Cloning Library of Prokaryote in Eutrophic TSCAR

PLOAD: Pollutant Load Model

SFKS1: South Fork Koukonahua Station 1

SFKS2: South Fork Koukonahua Station 2

SFKS3: South Fork Koukonahua Station 3

SRP: Soluble Reactive Phosphorus

TC: Total Carbon

TDS: Total Dissolved Solids

TE buffer: Tris-Cl, EDTA Buffer

T-RFLP: Terminal Restriction Fragment Length Polymorphisms

TMDLs: Total Maximum Daily Loads

TN: Total Nitrogen

TOC: Total Organic Carbon

TP: Total Phosphorus

TSCARs: Trophic State-Classified Algal Reactors

TSI: Carlson Trophic State Index

TSS: Total Suspended Solids

USEPA: US Environmental Protection Agency

WSFP: Wahiawa State Freshwater Park

WWTP: Wahiawa Wastewater Treatment Plant

LIST OF SYMBOLS

A_L = Area over a photoperiod, [L^2]

A_s = Area of reservoir surface, [L^2]

A_U = Area of land use type u, [L^2]

C=Output concentration of tracer, [ML^{-3}]

C_0 =Input concentration of tracer, [ML^{-3}]

C_g =Constant determined by grazing effect, [none]

C_U =Event mean concentration for land use type u, [ML^{-3}]

D=Dilution rate, [T^{-1}]

E =Evenness, [none]

H =Mean depth of lake water, [L]

H'=Shannon index, [none]

K=Carrying Capacity, [ML^{-3}]

Kb=Exponential constant, [none]

L_P=Pollutant load, [MT^{-1}]

N_j =Total peck height of intensity of jth lane, [none]

n_i =Intensity of peck height of ith band, [none]

P=Productivity, [$ML^{-2}T^{-1}$]

P_J =Ratio of storms producing runoff, [none]

P_r =Algal bioproductivity, [$ML^{-2}T^{-1}$]

$\overline{P_r}$ =Average algal bioproductivity, [$ML^{-2}T^{-1}$]

P_T =Areal algal product, [ML^{-2}]

p =SRP concentration, [ML^{-3}]

p_0 =Initial SRP concentration, [ML^{-3}]

p_{ij} =Importance probability of band at *i* pixel position on *j* lane, [none]

p_{ss} = SRP concentration under steady-state, [ML^{-3}]

Q =Flow, [L^3T^{-1}]

q =Hydraulic overflow rate, [LT^{-1}]

R=Respiration, [$ML^{-3}T^{-1}$]

R_f =Precipitation, [LT^{-1}]

R_{VU}=Runoff coefficient for land use type u, [none]

r_g =Specific algal growth rate, [T^{-1}]

r_n =A change in algal concentration during a unit time, [$ML^{-3}T^{-1}$]

r_s =Algal net growth rate, [T^{-1}]

S=Total band count on one lane, [none]

Sr=Species richness, [none]

T= Photoperiod, [T]

t =Time, [T]

t_R =Mean hydraulic resident time, [T]

V=Volume, [L^3]

v_s =Net settling velocity, [LT^{-1}]

W'=Areal loading of TP, [$ML^{-2}T^{-1}$]

W_t =Phosphorus loading from external sources, [MT^{-1}]

X =Chl *a* concentration of algae, [ML^{-3}]

X_i =Input Chl *a* concentration, [ML^{-3}]

X_λ =Concentration of the predator species populations, [ML^{-3}]

z =Depth of the bioreactor, [L]

μ_g =Algal gross growth rate, [T^{-1}]

λ_l =Algal net loss rate, [T^{-1}]

θ = t/t_R, [none]

τ_p = Phosphorus resident time, [T]

σ = First-order loss coefficient, [T^{-1}]

ρ_w = Flushing coefficient, [T^{-1}]

ϕ = First-order total elimination coefficient, [T^{-1}]

CHAPTER 1. INTRODUCTION

Eutrophication has been widely recognized as a primary water quality issue affecting aquatic systems, particularly for most of lakes in the world (Asai, Ootani, et al. 2003; Vollenweider 1971; Zison, Mills, et al. 1978). Although eutrophication has often been described as a natural aging process, most of the eutrophic process characterized by the state results from nutrient enrichment and is accelerated by human activities. Many undesirable side effects and economic issues have been raised regarding eutrophication problems. These effects are associated with the excessive plants growth (e.g. phytoplankton). Plankton plays a central role in man-made eutrophication problems. In lakes, the health-related symptom of eutrophication is algal bloom. Algal bloom causes bottom-water hypoxia, and the toxin product, resulting in fish death and health risk mentioned in some cases (Conley, Paerl, et al. 2009; Hasler and Swenson 1967). Many species of algae, including photosynthetic prokaryote and eukaryote, may produce toxic compounds when algal bloom occurs in freshwater. This suggested that one of the most significant relationships between phytoplankton growth and its dynamic composition may provide the solution to eutrophication in lakes.

Although many researches emphasized the importance of eutrophication and the

water quality management strategy of controlling nutrient loading received from heavily human-activated watersheds (Chou, Lee, et al. 2006; Schindler 1977; Schindler, Hecky, et al. 2008), many key questions of the scientific fundamentals in eutrophication were far from settled. Several attempts have been made on the scientific knowledge of eutrophication processes by investigating the relationship between the biological activity and variation of the plankton (Dodson 2000; Striebel, Behl, et al. 2009). Many documents indicated that the nutrient-stimulated eutrophication process is able to alter the species composition such as eukaryotic and prokaryotic community structure and its correlated bioproductivity (Chauhan, Fortenberry, et al. 2009; Horner-Devine, Leibold, et al. 2003; Yannarell, Kent, et al. 2003). For instance, Horner-Devine (2003) established microcosms by controlling the input of inorganic nutrient to assess the relationship between primary productivity and diversity. They found that algae and particular taxonomic groups of bacteria richness varied with primary productivity.

During 2008 to 2010, a general survey conducted showed the status of trophic levels of Lake Wilson. The Chl *a*, total carbon, total phosphorus and total nitrogen were monitored in the Lake Wilson. There were variations in Chl *a* concentrations in both temporal and spatial trends. The wide range of Chl *a* concentration (means, ±standard deviations) between 45.9 to 12.2 μg/L (mean=27.7, ±11.5, n=11) was monitored at WSFP

(Wahiawa State Freshwater Park) site. The data showed an average concentration of 410μg as N/L total nitrogen (±155, n=6), 69 μg as P/L total phosphorus (±15, n=6), and 3,077 μg as C/L inorganic carbon (±590, n=6). Based on the survey of 26 Nov. 2008, there exhibited the spatial variations in ratio of N:P and ratio of C:P in the water as shown in Table 1.1. The ratio of C:P was higher than 27 at all monitored locations. The ratio of N:P was higher than 7.2 at two locations: 1) Wahiawa State Freshwater Park and, 2) downstream of Wahiawa Wastewater Treatment Plant (WWTP). This observed data (Table 1.1) released the information about the spatial scale difference of the limiting nutrient. In general, the higher N:P ratio(e.g. >7.2) indicated that phosphorus was the limited element regulating algal bioproductivity in lake water (Chapra 1997). In practice, phosphorus control has succeeded in preventing eutrophication in many lakes (Schelske 2009; Schindler 1974). It is possible that the effluent contributed sufficient nitrogen nutrient to the lake from the sewage treatment plant. This implies that phosphorus limited the algae growth near the outfall of the sewage treatment plant due to the N:P ratio being higher than 7.2, that is, phosphorus regulates the algal growth in two locations of Lake Wilson.

Table1.1 Spatial distribution of limiting nutrient analysis of Lake Wilson

Location‡	TN (mg/l)	TP (μg/l)	TDS (mg/)	TOC (mg/)	TC (mg/l)	Chl *a* (μg/l)	*E. Coli* ($100ml^{-1}$)	Limiting nutrient analysis		
								TN:TP Ratio	TC:TP Ratio	Chl *a*:TOC Ratio*
SFKS1	0.23	66.0	55.0	4.13	6.54	3.5	75	3	36	1
SFKS2	0.22	89.1	65.0	3.36	5.75	19.9	107	2	27	6
SFKS3	0.41	82.5	80.0	3.48	6.55	31.3	4	5	37	9
WSFP	0.55	46.2	64.7	3.51	6.97	23.7	16	12	75	7
DSTP	0.58	62.7	82.0	3.53	7.40	18.8	10	9	61	5
NFKS	0.47	66.0	90.4	3.85	7.13	34.5	20	7	50	9

*: (μg Chl *a* mg C^{-1}); **: Data collected on 26 Nov. 2008. According to USGS SF Kaukonahua Rain Gage, there was no effective rainfall to cause a runoff into receiving water body prior to 72 hrs of the sampling date. Reservoir gage height was 21 m based on USGS 16210000 Wahiawa Reservoir at Spillway at Wahiawa.; ‡: SFKS1: South Fork Koukonahua station 1 (21°29'21''N, 158°00'37''W); SFKS2: South Fork Koukonahua station 2 (21°29'25''N, 158°00'44''W); SFKS3: South Fork Koukonahua station 3 (21°29'56''N, 158°01'00''W); WSFP: Wahiawa State Freshwater Park (21°29'48''N, 158°01'34''W); DSTP: Downstream of Sewage Treatment Plant (21°29'28''N, 158°02'30''W); NFKS: North Fork Koukonahua station (21°20'02''N, 158°01'45''W).

The field data described above clearly exhibited the existence of dynamically varying Chl *a* content in terms of biological activity, regulated by the limited nutrient of phosphorus. Based on the phosphorus limitation, a nutrient enrichment experiment for investigating algal bioproductivity was designed and performed in laboratory scale.

The objective of this research thesis is to investigate the relationships between lake bioproductivity and algal biodiversity in lakes. The relationships between lake bioproductivity and algal biodiversity was regarded as a central theme of scientific fundamentals in lake eutrophication. An integrating methodology including a traditional

assessment method and state-of-the-art molecular biotechnology was developed to assess this problem.

Based on the objective, the algal bioproductivity and lake biodiversity were investigated under the laboratory scale and a field study. The following tasks were fulfilled: (i) to establish the laboratory-scale experiment for simulating eutrophication process; (ii) to investigate the algal bioproductvity; (iii) to investigate the lake biodiversity of eukaryotes and prokaryotes in lakes by varying the trophic levels under stable state; (iv) to investigate lake biodiversity with time-varying algal bioproductivity; (v) to determine the structure of eukaryotic assemblage under differing the trophic status.

To ensure that the relevant tasks are able to accomplish the objective, Chapter 2 provides a comprehensive literature review of the importance of eutrophication and algal bloom in water resource management, the current knowledge of lake bioproductivity, the introduction of current molecular biotechnology in examining the biodiversity and the methods of identifying the eukaryotic structure among trophic levels. Chapter 2 reviews the traditional and current treatments in study of the man-made eutrophication problems and summarizes the critical control factors, limited nutrient such as the element of phosphorus or nitrogen in lake systems. Chapter 3 portrayed the methodology used for investigating the algal bioproductivity estimation and its lake biodiversity. A set of

methods contains the experimental concept, theorem of CSTR bioreactors, algal culture, the principle of bioproductivity calculation and the protocols of examining biodiversity, and illustrates the performance of bioreactors including the hydraulic behavior, algal growth patterns and the related utilization of phosphorus.

Chapter 4 represents the relationships of biodiversity-bioproductivity based on the bioreactors of the gradient trophic levels: oligotrophic, mesotrophic and eutrophic status. And the relationships of biodiversity-bioproductivity also were paralleled with field study in Lake Wilson. Chapter 5 illustrates how the lake biodiversity was regulated by biological activity factor, algal bioproductivity during the varying algal growth phases carried out in a eutrophic bioreactor. The observation was conducted by using time-varying analysis which demonstrates the general correlation between eukaryotic and prokaryotic biodiversity and its algal bioproductivity. Chapter 6 presents the composition of the eukaryote community, differentiated by trophic levels. These trophic levels were constructed out of three libraries: oligo, meso, and eutrophic libraries. Chapter 7 provides the conclusion and perspective of these efforts.

CHAPTER 2. LITERATURE REVIEW

To ensure that the relevant tasks are able to accomplish the objective, chapter 2 provides a comprehensive literature review. This review includes the importance of eutrophication and algal bloom in water resource management, the current knowledge of algal bioproductivity, an introduction to advance modern molecular techniques in examining the biodiversity, and identifies the eukaryotic structure among trophic levels. Chapter 2 reviews the traditional and current treatments in studies of the artificial eutrophication problems and summarizes the critical control factors, limited nutrients, such as the elements of phosphorus or nitrogen in lake systems.

2.1 Lake eutrophication and algal bloom

Eutrophication is organized as a pollution problem in lakes and reservoirs since it has a number of deleterious effects on aquatic systems (Bowie, Mill, et al. 1985; USEPA 1974). Many species of algae, including photosynthetic prokaryotes and eukaryotes, may produce toxic compounds when algal bloom occurs in marine and freshwater. Some reports have shown that these toxins have caused the fatal human poising and the death of nonhuman mammals in New Zealand, Japan, Canada, the United States and Great Britain (Ryoichi, Ootaini, et al. 2003). A number of eutrophication studies have primarily

focused on the development of the assessment method of using a single or multi index of eutrophication (Carlson 1977; Kasprzak Padisák, et al. 2008). Carlson Trophic State Index (TSI) has also been used to estimate the trophic state of reservoirs and lakes. Lakes were often classified based on its trophic state (Carlson 1977; Chou, et al. 2006; USEPA 1974; Vollenweider 1968).

The three trophic levels were frequently used (referred to in Table 2.1). The Carlson Trophic State Index (TSI) has also been used to estimate the trophic state of reservoirs and lakes. TSI is calculated by three parameters, including Secchi disk transparency, chlorophyll a and TP.

Table 2.1 Typical trophic-state classification

Variable	Oligotrophic	Mesotrophic	Eutrophic
Total phosphorus(μg-P L^{-1})	<10	10-20	>20
Chl *a* (μg L^{-1})	<4	4-10	>10
Secchi-disk depth(m)	>4	2-4	<2

Obviously, it is difficult to characterize the interaction between a biotic and abiotic environment. The viewpoint was proposed that the process of nutrient-stimulated eutrophication is able to alter the species composition such as the eukaryotic and

prokaryotic community structure, with its correlated bioproductivity (Horner-Devine, et al. 2003). Over the years, the trophic state of upland reservoirs subject to man-made pollution has been assessed by using the Vollenweider model (Vollenweider 1968) and/or Carlson Trophic State Index (TSI)(Carlson 1977). The Vollenweider model was formulated by assuming that the reservoir behaves as a completely stirred tank reactor (CSTR) and that phosphorus is the only limiting nutrient. It can be expressed as the following equation: $V\frac{dp}{dt} = W_t - v_s A_s p - Qp$, where V is the water volume of the reservoir; p is the concentration of TP; Q is the outflow; t is time; W_t is the phosphorus loading from external sources; v_s is the net settling velocity; A_s is the reservoir surface area. US Environmental Protection Agency (USEPA 1974) suggested that a reservoir is under an oligotrophic state while the average TP concentration is below 10 (μg L^{-1}), and a reservoir is under an eutrophic state while the average TP concentration is more than 20 (μg L^{-1}). At a steady state, a Vollenweider model can be solved to yield relationships between average annual TP loading (*W'*) and reservoir hydraulic properties represented by H/t_R, where H is the mean water depth and $t_R = (H A_s)/Q$ is the mean hydraulic residence time.

Limited nutrient and algal growth

Monod model and Droop model are well-known kinetics models that express the

relationship between nutrient uptake and algal growth. The Monod model described that the algal growth is governed by external nutrients. The Droop model stated that the algal growth kinetics is a function of internal nutrient such as phosphorus or vitamin (Droop 1974). In previously study, TP concentrations of lakes were investigated by Hudson and Taylor (2000) from 56 lakes in North America (Hudson, Taylor, et al. 2000). This research pointed out that the soluble reactive phosphorus (SRP) was often under picomolar concentration in lakes which along different trophic statuses. The SRP was increased along the ascent of trophic levels. Similar results were detected in TSCAR experiments. The result of laboratory experiments showed that the SRP concentrations of three trophic levels are 1.5~4.5, 2.5~6, and 8~20 μg/L for oligo, meso and eutrophic bioreactors, respectively. The SRP is the biologically availability nutrient for maintaining the primary productivity in lake. In general, the loading from runoff is recognized as the major source of nutrient in lake. Recently, the study showed that the available phosphorus for lake plankton growth obtained from internal source such as the plankton community, rather than from external loading (Hudson, Taylor, et al. 1999).

Table 2.2 SRP and TP in lakes and laboratory experiments

Lake/reactor	SRP(μg/L)	TP(μg/L)	Trophic level	Reference
Flathead Lake	0.4~0.5	2.4~4.6	oligotrophic	Graig N. Spencer and Bonnie K. Ellis 1998; Hudson, et al. 2000.
Jacks Lake	---	15.5	mesotrophic	Hudson, et al. 2000
Halfmoon Lake	---	80.6	eutrophic	Hudson, et al. 2000
Wahiawa Reservoir (Wahiawa State Freshwater Park)	8.7~12.7	26.5~46.0	eutrophic	This research
Oligotrophic bioreactror	1.5~4.0	3.30	oligotrophic	This research
Mesotrophic bioreactor	2.0~4.5	16.50	mesotrophic	This research
Eutrophic bioreactor	2.5~10.0	34.65	eutrophic	This research

The relationships between the total phosphorus and TN, Chl *a*, and total carbon have a positive linear relationship based on the study by Schindler (Schindler 1977). In order to control eutrophication in lake, we have to figure out which is the critical element of nutrients such as nitrogen (N) and phosphorus (P). Schindler et. al. (Schindler, et al. 2008) indicated that eutrophication in a lake can not be controlled by reducing the input total loading of nitrogen. They concluded that eutrophication management must be more concerned about the problem on the input loading of phosphorus from watershed. The

phosphorus loading from watersheds include a point source and a non point source. In this research we used PLOAD/BASINS (Pollutant Load/Better Assessment Science Integrating point & Non-point Sources) to estimate the phosphorus loading from watershed shown as Table 2.3.

Table 2.3 Phosphorus loading of Lake Wilson estimation

Inflow	Loading(tn/yr)	Estimated method
Non point source: Watershed runoff	3.1	PLOAD/ BASINS
Point source: Wahiawa wastewater treatment plant effluent	3.5	Data provided from WWTP

Algal growth kinetics is described based on three principle components: temperature effect, solar radiation, and nutrient effect. The first order reaction model and Michaelis-Menten model are the most common approaches to illustrate the algal growth kinetics. Laws and Chalup (1990) developed an approach of chlorophyll content basis that attended to integrate the effect of light and nutrient level in algal growth kinetics (Laws and Chalup 1990). In this approach, the behavior of algal growth kinetics such as growth rate and respiration rate is stimulated.

The half saturation rate of Michaelis-Menten model represents the affinity of the alge for the nutrient throughout transporter and receptor of cell wall to uptake or transport

nutrient from external water. A study shown that phosphate uptake rate in each species examined was a function of external phosphorus, substrate concentration and intracellular phosphorus level (Ivan and Rhee 1981). On the other hand, it was known that the metabolism of organic carbon within cells varies related based on phosphate uptake. TP concentration is often used to classify trophic status as mentioned above. However, it was known that phytoplankton's nutrient growth limitation is a function of its internal concentration rather than the nutrient concentration in water.

2.2 Algal bioproductivity

In common usage, the term "bioproductivity" is often used to expound the product of photosynthesis. For example, the book, *Technique in Bioproductivity and Photosynthesis*, edited by Coombs et. al. (1985) provides the introduction to methodologies for investigation of photosynthetic limitations to bioproductivity in natural and artificial systems (Coombs 1985). Bioproductivity was often used to describe the biomass yields of algae in field and bioreactors (Gordon and Polle 2007; Janssen, Tramper, et al. 2003). In this research, however, the bioproductivity can be considered as the increasing content of biomass per unit area and per unit time (e.g. mg m^{-2} day^{-1}) through the process of photosynthesis, and results in the conversion of light energy to chemical energy. Biomass can be expressed as conserved quantity, e.g. weight of Chl *a*,

organic matter, carbon, or energy. The bioproductivity can be measured by the variation of the amount of 14C (Yoo 2008), Chl *a* (Chapra 1997), dry weight (Gordon and Polle 2007) or dissolved oxygen (Odum 1956) per unit of time.

Carbon- based estimation

Compared with alternative methods, the 14C method represents the carbon fixed that it is a direct technique to measure algal bioproductivity. The estimate of bioproductivity is fundamentally different than productivity measured in other ecosystems (e.g., grasslands, forests). With the advanced measure technology, the easy-to-measure content (e.g. Chl *a* and DO) was utilized as an alternative in contrast to the direct measure (14C method). On the basis of literature, the range of algal bioproductivity is approximately 525~20,805g C/m^2/yr in eutrophic lakes (Gelin 1975) and 1~54.75 g C/m^2/yr in oligotrophic lakes (Waide, Willig, et al. 1999). Under lower temperature lakes, 11.3g C/m^2/yr in eutrophic lake and 4.1g C/m^2/yr in oligotrophic lake were observed (Kalff and Welch 1974).

Chlorophyll- based estimation

Kasprzak (2008) developed the Chlorophyll- based indicator which is able to describe the broad trophic gradient of lakes (Kasprzak, et al. 2008). Laws and Chalup (1990) developed an approach of chlorophyll content basis that attempts to integrate the

effect of light and nutrient level in algal growth kinetics (Laws and Chalup 1990). In this approach, the behavior of algal growth kinetics such as growth rate and respiration rate is stimulated.

The algal bioproductivity often is expressed as either the content of chlorophyll *a* (Chl *a*) (Ryther and Yentsch 1957) or by the rate of production (Schallenberg and Burns 1997). Some research showed that high Chl *a* is not guaranteed to reflect high bioproductivity because it can be merely the result of accumulation of Chl *a*. Therefore, it is necessary that algal bioproductivity should be defined as a rate term and be the amount of carbon fixed in unit area per unit time through photosynthesis. Kalff (1974) reported that based on the plot of observed Chl *a* indicated around 1~6 mg Chl *a* /m^2 in oligotractic Char Lake and around 2~40 mg Chl *a* /m^2 in eutrophic Meretta Lake (Kalff and Welch 1974).

DO- based estimation

Bioproductivity can be represented as the content of dissolved oxygen (DO) in an aquatic system. In the field study, the changes of diurnal DO can be observed, and then we can interpret its significance in the lake's water quality. In 1956, Odum pioneered in the curve of diurnal dissolve oxygen (DO). Odum used the diurnal changes of dissolved oxygen (DO) to calculate the component rate of bioproductivity and respiration. The

method was summarized as a procedure by Seeley (Seeley 1969). Odum (1956) also used the curve of diurnal DO to evaluate the productivity and respiration. He indicated that the ratio of the productivity and respiration can be used classify the freshwater communities into autotrophic and heterotrophic types.

The oxygen-oriented water quality factor is considerably influenced by photosynthesis, and respiration in particular, at the biological activity waterbody. Photosynthesis is the process which tranforms sunlight energyby green plants as chemical energy. It is also the most important biochemical pathway in aquatic ecosystem.

$$6H_2O + 6CO_2 \xrightarrow{48\text{photone}} C_6H_{12}O_6 + 6O_2 \qquad (2.1)$$

Diurnal DO variation has been recognized as an indicator related to phytoplankton and other aquatic green plants in a stream or reservoir (Odum 1956). Wang et al. (2003) used an extreme value method (EVM) which employed the maximum and minimum dissolved oxygen deficits affected by photosynthesis and respiration, to estimate metabolism rates in streams (Wang, Hondzo, et al. 2003). This method suggested that photosynthetic production could be estimated combining a mass balance model with diurnal oxygen measurements.

2.3 Lake biodiversity

An operational definition and an appropriate measuring approach are important in

lake biodiversity (LB) research. The term "biodiversity," also called biological diversity, was defined as the number of species in a given habitat (Encyclopedia 2009). The concept of biodiversity also implies the variation of number in respect to species and individual within the boundaries of a given space and time. Purvis (2000) explicated "biodiversity is the sum total of all biotic variation from the level of genes to ecosystems" (Purvis and Hector 2000). The hierarchical character of biodiversity showed the variety under higher and lower level. Norse et al. (1986) established a hierarchy of biological diversity at three levels: genetic diversity (within-species diversity), species diversity (number of species), and ecological diversity (diversity of communities) (Harper and Hawksworth 1994). To date, the study of biodiversity often investigates the three biodiversity levels which are of within species, between species and of ecosystems. The definition of three levels was expressed by the CBD (Convention on Biological Diversity). This definition of CBD: "Biological diversity" means the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic systems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystem" was widely cited. (https://www.cbd.int/ibd/2008/Resources/teachers/glossary.shtml). Some researcher have begun to investigate the interaction and linking on these levels of diversity (Magurran

2005, Vellend 2005). Lake biodiversity is the variety of eukaryote and/or prokaryote in defined space and a time of study. The definition of lake biodiversity which is used in studying lake eutrophication is: “The variation of the richness and evenness among eukaryotc and prokaryotic species in lakes.”

The measurement of biodiversity is well documented including theoretical and practical guides (Magurran 1988). Much of what is known about the models and indices was developed for addressing the temporal and spatial variation of biodiversity (Dunbar, Barns, et al. 2002). The application of models often confronted considerable debate due to the aspects of sampling (Gotelli and Colwell 2001; Magurran 1988), count technique (Caron, Countway, et al. 2004), and species determination (Templeton 1994). These problems of quantifying biodiversity were tackled by devising the indices and model (Purvis and Hector 2000). The census of individual species play important role for fitting the theoretical biodiversity models. For investigation of biodiversity, it is important for the survey of the number of species and the proportional abundance of species. The methods of surveys often depend on the level of biodiversity (Gaines, Harrod, et al. 1999). The survey techniques include remote sensing and geographic information system (GIS) for large-scale (Gaines, et al. 1999), D-net sweeps (Chase and Leibold 2002) for macroorganisms, and staining, pigment analysis, transmission electron microscopy

(Caron, et al. 2004; Guillou, Chre´tiennot-Dinet, et al. 1999) and DNA fingerprinting analysis (Templeton 1994) for picoplankton. The protocol of identification of the eukaryote and prokaryote usually is that they are isolated and extracted by specific culture media (e.g. sugar or agar) from their environment and then counted and identified by microscope or in hand. The limitation of studying lake biodiversity by using an optical instrument is that it can not promise to count the totality of species, due to little species that can be cultured on medium due to the tiny size and lack of distinctive taxonomic characters (Moon-van der Staay, De Wachter, et al. 2001). The biodiversity assessment by a DNA-based approach provides the a huge amount of invaluable species information (Caron, Countway, et al. 2009; Magurran 2005; Vellend 2005). Molecular approaches provide many advantages for biodiversity assessment. These approaches can be used to: identify the species according to the data of DNA sequences to know what species are the dominant; distinguish the species and subspecies to exhibit the phylogenetic position; and quantify the relative amount of species (Caron, et al. 2004; Karp, Edwards, et al. 1997). The molecular biotechnology (e.g. denaturing gradient gel electrophoresis, molecular cloning, sequence, and fragment analysis) can detect the variation in DNA level (Karp, et al. 1997).

In the past, numerous studies tried to use molecular biotechnology to explore a

more effective method to detect and diagnose the ecosystem. Nowadays, molecular signals have been used to distinguish organisms and represent their responses for the effects of a physical and chemical environment. The use of molecular signals as an assessment indicator for water quality comes with a few advantages which provide an early warning of possible environmental deterioration and sensitive measures of pollutant. A molecular tool is now in widespread used because it provides more detailed information on genetic content. This information from a specific gene sequence (e.g.18S rDNA) provides the basis for identification and quantification of eukaryotic micrograms. Ryoichi et al. (2003) developed a technique, PCR-based ribosomal DNA detection technique, to detect the bloom-forming genus of algae (Ryoichi, et al. 2003). The small subunit ribosomal DNA gene (e.g. 16S rDNA) sequence is used for analyzing phylogenetic relationships among the diversity taxa because it presents the properties of both highly conserved and variable region.

These are a number of approaches used to investigate genetic diversity. These approaches include denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphisms (T-RFLP) based on the DNA fragment length (Caron, et al. 2004). Muyzer G. et al.(1993) demonstrated an approach where they used DGGE to separate the specific gene sequence, 16S rDNA, obtained after PCR

(Polymerase Chain Reaction) amplification of genomic DNA extracted from microbial community population (Muyzer, Ellen, et al. 1993). Van Hannen et.al (1999) documented that nonmetric multidimensional scaling (NMDS) using statistical analysis to calculate the distance matrix converted from DGGE banding patterns provides a bidimensional plot to compare the migration of bacteria and eukaryotic community occurred after the change of condition (Van Hannen, Mooij, et al. 1999). Carrino-Kyker (2008) interpreted the importance of abiotic condition influence on the macroorganisms (Carrino-Kyker and Swanson 2008). They also used NMDS to analyze the temporal and spatial patterns of eukaryotic and bacterial communities. Muyzer (1998) presented the method of DGGE which is employed to construct the information of biodiversity on marine or freshwater (Muyzer and Kornelia 1998). They emphasized the significance of molecular biological technique particularly on detecting and identifying microorgams. The molecular marker (16S rDNA) or encoding gene is used to investigate the microbial diversity and analyses their structure of microbial communities.

In this study, DGGE technique shows an advantage that it has the ability of an immediate display of the population composition with less time-consuming and laborious. Lefranc M. et al. (2005) performed a study that they selected oligotrophic, mesotrophic, and eutrophic lakes and then used molecular biotechnology to analyze the diversity of

small eukaryotes. The study showed that the population composition of eukaryotes varied with the trophic levels (Lefranc, Thenot, et al. 2005).

The relationships between algal bioproductivity and lake biodiversity commonly are expressed concisely as four patterns (mono hump-shaped, U-shaped, positive, and negative) (Chase and Leibold 2002; Drake, Cleland, et al. 2008). The patterns were observed and concluded that highly related to the size of spatial scale (Chase and Leibold 2002), the level of nutrition (Lefranc, Thenot et al. 2005), the amount of available energy and the extent of invasion of non-native species (Chase and Leibold 2002).

Lake biodiversity (AB) is defined as the variety of eukaryotes and prokaryotes in the defined space and time of study. To investigate algal biodiversity, the method must be able to distinguish, identify and enumerate individual species. For this purpose, microscopic observation has been employed to study ecological diversity (Chase and Leibold 2002; Chien, Wu, et al. 2009; Gaines, et al. 1999). Yet the limitations of morphology and other classical taxonomic characters have been recognized for the use of the method of microscopic observation to study biodiversity (Caron, et al. 2004; Hammond 1994). The molecular observation methods probing specific genes (e.g. 16S r DNA and 18S rDNA) encoding particular species are useful tools to investigate biodiversity (Caron, et al. 2009; Karp, et al. 1997; Magurran 2005; Vellend 2005).

CHAPTER 3. METHODOLOGY

Chapter 3 represents the methodology used for investigating the algal bioproductivity and its related lake biodiversity in lakes. A set of methods contains experimental design, the use of CSTR bioreactors, algal culturing techniques, the estimates of bioproductivity and the protocols of examining biodiversity, and illustrates the performance of three trophic state-classified algal reactors (TSCARs) including the hydraulic behavior, algal growth patterns and the related utilization of phosphorus.

3.1 Experimental design

To study algal bioproductivity, the system of trophic state-classified algal reactors (TSCARs) was constructed and operated in the laboratory. The TSCARs provided a lake-like microcosm to perform a simulation of a gradient trophic state for lakes which promoted the algae growth. The design and operation parameters of the TSCARs system are summarized in Table3.1. The determination of hydraulic overflow rate (q) and areal loading of TP (W') is based on the Vollenweider model (Vollenweider 1976). According to the theorem of mass balance, under steady state this W' and q can be solved for $W' = 0.01(q+10)$ and $W' = 0.02(q+10)$ (Dillon 1975). Vollenweider (1976) suggested that the mesotrophc state may be restricted by TP concentration within 10 and

20 μg P/L. Thus, the trophic-state classifications based on a phosphorus-limited system are separated as three zones as shown in Figure 3.1.

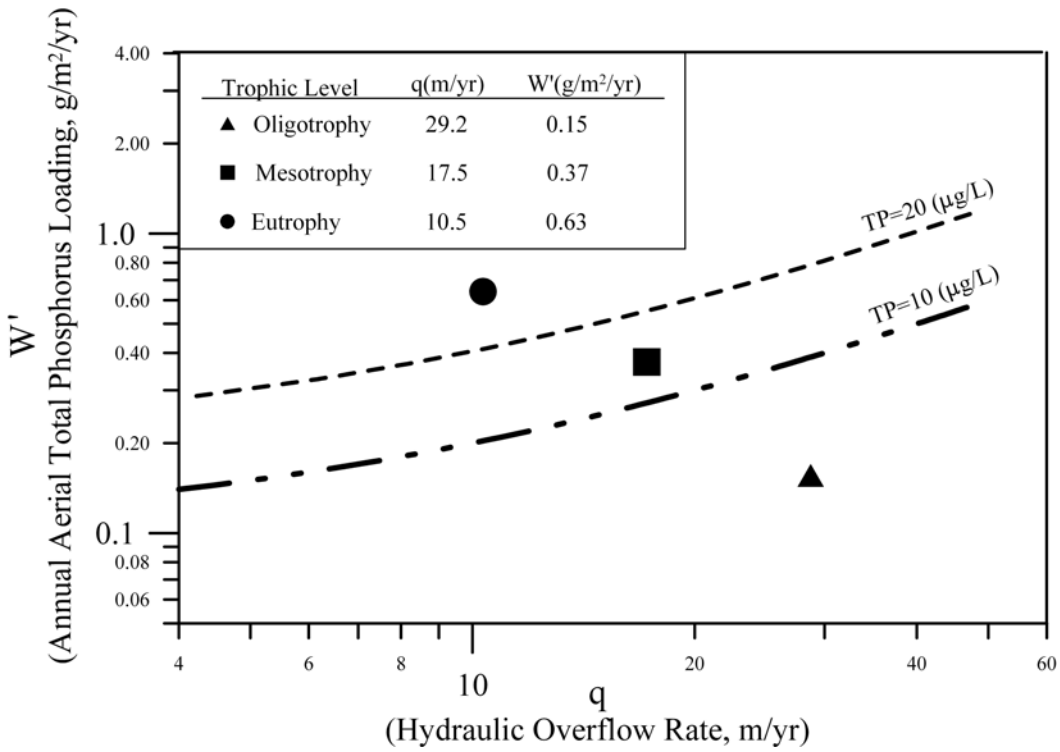


Figure 3.1 Experimental parameters of bioreactors based on Vollenweider model dividing the three classified trophic states. Average of TP concentration 10 and 20 (μg P/l) are plotted as dash dot dot and dash lines which commonly accepted to provide the upper limit for oligotrophic state and lower limit for eutrophic state in lakes.

Table 3.1 Experimental parameters of TSCARs based on the Vollenweider plot dividing the three categories of trophic levels

Bioreactor	Design parameters		Operation parmeters	
	Hydraulic overflow rate (q, m/yr)	Areal loading of TP (W', $g/m^2/yr$)	Flow rate (ml/min)	TP concentration (μg P/L)
Oligotrophic	29.2	0.15	2.6	5
Mesotrophic	17.5	0.37	1.5	15
Eutrophic	10.5	0.63	0.9	60

The reactor of TSCARs was a rectangular tank with a total volumetric capacity of 0.015 m^3 and a surface area of 0.045 m^2. A metal-halide light source (GE Muli-Vapor Lamp) was selected for the experiments to provide desirable light intensity ranges. A sterile growth medium at varying nutrient concentrations and flow rates was fed through bioreactors. The test apparatus utilized diffused-air agitation to simulate completely stirred tank reactors. The air diffuser was installed on the bottom of the culture vessel to facilitate air-bubble agitation throughout the culture vessel. The diffused air also supplied dissolved CO_2 for algal growth.

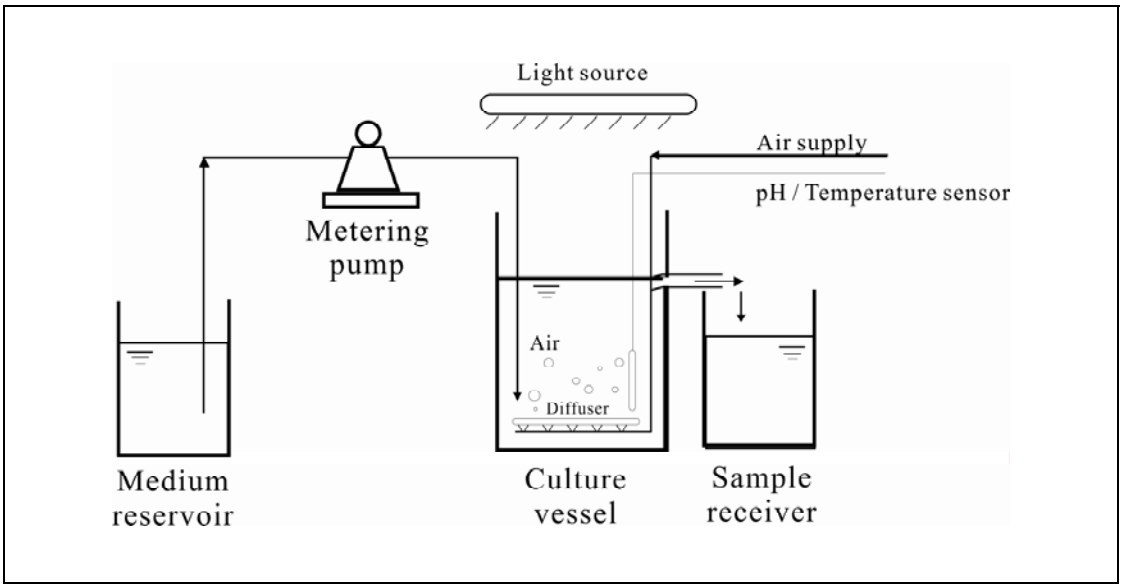


Figure 3.2 The system of trophic state-classified algal reactors (TSCARs)

TP was used as the limiting nutrient controlling different trophic states within the test apparatus. Determination of hydraulic overflow rate (q) and areal loading of total phosphorus (W') were based on the Vollenweider model (Vollenweider 1971). Bold's basal medium (BBM) (Bold 1949) was adopted for algae cultivation to create the limited-nutrient condition of phosphorus. TP were 5 μg P/l, 15 μg P/l, and 60 μg P/l of K_2HPO_4. BBM contains the mixture with the final concentration, 2.94 mM $NaNO_3$, 0.17 mM $CaCl_2$ 2 H_2O, 0.304 mM $MgSO_4$ 7H_2O, 0.428 mM NaCl, EDTA, Acidified Iron, $FeSO_4$ 7H_2O, 0.185 mM H_3BO_3, 30.7μM $ZnSO_4$ 7H_2O, 7.28μM $MnCl_2$ 4 H_2O, 4.93μM MoO_3, 6.29μM $CuSO_4$ 5H_2O, 1.68μM $CoCl_2$ 6H_2O and 5μM, 15μM, and 60μM K_2HPO_4 (KH_2PO_4) for forming the three trophic states. The modified BBMs were autoclave

sterilized at 121°C for 20 min before use. The test apparatus was continuously irradiated at 8900 W/m^2 with metal-halide lamp (400 Watt, GE Multi-Vapor Lamp). The photoperiod was 24 hours during whole period of cultivation. The inoculums of algae were introduced from the Lake Wilson sample and added to the three bioreactors using a ratio (1:10) of inoculum to BBM.

Parameters including algae cell numbers, Chl *a*, pH, TN, TOC, TP, SRP, TSS, and turbidity were monitored during the field investigation and laboratory experiments. The quantification of Chl *a* was achieved by adsorption measurements using three different wave lengths (664, 647, and 630 nm) by a spectrophotometer (DR/4000, HACH). Direct microscopy counting of algae cell numbers was accomplished using a hemocytometer (Hausser Scientific, Horsham, PA) and a bright-field microscope. TP and SRP were determined by using a PhosVer 3 kit and a DR/4000 Hach spectrophotometer following the procedure provided by the manufacturer. TN was measured using a TOC-VCPN instrument (Shimadzu Corporation, Kyoto, Japan) based on the oxidative combustion-chemiluminescence mechanism. Water-sample turbidity was measured using a Model 2100N Laboratory Turbidimeter with a working range of 0 to 4000 Nephelometric Turbidity Units. Total carbon (TC), inorganic carbon (IC), and TOC in water samples were measured using a TOC-VCPH/CPN instrument (Shimadzu

Corporation, Kyoto, Japan). Total suspended solids was determined based on the Standard Method 2540 D (Eaton, Clesceri, et al. 2005). Total dissolved solids (TDS) of water samples were determined using Standard Method 2540C (Eaton, et al. 2005). An Orion® 720A+ Dual Channel pH/ISE Meter (Thermo, Waltham, MA) was used for pH measurements. YSI 58 DO meter and YSI 600R DO Sonde (YSI, Inc., Yellow Springs, Ohio) were used for DO measurements.

3.2 Estimates of algal bioproductivity

The estimates of algal bioproductivity are based on the changes in algal biomass in terms of Chl *a* content which occurs in a given aquatic system during a unit of time. In the equation (3.1), the mass balance model represents the changes of Chl *a* in the given system during a unit time. Changes in Chl *a* content resulted from the algal growth and loss processes can be represented by algal net growth rate (r_s) consisting of the algal gross growth rate (μ_g) and algal net loss rate (λ_l). In the equation (3.2a), the first term expresses the gross growth rate (μ_g), and the second term represents the net loss rate (λ_l). The net loss rate combined with respiration rate, settling rate, decomposition rate and grazing rate is modeled by assuming which loss rate is directly proportional to the algal biomass.

The algal bioproductivity could be simulated by the mass balance model in

conjunction with the changes of Chl *a* concentration. Based on the mass balance equation, the algal bioproductivity model for a well-mixed lake can be written as:

$$\frac{dX}{dt} + (\frac{Q}{V} \cdot X - r_n) = \frac{Q}{V} \cdot X_i \tag{3.1}$$

$$r_n = r_s \cdot X \tag{3.2}$$

$$r_s = \mu_g - \lambda_l \tag{3.2a}$$

Where,

X_i = input Chl *a* concentration, mass/volume

X = Chl *a* concentration, mass/volume

V = volume of reactor, volume

Q = flow, volume/time

r_n = the change in Chl *a* concentration during a unit time, mass/volume/time

r_s = algal net growth rate, 1/time

μ_g = algal gross growth rate, 1/time

λ_l = algal net loss rate, 1/time

The input Chl *a* concentration (X_i) is assumed close to zero and Q/V is defined as dilution rate (D), then r_n can be calculated by equation (3.3).

$$r_n = (\frac{dX}{dt}) + D \cdot X \tag{3.3}$$

Algal bioproductivity can be expressed as P_r (mg Chl *a* / m^2/ day) based on the

definition: “The change in Chl *a* of algae occurred in a given area over a photoperiod”.

The P_r (mg Chl *a* / m^2/ day) of areal basis can be formulated as:

$$P_r = \frac{\text{Biomass at } t_{n+1} - \text{Biomass at } t_n}{t_{n+1} - t_n} \cdot \frac{1}{\text{Area}(A_L)} \tag{3.4}$$

The area (A_L) is determined by water volume divided by the depth (z) of euphotic zone which assumed water volume under a condition of uniform algal density and light intensity. Based on equation (3.4), P_r (mg Chl *a* / m^2/ day) can be expressed as equation (3.5) in the CSTR system.

$$P_r = r_n \cdot z \tag{3.5}$$

Where, z is the depth of the bioreactor (For a real lake, z is defined as the depth of euphotic zone in lake).

The steady-state condition ($dX/dt = 0$) is uncommon in lakes. The non steady-state conditions are especially present in a mixture culture of an algal community (e.g. lakes). The fluctuations in algal population are often observed due to the algal systems functioning on diurnal cycles, a seasonal succession, biological regulation and physical and chemical processes (such as runoff, temperature, light and nutrient). It results in the variety of primary producer (e.g. algal population) density during specific period. The algal bioproductivity, therefore, is described as a function of time: $P_r(t)$ called instantaneous algal bioproductivity. For this reason, the estimates of algal

bioproductivity must take time-dependency into account when a mathematic expression is derived from the period-integrated basis. This equation (3.6) proposes that algal bioproductivity under the non steady-state conditions, namely average algal bioproductivity ($\overline{P_r}$), can be obtained by integrating instantaneous algal bioproductivity ($P_r = r_n \cdot z$) over a given time period, T. In the equation (3.6), the integrated term, $\int_0^T P_r(t)dt$, is the amounts of areal algal product (P_T) formation over T period.

$$\overline{P_r} = (1/T) \cdot \int_0^T P_r(t)dt \qquad (3.6)$$

The areal algal product (P_T) may present either the positive or negative value during algal growth processes. Positive P_T indicates the primary producers dominate the algal system during the period; negative P_T indicates consumers dominate the algal system during the period.

The two principal components are summarized to develop the estimates of algal bioproductivity expressed as:

(i) Instantaneous algal bioproductivity (P_r)

Instantaneous algal bioproductivity is a theoretical notion. Moreover, since instantaneous Chl *a* change (dX/dt) cannot be measured directly, it is necessary to develop the daily arithmetic interpolation of algal bioproductivity. For practical use, it is necessary to transform instantaneous algal bioproductivity to daily algal bioproductivity.

The daily arithmetic interpolation procedure has been used in field works (Vadeboncoeur, Peterson, et al. 2008). The daily interval will be used in following the calculation. Based on finite differences as $dX/dt \approx \Delta X/\Delta t$, ΔX (mg/m^3) represents the change of Chl *a* under Δt, a uniform daily interval. In CSTR system, based on equation (3.5), the daily algal bioproductivity (P_r) can be obtained.

(ii) Average algal bioproductivity ($\overline{P_r}$)

The amount of areal algal product (P_T, mg/m^2) can be calculated by either Pr(t) function integration or equation (3.7). Because the Pr(t) is not commonly able to be expressed as a set of formulae due to under varying environment, the area-based sum method is developed and expressed as equation (3.7), which can be used to calculate approximately P_T. Then, the average algal bioproductivity ($\overline{P_r}$, mg/m^2/day) could be determined by P_T /T.

$$P_T = \sum_{n=1}^{T}(P_r)_n = z \cdot \sum_{n=1}^{T} r_n \cdot \Delta t_n = z \cdot \sum_{n=1}^{T}(\frac{\Delta X}{\Delta t})_n \cdot \Delta t_n + z \cdot D \cdot \sum_{n=1}^{T}(X)_n \cdot \Delta t_n \qquad (3.7)$$

Procedure of algal bioproductivity estimations:

An example is employed to illustrate the procedure of algal bioproductivity estimations. The procedure includes :(1) the sampling for obtaining the represent of biomass by Chl *a* content, (2) calculation of biomass change, (3) based on observed biomass and dilution rate to calculate the amount of washout in the CSTR system, (4)

calculation of r_n and plot of the time-varying Pr(t), (5) based on equation (3.7) to calculate areal algal product (P_T), and (8) calculate the average algal bioproductivity ($\overline{P_r}$).

The Figure 3.3 showed that the Chl *a* data observed results in the calculation of Chl *a* change in unit time and r_n, which used in this example. The data were collected from the eutrophic TSCAR bioreactor, which represents the algal growth in mixture culture.

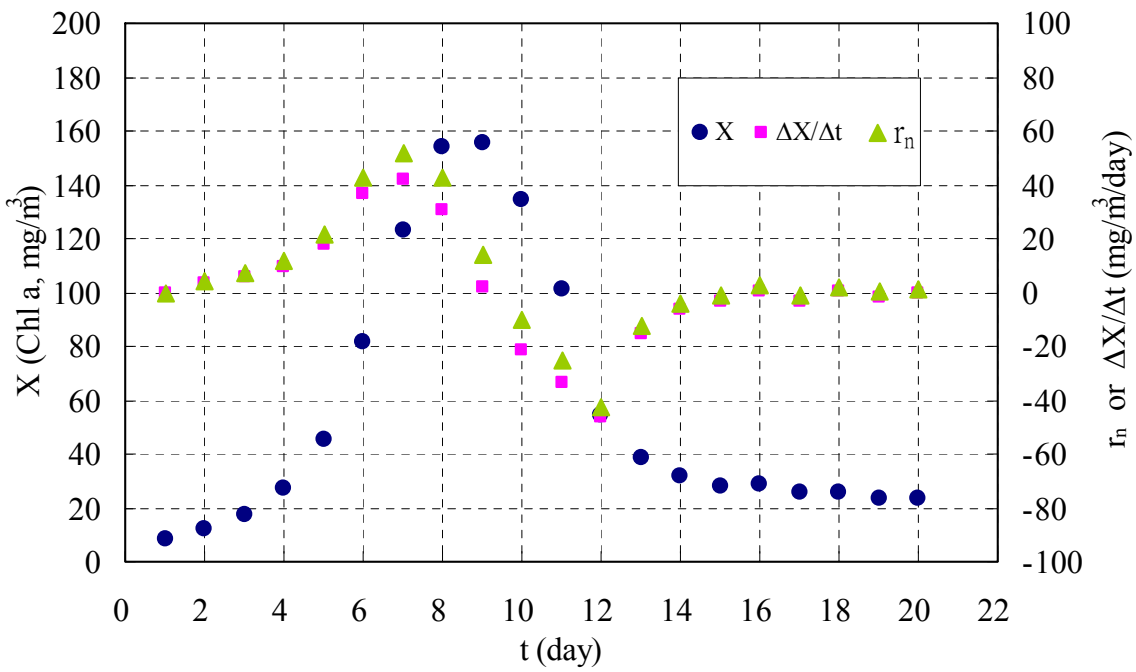


Figure 3.3 Chl *a* concentaction (X) collected from TSCAR eutrophic bioreactor and change of Chl *a* concentaction per day ($\Delta X/\Delta t$) and r_n which used in the algal bioproductivity calculation procedure.

1. Monitor X

Replicate samples for X (Chl *a*, mg/L) are taken which is recommended under regular interval at 1-day over T period. If an irregular interval is given, use interpolation to insert an intermediate term in a period of time. The length of T is dependent on the need of tasks. It can be collected in event base (e.g., algal bloom event), seasonal, or annual period.

2. Change of Chl *a* concentration per unit time: $\Delta X / \Delta t$

A set of rate-of-change data was constructed (mg Chl *a*/m^3 /day) by using 1-day intervals for calculation.

3. Washout term: DX

In CSTR, washout Chl *a* was determined as dilution rate multiplied by Chl *a* concentration at time, t.

4. Instantaneous algal bioproductivity: $P_r(t)$

Instantaneous algal bioproductivity was calculated by equation (3.4) and (3.5). The r_n could have a positive or negative value. As referred in Figure3.3, during the positive phase, the algal population is elevating (Day 1 to Day 7); during the negative phase, the algal population is alleviating (Day 8 to Day 20). In this example, the algal population in ups and downs was observed in the eutrophic bioreactor (referred to in Figure 3.4).

Figure 3.4 represents the phases in development of algal population. There are two major phases: accrual phase and loss phase. The change of phases should be caused by either biochemical compounds or interactions between populations while the environmental and nutrient conductions are in control of laboratory experiment. Based on the observed data, $P_r(t)$ can be summarized as a set of equations (3.8).

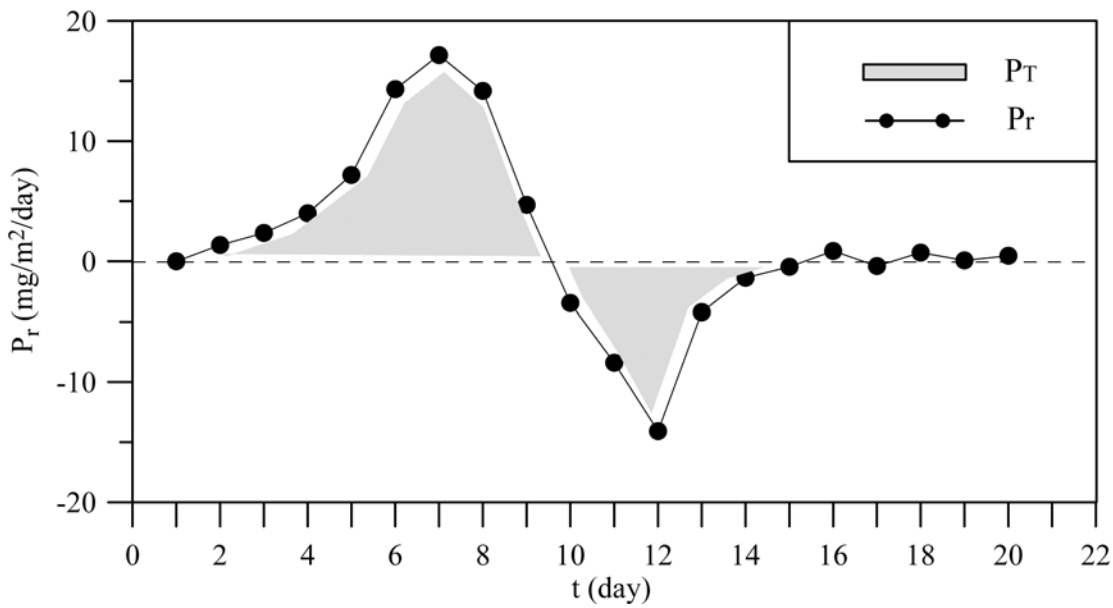


Figure 3.4 Demonstration for algal areal product (P_T, mg/m^2) based on $P_r(t)$ by using a set of $P_r(t)$ functions (3.8).

$$P_r(t) = \begin{cases} 0.478e^{0.535t} & \text{when} \quad 1 < t \le 7 \quad (R^2 = 0.987) \\ -6.632t + 64.685 & \text{when} \quad 7 \le t \le 12 \quad (R^2 = 0.985) \end{cases} \tag{3.8}$$

The algal bioproductivity (P_r) was exponential increase during the period of Day 1 to Day 7; its exponential constant, Kb, was 0.54 (1/day). Following the P_r was

decreased along the linear track, correlated with time during the period of Day 7 to Day 12. And at Day 9.7 the r_n was equal to zero (dX/dt=0). The maximum instantaneous bioproductivity (P_r) was 17.2 (mg as Chl *a*/ m^2/day) and occurred on Day 7.

5. Calculate the magnitude of areal algal product (P_T)

Based on step (6), plotted instantaneous bioproductivity (P_r) versus time (t) showed as Figure 3.4. With the use of equation (3.7), P_T can be calculated approximately. The area under instantaneous algal bioproductivity $P_r(t)$ curve was called areal algal product (P_T). The shaded area shows that P_T was 34.9 (mg Chl *a* /m^2) which was calculated by the sum of positive area (+67.3 mg Chl *a* /m^2) and negative area (-32.4 mg Chl *a*/m^2). Referred to Figure 3.5, the positive area represents that the P_T was accumulating during Day 1 to Day 9. In contrast, the period of the negative area showed that consumer dominated the system. Finally, P_T was maintained at the amounts of around 34.9 (mg Chl *a* /m^2) for the following seven days.

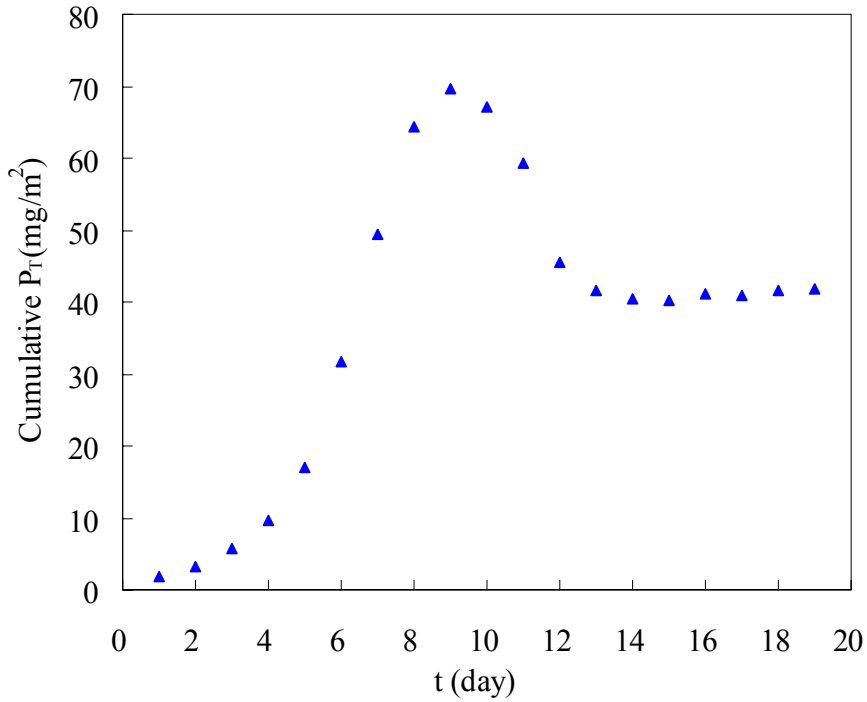


Figure 3.5 Cumulative P_T (mg Chl *a* /m^2) in the bioreactor over the time period.

6. Estimate average algal bioproductivity ($\overline{P_r}$)

$\overline{P_r} = P_T$ /T =34 (mg Chl *a*/ m^2)/20 =1.75 (mg Chl *a*/ m^2/day)

3.3 Estimates of lake biodiversity

For lake biodiversity analysis, the major techniques employed are: (1) DGGE method and (2) Molecular cloning. The methods involved the following steps: sampling, nucleic acid extraction, nucleic acid amplification by PCR, DGGE analysis, molecular cloning and biodiversity estimation by using the Shannon index. The molecular cloning was conducted to study lake biodiversity under different trophic levels.

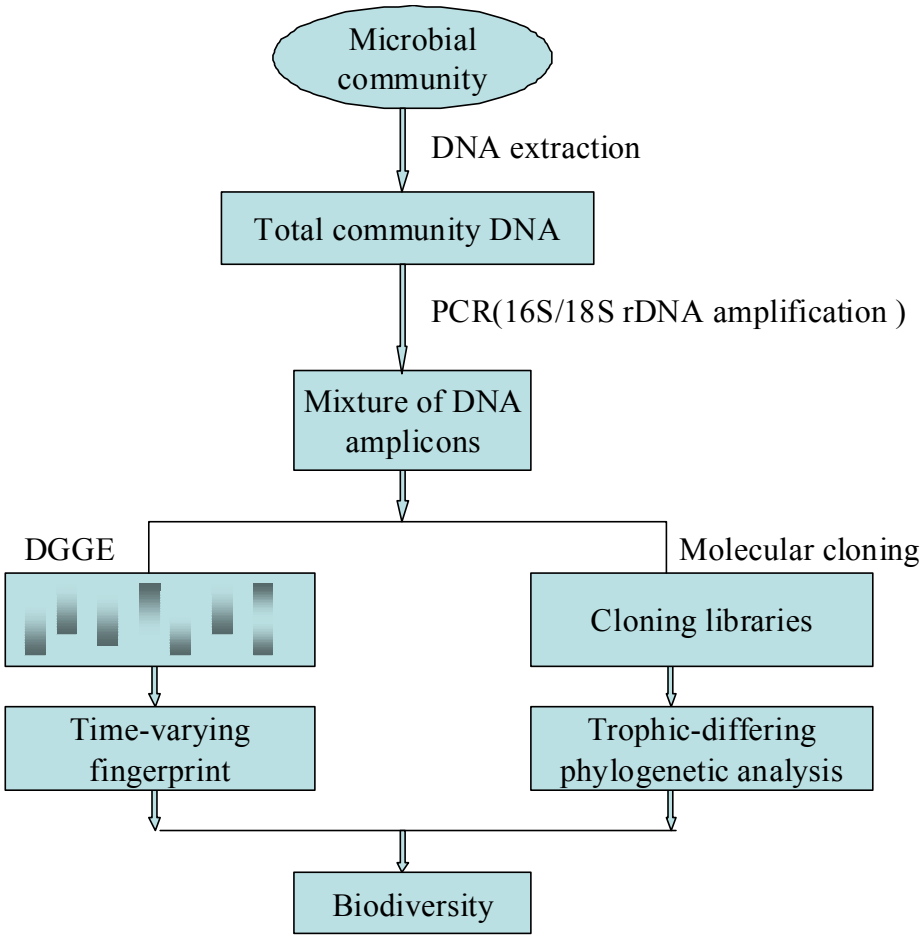


Figure 3.6 Flow diagram of the application of DGGE and molecular cloning to lake biodiversity estimation.

Methods

Sampling

Water samples were collected from three TSCARs' sample receivers during the experiment period. 300 ml volume of water samples were collected and then immediately filtered through 0.45 μm pore-size hydrophilic mixed cellulose ester filter (Pall Life Science). The filter was kept in 1.7ml microcentrifuge tube (VWR) and stored at -75 ^{o}C until nucleic acid extraction was conducted.

DNA extraction

The CTAB (Cetyl Trimethyl Ammonium Bromide) base protocol of nucleic acid extraction was used in this research. This was well developed, described as in previous studies (Lefranc, et al. 2005; Phillips, Celia, et al. 2001). The filters were moved from -75 ^{o}C to room temperature for 30 min. Cover the filters with TE buffer (10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0) and lysozyme solution (final conc., 250µg/ml). The filters were incubated at 37 ^{o}C for 30 min. Add sodium dodecyl sulfate (SDS, 10%) and Proteinase K (final conc. 100ug/ml) and then incubate at 37 ^{o}C for at least one hour. Add CTAB (final conc. 2% in a 0.7 M NaCl solution) and incubate at 65 ^{o}C for 10 min. Transfer the sample to a new tube (1.7ml microcentrifuge tube). Add an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Mix the contents of the tubes until the emulsion forms.

Centrifuge the mixture at 13,000 rpm for one min. Move upper phase to new tube. Add an equal volume of chloroform mixture (chloroform: isoamyl alcohol (24:1)). Estimate the volume of the nucleic acid solution. Move 495 µl to new tube and add the 5 µl NaCl 5M (final conc. 0.1M). The final volume is equal to 500 µl. Gently mix the solution. Add ice-cold ethanol, 2 volumes of nucleic acid solution mixture. Mix solution and store the ethanolic solution on ice to allow the precipitate of nucleic acid to form. Store the solution at -20 ^{o}C for 4 hours. Centrifuge at 13,000 rpm for 10 min. Fill the tube 1000 µl with 70% ethanol and re-centrifuge at 13,000 rpm for 2min. Then carefully remove the supernatant by standing the tube in an inverted position on a wipes layer. Store the open tube on the bench for 30 min. Add TE (1X) 50µl. The nucleic acid yield was quantified by a spectrophotometer (Biophotometer 6131, Eppendorf) with a plastic cuvette (10/2 mm, 220-1600nm, UVette, Eppendorf) detecting absorbance at 260nm. The nucleic acid extracts were stored at -20°C until use.

DGGE analysis

PCR amplification for DGGE experiments

PCR was performed to amplify the specific gene (16S rDNA and 18S rDNA). After extracting the nucleic acid, the fragment of DNA was performed in a PTC 100 Thermal Cycler (MJ Research) which was automated and programmable equipment. The

16S rDNA gene were amplified by the primers of BAC518R (5' ATT ACC GCG GCT GCT GCT GG 3') (Muyzer, G. et al., 1993) and BAC338F+GC (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG 3') which are specific for most *Bacteria*, *Archaea*, and *Eucarya*. PCR amplification was performed in a 50 µl volume containing approximately 5 ng of template DNA, 1X KCl Reaction Buffer (Bioline) containing 50mM KCl, 10mM Tris-HCl pH 8.8 (at 25°C), 1.5mM $MgCl_2$, and stabilizers, 0.2µM primer, 200µM each deoxynucleotide (Bioline), 400 ng of bovine serum albumin (Promega), and 1 U of *Taq* DNA polymerase (Bioline). PCR cycling was performed with a PTC 100 Thermal Cycler (MJ Research). The temperature cycling conditions were as follows: a 5 min initial denaturation at 94°C, 10 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30s, and 25 cycles of 92°C for 30 s, 55°C for 30 s, and 72°C for 30s, followed by 5 min at 72°C and incubation by 2 min at 4°C (Lapara, Nakatsu, et al. 2000). The 18S rDNA gene were amplified by the primers of F1427GC (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CTC TGT GAT GCC CTT AGA TGT TCT-3'-3') and R1616 (5'-GCG GTG TGT ACA AAG GGC AGG G-3'). The primers are specific for eukaryotic aquatic microorganisms (Van Hannen, Zwart, et al. 1999). The thermal cycling conditions were as follows: 5 min of initial denaturation at 94°C followed by 25 cycles of 94°C for 1 min, 52°C for 1 min,

and 72°C for 1 min and then a final extension step of 72°C for 5 min. The reaction conditions were as described above.

DGGE experiments

DGGE was performed as described previously (Klammer, Knapp, et al. 2008; Muyzer, et al. 1993). Briefly, 30µl volume of PCR products were loaded into a 1.5-mm-thick vertical gel containing 8% (w/v) polyacrylamide (acrylamide-bisacrylamide, 37.5:1) and conducted by the DCode University Mutation Detection system (BIO-RAD). Two linear gradient denaturing gels were casted for detecting the migration of DNA fragments. The linear gradient denaturing gels consisted of urea and deionized formamide (deionized by ion exchange resin, BIO-RAD G501-X8CD), ascending denatueant from 40% to 60% for the mutation of the 16S rDNA fragment and from 30% to 55% for the mutation of the 18S rDNA fragment. We prepared 100% denaturant which was defined as 7 M urea and 40% (v/v) formamide (deionized). Electrophoresis was performed at 60°C in a 0.5X TAE buffer, and 75 V of electricity (6V/cm) was applied to the gel for 16 hr. DNA were visualized by staining them for 30 min in 3X Gel Green (Biotium) staining solution containing 50 µl Gel Green, 17 ml 1M NaCl, and 150 ml di-water and then photographing the gel using the Fotodyne system (FOTODYNE Incorporated, Japan). Digitized images were adjusted by Quantity One

4.6.3(BIO-RAD) PC image software.

Molecular Cloning

PCR amplification

PCR was performed with primer EukA (5'-ACCTGGTTGATCCTGCCAG-'3) and EukB (5'-TGATCCTTCYGCAGGTTCAC-'3) (Medlin, Elwood, et al. 1988; Moon-van der Staay, Staay, et al. 2000), which amplified approximately 1.7kb of full-length 18S rRNA gene. PCR program involved a 3 min initial denaturation at 94°C followed by 30 cycles of 94°C for 45 min, 55°C for 1 min, and 72°C for 3 min and then a final extension step of 72°C for 5 min (Moon-van der Staay, et al. 2001). PCR amplification was performed in a 50 µl volume containing 1µl DNA extraction product as template DNA, 1X KCl reaction buffer (Bioline), 1.5mM $MgCl_2$, 0.2mM primer, 0.2mM each deoxynucleotide (Bioline), and 0.3U of *Taq* DNA polymerase (Bioline). PCR cycling was performed with a PTC 100 Thermal Cycler (MJ Research). The 1% low-melt agarose gel (Promega) was used to recover DNA fragments of PCR product and then purified by Wizard SV Gel and PCR Clean-up system (Promega) for ligation reaction use.

Transformations

E.Coli strain DH10B cell were prepared described as in previously study

(Sambrook and Russell 2001). The purified PCR products were ligated into the pGEM-T plasmid vector (Promega, Madison, Wis) and transformed into DH10B cell. Electroporation were carried out by Electroporator 2510 (Eppendorf, USA) set at 1700 V pulse discharge. Ligation reactions were performed according to manufacturer's recommendations (Promega, Madison, Wis). Clones containing insert DNA of the correct size were stored in glycerol (15%) at -20 °C for later use.

Clone Libraries

Three eukaryotic libraries (Oligotrophic, mesotrophic and eutrophic bioreactors) and one prokaryotic library (eutrophic bioreactor) were constructed, designated as Ek4 library (Oligotrophic), Ek5 library (Mesotrophic), Ek6 library (Eutrophic), and Pk6 library (Eutrophic). Around 62 clones from each library were randomly picked from different LB plates with ampicillin/IPTG/X-Gal. These clones containing insert DNA of the correct size were picked, incubated at 37 °C overnight and stored in glycerol (15%) at -20 °C for later use. The size of inserts DNA of 18S /16 S rDNA in positive colonies were checked by PCR amplification by using primers M13f (5'GT TTT CCC AGT CAC GAC'3) and M13r (5'CA GGA AAC AGC TAT GAC'3). The correct size of inserts DNA was amplified by PCR using the primers EK1427f (GC) and EK1616r described previously. These PCR products were loaded into DGGE gel for screening clone libraries

(Kowalchuk, Stephen, et al. 1997; Rowan, Snape, et al. 2003; Van Hannen, et al. 1999). It provides a pre-filtered strategy for analyzing a numerous clones. The presence of same position in DGGE gel was be grouped together and considered representative member in each library (Muyzer and Kornelia 1998).

DNA Sequence

One of insert DNA of each group was sequenced as a representative member. The plasmid from selected clones were extracted by using Mine-prep protocol (Sambrook and Russell 2001). The primers Eku A and Eku B were used for sequencing. The sequencing reactions were carried out by Biotech Core (http://core.biotech.hawaii.edu/default.htm). Automatic pairwise alignment of all sequences was performed by Bioedit Sequence Alignment Editor Program (available for download at http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Phylogenetic analyses

The sequence of three bioreactors were compared to SSU rDNA sequence in GenBank using BLAST (Basic Local Alignment Search Tool) (Zhang, Schwartz, et al. 2000) analysis to determine their phylogenetic affiliation.

Determination of Biodiversity

DGGE experiments

Image of DGGE gel was used to determine the lake biodiversity of three TSCARs by the Shannon index. The formula of the Shannon index (H') was published by Claude E. Shannon (Shannon 1948). The Shannon index is one of the nonparametric measures of biodiversity based on two assumptions: (1) the individuals are randomly sampled from an infinite community; (2) all species are represented in the sample (Hutcheson 1970). The banding data of the DGGE gel was digitized by using the TotalLab software (Nonlinear, USA). The digital banding data consisted of the number of band and peck height intensity of band. An example performed by TotalLab is shown as Appendix A which illustrated the procedure of digitizing bands. Equation (3.9) was used to calculate biodiversity based on the importance probability of band (p_{ij}) at *i* pixel position on *j* lane. The p_{ij} ($=n_i/N_j$) was determined by intensity of peck height (n_i) of ith band divided by total peck height (N_j) of intensity of jth lane (Eichner, Erb, et al. 1999). Based on several assumptions adopted as described previous studies (Fromin, Hamelin, et al. 2002; Murray, Hollibaugh, et al. 1996), digital banding data of DGGE gel can be applied in fingerprinting analysis. For interpreting DGGE fingerprinting, each discrete band of DGGE gel was regarded as a unique OUT (or phylotype) and the peck height of each band was directly related to density (or abundance) of OUT (or phylotype) in assemblage (Murray, et al. 1996). Thus, the banding data obtained from gel image can be applied in the calculation of statistic

index of biodiversity (Fromin, et al. 2002). The formula for calculating algal biodiversity by the Shannon index is:

$$H' = -\sum p_{ij} \ln p_{ij} \tag{3.9}$$

$$p_{ij} = \frac{n_i}{N_j} \tag{3.10}$$

$$E = \frac{H'}{\ln S} \tag{3.11}$$

Where,

H' = Shannon index; p_{ij} = importance probability of band; n_i = peck height of i th band; N_j = sum of peck height of all band on j th lane; E = Evenness; S = total band count on one lane.

TotalLab software was used to banding detection of DGGE gel of 18S rDNA fragments amplified from Lake Wilson water samples from WSFP (gel C) and DSTP (gel D) stations collected on April 13, 2011 (Refered to Appendix A). Using equaton (3.9)~(3.11), eukaryotic biodiversity was calculated and shown as Table 4.8. The variance in biodiversity of two sites can be estimated using the t-test method developed by Hutcheson (Hutcheson 1970). Hutcheson (Hutcheson 1970) presented a t-test method for comparing the biodiversity of Shannon index. If absolute t-value is larger than the two-tailed critical value of t ($t_{\alpha(2),df}$, where $\alpha_{(2)}$ = two-tailed probability of α; d.f.= degrees of freedom), it indicated that two sites are significantly different at the 5% level.

Based on the DGGE banding data of FigureA.1 in Appendix A, for instance, the biodiversity of DSTP site was H'=1.725 while the biodiversity of WSFP site was H'=2.571. The t-test showed that the two sites were significantly different under 5% level ($t=14$, $\alpha_{(2)}=0.05$, d.f.=764).

3.4 Discussion

In order to diagnose and evaluate the performance of bioreactors in terms of the physical and biological aspects, the tracer experiment and image of DGGE were used. Tracer experiments can be used to diagnose whether the system was under perfect mixing. DGGE banding data can be used to evaluate that the species introduced from Lake Wilson in the artificial system.

Behavior of fluid flowing through the bioreactors

A tracer experiment was conducted in order to determine the flow behavior of this TSCARs system either under plus flow or well-mixed flow. The three bioreactors were deviated from the ideal flow model and the operation parameters referred to in Table 3.1. The tracer experiment was used to confirm the actual system and evaluate the efficiency of bioreactors. A conservative substance, potassium chloride (KCl), was used as a tracer in the experiment. The concentration C_0 (0.01 M, KCl) of step tracer input was injected continuously, and the concentration of tracer output (C) and the volumetric rate of

outflow (Q) were measured daily. The fraction of KCl in the outflow at time t was C/C_0. The mean hydraulic resident time was defined as $t_R = V/Q$. V was the volume of bioreactor. The plot of (C/C_0) versus θ (= t/t_R) showed that the representative type of systems was well-mixed system (referred to Figure3.8). The observed data were represented as the equation, $C(t) = C_0(1 - e^{-t/t_R})$ in three bioreactors.

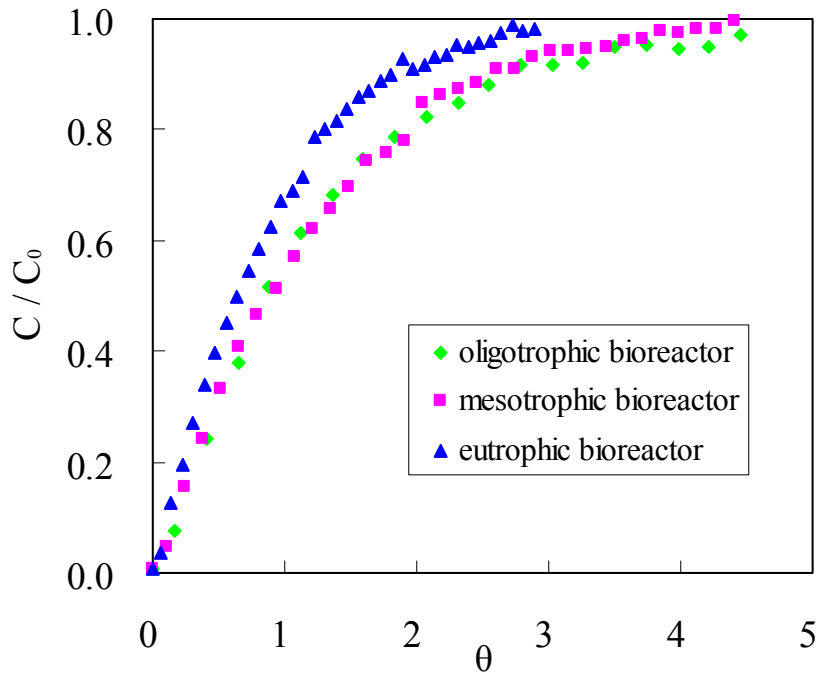


Figure 3.8 The plot of observed data by using step tracer input for the three bioreactors: oligotrophic bioreactor, mesotrophic bioreactor, and eutrophic bioreactor, in response to a continuous input.

The mean hydraulic residence times (t_R) of three bioreactors determined based on

tracer observed data are: 4.8 day, 8.6day, and 10.4 day for oligo, meso, and eutrophic bioreactors, respectively. For a non-conservative substance like phosphorus, the resident time must be taken the internal losses and hydraulic losses into account in the CSTR system (Sonzogni, Uttormark, et al. 1976). The phosphorus resident time (τ_p) can be determined by $\tau_p = 1/(\rho_w + \sigma)$ (see Chapter4).

The evaluation of the Cultivation of eukaryotic and prokaryotic algal in three bioreactors

The artificial lake water, BBM media, was used for algal culture experiments. The BBM medium described previously was widely used for many algae culture except vitamin-required algal. The evaluation indicates that in the artificial system the population dynamic which includes growth and extinction of the algae introduced from the nature system. Based on community analysis (see Chapter6), the artificial lake water, BBMM media, was able to provide an environment for prokaryotic and eukaryotic community structure when compared with real lakes.

Patterns of algal growth in TSCARs

Three patterns of algal growth were observed in three CSTR bioreactors (graphed in Figure 3.9). Pattern I represents that the equilibrium may be achieved. Pattern II and pattern III showed the non-equilibrium condition in the system.

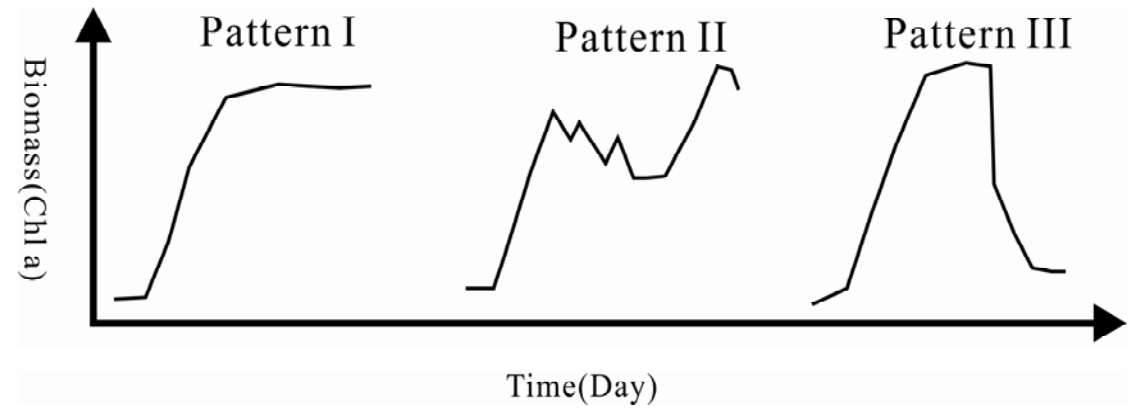


Figure 3.9 Algae growth patterns observed in three TSCAR bioreactors

CHAPTER 4. LAKE BIODIVERSITY UNDER DIFFERENT TROPHIC LEVELS

Lake biodiversity of the different trophic levels was examined by conducting denaturing gradient gel electrophoresis (DGGE) experiments. In the laboratory, three trophic states formed in TSCARs based on the total phosphorus loading concept of the Vollenweider model. This is representative of the trophic states of the lake described in Chapter 3. DGGE fingerprints of 18 S ribosomal DNA (eukaryote) and 16S ribosomal DNA (prokaryote) fragments were used to investigate the lake eukaryotic and prokaryotic biodiversity in samples obtained from TSCARs. On the other hand, a comparative filed investigation was conducted by sampling from two distinct trophic-state stations of Lake Wilson. In the field study, the survey in the lake was conducted including the measurement of water quality such as TP, SRP, TN, TC, TSS, pH, DO, conductivity, and Chl *a*. The yield of point and nonpoint sources of TP loading from Lake Wilson watershed was estimated by the observed data of the Hawaii state government and Basins/PLoad Model. This case study allowed us to relate the empirical lake biodiversity to different trophic levels.

4.1 Laboratory investigation

The artificial, simplified algal microcosms used to simulate different trophic states

of the lake were common methods in investigating the relationship of trophic level and its biodiversity (Jardillier, Boucher, et al. 2005; Kassen, Buckling, et al. 2000). Three algal microcosms formed by TSCARs in the laboratory were regarded as the lakes classified according to trophic states (oligotrophic, mesotrophic and eutrophic) and then the biodiversity was examined by DGGE. The details of TSCARs were described in Chapter 3. The results of TSCARs showed that three trophic states were clearly demonstrated according to TP concentration which is in response to variation in nutrient enrichment.

4.1.1 Results of TSCARs experiments

The TSCARs were designed based on the Vollenweider model (Vollenweider 1976) and this model was expected to predict trophic status in lakes. Based on the experimental design (referred to Table 3.1), the TSCARs phosphorus-limited system conducted under two designated parameters: hydraulic overflow rate (q, m/yr) and areal loading of TP (W', $g/m^2/yr$) was expected to create the three distinct trophic states identified by TP concentration as shown Table 4.1.

After replicate experiments, TSCARs formed a sufficient basis for describing the algal microcosms in which the system presents the attributes of trophic states. In the three trophic states, algal productivity in the three algal microcosms was estimated and grouped into three trophic degrees, in which the magnitude of algal bioproductivity

compared with literature in field studies of lakes (Waide, et al. 1999; Wetzel 2001). The other characteristics of TSCARs such as physical (temperature, light, turbidity), chemical (TN, pH, TC, TDS, TSS) factors were measured during TSCARs operation period.

Table 4.1 Trophic-state classification based on total phosphorus concentration

Trophic classes	TP (μg P/l)
Oligotrophic	<10
Mesotrophic	10-20
Eutrophic	>20

Phosphorus kinetics in TSCARs

During the experiment, the SRP and TP of TSCARs were measured under the experimental treatments conducted based on Table3.1. The TP content is the sum of dissolved phosphorus and phosphorus in suspension. The SRP was the dissolved inorganic phosphorus in filtrate. Figure4.1 shows that the results declined with low SRP concentrations were exhibited during the experimental period. In the oligotrophic bioreactor, SRP keep low concentration (mean = 2.75 μg P/l, n=12, sd.=1.53) on the whole period. In the mesotrophic bioreactor, SRP were decreased from 13.7 to 3.7 (μg P/l) within Day1 and then remain low concentrations (mean=3.41μg P/l, n=11, sd.=1.29) during the following 10 days. In the eutrophic bioreactor, gradual lessening of SRP was

observed from Day1 to Day4 (from 63.6 to 6.7 μg P/l) and then presents at low level concentrations (mean=6.02 μg P/l, n=7, sd.=1.79). Among the three reactors, there was general agreement that SRP concentrations present lower levels as the SRP concentrations reach stable state.

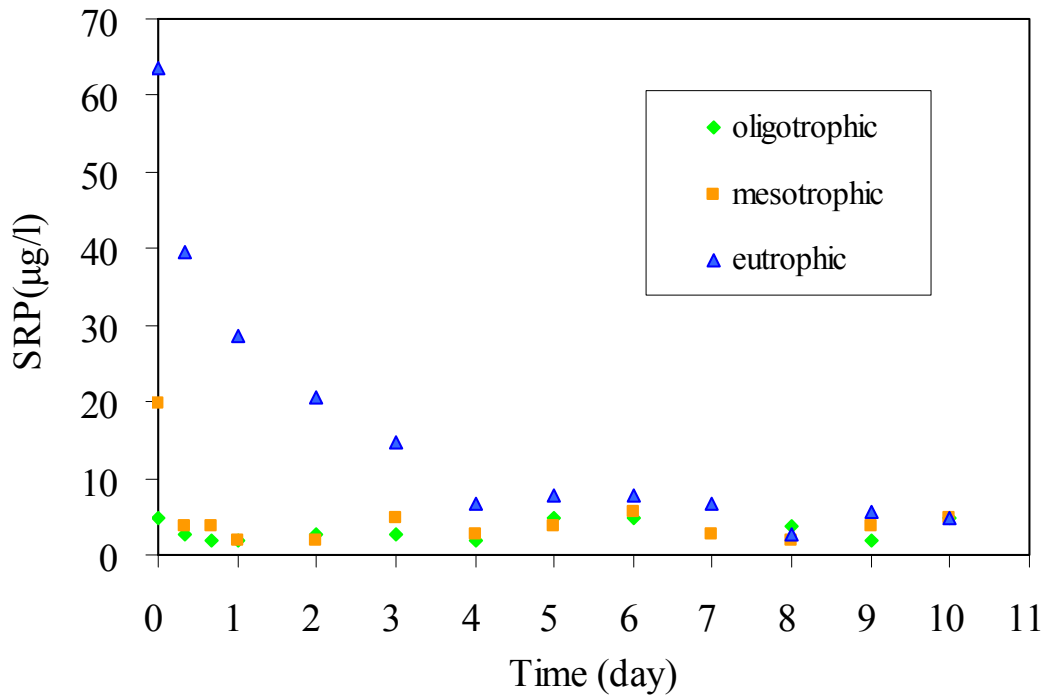


Figure 4.1 Kinetics of SRP in TSCARs.

A first-order nutrient loading model developed for lakes was commonly used to simulate the behavior of phosphorus in CSTR system (Snodgrass and O'Melia 1975; Vollenweider 1976). This model can be applied based on the basic assumptions Vollenweider (1976) proposed. The change in mass of phosphorus was expressed as the

difference between the sums of the inputs and outputs in CSTR system. The input terms were the total mass of SRP into reactor. The output terms includes internal losses (e.g., sedimentation, biological uptake, absorption) and hydraulic losses. In TSCARs system, a basic mass-balance model for SRP can be written as (4.1). Equation (4.2) was integral form of equation (4.1).

$$dp/dt = W^{'}/z - (Q/V)\cdot p - \sigma \cdot p \qquad (4.1)$$

$$p(t) = p_0 \cdot e^{-\phi \cdot t} + (\frac{W^{'}}{z}\cdot\frac{1}{\phi})(1 - e^{-\phi \cdot t}) \qquad (4.2)$$

Where,

p = SRP concentration (mg P/m^3), p_0 = initial value of SRP concentration (mg P/m^3), $W^{'}$ = areal loading of SRP (mg P/m^2/day), t = time (day), z = depth of reactor (m), V = volume (m^3), Q = outflow (m^3/day), σ = first-order loss coefficient (1/day), ρ_w = flushing coefficient (1/day), ϕ = first-order total elimination coefficient(1/day)

In the eutrophic reactor, a linear regression was used by plotting dp/dt versus p and then a straight-lined equation $dp/dt = 10.36 + 1.55p$ (n=12, r^2=0.74) was given by using the observed data as shown in Figure 4.1. The $W^{'}$ and ϕ can be calculated based on the slope and intercept of this straight-lined equation. Then, the kinetic equation $p(t)$ of SRP can be obtained. The gradual lessening exponentially equation with its initial SRP concentration p_0 (=63.5 mg as P/m^3) and z (=0.34 m) can be

expressed as $p(t) = 63.5 \cdot e^{-1.5502 \cdot t} + 6.68(1 - e^{-1.5502 \cdot t})$. When the t>> phosphorus residence time (theoretically around 3 times of ϕ^{-1}), the exponential terms approached zero, and the steady-state SRP concentration (p_{ss} =6.68 mg as P/m^3) was given. Because the mesotrophic and oligotrophic reactors reach steady state within one day, the first-order loss coefficient (σ) of mesotrophic and oligotrophic reactors were calculated as $p(t)$ approaches steady-state concentration (p_{ss}). The steady-state equation: $p_{ss} = \frac{W'}{z} \cdot \frac{1}{\sigma + \rho_w}$ can be used to calculate σ (Jones and Bachmann 1976). The first-order kinetic parameters of SRP in three trophic states were summarized in Table4.2.

Table 4.2 Parameters and coefficients of first-order kinetic SRP model in three trophic states based on the data of Figure4.1.

Trophic classes	W' (g as P/m^2/yr)	p_{ss} (mg as P/m^3)	ϕ (1/day)	σ (1/day)	ρ_w (1/day)
Oligotrophic*	0.15	2.75	0.447	0.239	0.208
Mesotrophic*	0.37	3.41	0.877	0.761	0.116
Eutrophic**	1.28	6.68	1.550	1.454	0.096

*: Steady-state equation: $p_{ss} = (W'/z) \cdot 1/(\sigma + \rho_w)$ was used to calculate σ in oligotrophic and mesotrophic reactors based on experimental treatments.

**: Gradual lessening exponentially equation: $p(t) = 63.5 \cdot e^{-1.5502 \cdot t} + 6.68(1 - e^{-1.5502 \cdot t})$ was used to calculate W', p_{ss} and σ of eutrophic reactor.

***: ρ_w was calculated by $1/t_R$, and here the hydraulic residence times (t_R) of three bioreactors were: 4.8, 8.6, and 10.4 day for oligo, meso, and eutrophic reactors, respectively.

Basically, the trophic state of lakes can be classified by total phosphorus concentration (Chapra and Tarapchak 1976; Dillon 1975; Vollenweider 1976). It was proposed that the dividing lines of 10 and 20 (μg P/l) of TP concentrations were lower and upper tolerance limits for mesotrophic state and based on that lakes can be classified as three trophic states. In this experiment, the daily concentrations of TP were measured for three trophic reactors (data as shown Figure 4.2). The mean TP concentration of the oligotrophic reactor was 7.5 (μg P/l) (n=5, sd.=7.1) below TP tolerance value 10 (μg-P/l), which was regarded as an oligotrophic state. The mean TP concentration of mesotrophic reactor was 18.5 (μg P/l) (n=10, sd.=5.9), which falls into the mesotrophic state. And for

the eutrophic reactor, it presented around 42.2 (μg P/l) (n=10, sd.=5.7) above 20 (μg P/l) which is affiliated with an eutrophic state. The observation of TP concentrations in TSCARs provided the clear evidence, which showed that the treatment of experiments by regulating the hydraulic overflow rate (q) and areal loading of TP (W') was able to form different trophic states identified by TP concentration.

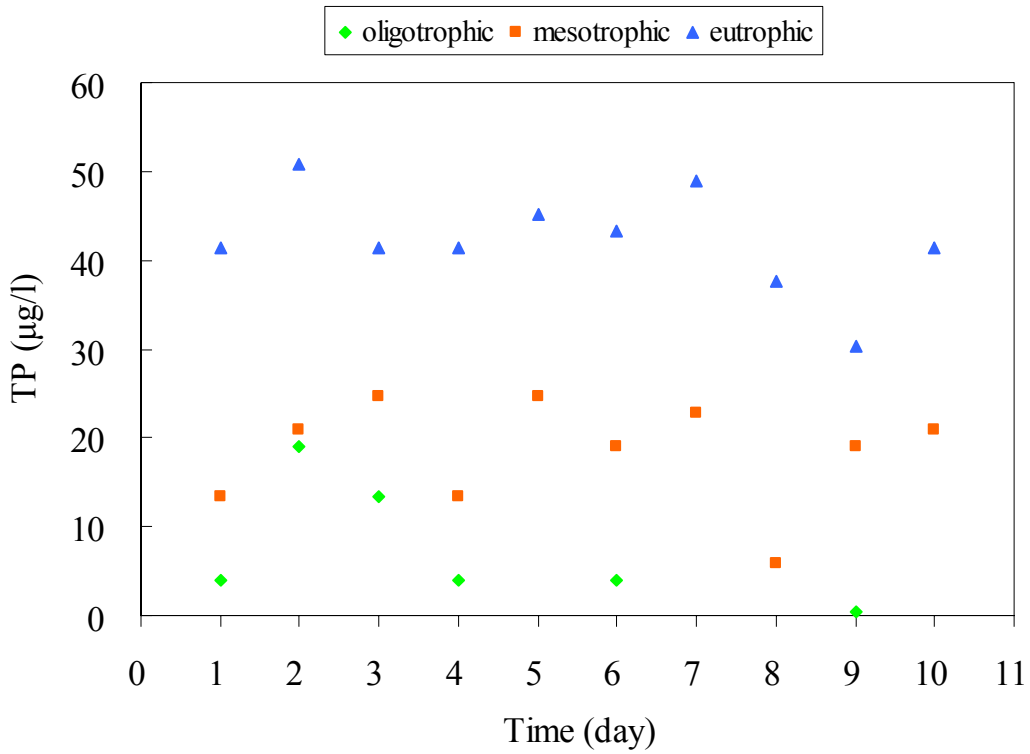


Figure 4.2 Observed data of TP concentration in TSCARs.

Estimates of the algal bioproductivity in three trophic levels

The algal bioproductivity was estimated by using the procedure described in

Chapter 3. The algal bioproductivity of three trophic states were estimated by using the data of Chl *a* collected from TSCARs (data shown in Figure 4.3). Primary procedures were described as following:

1. Samples for X (Chl *a*, mg/L) were taken at 3 or 4 days interval over the period of 20 days. Due to irregular sampling interval, the method of interpolation was used to insert intermediate value into the sampling interval for calculating daily Chl *a* change (dX/dt).

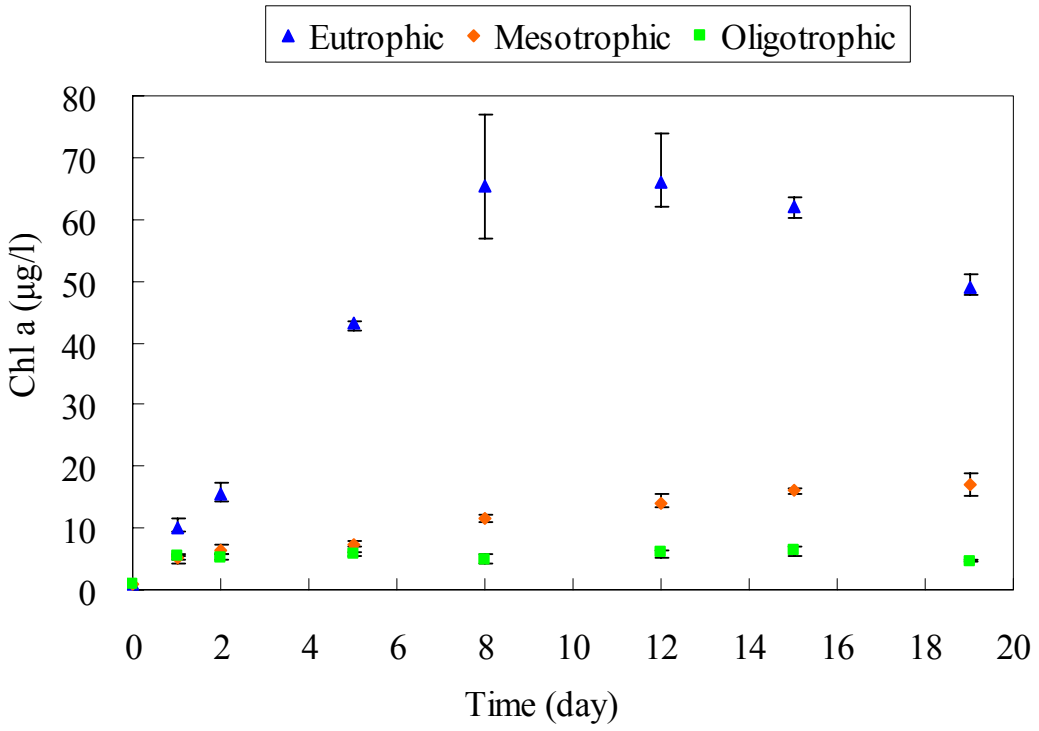


Figure 4.3 Chl *a* concentration observed in TSCARs from Day1 to Day19.

2. Washout terms of three CSTR reactors were calculated by DX. Dilution rate (D) was

defined as $1/t_R$, and t_R of three reactors are: 4.8, 8.6, and 10.4 day for oligo, meso, and eutrophic reactors, respectively.

3. Based on step1 and step 2, r_n can be calculated by using equation (3.3). And Pr can be determined by using equation (3.5).

4. The amount of areal algal product (P_T, mg/m^2) can be calculated by equation (3.7). The results of cumulative areal algal product (P_T, mg/m^2) along time was shown as Figure 4.4.

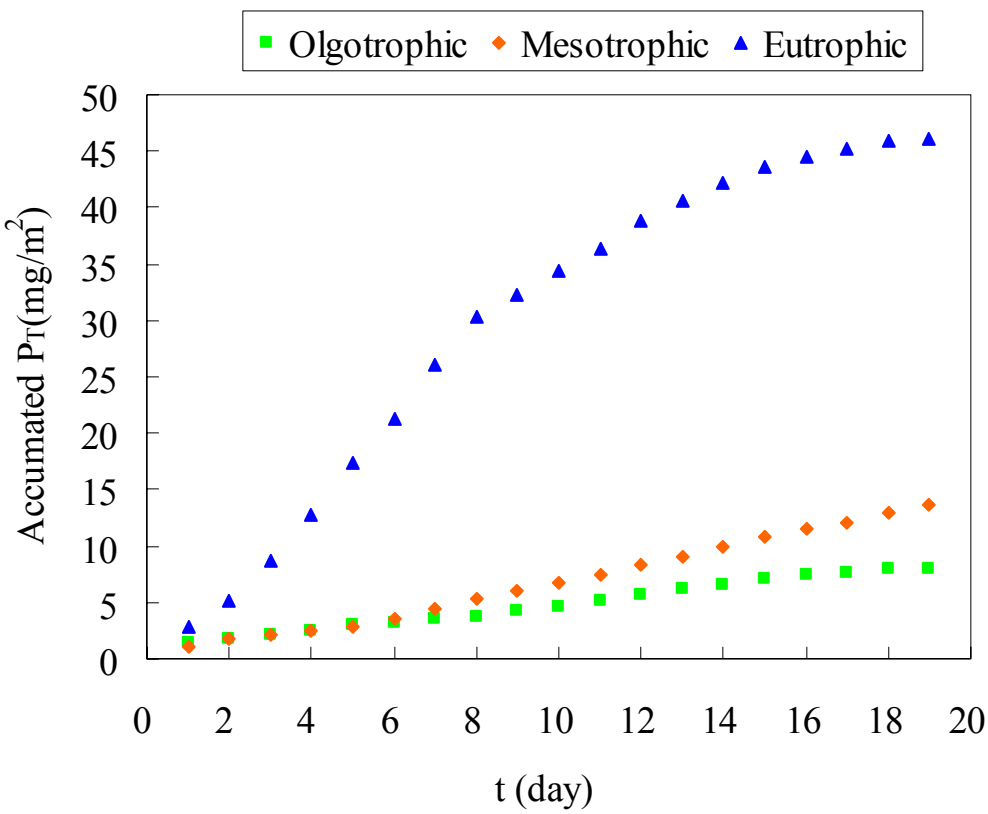


Figure 4.4 Cumulative P_T (mg as Chl *a* /m^2) in the bioreactor over the time period.

5. Average algal bioproductivity ($\overline{P_r}$, mg/m^2/day) could be determined by P_T /T.

In this study, the average algal bioproductivity ($\overline{P_r}$) and P_T is shown as Table 4.3. The daily Chl *a* content can be converted to annual carbon bioproductivity by using the ratio of carbon/Chl *a* between 10 to 182 (Bowie, et al. 1985; Yacobi and Zohary 2010). The annual algal bioproductivity of three trophic levels can be estimated to be 2~26, 3~47 and 9~159 C g/ m^2 /yr for the oligotrophic level, mesotrophic level, and eutrophic level, respectively. According to the literature, the range of algal bioproductivity was approximately 10~25g C/ m^2/yr at oligotrophic lake (Findenegg 1964; Waide, Willig et al. 1999), 15~45g C/ m^2/yr at mesotrophic lake and 60~110g C/ m^2/yr at eutrophic lake (Findenegg 1964; Gelin 1975). Average algal bioproductivity is approximately the value of literature.

Table 4.3 Total phosphorus concentration and algal bioproductivity observed in the three trophic levels.

Variable	Oligotrophic	Mesotrophic	Eutrophic
TP (μg P/l)	2.6	18.5	42.2
P_T (mg as Chl *a* /m^2)	6.7	12.5	43.3
$\overline{P_r}$ (mg as Chl *a* /m^2 /day)	0.37	0.69	2.40

Characterization of reactor water observed in TSCARs

The parameters of reactor water considered in this research were summarized in Table 4.4. The TP, SRP, Chl *a*, turbidity, pH, water temperature and flow rate of three TSCARs were monitored daily. The TN, TDS, TSS, TOC, TC, and IC were examined at equilibrium state. The pH of 7.5±0.5 and water temperature of 23±1 ^{o}C were observed through operation period. The higher ratio of TN:TP (>20) indicates that the TSCARs were under the phosphorus-limited condition. The increasing values of parameters listed in Table 4.4 were observed along the gradient trophic states.

Table 4.4 Characteristics of receiving water samples collected from TSCARs

Characteristic	Oligotrophic	Mesotrophic	Eutrophic
TN (mg N/L)	55.69	56.69	59.15
TP (μg P/L)	2.6	18.5	42.2
TDS (mg/L)	483.33	485.56	513.89
TSS (mg/L)	0.31	3.94	4.47
TOC (mg/L)	25.66	26.89	30.96
TC (mg/L)	28.45	30.31	36.04
IC (mg/L)	2.79	3.42	5.09
Chl *a* (μg/L)	5.5	14.7	60.6
Turbidity (NTU)	0.57	1.66	5.02
SRP (μg P/L)	1.5~4.0	2.0~4.5	2.5~10.0

4.1.2 Investigation of the lake biodiversity in TSCARs by DGGE

DGGE profiles of 16s rDNA and 18s rDNA fragments amplified from three

trophic state of TSCARs samples were shown in Figure 4.5. According to DGGE image, seven, thirteen and ten of bands of eukaryotes were detected for oligotrophic, mesotrophic and eutrophic bioreactors, respectively. For prokaryotes, seventeen, fifteen and eighteen of bands were detected for oligotrophic, mesotrophic and eutrophic bioreactors, respectively.

DGGE data was used to calculate the eukaryotic and prokaryotic biodiversity sampled from the TSCARs at Day 12. The DGGE analysis of eukaryotes showed Day 12 in mestrotrophic level was higher than the oligo and eutrophic levels (Table 4.5 and Figure 4 .5(a)). For prokaryotic biodiversity analysis (Table 4.5 and Figure4 .5(b)) the DGGE pattern showed that the biodiversity of the mestrophic level was lower than the oligo and eutrophic levels.

Table 4.5 Lake biodiversity of three trophic levels based on DGGE banding data analysis of 18 rDNA/16 rDNA fragment sampled from TSCARs

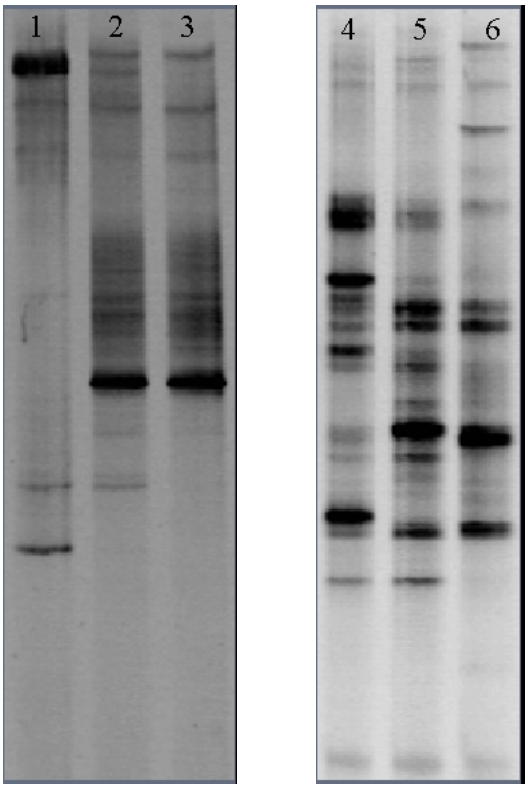
Trophic levels	Eukaryote				Prokaryote			
	Lane	H’	Sr	E	Lane	H’	Sr	E
Oligotrophic	1	1.762	7	0.905	4	2.741	17	0.968
Mesotrophic	2	2.390	13	0.932	5	2.410	15	0.890
Eutrophic	3	2.113	10	0.918	6	2.661	18	0.921

H’: Shannon index; Sr: Species richness; E: Evenness

The significant difference of eukaryotic and prokaryotic biodiversity in three

trophic level bioreactors was analyzed by t-test. These results showed that the biodiversity among the oligotrophic, mesotrophic and eutrophic bioreactors was significantly different at the 5% level.

The DGGE analysis showed that the biodiversity of prokaryotic and eukaryotic communities in oligotrophic, mesotrophic and eutrophic bioreactors were significantly different based on t-test. The Shannon index indicated that the eukaryotic biodiversity at the mestrophic level was higher than that of oligo and eutrotrophic levels. The biodiversity of prokaryote at the mestrophic level was lower than that of oligo and eutrotrophic levels.



(a)Eukaryote **(b)Prokaryote**

Figure 4.5 Negative image of DGGE gel of 18/16S rDNA fragment for determining the biodiversity. Image (a): DGGE analysis of 18S rDNA fragment. Image (b): DGGE analysis of 16S rDNA fragment. Wahiawa reservoir sample collected on 26 Nov. 2008. lane1, lane2, and lane3 showed the DGGE band of 18S rDNA fragment collected from oligotrophic, mesotrophic, and eutrophic bioreactor which samples collected on Day12. lane4, lane5, lane6 showed DGGE band of of 16S rDNA fragmen from oligotrophic, mesotrophic, and eutrophic bioreactor collected on Day12.

4.2 Field investigation

Lake Wilson was selected as a study site for examining the relationship between lake biodiversity and trophic levels. Based on the literature, several studies had been conducted about 30 years ago for Lake Wilson. It provided an overview for Lake Wilson (Lum and Young 1976; Moore, Lowry, et al. 1981; Young, Dugan, et al. 1975). Lake Wilson has a maximum capacity of 14.2×10^6 m^3, watershed area around 41.4 km^2, a water surface area of around 1.2 km^2 at maximum water level, mean depth of 8 m and a flushing time of 73 days (Young, et al. 1975). The dam withholds the impounded water from the North and South Forks of the Kaukonahua stream. Recently, several investigations were conducted in order to develop the TMDLs (Total Maximum Daily Loads) of pollutants into Lake Wilson. The yield of pollutants (TP, TN, and TSS) from the North and South Forks of the Kaukonahua watershed were estimated by Tetra Tech (Tetra Tech 2009; Tetra Tech 2006). According to the water quality standards classification, Lake Wilson was classified as Class 2 which permitted the use of water for recreational purposes, propagation of fish and other aquatic life and as agricultural and industrial water supply (State of Hawaii 2004). Although previous investigations provided the informed description of Lake Wilson, the lake biodiversity was poorly known.

4.2.1 Results of field investigation in Lake Wilson

On 26 Nov. 2008, a lake-wide survey conducted by dry season sampling at six locations of Lake Wilson exhibited spatial variation shown in Table1.1. The preliminary results will serve to illustrate the locations selected for investigating lake biodiversity. The TP concentration of six locations was under higher concentration (range from 42.2 to 89.1 (μg P /L)). The highest TN peak was observed at DSTP (Downstream of Sewage Treatment Plant) station. Moving upstream of South Fork Koukonahua from DSTP to SFKS1 (South Fork Koukonahua station 1), there was a decrease in TN. It was possible that the effluent contributed the sufficient nitrogen nutrient to the lake from the sewage treatment plant. The rainfall condition and water level of Lake Wilson were monitored by two USGS stations: USGS SF Kaukonahua rain gage and USGS 16210000 Wahiawa Reservoir at Spillway. Sampling date 26 Nov. 2008 was considered under dry weather and lower water levels. According to USGS SF Kaukonahua rain gauge, there was no effective rainfall to cause a runoff into receiving water body prior to 72 hrs of the sampling date 26 Nov. 2008. Reservoir gauge height was 21 m based on USGS 16210000 Wahiawa Reservoir at Spillway at Wahiawa dam.

According to the survey of 26 Nov. 2008, there were spatial variably ratio of TN:TP, ratio of TC:TP, and Chl *a*:TOC ratio in the lake water. The ratio of TC:TP was

higher than 27 at all monitored locations. The ratio of TN:TP was higher than 7.2 at two locations: 1) Wahiawa State Freshwater Park and, 2) downstream of Wahiawa Wastewater Treatment Plant (WWTP). Chl *a*-to-TOC ratio ranges from 9.0 to 0.8 (μg Chl *a*/mg C). This observed data (Table 1.1) released the information regarding the spatial scale difference of the limiting nutrient. In general, the higher TN:TP ratio >7.2 indicates that phosphorus was the limited element regulating algal growth in lake water (Chapra 1997). This implies that phosphorus limited the algal bioproductivity near the outfall of the sewage treatment plant due to the TN:TP ratio being higher than 7.2. That suggests that phosphorus regulates the algal growth in two locations of Lake Wilson.

As a conclusion, several reasons were considered for Lake Wilson as a study site to examine the relationship between lake biodiversity and trophic levels. Firstly, Lake Wilson is regarded as an eutrophic lake, and its eutrophic state is caused by enriched nutrients from the point source (effluent of sewage treatment plant) and non-point source. Secondly, a hypothesis was proposed that trophic levels gradually increases downstream of Lake Wilson due to significant nutrient contribution from the sewage treatment plant and longer hydraulic retention time downstream of Lake Wilson. Finally, the Lake Wilson watershed had been installed with rain and flow gauge stations and then provided long-term monitored data for analysis.

Sampling Locations

Based on the lake-wide survey, two sampling stations in Lake Wilson were selected: (1) Downstream of Sewage Treatment Plant (DSTP) located Wahiawa Wastewater Treatment Plant (WWTP) outfall; and (2) Wahiawa State Freshwater Park (WSFP) located upstream of WWTP (referred to Figure 4.6).

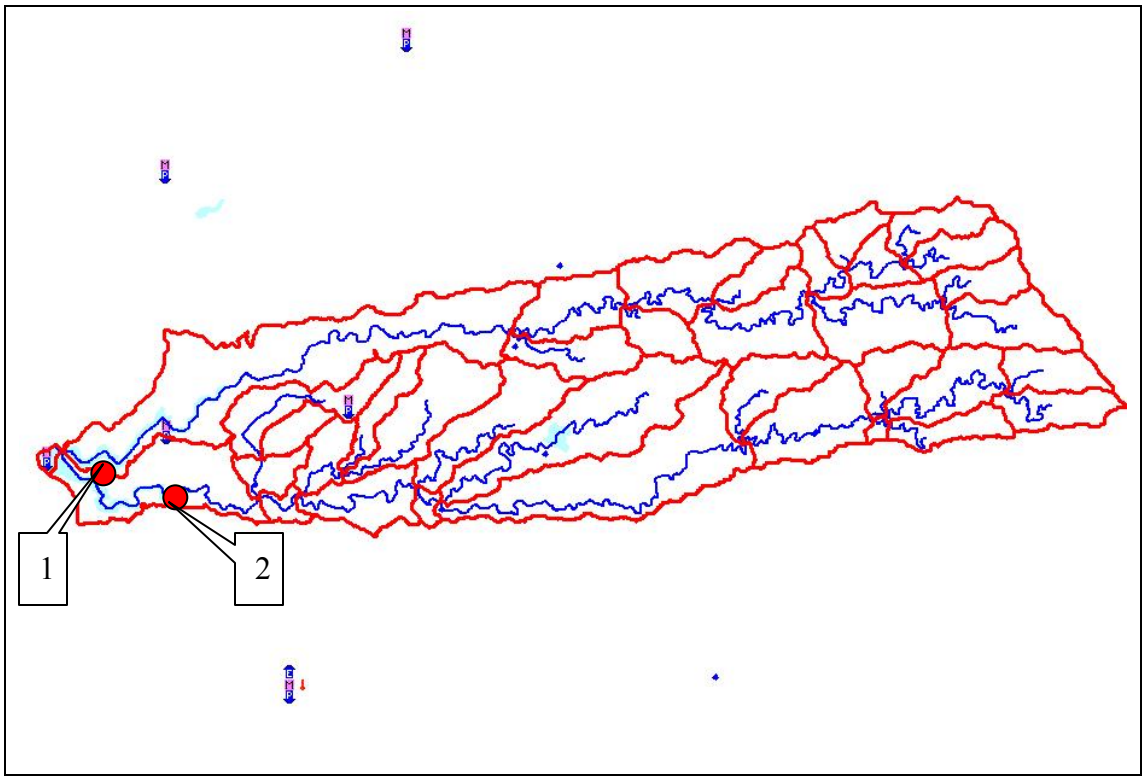


Figure 4.6 Map of Lake Wilson watershed and sampling locations. 1: Downstream of Sewage Treatment Plant (DSTP); 2: Wahiawa State Freshwater Park (WSFP).

Due to the continuous and higher nutrient concentration from the effluent of WWTP and lower nutrient concentration from the freshwater of upper streams, it is

possible to cause the trophic gradient from downstream (WWTP) toward the upstream (DSTP) of South Fork of Kaukonahua. The trophic gradient in rivers was observed in several field studies (Basu and Pick 1995). Vollenweider et al. (1974) indicated that average Chl *a* distribution presents inshore (higher lever) and offshore (lower lever) patterns in Lake Huron. Trophic levels gradually increases downstream of Lake Wilson. Several trophic-level related factors were examined as shown in Table 4.6. Samplings were conducted offshore of Lake Wilson on April 13, 2011 for DSTP and WSFP. As displayed in Table 4.6, the DSTP station has higher biomass content (Chl *a*) and nutrient (TN, TP and SRP) than WSFP station. Thus, an act of increasing in these parameters between two stations may suggest that higher trophic levels appeared at the DSTP station.

Table 4.6 Lake water parameters of two stations for investigation of lake biodiversity

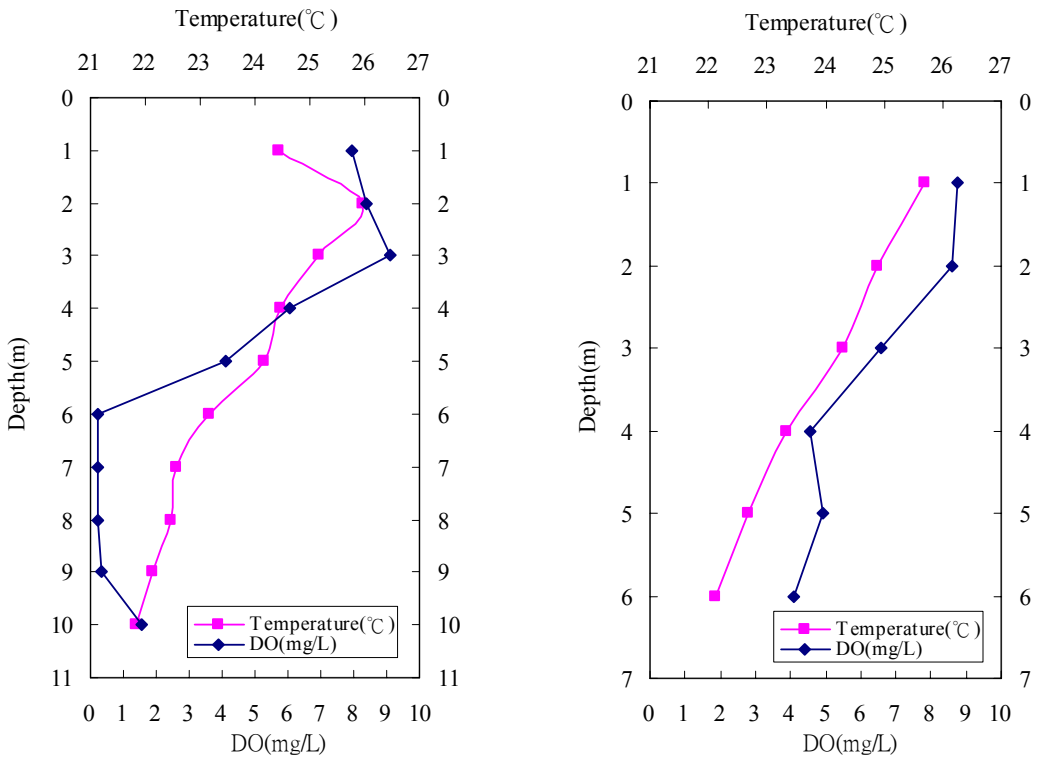
Locations‡	Chl *a* (mg/m^3)	TN (mg/L)	TOC (mg/L)	TP (μg/L)	SRP (μg/L)	TSS (mg/L)
DSTP	23.9	0.35	4.14	58.3	17.7	5.0
WSFP	17.4	0.24	3.35	32.1	11.7	4.3

*: Data collected on 13 Apr. 2011for DSTP and WSFP. According to USGS SF Kaukonahua Rain Gage, there was no effective rainfall to cause a runoff into receiving water body prior to 72 hrs of the sampling date. Reservoir gage height was 22 m based on USGS 16210000 Wahiawa Reservoir at Spillway at Wahiawa. ‡ DSTP: Downstream of Sewage Treatment Plant (21°29’28’’N, 158°02’30’’W); WSFP: Wahiawa State Freshwater Park (21°29’48’’N, 158°01’34’’W).

Profile of DO and temperature at three study sites

Dissolved oxygen (DO) content is the fundamental characteristic of lakes because photosynthetic production could be estimated by DO (Chapra 1997; Odum 1956; Wetzel 2001). Varying trophic levels related to diurnal cycle of DO curves have been proposed and investigated in rivers and lakes (Odum 1956; Wang, et al. 2003). The vertical distribution of DO patterns were found during the four seasons of different trophic level lakes. The vertical distribution of DO of Lake Wilson was investigated by Moore et al. (Moore, et al. 1981).

In this study, a real-time approach was used to measure DO variation in two study locations. YSI 600R DO Sonde (Yellow Springs, Ohio) was used to measure nearly instantaneous DO in situ. The DO data collected on April 13, 2011 in DSTP and WSFP. As shown in Figure4.7, the DO vertical profile presents that the higher clino-grade oxygen curve in DSTP than WSFP of Lake Wilson. The lower zone (depth 6-10m) of DSTP was an anaerobic environment and DO content increased to 9.5mg/l at the upper zone (depth 3m). This indicated that the lower zone of this site (DSTP) may have intensive biochemical oxidation processes in the very productive water.



(a) DSTP (b) WSFP

Figure 4.7 Vertical profile of DO and temperature at two sites of Lake Wilson.

The observed data shown in Table 4.6 and Figure 4.7 represent that nutrient (TP, TN, TOC, SRP), biomass (Chl *a*, DO) was consistent in gradual change between DSTP and WSFP stations. This comparison has been suggested by the fact that the visible trophic difference between the two stations DSTP and WSFP have occurred. Therefore, the two (DSTP and WSFP) stations provided different trophic condition in which we were allowed to investigate the relationship between lake biodiversity and trophic level.

Phosphorus Loading Assessment

In this research, PLOAD/BASINS4.0 (Pollutant Load/ Better Assessment Science Integrating point & Non-point Sources) was used to estimate TP non-point source pollutants loading from watershed contributed to Lake Wilson. Annual pollutant loads can be calculated for each watershed using simple methods in the model of PLOAD/BASINS 4.0 (USEPA). The governing equation of simple methods is: $L_P = \sum (R_f * P_J * R_{VU} * C_U * A_U * 2.72 / 12)$. Where, L_P = Pollutant load (lbs), R_f = Precipitation (inches/year), P_J = Ratio of storms producing runoff (default = 0.9), R_{VU}= Runoff coefficient for land use type u, (runoff inches /inches rainfall), C_U = Event Mean Concentration for land use type u (mg/l), A_U = Area of land use type u (acres).

The input data for PLOAD included GIS (Geographic Information Systems) data and tabular date. Digital data of GIS such as a watershed boundary, stream, digital elevation model (DEM), and land use can be downloaded from the database of Hawaii government and USEPA (http://hawaii.gov/dbedt/gis/download.htm). As displayed in the results in Table 4.7, the yield of TP from the non-point source was 3.1 (tn/yr) and from point source was 3.5 (tn/yr). This is based on surface area 1.2 km^2 of Lake Wilson, the areal loading of TP (W', $g/m^2/yr$) was 5.5 ($g/m^2/yr$).

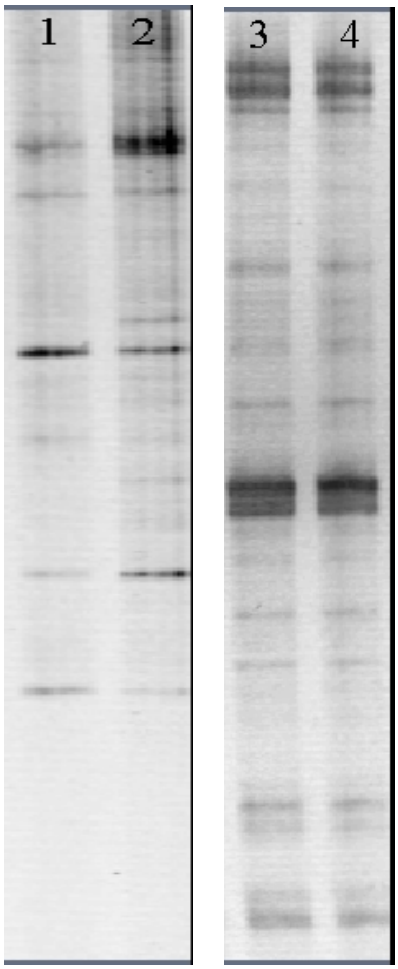
Table 4.7 Estimation of TP loading of Lake Wilson

Inflow	Loading(tn/yr)	Estimated method
Watershed runoff	3.1	PLOAD/ BASINS*
Wahiawa wastewater treatment plant effluent	3.5	Data provided from WWTP**

*: Annual mean precipitation: 50.54 (inches/yr) at dam 863 gage(Young, et al. 1975); TP Event Mean Concentration: undeveloped area such as conservation (0.2 mg/l) and water (0 mg/l), developed area such as urban, agriculture (0.5mg/l); **: Data of daily effluent data of flow (m^3/day) and TP (mg/m^3) concentration provided from WWTP during 2007, 2008, and 2009 period.

4.2.2 Investigation of lake biodiversity in filed study

The DGGE profiles of 18S rDNA and 16S rDNA fragments amplified from water samples of DSTP and WSFP stations collected on April 13, 2011 are shown in Figure 4.8. The detection of bands is referred to in Figure 3.7. According to Figure 4.8, the DGGE profiles, twelve and twenty of bands of eukaryty, was detected for DSTP and WSFP stations, respectively. For prokaryote, twenty five and twenty one of bands were detected for DSTP and WSFP stations. DGGE data is used to calculate the eukaryotic and prokaryotic biodiversity for two stations. The DGGE analysis of the eukaryote showed WSFP station held higher biodiversity than the DSTP station (Table 4.8). For prokaryotic biodiversity analysis (Table 4.8) the DGGE pattern showed that the biodiversity of the DSTP was lower than the WSFP station.



(a) (b)

Figure 4.8 DGGE analysis of 18/16S rDNA fragment for determining the biodiversity in Lake Wilson by using DGGE banding pattern. Image (a): DGGE analysis of 18S rDNA fragment. Image (b): DGGE analysis of 16S rDNA fragment. Lane1 and Lane3 represented the samples collected from DSTP of Lake Wilson; Lane2 and lane4 represented the samples collected from WSFP of Lake Wilson.

Table 4.8 Lake biodiversity based on DGGE banding data analysis of 18 rDNA/16 rDNA fragment of Lake Wilson.

Location	Eukaryote				Prokaryote			
	Lane	H’	S	E	Lane	H’	S	E
DSTP	1	1.725	9	0.7850	3	2.861	25	0.8855
WSFP	2	2.571	20	0.8582	4	2.658	21	0.8729

The eukaryotic biodiversity of DSTP site was H’=1.725 while the eukaryotic biodiversity of WSFP site is H’=2.571. The t-test showed that the two sites are significantly different at 5% level (t=14, d.f.=764). On the other hand, the prokaryotic biodiversity of DSTP site was H’=2.861 while the prokaryotic biodiversity of WSFP site was H’=2.685. The t-test showed that the two sites are different at 5% level (t=5, d.f.=1391).

4.3 Discussion

The experimental investigation has been developed as a protocol of determining the relationship between the bioproductivity and biodiversity in a gradient trophic condition. The designated gradient trophic condition was successfully formed in three completely stirred tank reactor (CSTR) systems and depended on the phosphorus mass-balance model. Preliminary results showed the mestrophic state held a higher biodiversity than the oligo and eutrophic states, depending on the DGGE banding data of 18S rDNA (Eukaryote). On the other hand, the prokaryote of the mesotrophy has less

biodiversity than the oligo and eutrophic states. The DGGE analysis showed that the biodiversity of prokaryotic and eukaryotic communities in oligotrophic, mesotrophic and eutrophic bioreactors are significantly different based on the t-test.

The algal bioproductivity often was expressed as either the content of Chl *a* (Ryther and Yentsch 1957) or by the rate of production (Schallenberg 1997). Some research works showed that high Chl *a* was not guaranteed to reflect high bioproductivity because it can be merely the result of accumulation of biomass (Chl *a*). Therefore, it is necessary that algal bioproductivity should be defined as a rate term and be the amount of carbon fixed in unit area per unit time through photosynthesis. In this study, the average algal bioproductivity (average Pr) can be converted to annual carbon bioproductivity by using the ratio of carbon/Chl *a* between 10 to 182 (Bowie, et al. 1985; Yacobi and Zohary 2010). Experiment results showed that the average algal bioproductivity of three trophic levels, oligotrophic, mesotrophic, and eutrophic levels were 0.4, 0.7 and 2.4 mg as Chl *a* /m^2 /day, respectively. The annual bioproductivity of three trophic levels can be estimated to be 2~26, 3~47 and 9~159 C g/m^2 /yr for the oligotrophic level, mesotrophic level, and eutrophic level, respectively. According to the literature, the range of algal bioproductivity was approximately 10~25g C/m^2/yr at oligotrophic lakes (Findenegg 1964; Waide, et al. 1999), 15~45g C/m^2/yr at mesotrophic lakes and 60~110g C/m^2/yr at

eutrophic lakes (Findenegg 1964; Gelin 1975). Average algal bioproductivity was approximately the value of literature.

CHAPTER 5. LAKE BIODIVERSITY WITH TIME VARYING ALGAL BIOPRODUCTIVITY

Chapter 5 illustrates that lake biodiversity was regulated by algal bioproductivity during the period of varying algal growth phases, carried out in an eutrophic TSCAR. The experiment was conducted during 20 days of time-varying observation on mixture culture in algal bloom. This observation can be used to demonstrate the correlation between lake biodiversity and algal bioproductivity. In this eutrophic TSCAR, an algal bloom has been induced by the treatment described in Chapter 3. DGGE fingerprinting technique was employed to investigate the lake biodiversity along temporal variation in terms of varying algal bioproductivity during 20 days of laboratory experiment. The evidence indicates that lake biodiversity can be displayed with time variation in algal bioproductivity. Consequently, there is a general trend that when data were plotted by eukaryotic biodiversity (H') against algal bioproductivity (Pr), the relationship was positive relation following hump-shaped pattern, whereas when data were plotted by prokaryotic biodiversity (H') against algal bioproductivity (Pr), the relationship was negative relation following U-shaped patterns. Several critical timing points of algal growth in terms of time-varying Pr(t) revealed a shift in lake biodiversity.

5.1 General variation in biodiversity

Many hypotheses attempting to explain the biodiversity variation have recently been reviewed by Connell and Orias (Connell and Orias 1964), Waide et al. (Waide, et al. 1999), Fukami and Morin (Fukami and Morin 2003) and Smith (2007). These hypotheses have been examined and account to the interactive effects between the biodiversity and its environmental conditions. Indeed, biodiversity is regulated by many factors such as global loss (latitudinal gradient), climatic change (precipitation), spatial factor (heterogeneity), availability of nutrient, and solar energy (primary bioproductivity) (Pianka 1966). The relationship between the biodiversity and the bioproductivity attracted more attention in the biodiversity studies of Pianka. The biodiversity can either positively or negatively be corrected with bioproductivity (Fukami and Morin 2003; Kassen, et al. 2000; Morin 2000). In fact, six patterns of the biodiversity-bioproductivity were observed and summarized from experiment of laboratory and natural environment: random, positive, flat, negative, hump-shaped, and U-shaped (Smith 2007; Waide, et al. 1999). The hump-shaped relationship of biodiversity-bioproductivity is a widely observed pattern in which the peak of diversity is at the intermediate bioproductivity level (Kassen, et al. 2000).

In this study, the hypothesis that “the algal bioproductivity correlates closely with

lake biodiversity" was examined to explain if the general patterns of biodiversity-bioproductivity were caused due to competition for limited nutrients in an eutrophic freshwater lake. With time-varying analysis, it provided interpretation that the man-made influx of nutrient often resulted in not only a decrease of eukaryotic biodiversity but also an increase of prokaryotic biodiversity.

5.2 Results of a time-varying experiment on the eutrophic TSCAR

According to the laboratory experiments described previously (Chapter 3), the eutrophic environment was formed and the most of the members of phytoplankton were able to reproduce and build up the algal population in the TSCAR bioreactor. The daily change of Chl *a* concentration was measured and the bioproductivity can be calculated by using the CSTR mass balance equation.

In order to investigate the algal bioproductivity and lake biodiversity, TSCAR bioreactors were designed and constructed in a laboratory scale experiment. An experiment was conducted from Sept 23, 2011 through Oct. 4, 2011 in three different trophic-level TSCAR bioreactors. The samples of TSCARs in oligotrophic, mesotropnic (data not shown in this chapter) and eutrophic TSCAR bioreactors were collected and analyzed. The eutrophic TSCAR bioreactor has been set for the initial condition of the concentration of 60μg as P/l limited-phosphorus nutrient in culture mediums which

operated under the conditions shown in Table 3.1. These conditions were designed to simulate the lake-like eutrophic environment. The SRP and Chl *a* were measured. Moreover, samples for DNA extraction of the eukaryotic and prokaryotic DGGE experiment were collected under a daily interval. In this experiment, an algal bloom in eutrophic TSCAR was observable and its algal bioproductivity was estimated by Chl *a* in the CSTR system during 20 days period. The 12-day time-varying lake biodiversity was investigated by DGGE profile.

5.2.1 Algal bloom in eutrophic TSCAR

An observation of algal bloom process can be used to exhibit the change of lake biodiversity along with algal population growth phases in one CSTR system where regarded as a lake-like microcosm. It could be an example to demonstrate that the relationship of lake biodiversity and algal bioproductivity in controlled CSTR system, eutrophic TSCAR bioreactor. A typical algal bloom was formed based on the treatment of Table3.1 and algal X (mg as Chl *a*/ m^3) against time (t, Day) was plotted shown as Figure 5.1.

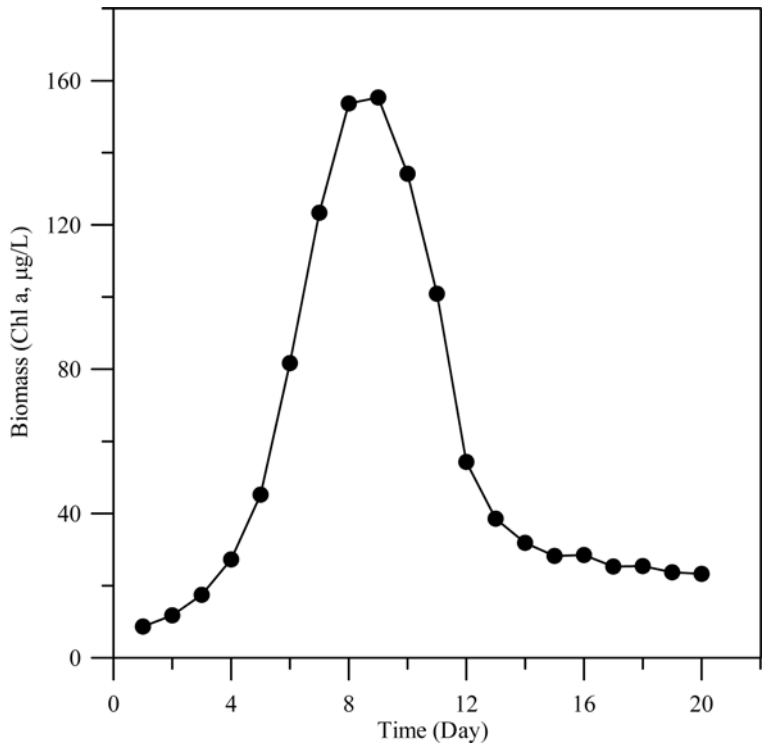


Figure 5.1 Observed data showed the algal biomass concentration X against time, t. Observed data of algal populations size represented Chl *a* in eutrophic bioreactor. On Sep. 23 2011, 1.5 liter of lake water from Lake Wilson was introduced into eutrophic TSCAR bioreactor described Chapter 3. The biomass by Chl *a* were collected daily until 2011, Oct. 4.

Based on the procedure of estimation described as in Chapter 3, time-varying algal bioproductivity, Pr(t), was estimated and plotted shown as Figure 3.4. As the results, the average algal bioproductivity ($\overline{P_r}$) was 1.75 (mg Chl *a*/ m^2/day). The algal areal

product (P_T) was 34.9 (mg Chl *a*/ m^2). The maximum instantaneous bioproductivity (Max Pr) was 17.2 (mg as Chl *a*/ m^2/day). The quantity of algal growth over the period of 20 days was summarized in Table 5.1. The time-varying Pr(t) was plotted shown as Figure 3.4. The time-varying r_s (t) was plotted shown as Figure 5.2.

Table 5.1 Quantity of algal growth based on observed data collected from the eutrophic TSCAR

Quantity	Symbol	Unit	Value
Algal areal product	P_T	mg as Chl *a* /m^2	34.9
Average algal bioproductivity	$\overline{P_r}$	mg as Chl *a* /m^2 /day	1.75
Max algal bioproductivity	max Pr	mg as Chl *a* / m^2/day	17.2
Max specific algal net growth rate	max r_s	1/day	0.53
Min specific algal net growth rate	min r_s	1/day	-0.78
Carrying capacity	K	mg as Chl *a* /m^3	145

Algal growth in mixture culture

When a few phytoplanktons are introduced into an unsaturated space, the rate of growth among the increasing populations has frequently been observed. After the acceleration of growth phase, it soon slows down gradually due to the environmental resistance (the slow deceleration phase). The growth of the algal population can be affected by itself density and other populations due to a force “potential environmental

resistance" that acts to retard growth. The potential environmental resistance may result from competition to gain the limited nutrient and predation or grazing process by other species. The classification of algal growth phases was plotted based on algal net growth rate (r_s) referred to in Figure 5.2. When the algal populations complete their growth (Phase I and Phase II), algal population density in terms of algal biomass (X) tends to decline below the carrying capacity (K) level which moves towards phase III. The lake biodiversity of three phases (phase I, phase II and phase III) was investigated in order to realize the lake biodiversity variation in algal bioproductivity.

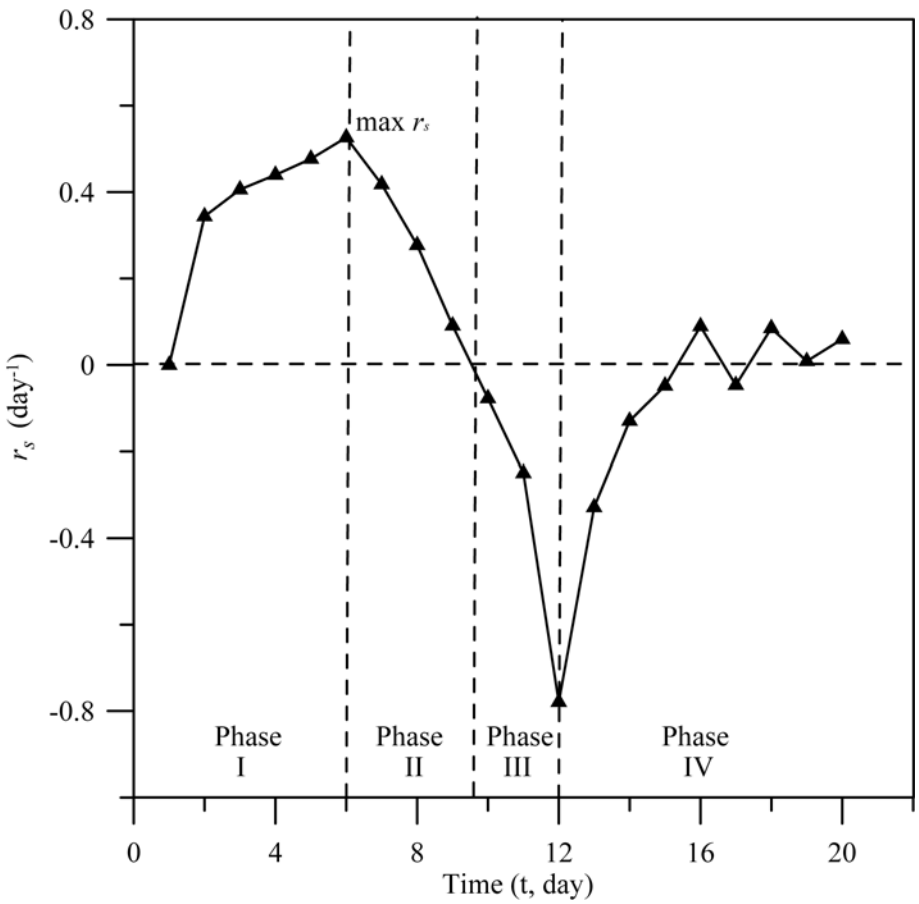


Figure 5.2 Time-varying curve of algal net growth rate (r_s) calculate from the observed data of Figure 5.1. Phases of growth are defined as: phase I: positive algal net growth rate ($+r_s$) in act of increasing; phase II: positive algal net growth rate ($+r_s$) in act of decreasing; phase III: negative algal net growth rate ($-r_s$) in act of decreasing; phase IV: negative algal net growth rate ($-r_s$) in act of increasing.

5.2.2 Time-varying lake biodiversity in eutrophic TSCAR

Over a time period of 12 days, the lake biodiversity could be illustrated with the

images of DGGE gel shown as Figure 5.3 for prokaryotic biodiversity and Figure 5.4 for eukaryotic biodiversity. The lake biodiversity was investigated with the variation of algal bioproductivity based on observed data of Figure 5.1. During the 12-day period (excluding Day3), DGGE image demonstrated the time-varying fingerprinting of the lake biodiversity in terms of prokaryotic and eukaryotic biodiversity under phase I, phase II, and phase III. The three phases are in conjunction with an algal blooming. The banding data of DGGE gel is used to determine the prokaryotic and eukaryotic biodiversity by Shannon index (Fromin, et al. 2002; Shannon 1948). The banding data of DGGE gel was digitized by using the TotalLab software (Nonlinear, USA). The digital banding data transformed from DGGE gel image consisted of the number of band (S) and peck height (n_i) of i th band on j th lane. The Shannon index (H') and evenness (E) were calculated as described in Chapter 3.

(i) prokaryotic biodiversity

For comparison, the banding data found for 11 samples collected from eutrophic TSCAR under the period of 12-day period. As the Figure 5.3 indicates, the variations in bands were described as present, absent, gradually increasing and decreasing pixel intensity along each day of the experiment. Nine bands are interested and excised which marked *a, b, c, d, e, f, g, h* and *i* in Figure 5.3. Band *a*, band *b*, and band *c* with the high

intensity of pixel can be regarded as dominant bands. Band *a* was present from Day 1 to Day10 and with less intensity on Day 9 to Day 10; Band *b* was present from Day 1 to Day 12 and became less on Day 12; band *c* was present on Day 10 to Day 12 and became less on Day 12; band *d* was present on Day 4 to Day 6; band *e* was present on Day 2 to Day 12 and on Day 12 had higher intensity; band *f* was present and had gradually decreasing band intensity from Day 1 to Day 6; band *g* was present and had gradually increasing band intensity from Day 1 to Day 12; band *h* was present on Day 12; band *i* was present on Day 5, 6, 7 and Day 12.

The implication of the variation in bands over 12 days is that the band *a* and band *b* may highly relate to Chl *a* biomass due to gradually increasing from Day 1 to Day 9 and then decreasing to Day 12 which is similar to the change of Chl *a*. The variation in band *g* may only depend on varying of time. In addition, once band *a* was absent, band *c* will be present. Under the 12-day period, approximately, prokaryotic H' decreased in value from Day 2 to Day 9 and then increased from Day 9 to Day 12. Day 9 showed the lowest prokaryotic H'.

Table 5.2 Variation in prokaryotic biodiversity calculated from DGGE gel image of Figure 5.3.

Day	S	N_j	H'	E
1	14	396	2.230	0.8451
2	19	475	2.378	0.8077
4	17	495	2.269	0.8010
5	16	510	2.277	0.8211
6	13	555	2.233	0.8705
7	15	504	2.280	0.8420
8	13	497	2.186	0.8521
9	10	497	1.865	0.8102
10	11	454	1.953	0.8145
11	13	445	1.969	0.7676
12	16	426	2.525	0.9107

*: S = total band count on one lane; N_j = sum of peck height of all band on j^{th} lane; H' = Shannon index; E = Evenness.

Thirty-three distinct bands were detected in total bands of 157 on 11 lanes of the DGGE gel for prokaryotic experiment. Day 2 with maximum (S=19) and Day 9 with minimum (S=10) total band count were detected on one lane. Maximum prokaryotic biodiversity and evenness was found in Day 12; Minimum prokaryotic biodiversity is found in Day 9. Based on the Table 5.2, a U-shaped diagram could be found by plotting prokaryotic H' against time.

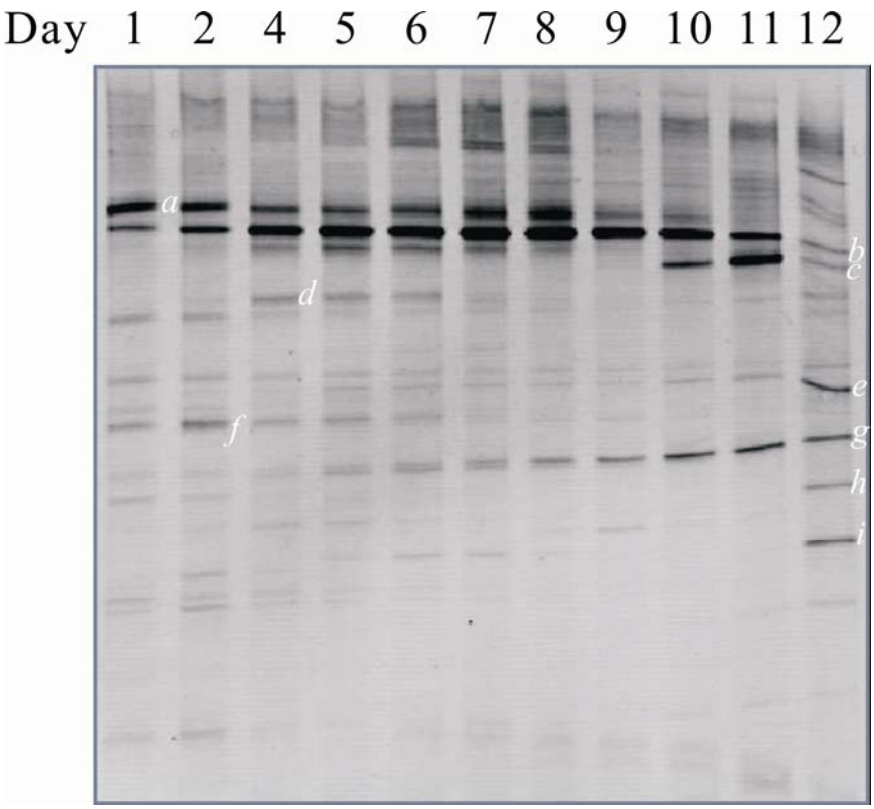


Figure 5.3 Negative image of DGGE gel of 16S rDNA fragment for determining the prokaryotic biodiversity in TSCARs by using DGGE banding data. Nine bands are interested which marked *a, b, c, d, e, f, g, h* and *i*.

(ii) eukaryotic biodiversity

As shown in Table 5.3, the variation in eukaryotic biodiversity (H') was obtained from the DGGE banding data of Figure 5.4. The eukaryotic H' increased in value from Day 1 to Day 9 (except Day 7) and then decreased from Day 9 to Day 12. Thus, a diagram of humped shape could be obtained by plotting eukaryotic H' against time. The maximum eukaryotic H' was found on Day 9. The minimum eukaryotic H' was

found on Day 12.

Day 6, Day 7, Day 9, and Day 12 seemed to be a milestone to signal for the variation in eukaryotic biodiversity. The implication of Day 6, Day 7, Day 9, and Day 12 can be interpreted by algal bioproductivity. It indicated that the variation in eukaryotic biodiversity depended on phases of algal growth which were determined by algal bioproductivity.

Table 5.3 Variation ineukaryotic biodiversity calculated from DGGE gel image of Figure 5.4.

Day	S	N_j	H'	E
1	5	589	1.576	0.9791
2	6	669	1.776	0.9910
4	7	910	1.893	0.9726
5	9	1227	2.131	0.9697
6	11	1416	2.320	0.9676
7	7	830	1.885	0.9687
8	11	1305	2.338	0.9748
9	12	1305	2.433	0.9790
10	11	1385	2.357	0.9831
11	9	1121	2.188	0.9960
12	6	892	1.734	0.9680

*: S = total band count on one lane; N_j = sum of peck height of all bands on j^{th} lane; H' = Shannon index; E = Evenness.

Compared with 12-day period, Day 12 showed dramatic changes of the pattern on

banding. Five bands *E1, E2, E3, E4* and *E5* of Day 12 were interested and identified. *E1*, *E2* and E5 showed high intensity. *E1* was only present on Day 12. *E2* was present from Day 1 to Day 12, and it gradually decreased and then gradually increased. *E2* seems to be inversely related to the change of Chl *a* biomass. *E3* with less intensity was present on Day 1 to Day 3 and Day 8 to Day 12. *E4* was only present Day 12. *E5* was present from Day 9 to Day 12 with increasing intensity. The varying bands were present, absent and gradually changing which might be used to explain the coexisting and grazing between phytoplankton and herbivorous zooplankton dynamics.

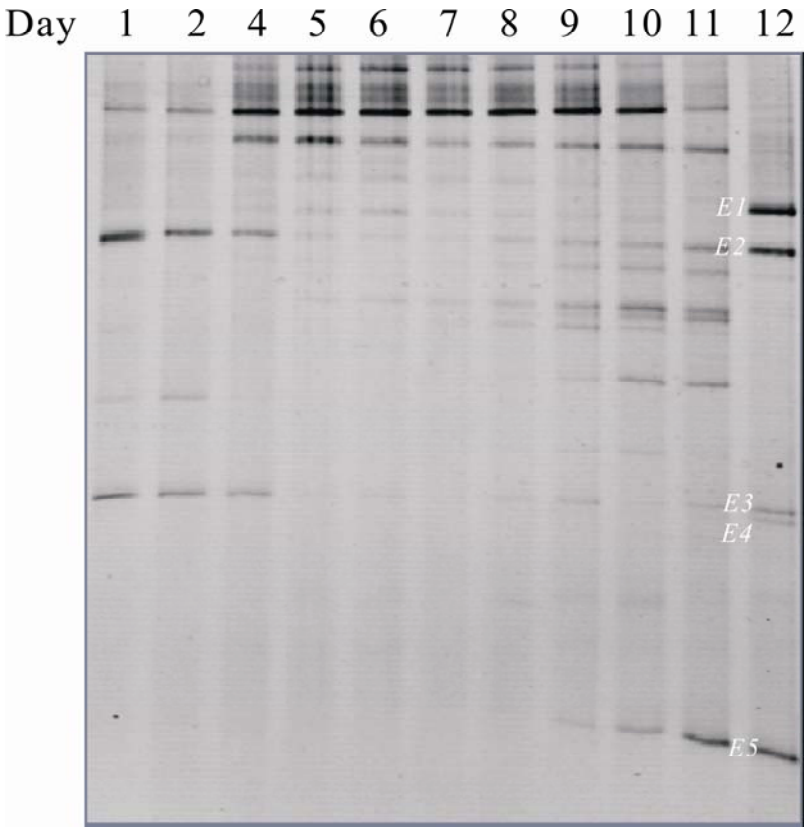


Figure 5.4 Negative image of DGGE gel of 18S rDNA fragment for determining the eukaryotic biodiversity in TSCARs by using DGGE banding data. Five bands are interested which marked *E1, E2, E3, E4* and *E5* on Day 12.

5.2.3 Relationships between lake biodiversity and algal bioproductivity

Based on the time-varying analysis, lake biodiversity might be expected at variations not only in temporal scale, but also in Pr(t) and r_s (t). Mathematically, the relationship between Pr(t) and r_s (t) is: Pr(t) $= z \cdot X(t) \cdot r_s(t)$. Here, z is constant and Pr, X,

and r_s are the function of time(t). The lake biodiversity relationships with algal growth were demonstrated by plotting H' against Pr(t) and r_s (t). As a general trend, the results showed that when time-varying data were plotted by eukaryotic biodiversity (H') against algal bioproductivity, the relationship was hump-shaped, whereas when data were plotted by prokaryotic biodiversity (H') against algal bioproductivity, the relationship was U-shaped.

However, based on time-varying analysis, Day 6, Day 7, Day 9, and Day 12 of this experiment seemed to be a key point in time which predicted a shift in lake biodiversity along algal bioproductivity. The implication of Day 6, Day 7, Day 9, and Day 12 can be interpreted by four algal growth phases separated by r_s (t) referred to Figure 5.2. The Day 6, Day 9 and Day 12 were the demarcation of the four phases: phase I, phase II, phase III and phase VI, respectively. The interpretation for the critical timing is: Day 6 displayed the maximum r_s ; Day 9 displayed the maximum K; Day 12 reached the minimum r_s ; Day 7 presented the maximum Pr.

Assumption

The concept of the algal growth phase-dependency is proposed to give an explanation for the varying relationship between between lake biodiversity and algal bioproductivity. The primary assumptions involved in the explanation of the concept of

algal growth phase-dependency control as follow.

(1) It is assumed that algal population is limited in the closed space.

(2) The algal populations are assumed to be growing in the environment with the carrying capacity (K) due to the available resources such as the limited nutrient phosphorus.

(3) The distribution of species-abundance is a lognormal distribution on the given microcosm.

(i) the relationships between prokaryotic biodiversity and algal bioproductivity

The relationships between prokaryotic biodiversity (H') and algal bioproductivity were demonstrated by plotting prokaryotic biodiversity (H') versus r_s (t) and Pr(t). The result in the Figure 5.5 r_s (t) showed that three varying phases in prokaryotic biodiversity (H'). During phase I (Day 1 to Day 6), the positive r_s (t) was increasing and H' was decreasing in a straightforward fashion. When switched to phase II, the positive r_s (t) was decreasing and H' was also straightforward decreasing during the three days (Day 7 to Day 9). After Day 9, the negative value of r_s (t) was increasing and H' shifted phase II to phase III with increasing H'value during the three days (Day 10 to Day 12).

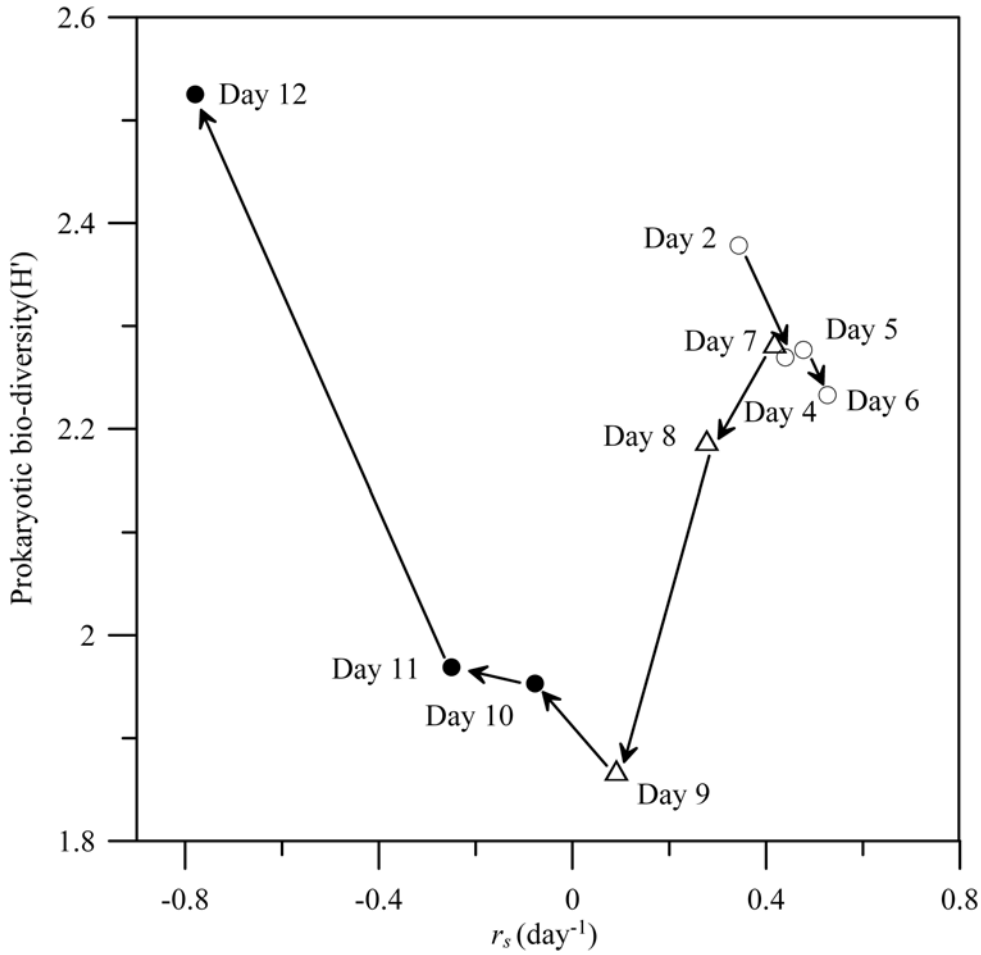


Figure 5.5 Phased relationships of prokaryotic biodiversity and algal specific net growth rate. The open circle, open triangle and solid circle denote phase I (Day 1 to Day 6), phase II (Day 7 to Day 9) and phase III (Day 10 to Day 12), representatively.

When compared with r_s (t) in Pr(t) was found the similar trend (see Figure 5.6). The prokaryotic community has the lowest biodiversity (H') at intermediate algal bioproductivity. Referred to the Figure 5.5, a diagram of U shape was found by plotting prokaryotic H' against Pr(t). In the previous study, the bacteria belonging to

α-proteobacteria were found to have the U-shaped relationship with primary productivity (Horner-Devine, et al. 2003). According to this investigation, the algal bioproductivity could influence the prokaryotic biodiversity in terms of the composition and their abundance.

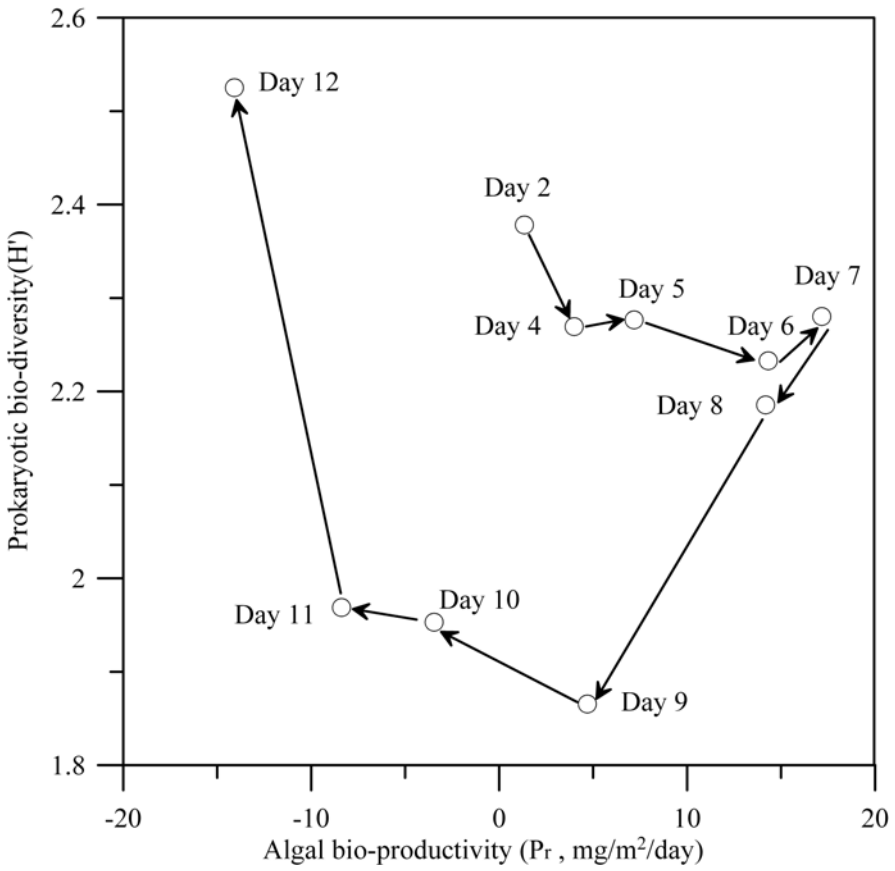


Figure 5.6 The change of prokaryotic biodiversity resulted from algal bioproductivity based on observational study. Open circle is the sampling day. The values of Pr are calculated using the observed date of Figure 5.1.

(ii) relationships between eukaryotic biodiversity and algal bioproductivity

Based on Figure 5.7, it showed the phased relationships of the eukaryotic biodiversity (H') with r_s (t). During phase I (Day 2 to Day 6), an increase of eukaryotic biodiversity with r_s (t) was exhibited. After Day 6 (max r_s), a break point was found on Day 7 when the eukaryotic biodiversity suddenly dropped from high (Day 6) to very low (Day 7) value. During phase II (Day 7 to Day 9), the eukaryotic biodiversity was recovering and reached the highest value at Day 9, as the positive r_s (t) was slowly decreasing and approaching to zero. As r_s (t) entered phase III (Day 10 to Day 12), the eukaryotic biodiversity was decreasing.

As conclusion, the eukaryotic biodiversity has been a positively correlated with r_s (t) during phase I. And its humped-shape correlated with r_s (t) presented in phase II and phase III.

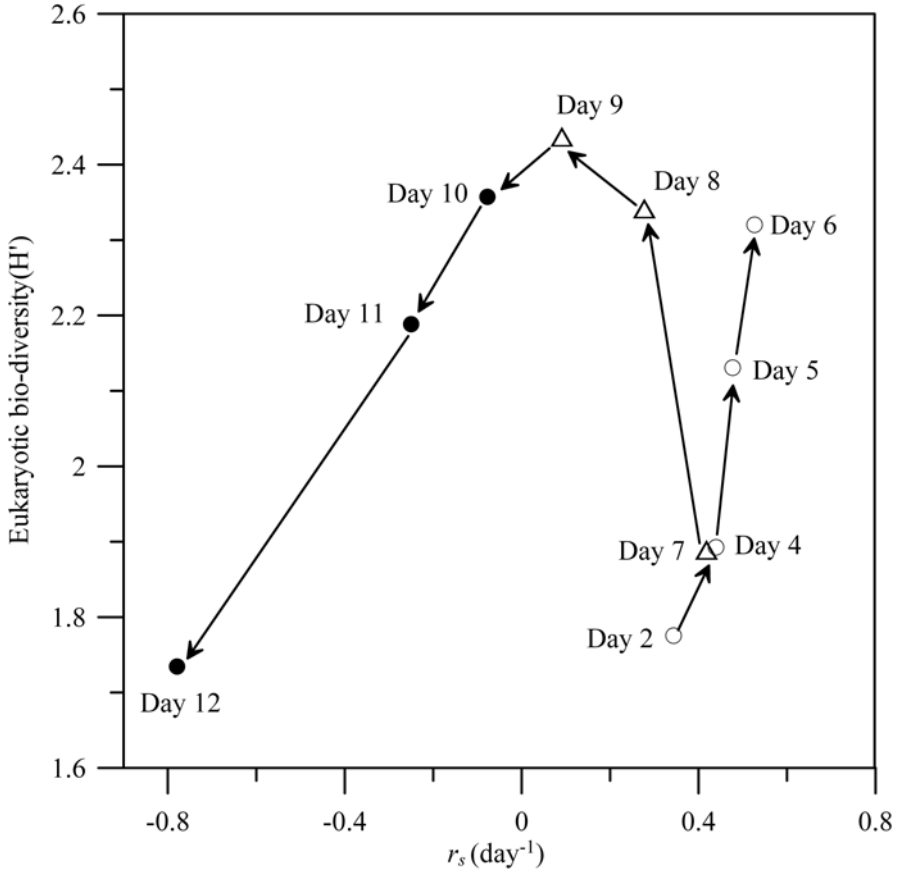


Figure 5.7 Phased relationships of eukaryotic biodiversity and specific algal net growth rate. The open circle, open triangle and solid circle denote phase I (Day 1 to Day 6), Phase II (Day 7 to Day 9) and Phase III (Day 10 to Day 12).

Figure 5.8 unveiled the overview of the relationship between eukaryotic biodiversity (H') and algal bioproductivity (Pr). During the early period of algal growth, the eukaryotic biodiversity (H') has positively correlated with algal bioproductivity (Pr). After Day 6, a relatively low eukaryotic biodiversity (H') was observed. This indicated

that eukaryotic biodiversity (H') has a break point when Pr reached maximum value. During the period of Day 7 to Day 12, humped-shape relationship between H' and Pr was formed. As Pr was approaching to zero, the peak of eukaryotic biodiversity (H') occurred.

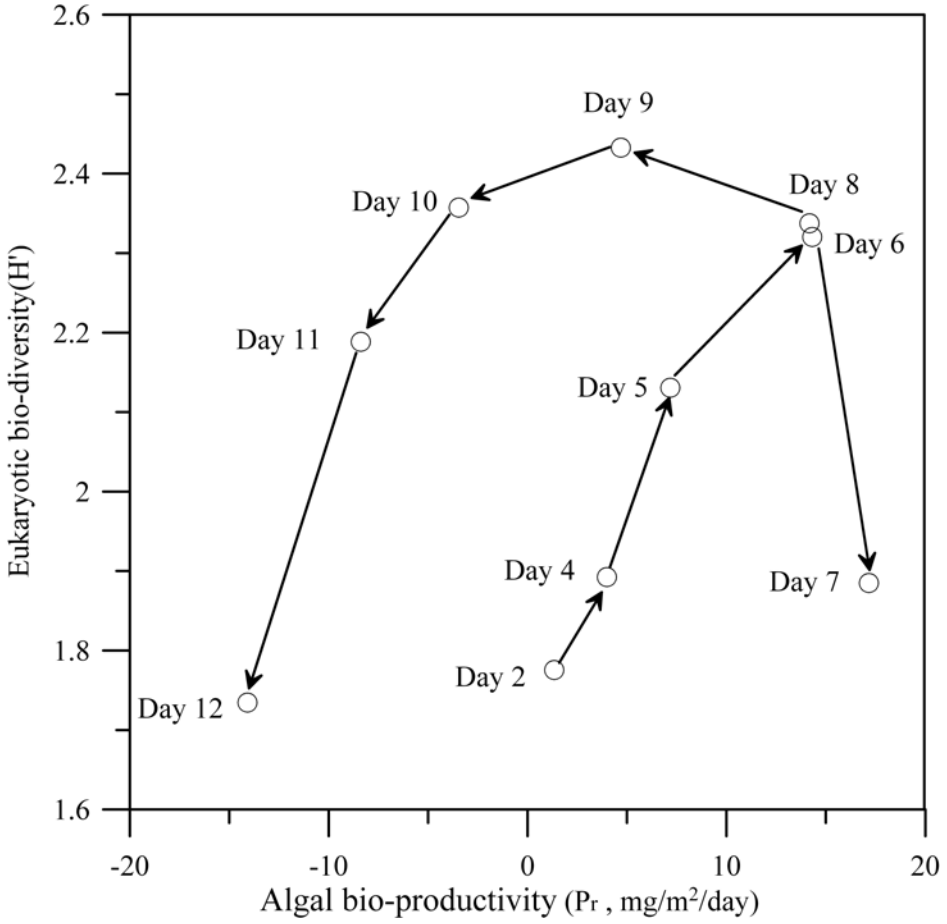


Figure 5.8 The act of changing eukaryotic biodiversity resulted from algal bioproductivity based on observational study. Open circle is the sampled day. The values of Pr are calculated using the observed date of Figure 5.1 referred to the procedure of Pr estimation in Chapter 3.

5.3 Discussion

The development of algal phytoplankton community is a dynamic process. The many physical and biological mechanisms could potentially influence the patterns of biodiversity-bioproductivity in the algal phytoplankton community. The factors correlated with biodiversity are the basis in understanding how biodiversity is regulated.

Algal population dynamics of CSTR system can be expressed by algal net growth rate (r_s).The algal net growth rate (r_s) can increase and decrease due to the algal gross growth rate (μ_g) and algal loss rate (λ_l). In the CSTR system the algal populations are introduced and incubated under an environment where nutrients and physical conditions are kept consistent. A situation is the fact that the algal population dynamics can be affected by its population size, environmental resistance and the biological factor of other populations. The concept of algal population dynamics is essential for describing algal growth rate, related interaction and regulation among the populations during growth phases. Depending on the algal bioproductivity, algal population dynamics correlates closely with the algal net growth rate. The algal growth can be expressed as the algal growth under unlimited conditions, following the potential environmental resistance, and then the negative growth rate caused by other species interactions. The concept of considering a population growth rate depending on population size is used to express the

variation in the growth rate and involved algal individual.

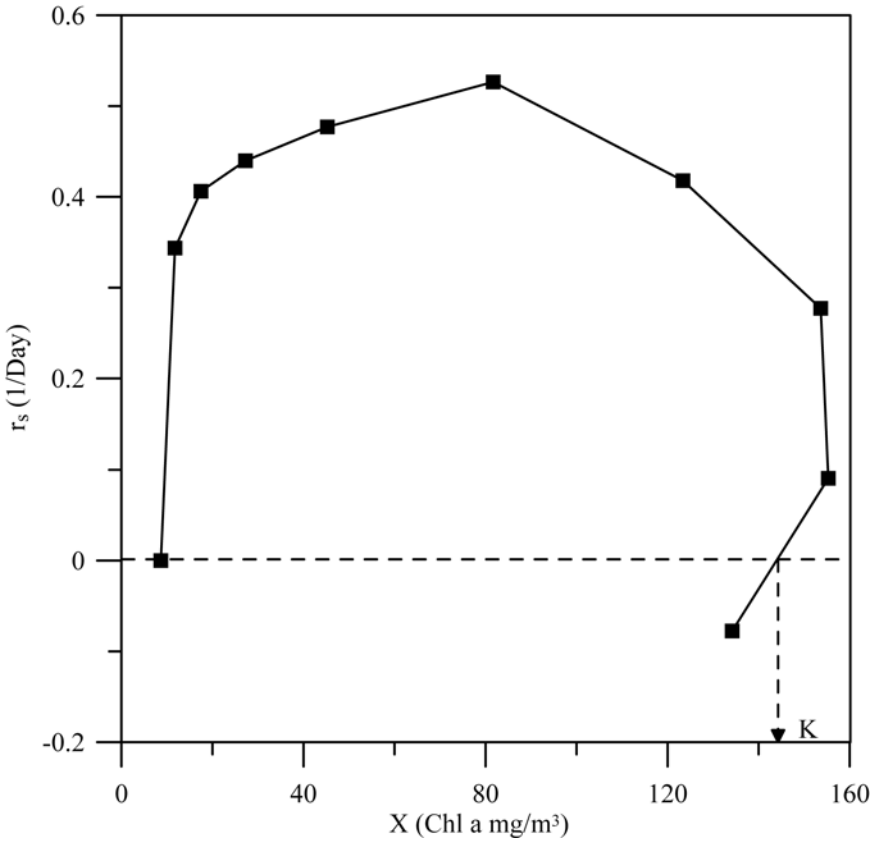


Figure 5.9 Algal population growth showing the relation between the algal net growth rate and algal population size *X* (Chl *a*). K is carrying capacity.

In limnology, algal bioproductivity is often used to classify the trophic levels of a lake. It is commonly observed that the higher trophic level results from excessive and rapid growth of algae, in terms of higher algal bioproductivity. However, the negative relationship of bioproductivity-biodiversity was observed in higher trophic level (e.g.

eutrophication). The unexpected phenomenon was called “the paradox of the plankton.” (Hutchinson 1961). The time-varying analysis of eukaryotic biodiversity may provide the best interpretation for this unexpected phenomenon. When the maximum algal bioproductivity presents, a sudden loss of eukaryotic biodiversity may be expected.

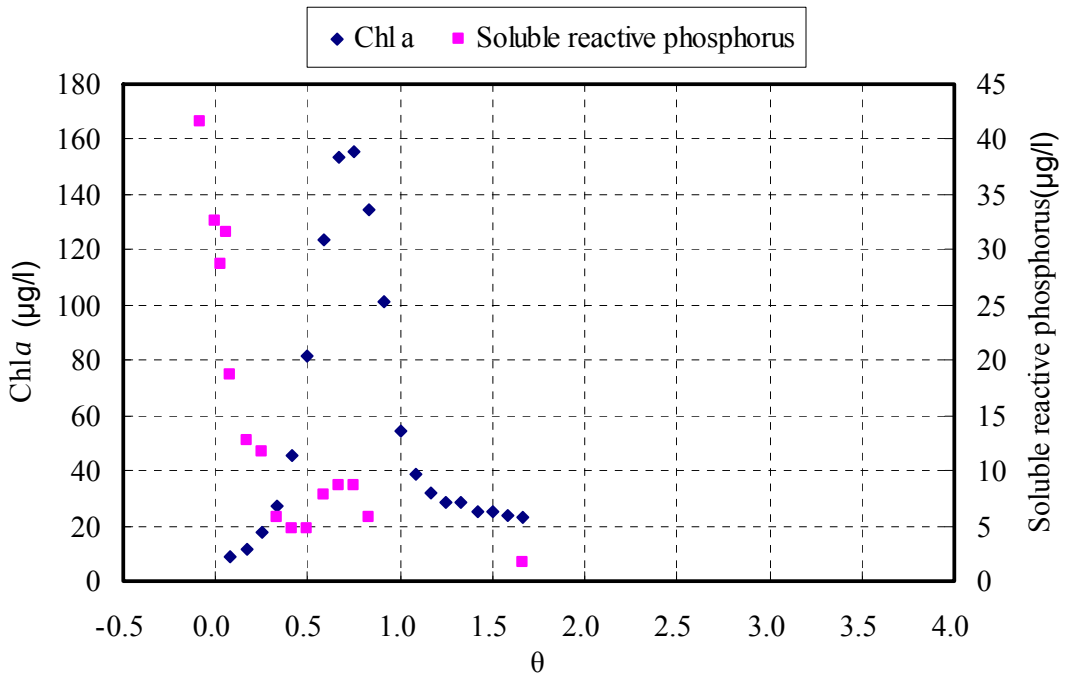


Figure 5.10 Experiment results conducted at eutrophic TSCAR during Sept. 13, 2010 to Sept. 29 2010.

The soluble reactive phosphorus has a increase while Chl *a* approaching peak under eutrophic TSCAR (refered to Figure 5.10) and mesotrophic TSACR bioreactor. The cause was stills little known. Hudson et. al (1999) reproted that the regeneration of planktonic phosphorous is a central proces of algal growth. The recycling phosphorus

supplied the major nutrient for primary productivity. Figure 5.10 showed that it may have resulted in the regeneration of phosphorus. It suggests that huge population may be digested and then released phosphorus. It could be evidence to describe how the lake biodiversity presents huge losses before reaching peak biomass.

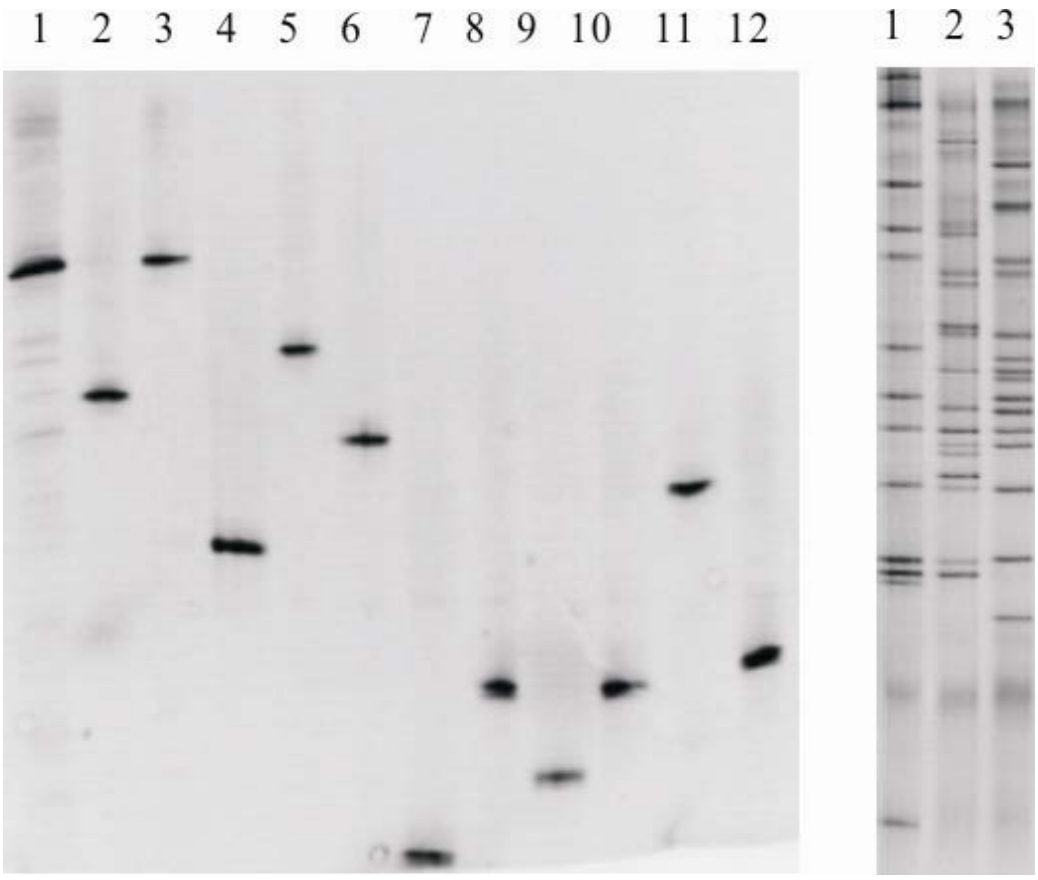
CHAPTER 6. MOLECULAR PHYLOGENY OF EUKARYOTIC ASSEMBLAGE UNDER VARYING TROPHIC LEVELS

The eukaryotic biodiversity was examined by molecular cloning and sequencing the 18S ribosomal DNA in three bioreactors differing by their trophic levels (oligotrophic, mesotrophic, and eutrophic levels). The results of sequence analysis showed that the composition of the eukaryotic community and lineages were subjected to the lake trophic status. The Ek4 library reflected higher equitability than EK5 and EK6 by used Shannon evenness, in other words, lower clone dominance was observed in the oligotrophic assemblage survey. The Shannon index of Ek5 appeared more diverse than Ek4 and Ek6. The discovery of unknown species and new phylogentic groups identified were found in PK6, EK4, EK5, and EK6 libraries.

6.1 Results

The results of the clone library provided the informative phylogeny characterization of eukaryotic and prokaryotic assemblage (Lopez-Garcia, Rodriguez-Valera, et al. 2001; Moon-van der Staay, De Wachter, et al. 2001). Each library (Ek4, Ek5 and Ek6) randomly picked up approximately 63 positive clones, except for Pk6 library samples where only 37 positive clones were analyzed. For prokaryotes, the Pk6 library constructed was analyzed, producing 10 different operational taxonomic units

(OTUs) (refer to Figure 6.1 (a)). For eukaryote libraries, the EK4, EK5, and EK6 libraries constructed were analyzed, producing 17, 22, and 18 different OTUs for oligotrophic, mesotrophic and eutrophic bioreactors (refer to Figure 6.1 (b), Table 6.2, Table 6.3 and Table 6.4). 57 OTUs were identified based on a DGGE profile using a total of 187 clones obtained from three eukaryotic libraries. A representative clone of each OUT in each library was sequenced. 45 OTUs having full-length approximately1784 bp and 12 OTUs having approximately 892 bp sequencing were yielded.



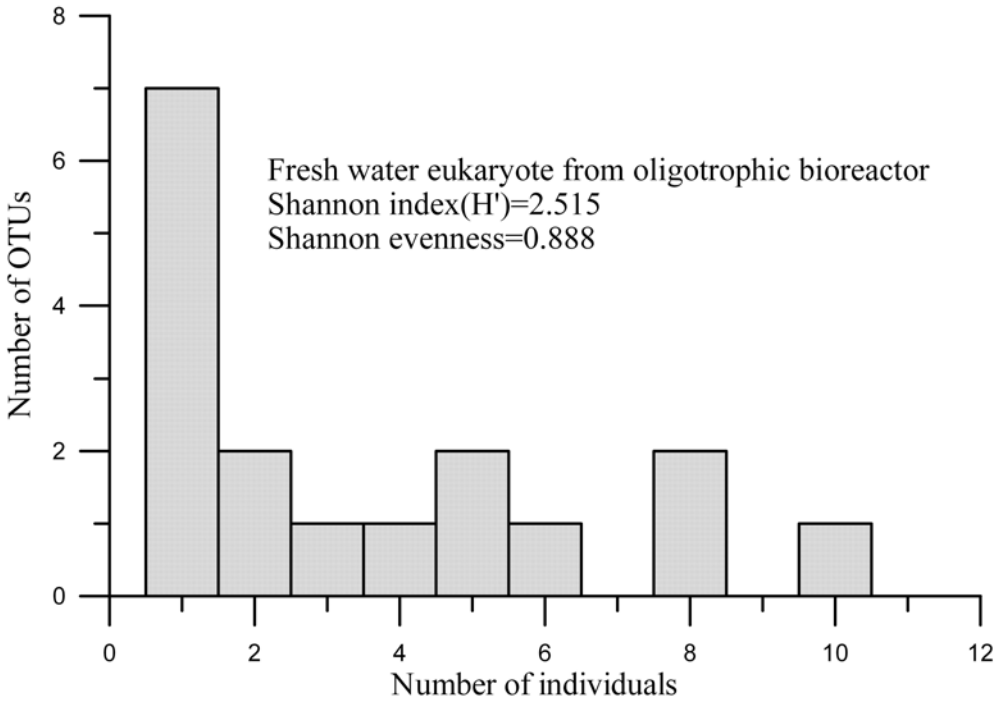
(a) (b)

Figure 6.1 DGGE profile image of prokaryotic and eukaryotic cloning libraries termed Pk6, Ek4, Ek5, and Ek6 with representative clones. (a) Comparison of DGGE profile of the eutrophic bioreactor mixed community to prokaryotic clones containing insert 16S rDNA gene. Lane1, eutrophic bioreactor mixed community; Lane2, EP 3; Lane3, EP 5; Lane4, EP 13; Lane5, EP 15; Lane6, EP 16; Lane7, EP 18; Lane8, EP 19; Lane9, EP 29; Lane10, EP 30; Lane11, EP 61; Lane12, EP 62. (b) DGGE image demonstrates that the DGGE banding data depicted the OTUs of each eukaryotic libraries (lane1 :Ek4, lane2 :Ek5, and lane3: Ek6)

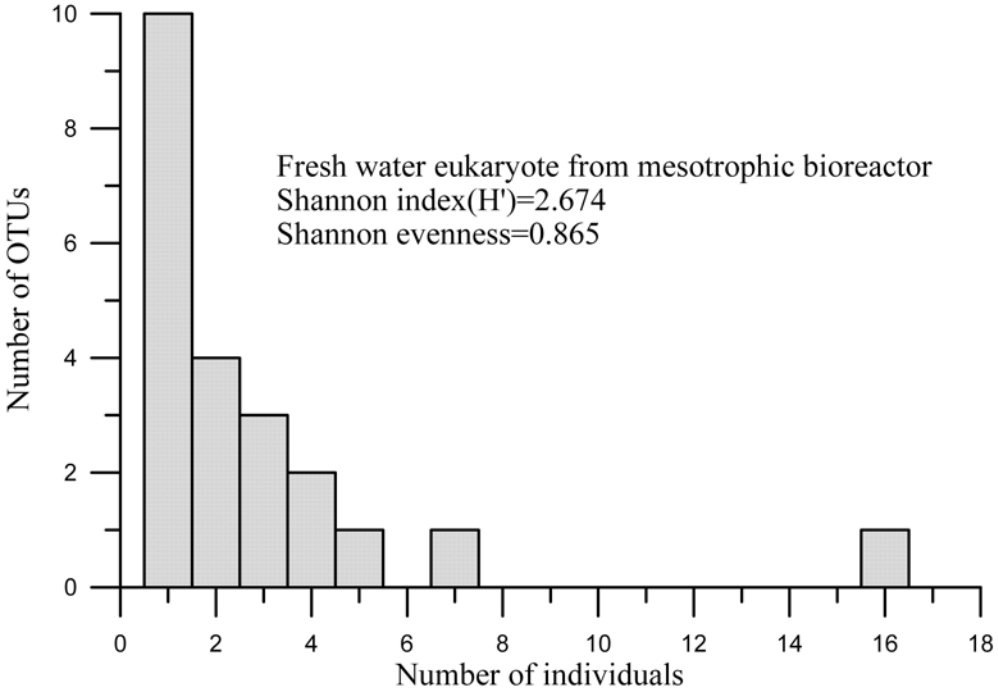
6.1.1 Eukaryotic libraries in TSCARs

The overall composition of the eukaryotic community often was interpreted by species richness (number of species), abundance (number of individuals) and evenness (level of dominance) in classical ecological biodiversity studies (MacArthur and Wilson 1967; May 1975, Pielou 1975). Contemporary studies on eukaryotic biodiversity based on the method of sequencing 18S rDNA were often used for analyzing the composition of the community and the OTUs were regarded as the surrogates of taxa or species (Bent and Forney 2008; Curtis, Head, et al. 2006). Indeed, the method allowed for an improved understanding of the common and rare species in lake biodiversity, especially the discovery of unknown species and new phylogentic groups (Lefèvre, Bardot, et al. 2007; Richards, Vepritskiy, et al. 2005).

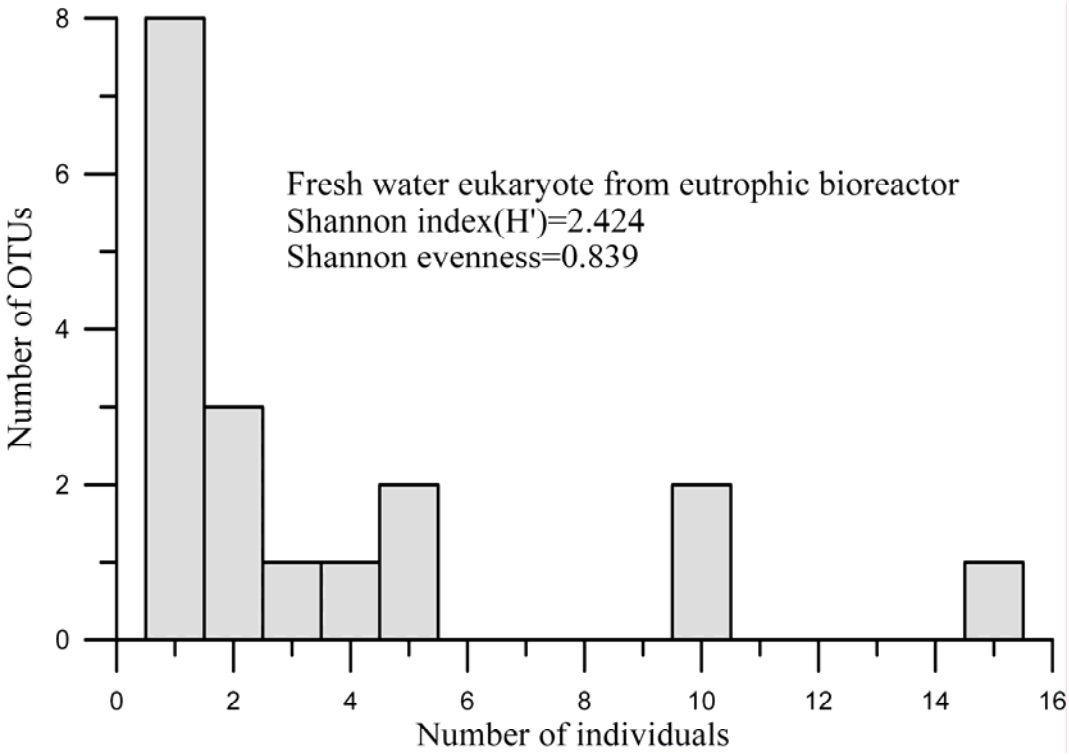
In this clone library study, the raw data (plotted Figure 6.2) shows the relationship between species richness and relative abundance in three clone libraries (Ek4, Ek5, and Ek6). The plots provided information on a number of species and on their relative abundance as well as the potential dominance species. Figure6.2 illustrates that in the data sets it was found that a few species were particularly abundant, such as Ek4.16, Ek5.1 and Ek6.9, some species of varying intermediate degree, while most of the species appeared as a few individuals.



(A) Ek4 clone library



(B) Ek5 clone library



(C) Ek6 clone library

Figure 6.2 The plots demonstrate that the relationship between number of OTUs and number of individual in three trophic-level assemblages.

The Ek4 library reflected higher equitability than the other two by used Shannon evenness, in other words, lower clone dominance was observed in the oligotrophic assemblage survey. Based on the Shannon index, Ek5 appears more diverse than Ek4 and Ek6.

6.1.2 Prokaryotic library in eutrophic TSCAR

In the PK6 library, ten phylotypes were found and defined as three potential

groups: Proteobacteria, Cyanobacteria, and unidentified environmental samples (see Table 6.1). The bacterial communities in lakes play a central role and obtained more attention in lake field investigations. Van et al. reported that the groups (such as *Actinobacteria*, *Betaproteobacteria*, *Bacteroidetes*, and *Cyanobacteria*) of bacterial communities in freshwater lakes related to the food-wed variation.(Van der Gucht, Vandekerckhove, et al. 2005). The *Cyanobacteria* bloom obtained more attention among those function groups (Eiler and Bertilsson 2004) due to water quality issues. Limited in data of sequence demonstrated of the PK6 were merely preliminary in the community structure analysis. Based on the sequencing data, PK6 was dominated by EP5. In environmental sample group, fourteen positive clones clustered with EP5 with 95% similarity related to uncultured bacterium clone A5-083. The high percentage of unidentified environmental samples occupied PK6. It pointed out that additional investigation of prokaryotic biodiversity are necessary.

Table 6.1 Sequence length and closest phylogenetic affiliation of the PK6 clone library analysis.

Taxon	OUT	Sequence length (bp)	Potential relative Species	Maximum Identity (%)	Number of clones
Proteobacteria	EP 3	1529	*Pseudomonas putida* strain GMC1 (*Pseudomonas putida* strain 31920-1)	98	1
	EP 15	1523	*Pseudomonas putida* strain 31920-1	99	5
Cyanobacteria	EP 29	1447	*Leptolyngbya foveolarum* VP1-08	98	1
Environmental samples	EP 5	1573	Uncultured bacterium clone A5-083	95	14
	EP 13	1525	Uncultured organism clone ctg_CGOF247	96	3
	EP 16	1500	Uncultured bacterium clone aab57d02	98	5
	EP 18	677	Uncultured bacterium clone NN13	98	1
	EP 19	1522	Uncultured bacterium clone NN25	95	5
	EP 61	1467	Uncultured bacterium clone 3C003679	98	1
	EP 62	1497	Uncultured bacterium clone NN13	94	1

6.2 Phylogenetic analyses

A total of fifty-seven insert 18S rDNA consisting of forty-six completely and eleven partially sequences were used in the phylogenetic analyses for all three libraries EK4, EK5 and EK6.

EK4 cloning library

In the EK4 library, seventeen phylotypes were found and defined as ten phylogenetic groups: Chlorophyta, Heterolobosea, Cryptophyta, Stramenopiles, Cercozoa, Fungi, Rotifera, Amoebozoa, Alveolata and unidentified eukaryote (Table 6.2). In several field investigations, these phyla were often present in oligotrophic lakes (Lefranc, et al. 2005; Richards, et al. 2005). According to analysis, two main phyla, Chlorophyta (27%) and Stramenopiles (25%) were clearly observed in the EK4 library. In Chlorophyta group, thirteen positive clones clustered with EK4.14 and EK4.5 with 97% similarity related to *Coccomyxa* sp. and four clones belonging to EK4.1 with 98% similarity related to *Botryococcus braunii*. The *Botryococcus braunii* (4 clones) and *Coccomyxa* sp. (13 clones) are detected in the EK4 library. Both of the two nonmotile greens are characteristic of oligotrophic lakes. The *Botryococcus braunii* was found frequently in lakes of low productivity (Huszar, Kruk, et al. 2003; Wake and Hillen 1981) and low saline waters (Davis, Kolva, et al. 1977).

Table6.2 Number of clones in Ek4 library and the phylogenetic affiliations based on clones sequenced

Taxon	OUT	Sequence length (bp)	Potential relative Species	Maximum Identity (%)	Number of clones
Chlorophyta	Ek4.1	1758	*Botryococcus braunii*	98	4
	Ek4.5	1760	*Coccomyxa* sp.CCAP 211/97	99	5
	Ek4.14	1764	*Coccomyxa* sp.CCAP 211/97	97	8
Heterolobosea	Ek4.4	1036	Uncultured heterolobosean clone LC103_5EP_3	78	2
Cryptophyta	Ek4.12	1728	*Campylomonas reflexa*	98	2
	Ek4.17	1727	*Campylomonas reflexa*	97	1
Stramenopiles	Ek4.16	1770	*Spumella*-like flagellate JBNZ39	98	10
	Ek4.34	1812	Uncultured chrysophyte clone PR4_4E_14	95	5
Cercozoa	Ek4.18	1774	Uncultured freshwater cercozoan clone PCG5AU2004	98	1
Fungi	Ek4.63	966	Uncultured eukaryote clone KRL01E36	92	1
Rotifera	Ek4.68	1783	*Lepadella patella*	98	1
	Ek4.76	1785	*Cephalodella forficula*	98	3
Amoebozoa	Ek4.77	1600	*Flamella fluviatilis*	96	1
Alveolata	Ek4.79	1725	*Coleps* sp. small subunit 16S-like rRNA.	99	1
Unidentified eukaryote	Ek4.2	788	Uncultured eukaryote clone T0-euc.b.01	97	8
	Ek4.3	1746	Uncultured eukaryote clone TKR07E.13	98	6
	Ek4.21	1785	Uncultured eukaryote clone TKR07M.62	98	1

The most abundant *Coccomyxa* sp. in the EK4 assemblage belongs to endosymbiotic green algae and many ciliates and invertebrates with symbiotic green algae have been investigated. The endosymbiotic association is firmly observed in

previous studies (Luo, et al. 2011; Reisser 1984), implying that the endosymbiotic association of green algae (*Coccomyxa* sp.) with their potential hosts such as *Coleps* sp. (Ek4.79) and *Flamella fluviatilis* (EK4.77) was formed in this oligotrophc system(EK4 assemblage).

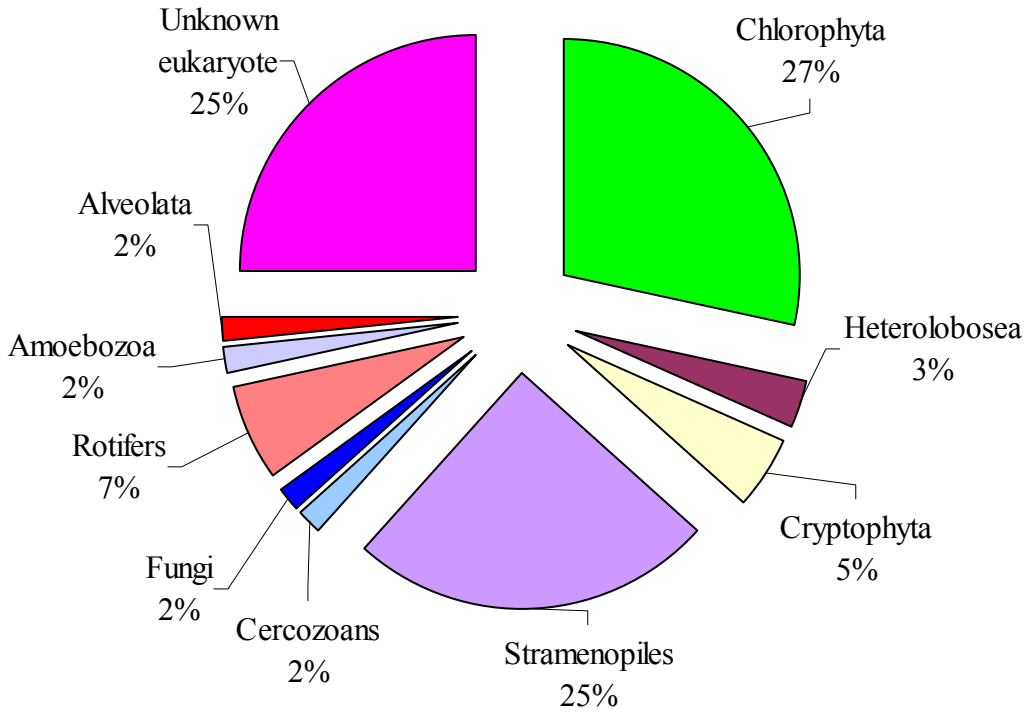


Figure 6.3 Relative abundance of clones represented in the nine phylogenetic groups in EK4 library.

The second most abundant group in the EK4 assemblage was Stramenopiles (25%)

with ten of clones. This group was related to *Spumella*-like flagellate (Ek4.16) with 98% similarity. Five of the clones were found close to the uncultured chrysophyte clone PR4_4E_14 with 95% similarity OUT (EK4.34). Lara et al.(2011) reported that the sequence of PR4_4E_14 is associated with *Spumella*-like flagellate (Lara, Mitchell, et al. 2011). The *Spumella*-like flagellate belonging to colorless, heterotrophic nanoflagellate (HNF), plays important role in carbon transfer and nutrient regeneration of aquatic food webs. However, the unexpected diversity was only recently investigated. Boenigk et al.(2005) reported that the *Spumella* of HNF are a high diverse group and commonly found in different geographic regions (Boenigk, Pfandl, et al. 2005). Several field investigations indicated that this function group, comprised of HNF, bacteria and ciliates, is associateed with lake productivity (Auer and Arndt 2001).

The Cryptophyta were detected two OTUs (EK12 and EK17), belonging to *Campylomonas reflexa*, one of primary producers, from EK4 assemblage which occupied 5% of clonal abundance. The lower proportion of clonal abundance are also found in recently lake and coastal studies, for instance, 7% of clonal abundance was observed in Lake Godivelle (Lefranc, et al. 2005), 10% in Lake Fuschlsee (Medinger, Nolte, et al. 2010), and 4% in Blanes Bay (Massana, Balagué, et al. 2004). In spite of the limitation in evidence found in the observation, the results suggested that the lower abundance of

Cryptophyta may associate with the trophic status in lakes. Auer (2001) indicated that the Cryptophyta group are positively correlated with lake bioproductivity in field investigation by non-molecular analysis (Auer and Arndt 2001).

Alveolate is a super phylum comprising ciliates, dinoflagellates, the parasitic apicomplexa and perkinsozoa (Moreira and Lpóez-Garca 2002). In this EK4 assemblage, they are represented by one OUT (EK4.79) associated with heterotrophic ciliophora: *Coleps* sp.. Field investigations indicated that *Coleps* sp. was the low grazing rates of detritofagous species (Sanders, Porter, et al. 1989; Simek, Bobkova, et al. 1995). Although EK4.79 was relatively low in abundance (2%), the *Coleps* sp. of heterotrophic ciliates plays an important role in parasites of green algae.

Two OTUs were grouped within the Rotifera which was regarded as a significant consumer of bacteria in freshwaters. EK4.68 and EK4.76 obtained 98% similarity with *Lepadella patella* and *Cephalodella forficula*, respectively. Both were found in an oligotrophic lake(Casper, Flobner, et al. 1985).

Three unidentified OTUs (Ek4.2, Ek4.3 and Ek4.21) were found in the EK4 library. Ek4.2, Ek4.3 and Ek4.21 belonged to environmental samples: uncultured eukaryote clone T0-euc.b.01, uncultured eukaryote clone TKR07E.13 and uncultured eukaryote clone TKR07M.62 with 97%, 98%, and 98% similarity, respectively. The two

sequences (uncultured eukaryote clone TKR07E.13 and uncultured eukaryote clone TKR07M.62) were originally detected in the oligotrophic Lake Tanganyika (Tarbe, Stenuite, et al. 2011).

Based on the species abundance distribution (referred to Figure 6.2(a)), the three OTUs (EK4.14, EK4.16 and EK4.2) affiliated with *Coccomyxa* sp.*, Spumella-like* flagellate and unidentified eukaryote (EK4.2) were recognized as the certain members of the EK4 library. The six OTUs (Ek4.1, Ek4.4, Ek4.12, Ek4.34, Ek4.76 and Ek4.3) are arranged in the moderately relative common groups, including *Botryococcus braunii,* uncultured heterolobosean clone LC103_5EP_3, *Campylomonas reflexa,* uncultured chrysophyte clone PR4_4E_14*, Cephalodella forficula,* unidentified eukaryote (EK4.3). Rare species were classified as those represented by single number of OUTs in taxon groups. Approximately 41% of OTUs (7 species observed) is rare in the EK4 library.

<u>EK5 cloning library</u>

The composition of a eukaryotic assemblage in a mesotrophic bioreactor (EK5 library) was investigated showing nine different phylogenetic groups: Chlorophyta, Heterolobosea, Cryptophyta, Stramenopiles, Cercozoa, Fungi, Metazoans, Alveolata, and LKM11 (Referred toTable 6.3). The dominant group was the Cryptophyta group which occupied 26% of clonal abundance observed in the EK5 cloning library. The Chlorophyta,

Fungi and LKM11 groups occupied 17%, 17% and 14% of clonal abundance, respectively. The five other groups: Heterolobosea, Stramenopiles, Cercozoa, Metazoans, and Alveolata obtained less then 10% of clonal abundance (see Figure 6.4).

In Cryptophyta group, sixteen positive clones clustered with EK5.1 obtained 97% similarity with *Campylomonas reflexa*, a most abundant individual in the EK5 library. *Campylomonas reflexa* is often found in diversely trophic-level lakes (Muller Ogle, Peterson, et al. 1999). Cryptophyta grown in light-limited and ice-covered lakes have been observed in field investigations (Pasztaleniec and Lenard 2008; Phillips and Fawley 2002). The Chlorophyta group occupied 17% of relative abundance in the EK5 library is also well presented. Seven OTUs were detected including *Makinoella tosaensis*, *Botryococcus braunii*, *Coccomyxa* sp.CCAP 211/97, *Scenedesmus regularis*, *Kirchneriella obesa* strain ACOI 3125, *Botryococcus braunii* and uncultured eukaryote clone KRL01E41 with more then 94% similarity. The proportions of Chlorophyta are highest among the three trophic-level libraries, contributing to 32% of OTUs in EK5. It suggests that the highest species richness of Chlorophyta represented in the mesotrophic lake. Compared with EK4 library, the Chlorophyta increased in number of species, but decreased in number of individuals in the EK5 library.

Table6.3 Number of clones in EK5 library and the phylogenetic affiliations based on clones sequenced

Taxon	OUT	Sequence length (bp)	Potential relative species	Maximum Identity (%)	Number of clones
Chlorophyta	Ek5.3	1749	*Makinoella tosaensis*	94	1
	Ek5.16	1758	*Botryococcus braunii*	98	1
	Ek5.33	1760	*Coccomyxa sp.*CCAP 211/97	99	2
	Ek5.90	1735	*Scenedesmus regularis*	94	2
	Ek5.127	1756	*Kirchneriella obesa* strain ACOI 3125	97	1
	Ek5.65	969	*Botryococcus braunii*		1
	Ek5.7	1749	Uncultured eukaryote clone KRL01E41	96	3
Heterolobosea	Ek5.25	1146	Uncultured heterolobosean clone LC103_5EP_3	83	5
Cryptophyta	Ek5.1	1727	*Campylomonas reflexa*	97	16
Stramenopiles	Ek5.85	1629	*Spumella* sp. TGS6	99	3
Cercozoa	Ek5.39	1050	*Lecythium* sp.	95	1
	Ek5.70	1774	Uncultured freshwater cercozoan clone PCG5AU2004	98	2
	Ek5.82	1672	Uncultured freshwater cercozoan clone PCH11AU2004	97	2
Fungi	Ek5.26	926	Uncultured fungus clone D53	98	3
	Ek5.71	966	Uncultured eukaryote clone KRL01E36	92	4
	Ek5.31	1750	Uncultured eukaryote clone PF7AU2004	99	4
Metazoans	Ek5.122	699	*Capsaspora owczarzaki*	95	1
Alveolata	Ek5.80	1724	Uncultured ciliate clone AY2009C19	98	1
	Ek5.130	1707	*Coleps hirtus*	98	1
Unidentified eukaryote (LKM11)	Ek5.27	1750	Uncultured eukaryotic picoplankton clone P34.42	94	7
	Ek5.78	732	Uncultured eukaryotic picoplankton clone P34.42	97	1
	Ek5.116	1716	Uncultured eukaryotic picoplankton clone P34.42	94	1

According to the literature, the field investigations of mesotrophic lake showed that the proportion of Chlorophyta represent 17.9% of total eukaryoyic assemblage (Mangot, et al. 2009).

The fungi group of Ek5 occupied 17% of clones, which formed three different OTUs (EK5.26, EK5.71, and EK5.31) which is similar to the result (11.4%) conducted by Mangot, Lepère et al. (2009). Three clones of EK5.26 were related to clone D53 with 98% similarity; four clones of EK5.71 were traced to uncultured eukaryote clone KRL01E36 based on 94% similarity. Moreover, four clones of EK5.31 was closely linked to uncultured eukaryote clone PF7AU2004 with 98% similarity affiliated with zoosporic fungi based on the investigation in Lake Pavin (Lefèvre, Jobard, et al. 2010). Compared with EK5.26, the highly similar observation of clone PFD5AU2004 clustered to novel clade of fungi with 98% similarity was made (Lefèvre, et al. 2007) and Lefèvre et al.(2007) highlighted that fungal zooflagellates are important member of the functional groups in oligomesotrophic Lake Pavin. The three unknown sequences could not have been identified as species level of fungi based on current data, because they are limited by the lack of species descriptions. These small fungi (<5μm) are recognized as important members in lake systems (Lefèvre, et al. 2007), since the function groups are considered to be parasites of the phytoplankton (Kagami, Donk, et al. 2004; Lepère, Domaizon, et al.

2008), consumers of algae, cyanobacteria and particulate orgasmic material (Van Hannen, et al. 1999) and a prey of larger zooplanktons. This group, therefore, provided the function of transfer energy (carbon biomass) into higher trophic levels in lake systems.

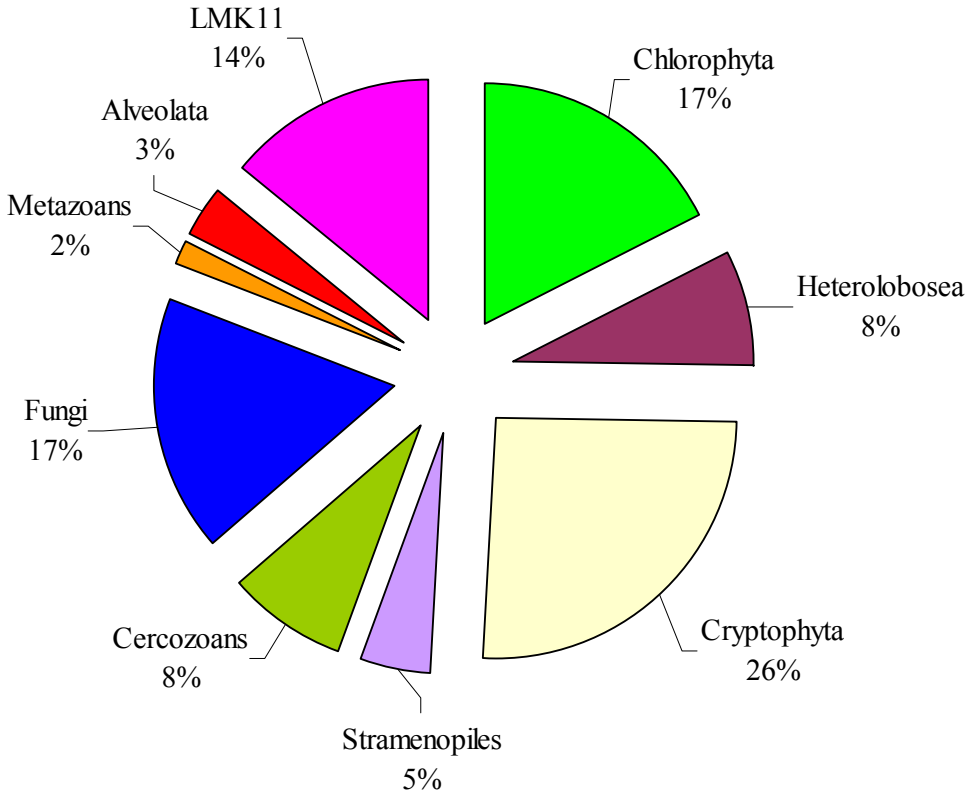


Figure 6.4 Relative abundance of clones represented in the 9 phylogenetic groups in EK5 library.

In the EK5 clone library, 14% of clones belonged to unidentified eukaryotes composed of three OTUs (Ek5.27, Ek5.78 and Ek5.116). Nine clones of the three OTUs were clustered with more then 94% similarity to uncultured eukaryotic picoplankton

clone P34.42 detected in oligomesotrophic Lake Pavin (Lefranc, et al. 2005). The P34.42 was affiliated with the clade LMK11 defined by Van Hannen et al. (Van Hannen, et al. 1999). Mangot, Lepère et al. (2009) reported that the LMK11 found 4.5% of clones relative abundance in the mesotrophic Lake Bourget (Mangot, et al. 2009). According to phylogenetic analysis (Van Hannen, et al. 1999), LMK11 group is regarded as a novel cluster and is strongly associated with Fungi. LMK11 is often found in the oligotrophic and mesotrophic lakes (Lefranc, et al. 2005; Lepère, et al. 2006; Lepère, et al. 2008; Mangot, et al. 2009).

Three OTUs (Ek5.39, Ek5.70 and Ek5.82) were grouped within Cercozoa phylum with more then 95% similarity. The sequence of EK5.39 linked to the core cercozoa: *Lecythium* sp., a small (5-10μm in size) heterotrophic filose amoeba known as a bacterial feeder (Nikolaev, Berney, et al. 2003). The EK5.70 and EK5.82 are closely related to uncultured freshwater cercozoan clone PCG5AU2004 and uncultured freshwater cercozoan clone PCH11AU2004 belonged to novel clade II of Cercozoa. The two sequences of novel clade II were firstly defined by Lefevre et al. (2007) based on the investigation of HF in Lake Pavin (Lefèvre, et al. 2007). EK5.25 obtained 8% of the clones abundance and belonged to the Heterolobosea phylum. The sequence of EK5.25 were related to the sequence of clone LC103_5EP_3 with lower similarity (83%)

identified by López-García et al. (López-García, Vereshchaka, et al. 2007).

The population of Stramenopiles (5%), Metazoans (2%) and Alveolata (3%) in EK5 library is rare. Three clones were detected belonging to Ek5.85 grouped into Stramenopiles and traced to *Spumella* sp. TGS6 with 99% similarity. In the previous studies, the heterotrophic *spumella* of small Chrysophyceae were common observed in freshwaters, but *spumella*-like flagellates did not dominate the mesotrophic lakes (Lefranc, et al. 2005; Lepère, et al. 2006).

Two OTUs (Ek5.80 and Ek130) were clustered into Alveolata (3% of clones). Ek5.80 had 98% similarity to uncultured ciliate clone AY2009C19 first identified by Monchy et al.(2011) in Lake Aydat (Monchy, Sanciu, et al. 2011). The sequence of Ek5.130 was related to *Coleps hirtus hirtus* based on 98% similarity. Barth, et al. (2008) reported that the *Coleps hirtus hirtus* was found only in autumn based on the samples of *Coleps* isolated from mesotrophic reservoir Saidenbach (Germany) (Barth, Tischer, et al. 2008).

<u>EK6 cloning library</u>

According to the phylogenetic analysis, eight groups: Chlorophyta, Heterolobosea, Cryptophyta, Stramenopiles, Apusozoa, Fungi, Alveolata, and uncultured eukaryote, were detected in the EK6 cloning library, which are commonly found in eutrophic lakes

(Huszar, et al. 2003; Lefranc, et al. 2005; Nixdorf, Mischke, et al. 2003). The diagram (Figure 6.6) shows one major phylum, Cryptophyta (48% of clone abundance) in the EK6 library. The Apusozoa and Chlorophyta are well represented, according for16% and 10% of clone abundance, respectively. Anther five groups: Heterolobosea, Stramenopiles, Fungi, Alveolata, and uncultured eukaryote are less then 8% of clone abundance.

Among the taxa groups, the EK6 library was clearly dominated by Cryptophyta group occupied 48% of clone abundance. Cryptophyta phylum of EK6 cloning library comprises two subgroups: Cryptomonadales (EK6.9 and EK6.35) and Pyrenomonadales (EK6.38). Fifteen clones belonged to EK6.9 with 96% similarity related to Cryptomonas marssonii strain WCK01 identified by Kim et al. (Kim, Boo, et al. 2007); four clones of EK6.35 exhibited 98% similarity to Campylomonas reflexa; ten clones of EK6.38 are close to Komma caudate based on 98% similarity. The morphological features between Cryptomonas marssonii and Campylomonas reflexa is very similar based on Hill's description (Hill 1991). However, the main distinguish between the two is the pyrenoid, a center of carbon dioxide fixation, that is, Campylomonas reflexa possesses pyrenoids, but Cryptomonas marssonii does not (Hill 1991; Javornicky 2003). Recently, based on molecular technology more novel species and its diversity are discovered (Marin, Klingberg, et al. 1998; Metfies, Gescher, et al. 2010).

Table6.4 Number of clones in Ek6 library and the phylogenetic affiliations

Taxon	OUT	Sequence length(bp)	Potential relative	Maximum Identity (%)	Number of clones
Chlorophyta	Ek6.1	1760	*Pectinodesmus pectinatus*	95	2
	Ek6.62	1751	*Chlamydomonas reinhardtii*	96	3
	Ek6.69	1758	*Micractinium* sp. TP-2008a	98	1
	Ek6.72	1749	Uncultured eukaryote clone KRL01E41	96	1
Heterolobosea	Ek6.33	930	Uncultured heterolobosean clone LC103_5EP_3	82	4
	Ek6.47	974	Uncultured heterolobosean clone LC103_5EP_3	82	1
Cryptophyta	Ek6.9	1741	*Cryptomonas marssonii* strain WCK01	96	15
	Ek6.35	1744	*Campylomonas reflexa*	98	4
	Ek6.38	1744	*Komma caudata*	98	10
Stramenopiles	Ek6.50	1141	*Spumella* sp. TGS6	95	1
	Ek6.23	1705	Uncultured chrysophyte clone PR4_4E_14	95	3
Apusozoa	Ek6.2	1779	*Ancyromonas sigmoides* strain HFCC327	98	10
Fungi	Ek6.28	1763	Unidentified fungus WIM48	92	1
	Ek6.40	1604	Chytridiomycota clone PA2009C3	95	1
	Ek6.3	966	Uncultured eukaryote clone KRL01E36	93	2
Alveolata	Ek6.67	1724	Uncultured ciliate clone AY2009C19	98	2
	Ek6.22	1718	*Ancistrum sp.* ZZ-2011	96	1
Uncultured eukaryote	Ek6.54	1773	Uncultured eukaryote clone TKR07M.62	97	1

Cryptophyta, unicellular, biflagellate protists, are permanent residents, important primary producer in freshwater. In EK6 cloning library, it demonstrates that the Ek6.9 is dominant and Ek6.38 is co-dominant. It may imply that species belonging to two classes: Cryptomonadales (e.g. EK6.9 and EK6.35) and Pyrenomonadales (e.g. EK6.38) are high potential as the dominant in eutrophic lake.

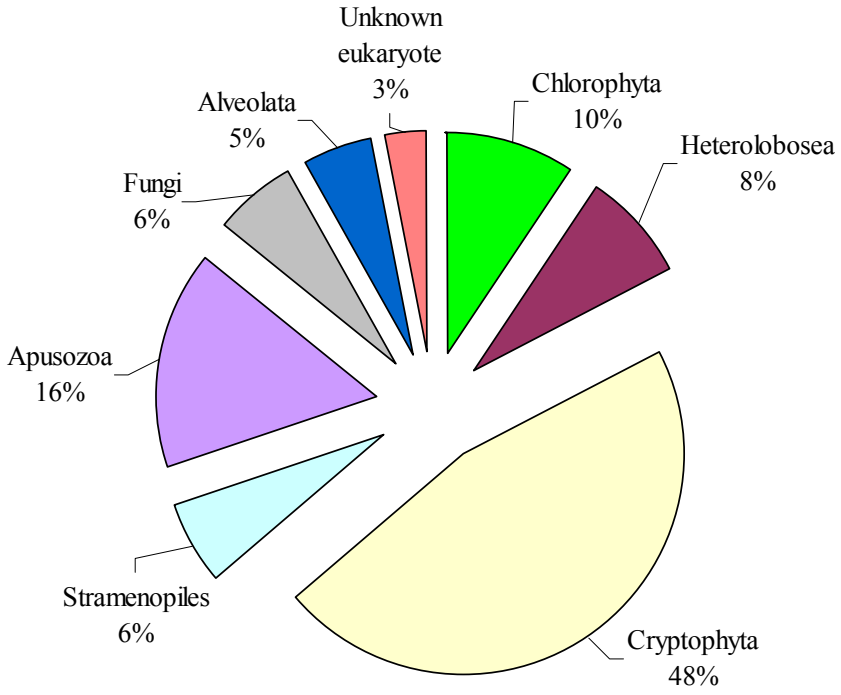


Figure 6.5 Relative abundance of clones represented in the 9 phylogenetic groups in EK6 library.

It is very common to describe the population dynamics of cryptophyta in lakes, but the relationship between cryptophyta and trophic status in lakes is poorly known. Based on three cloning libraries (EK4, EK5 and EK6), it clearly exhibits that the populations of the cryptophyta are positively correlated with trophic levels.

The second most abundant group was Apusozoa which obtained 16% of the Ek6 cloning library. In Apusozoa group, 10 clones belonged to the Ek6.2 based on 98% similarity related to *Ancyromonas sigmoides* strain HFCC327 identified by Scheckenbach et al.(Scheckenbach, Wylezich, et al. 2006). The heterotrophic flagellate *A. sigmoides* feed on another flagellate as well as bacteria. *A. sigmoides* has been found in eutrophic and mesotrophic freshwaters (Kosolapova and Kosolapov 2011; Kosolapova and Kosolapov 2009; Scheckenbach, et al. 2006).

In Chlorophyta group, four OTUs (Ek6.1, Ek6.62, Ek6.69 and Ek6.72) were detected. These OTUs obtained more then 95% similarly related to following sequences: *Pectinodesmus pectinatus, Chlamydomonas reinhardtii, Micractinium* sp. TP-2008a and uncultured eukaryote clone KRL01E41, respectively. The potential species are never detected in other two cloning libraries (EK4 and EK5), except uncultured eukaryote clone KRL01E41 found in EK5. According to BLAST, three potential species sequences (*Pectinodesmus pectinatus*, *Scenedesmus regularis* and *Scenedesmus pectinatus*) are

closely related to Ek6.1 based on 95% similarity. *S. regularis* and *S. pectinatus* are belonged to the Scenedesmus plankton, which is commonly observed in eutrophic lakes and ponds (An, Friedl, et al. 1999).

The OUT (EK6.62) is closely related to the sequence of *Chlamydomonas reinhardtii* based on 96% similarity. *C. reinhardtii*, a heterotrophic green alga with two flagella, is widespread in freshwaters. It is well known to serve as a model organism in response to the change of environments (e.g. light, nutrient conditions). A study pointed out that the regulation effort of *C. reinhardtii* was observed for the yield of algal toxic while cyanobacterial blooming (Kearns and Hunter 2000). EK6.69 obtained 98% similarity to the sequence of *Micractinium* sp. TP-2008a identified by Luo et al. (Luo, Pröschold, et al. 2010). *Micractinium* sp. serves as waste-water-grown algal and commonly occur in waste water treatment systems (Park, Craggs, et al. 2011). Ek6.72 is related to the sequence of eukaryote clone KRL01E41 sampled from eutrophic Lake Koronia (Greece) and environment sample KRL01E41 is highly correlated with *Carteria radiosa* (D86500) based on the phylogenetic analysis by Oikonomou et al. (Oikonomou, Katsiapi, et al. 2012).

Ek6.33 and Ek6.47 are affiliated with Heterolobosea phylum and identified only 82% to known sequence (uncultured heterolobosean clone LC103_5EP_3). The sequence

of LC103_5EP_3 is detected by López-García et al. (López-García, et al. 2007). It occurred in extreme environments (e.g. rich carbonate and thermal seawater) according to López-Garcíass' work and is also found in EK5 and EK6 cloning libraries in this study.

In Stramenopiles group, two OTUs (Ek6.50 and Ek6.23) were detected and related to the sequences of *Spumella* sp. TGS6 and uncultured chrysophyte clone PR4_4E_14 with 95% similarity. A field study showed that *Spumella* is abundant in eutrophic lake (Zhao, Yu, et al. 2003). Rothhaupt's experiments showed that the heterotrophic nanoflagellate (HNF) *Spumella* sp. plays a significant function in the group role in the transfer of nutrient from bacteria to phytoplankton (e.g. *Cryptomonas*) (Rothhaupt 1992). The sequence of PR4_4E_14 is identified by Lara et al.(2011) and sampled from pristine peat bog under a extreme condition (e.g. low pH value, high concentration of humic acids, and lack center nutrient) (Lara, et al. 2011). Based on the Lara's phylogenetic analysis, although the sequence of the PR4_4E_14 is close related to *Spumella*-like, it is assigned to the unidentified eukaryotic lineage, demonstrating that it is possible to belong the novel clade.

Four clones, comprising three OTUs (EK6.28, EK6.40 and EK6.3), are assigned to Fungi group which occupied 5% clone abundance in the EK6 cloning library. The EK6.28 is related to the sequence of fungal WIM48 (novel clade classified) identified by

Moon-van der Staay et al. (Moon-van der Staay, Tzeneva, et al. 2006) based on 92% similarity. In addition, two sequences: KRL01E5 (Oikonomou, et al. 2012) and fungus clone ESS270706.065 (Masquelier, Hoffman, et al. submitted) found in eutrophic and mesotrophic lakes, are related to EK6.28. The Ek6.40 related to the sequence of PA2009C3 identified by Monchy S. et al. (Monchy, et al. 2011). The Ek6.3 linked to eukaryote clone KRL01E36 (Oikonomou, et al. 2012). According to this study, EK6.3 is present in three trophic levels (EK4, EK5and EK6 libraries).

In Alveolata group, two OTUs (Ek6.67 and Ek6.22) are detected and closely related to uncultured ciliate clone AY2009C19 (Monchy, et al. 2011) and Ancistrum sp. ZZ-2011. Ek6.54 is closely related to the sequence of unidentified eukaryote clone TKR07M.62 originally found in the oligotrophic Lake Tanganyika (Tarbe, et al. 2011).

6.3 Discussion

It is very common in describing the population dynamics of cryptophyta in lakes, but the relationship between cryptophyta and trophic status in lake is poorly known. Based on three cloning libraries (EK4, EK5 and EK6), it clearly exhibits that the populations of the cryptophyta are positively correlated with trophic levels. Compared with real lakes, the EK4, EK5 and EK6, and PK6 are very similar prokaryotic and eukaryotic community structure.

The species abundance distribution plays a significant role in revealing the additional dimension of the lake biodiversity. In given assemblage, the distribution patterns may represent the following desirable aspects: the extent of evenness, which implies the degree of dominance of certain members, the reason of population fluctuation, which deduce that physical environment consist of either opportunity species (occasional species) or equilibrium species (permanent members) and the outcome of population size stemmed from the mechanisms of competition and predation. The literatures are rich in respect of the application and mathematic modeling of the species abundance distribution in the classical ecological diversity study (May 1975; Pielou 1975; Preston 1948), but limited in respect of the underlying species abundance distribution for eukaryotic and prokaryotic taxa in lakes. Recently, due to the great progress in the molecular genetic techniques, the modern ecological tools has offered the contribution to indentify and number individual and species (Caron, et al. 2004; Caron, et al. 2009). Indeed, more recently, the OTUs-based species identification and qualification are in widespread use. For example, the environmental samples collected from different trophic lakes (Lefèvre, et al. 2007; Lepère, Boucher, et al. 2006; Lepère, Domaizon, et al. 2007; Lepère, Masquelier, et al. 2010; Luo, Bock, et al. 2011; Mangot, Lepere, et al. 2009), ocean (Lepère, Vaulot, et al. 2009; Lopez-Garcia, et al. 2001; Moon-van der Staay, et al. 2001)

and soil (Bartram, Lynch, et al. 2011; Dunbar, Takala, et al. 1999) have been analyzed by this approach. The data of OTUs numbered has been regarded as the basic component in estimating biodiversity index and developing the modeling. For instance, Dunbar (Dunbar, et al. 2002) has attempted to mathematically describe the relationship between the number of species and number of individual in soil assemblage by using clones 16S rDNA gene library.

CHAPTER 7. CONCLUSION

The man-made eutrophication problem was expected to be solved through investigating the relationships between bioproductivity and biodiversity. To date, many key questions of scientific fundamentals in eutrophication related to the relationships were far from settled. Using traditional and molecular methods, this research made an effort to build up reliable laboratory experiments in order to simulate the eutrophic lake status, to develop a procedure of algal bioproductivity estimates, to unveil the biodiversity paradox, to obtain more knowledge in trophic levels and their biodiversity, to analyze the variance in algal bioproductivity and lake biodiversity and to explore the composition of eukaryotic community under different trophic levels.

The Vollenweider model was adopted as the basic principle to design the lake-like microcosms called TSCARs. The TSCARs, a CSTR system, provided an environment in control for algal growth and a basis for estimating algal bioproductivity in the laboratory. Many behaviors of algal growth and their performance were observed in the system underlying replicated experiments. Based on community analysis (see Chapter 6), artificial lake water, BBMM media, is able to provide an environment for prokaryotic and eukaryotic community structure when compared with real lakes. The three algal growth

patterns: pattern I, pattern II, and pattern III were observed and proposed which related to lake trophic levels (see Chapter3). The phosphorus has a small increase while biomass (Chl *a*) approaches its peak when observed by time-varying analysis in higher trophic levels' bioreactors. It may have resulted from the regeneration of phosphorus. This suggested that huge populations may be digested and then provide for phosphorus. It could be evidence to describe how the prokaryotic biodiversity presents huge losses before reaching a peak of biomass.

A series of equations were developed for the estimates of algal bioproductivity which were described in Chapter 3. These equations are easy to apply to laboratory and field investigation.

Based the treatments of experimental design, the algal bioproductivity were calculated for three trophic levels under TSCARs. The average algal bioproductivity ($\overline{P_r}$) of oligo, meso, and eutrophic levels were 0.37, 0.69, and 2.40 (mg as Chl *a* /m^2 /day); areal algal product (P_T) of oligotrophic, mesotrophic, and eutrophic levels were 6.7, 12.5, and 43.3 (mg as Chl *a* /m^2). The average algal bioproductivity ($\overline{P_r}$) can be converted to annual carbon bioproductivity by using the ratio of carbon/Chl *a* between 10 to 182. Average algal bioproductivity are approximately in agreement with the value of literature.

The lake biodiversity of three trophic levels was investigated. The Shannon index indicated that the eukaryotic biodiversity of mestrophic level was higher than that of oligo and eutrotrophic levels. The prokaryotic biodiversity of mestrophic level was lower than that of oligo and eutrotrophic levels. In comparison, two distinct trophic levels were identified in Lake Wilson based on TP concentration. Based on investigation, it indicated that phosphorus regulates the algal growth in two locations (DSTP and WSFP sites) of Lake Wilson. The eukaryotic biodiversity of DSTP site (higher trophic level) is H'=1.725 while the eukaryotic biodiversity of WSFP site (lower trophic level) is H'=2.571. On the other hand, the prokaryotic biodiversity of DSTP site is H'=2.861 while the prokaryotic biodiversity of WSFP site is H'=2.685.

In this dissertation, the phase-oriented concept of the algal growth was proposed to interpret the varying relationships between lake biodiversity and algal bioproductivity. The evidence indicated that lake biodiversity can be displayed with variation in algal bioproductivity. Based on the analysis, there is a general trend that when data were plotted by eukaryotic biodiversity (H') against algal bioproductivity (Pr), the relationship was hump-shaped under phase II and phase III, whereas when data were plotted by prokaryotic biodiversity (H') against algal bioproductivity (Pr), the relationship was U-shaped. During phase I, the eukaryotic biodiversity positively correlated with algal

bioproductivity, whereas the prokaryotic biodiversity negatively correlated with algal bioproductivity. Moreover, several critical timing points of algal growth history in terms of time-varying Pr(t) revealed a shift in lake biodiversity were proposed. It is a finding described as the lake biodiversity was deeply affected by the history of algal growth.

By conducting molecular cloning, four libraries (EK4, EK5, EK6, and PK6) were produced. The community structures sampled from the TSCARs (lake-mimicked microcosms) were very similar to that of nature lakes. It may provide a direction on an aspect of methodology for investigating lake eutrophication. In the PK6 library, ten phylotypes were found and defined as three potential groups: Proteobacteria, Cyanobacteria, and unidentified environmental samples. In the EK4 library, seventeen phylotypes were found and defined as ten phylogenetic groups: Chlorophyta, Heterolobosea, Cryptophyta, Stramenopiles, Cercozoa, Fungi, Rotifera, Amoebozoa, Alveolata and unidentified eukaryote. The composition of eukaryotic assemblage in a mesotrophic bioreactor (EK5 library) was investigated showing nine different phylogenetic groups: Chlorophyta, Heterolobosea, Cryptophyta, Stramenopiles, Cercozoa, Fungi, Metazoans, Alveolata, and LKM11. In the EK6 cloning library, the eight phylogenetic groups: Chlorophyta, Heterolobosea, Cryptophyta, Stramenopiles, Apusozoa, Fungi, Alveolata, and uncultured eukaryote, were detected, which were

commonly found in eutrophic lakes. These libraries demonstrated their unique and diverse functions.

Finally, a minor finding is worthy of note in the population dynamics of cryptophyta in lakes. According to three cloning libraries (EK4, EK5 and EK6), it clearly exhibited that the populations of the cryptophyta were positively correlated with trophic levels in TSCARs.

Appendix A: Additional information for Chapter 3

Banding detection

TotalLab software was used for banding detection. Firstly, lanes were created automatically. Each lane represents one sample. Automatic band detection was performed by setting the minimum slope 240 and noise reduction 5. The peaks less than 1% of max peak were excluded. Background noise was subtracted by using rolling disc with rolling disc radius 200. Finally, bands were assessed and corrected manually. A and B were the results of DGGE gel C and gel D by using TotalLab band detection. Boxed numbers of A and B indicated values of total band count (S) on gel C (S=9) and gel D(S=20) lane.

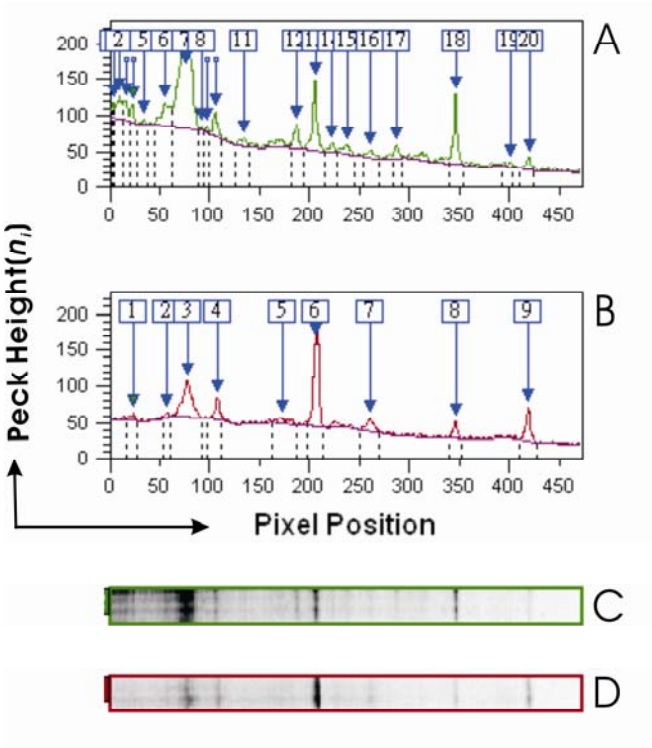


Figure A.1 Digital banding data of DGGE gel.

Appendix B: Supplement to Chapter 5

DNA sequence of DGGE shown in Figure 5.3

Band *a*
GGCTGGCTTCTGTCGGTACGTCAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGA
AGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGG
GGGCGGGGCCCC
Band *b*
CTATCTGTATCAGTAACGTCATTTTATTCCTCCCTGATAAAAGAGGTTTACAACCCGTAGGCATTCTTCCCTCACGCGGTAT
TGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAGGGGGGCGGGGGCGGCGGGCCGGGC
GCGGGGCGCGGCGGGC
Band *c*
GGTATCTTCTCAGTACGTCAGCTCCCACTCGTGAGAGGGTTTCTTCCTGTATAAAAGCAGTTTACAATCCATAGGACCGTC
TTCCTGCACGCGGCATGGCTGGTTCAGTCTTCCGACCATTGACCAATATTCCTCACTGCTGCCTCCCGTAGGGGGGCGGG
GG
Band *d*
CGTGCCCATCTAGGTACGTCATGGACCCCCTTTATTAAAGGAGTCTTTTCGTTCCGTACAAAAGCAGTTTACAACCCGAA
GGCCTTCATCCTGCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGG
GGGCGGGGGCGGCGGGCCGGGCGCGGGGCGCGGCGGGCG
Band *e*
CGGCGATCTCAGATACCGTCAGATCTTCTTCTCTGAGAAAGGAGTTTACGACCCACAGGCTTTCATCCTCCACGCGGCAT
TGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCTCACTGCTGCCTCCCGTAGGGGGGCGGGGGCGGCGGGCCGGGC
GCGGGGCGCGGCGGGCGCGCGCGCCGCGCCCGGC
Band *f*
CCTGGCGAATCTAGGGTACGTCATTAGCAACTGTATTAAAGCTCGCCGTTTCGTTCCGTACAAAAGCAGTTTACAACCCG
AAGGCCTTCATCCTGCACGCGGCATTGCTGGATCAGGCTTGCGCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAG
GGGGGCGGGGGCGGCGGGCCGGGCGCGGGGCGCGGCGGGCG
Band *g*
CCTAGCTTCTAGTACGTCATTCGCAGACTGTGTTAGAGCTGCTGTTTCTTCCCTGACAAAAGAGCTTTACAACCCGAAGG
CCTTCTTCACTCACGCGGCATTGCTGGATCAGGGTTGCCCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGGGG
GCGGGG
Band *h*
GTGCTATCGATTGTACGTCAATTCTCACGCATGAGCTGCTTTCTTCCCATCAAAAGCAGTTTACAACCCACAGGGCCGTCT
TCCTGCACGCGGCGTGGCTGGGTCAGGCTTCCGCCCATTGCCCAATATTCCCTACTGCTGCCTCCCGTAGGGGGGCGGGG
Band *i*
AGGGTCTATCTCGGTACGTCATCCCCGCAGGATATTAGCCCACAGGATTTCTTCCCTGACAAAAGTGCTTTACAACCCGAA
GGCCTTCTTCACACACGCGGCATTGCTGGATCAGGGTTTCCCCCATTGTCCAAAATTCCCCACTGCTGCCTCCCGTAGGG
GGGGCGC

DNA sequence of DGGE shown in Figure 5.4

Band *E1*
AAATCGCTGATGACTGCGTTTACTAGGATTCCTCGTTGAGATCAAGAATTGCAAGAATCTATCCCCATCACGACTGGCGTT
CCAAGATTACCCAGACCTGTCGGAGCCCAGGGTGAACTCGTTGAACCAGTCAGTGTAGCGCGCGTGCGGCCCAGAACAT
CTAAGGGCATCACAGAGGGGCGGGGGCGGCGGGCCGGGGGCGGGGCGCG
Band *E2*
CAAACAACTGATGATTGCGCTTACTAGGCATTCCTCGTTGAGATTAATAATTGCAATAATCTATCCCCATCACGACGCAGTT
TAAAGATTACCCGGGCCTTCCGGCCAAGGACAGCTCGTTGGTTGCGTCAGTGTAGCGCGTGTGCGGCCCAGAACATCTA
AGGGCATCACAGAGGGGCGGGGGCGGCGGGCCGGGCGCGGGGCGCGG
Band *E3*
ATGCAGCTATGAGGCGCTTACTAGGCATTCCTCGTTGAGATGAATAATTGCAATCATCTAGCCCCATCACGATGCAGTTTCC
CAGATTACCCGGACCTCTCGGTCAAGGCTAGGCTCGTTGCATGCATCAGTGTAGCGCGCGTGCGGCCCAGAACATCTAAG
GGCATCACAGAGGGGCGGGGGCGGCGGGCCGGGCGCGGGGCGCGG
Band *E4*
ATAGCAGCTGATGATTGCGCCTACTAGGATTCCTCGTTGATGATCATAATTACAAAGATCAATCCCCATCACGACGAAGGT
TCCAAGATTACCCACACCTTCCGGTGAAGGTTATAAACTCGCTGTCTTCTTCAGTGTACCGCGCGTGCGGCCCAAAACAT
CTAAGGGCATCACAAAGGGGCGGGGGCGGCGGCCCGGGCGCGGGGCCCGGAACCGGTGTGCTGGGAAAGATAGGTAAT
TTCGGTTTCAAAGAAATCAAGTAGGCGCGGGTGTCGCTGGCTATAGTCCTCCTTTGTAGGCCACTAATT
Band *E5*
CTGCGGCTATGATCGCGCTTACTGGGATTCCTCGTTCACGGAGAATAATTTCAAGCCCCGATCCCAATCACGAAGGGGTTT
CAGCGGATTACCCACTCCTTTCGGAGCAGGAAAAACACGCTTATCCCTTCAGTGTAGCGCGCGTGCGGCCCAGAACATCT
AAGGGCATCACAGAGGGGCGGGGGCGGCGGGCCGGGCGCGGGGCGCGG

Appendix C: Supplement to Chapter 6

DNA sequence

EK4 OLIGOTROPHIC LIBRARY
EK4.1
TAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCTGTTTATACTGTGAAACTGCGAATGGCTCATT
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AATTGGAATGAGTACAATCTAAAATCCTTAACGAGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCC
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EK4.2
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EK4.4
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EK4.12
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EK4.16
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EK5 MESOTROPHIC LIBRARY
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EK6 EUTROPHIC LIBRARY
EK6.1
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EK6.3

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CC

EK6.9

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EK6.22

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EK6.23
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EK6.28
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EK6.33
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EK6.35
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EK6.38
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EK6.40
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EK6.47
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EK6.50
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