REMOVAL OF NITROGEN AND PHOSPHORUS FROM MUNICIPAL WASTEWATER USING MICROALGAE IMMOBILIZED ON TWIN-LAYER SYSTEM

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Jing Shi

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Berichterstatter: **Prof. Dr. Michael Melkonian**

Prof. Dr. Werner Rammensee

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Nonnenstieg 8, 37075 Göttingen
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Telefax: 0551-54724-21
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CHEMICAL LIST

NAME	FORMULA	MANUFACTURER
Ammonium heptamolybdate Tetrahydrate	$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$	Merck
Ascorbic Acid	$C_6H_8O_6$	Carl Roth
Biotin	$C_{10}H_{16}N_2O_3S$	Serva
Boric Acid	H_3BO_3	Merck
Calcium Chloride Dihydrate	$CaCl_2\ 2H_2O$	Merck
Calcium Nitrate Tetrahydrate	$Ca(NO_3)_2\ 4H_2O$	Merck
Citric Acid	$C_6H_8O_7$	Heitmann
Cobalt Chloride Hexahydrate	$CoCl_2\ 6H_2O$	Merck
Cobalt (II) Nitrate	$Co(NO_3)_2$	Merck
Copper Sulphate Pentahydrate	$CuSO_4\ 5H_2O$	Merck
Diammonium Hydrogen Phosphate	$(NH_4)_2HPO_4$	Merck
Dipotassium Hydrogen Phosphate	K_2HPO_4	Merck
Ethyl Alcohol	C_2H_6O	Merck

Ferric ammonium citrate	$Fe_3(NH_4)$-Citrat	Merck
Ferrous Sulphate Heptahydrate	$FeSO_4\ 7H_2O$	Merck
HEPES(4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid)	$C_8H_{18}N_2O_4S$	Carl Roth
Manganese Chloride Tetrahydrate	$MnCl_2\ 4H_2O$	Sigma
Magnesium Sulfate Heptahydrate	$MgSO_4\ 7H_2O$	Merck
Meat Extract		Merck
Niacinamide	$C_6H_6N_2O$	Sigma
Peptone		Merck
Phenol Solution	C_6H_6O	Merck
Potassium Antimony (III) Oxide Tartrate Hemihydrate	$K(SbO)C_4H_4O_6\ 0.5H_2O$	Merck
Potassium Dihydrogen Phosphate	KH_2PO_4	Merck
Potassium Hydoxide	KOH	Merck
Potassium Nitrate	KNO_3	Merck
Sodium Chloride	NaCl	Merck

Sodium Hydroxide	NaOH	Merck
Sodium Hypochlorite	NaClO	AppliChem
Sodium Molybdenate Dihydrate	Na_2MoO_4 $2H_2O$	Serva
Sodium Nitrate	$NaNO_3$	Merck
Sodium Nitroprusside	$Na_2[Fe(CN)_5NO]$	Sigma
Sulfanilic Acid	$NH_2C_6H_4SO_3H$ H_2O	Merck
Thiamin	$C_{12}H_{17}N_4OS$	Sigma
Thiamine-HCl	$C_{12}H_{17}ClN_4OS$-HCl	Serva
Tiriplex II	$C_{10}H_{16}N_2O_8$	Merck
Tiriplex III	$C_{10}H_{14}N_{2.}Na_2O_8$	Serva
Urea	CH_4N_2O	Carl Roth
Vitamin B_{12}	$C_{63}H_{87}CoN_{14}O_{14}P$	Serva
Zinc Sulphate Heptahydrate	$ZnSO_4 \cdot 7H_2O$	Merck

ABBREVIATION

AMe	ammonium uptake efficiency, %
AM_0	concentration of ammonium at the beginning, mg l^{-1}
AM_i	concentration of ammonium at day *i*, mg l^{-1}
BOD	biological oxygen demand, mg O_2 l^{-1}
C	carbon
Chl *a*	chlorophyll *a*
Chlap	chlorophyll *a* productivity, µg cm^{-2} day^{-1}
COD	chemical oxygen demand, mg O_2 l^{-1}
DeN	denitrification
DW	dry weight
DWp	dry weight productivity, g m^{-2} day^{-1}
EU	European Union
GFM	glass fibre mesh
LGR	linear growth rate
MWTP	municipal wastewater treatment plant
N	nitrogen
NOe	nitrate uptake efficiency, %
NO_0	concentration of nitrate at the beginning, mg l^{-1}
NO_i	concentration of nitrate at day *i*, mg l^{-1}
P	phosphorus

PE	polyester
Pe	phosphate uptake efficiency, %
P_0	concentration of phosphate at the beginning, mg l^{-1}
P_i	concentration of phosphate at day *i*, mg l^{-1}
PTFE	polyetrafluroethylene
r^2	regression coefficiencies
WWTP	wastewater treatment plant
W+Si	Waris-H medium with silicate
W+3V	Waris-H medium with triplicate vitamin
ΔF/Fm’	effective quantum yield of photosynthetic energy conversion,

ABSTRACT

Excessive nitrogen and phosphorus loading from municipal wastewater treatment plants is an ongoing threat to water quality, which leads to more stringent environmental regulations in different countries. Microalgal mediated twin-layer system offers an interesting alternative for nitrogen and phosphorus removal since it provides a treatment to remove both nutrients efficiently coupled with the production of potentially valuable biomass. In the twin-layer system, microalgae are immobilized by self-adhesion on a wet, microporous, ultrathin substrate (the substrate layer). Subtending the substrate layer, a second layer, consisting of a macroporous fibrous material (the source layer), provides the growth medium. Twin-layer effectively separate microalgae from the bulk of their growth medium, yet allow diffusion of nutrients.

Nylon filter cloth and glass grid reinforced lamina were selected as the suitable substrate and source layer for twin-layer system, respectively. Immobilized green algae *Scenedesmus rubescens* and *Chlamydomonas terricola* showed highest biomass productivities among 23 algae supplied by synthetic secondary wastewater and were recommended for wastewater treatment in general. Removal of nitrogen and phosphorus from the real municipal wastewater by *S. rubescens* was investigated. During 54 days, *S. rubescens* grew well (1.05 g dry weight m^{-2} day^{-1}), remained immobilized and removed phosphorus, nitrate and ammonium efficiently from four types of wastewater within short retention time (1-2 days), e.g. the residual phosphate-P was < 0.22 mg l^{-1} and nitrate-N was < 2 mg l^{-1} within one day in the secondary settled wastewater. Three scenarios were proposed on integrating twin-layer system into wastewater treatment plants. In one of the three, removing phosphorus and ammonium from 100 m^3 primary settled wastewater to meet the discharge requirement i.e. $\leq$ 1 mg l^{-1} P will need a minimal ground area of greenhouse of about 1369 m^2 and 16.6 kWh electricity daily. It is concluded that immobilization of *S. rubescens* on twin-layers is an effective means to reduce nitrogen and phosphorus levels in the wastewater. More efforts should be focus on the selection of microalgae and co-immobilization of microalgae with microalgae/other microorganisms to reduce organic substances.

Key words: ammonium, *Chlamydomonas terricola*, microalga, nitrate, nylon filter, phosphorus, *Scenedesmus rubescens*, twin-layer, municipal wastewater treatment

KURZZUSAMMENFASSUNG

Hohe Stickstoff- und Phosphateinträge aus kommunalen Abwasseraufbereitungsanlagen stellen eine anhaltende Gefährdung der Wasserqualität dar, die in verschiedenen Ländern zu einer Verschärfung der entsprechenden Umweltrichtlinien führt. Die Twin-Layer Technologie zur Kultivierung von Mikroalgen bietet eine interessante Alternative zur Entfernung von Stickstoff und Phosphor, da eine effiziente Abwasseraufreinigung mit der Produktion von wertvoller Biomasse gekoppelt werden kann. Das Twin-Layer System ermöglicht die Immobilisierung von Mikroalgen auf einem feuchten, mikroporösen Ultradünnschichtsubstrat (Substratschicht). Eine zweite, darunter befindliche Schicht aus einem gröber strukturierten Fasermaterial (Versorgungsschicht) dient der Versorgung mit Kulturmedium. Die Twin-Layer Konfiguration trennt die Mikroalgen vom Großteil des Kulturmediums, ohne jedoch die Diffusion der notwendigen Nährstoffe zu verhindern.

Nylonfiltergewebe und verstärktes Glasfasergitter wurden als Substrat- bzw. Versorgungsschicht ausgewählt. Die immobilisierten Grünalgen *S. rubescens* und *Chlamydomonas terricola* zeigten höchste Biomasseproduktivitäten während 23-tägiger Versorgung mit sekundärem kommunalem Abwasser und werden im Allgemeinen zur Abwasserbehandlung empfohlen. Die Entfernung von Stickstoff und Phosphat aus kommunalem Abwasser durch *S. rubescens* wurde über einen Zeitraum von 54 d untersucht. *S. rubescens* war gut immobilisierbar und zeigte eine mittlere Wachstumsrate von 1,05 g m^{-2} d^{-1}. Phosphat, Nitrat und Ammonium konnten innerhalb einer kurzen Retentionszeit (1-2 d) effektiv aus vier verschiedenen Abwassertypen entfernt werden. Z.B verblieben nach eintägiger Behandlung von abgesetztem sekundärem Abwasser < 0.22 mg l^{-1} Phosphat-P und < 2 mg l^{-1} Nitrat-N im Medium. Für die Integration von Twin-Layer Systemen in Abwasseraufbereitungsanlagen werden drei Scenarios vorgeschlagen. Für eine Aufbereitung von 100 m^3 abgesetzten primären Abwassers im Rahmen der Grenzwerte von ≤ 1 mg l^{-1} P wäre eine minimale Gewächshausgrundfläche von 1369 m^2 sowie ein täglicher Energiebedarf von 16,6 kWh erforderlich. Die Immobilisierung von *S. rubescens* ist folglich ein effektives Mittel die N- und P-Gehalte in Abwässern zu reduzieren. Weitere Untersuchungen sollten mit dem Fokus auf die Auswahl der Mikroalgen sowie die Co-Immobilisierung von Mikroalgen mit anderen Mikroorganismen zur Reduktion der organischen Anteile im Abwasser durchgeführt werden.

Key words: Ammonium, *Chlamydomonas terricola*, Glasgitterverstärkte Laminate, Mikroalge, Nitrat, Nylonfilter, Phosphor, *Scenedesmus rubescens*, Twin-Layer, Kommunale Abwasseraufbereitung

1. INTRODUCTION

1.1 Municipal Wastewater Treatment Plants (MWTPs)

Municipal wastewater means the discharge of effluent from wastewater treatment plants which receive wastewater from households, commercial establishments, industries and combined sewer/separate storm overflows (European Environment Agency, http://www.eea.eu.int). Wastewater treatment plants are common worldwide and a necessary step to improve the quality of wastewater before it discharges to surface or groundwater and re-enters water supplies (Carey and Migliaccio, 2009).

The conventional municipal wastewater treatment plants (MWTPs) usually include two main treatment processes: primary treatment and secondary treatment. Figure 1-1 illustrates the main components of a conventional municipal wastewater treatment plant.

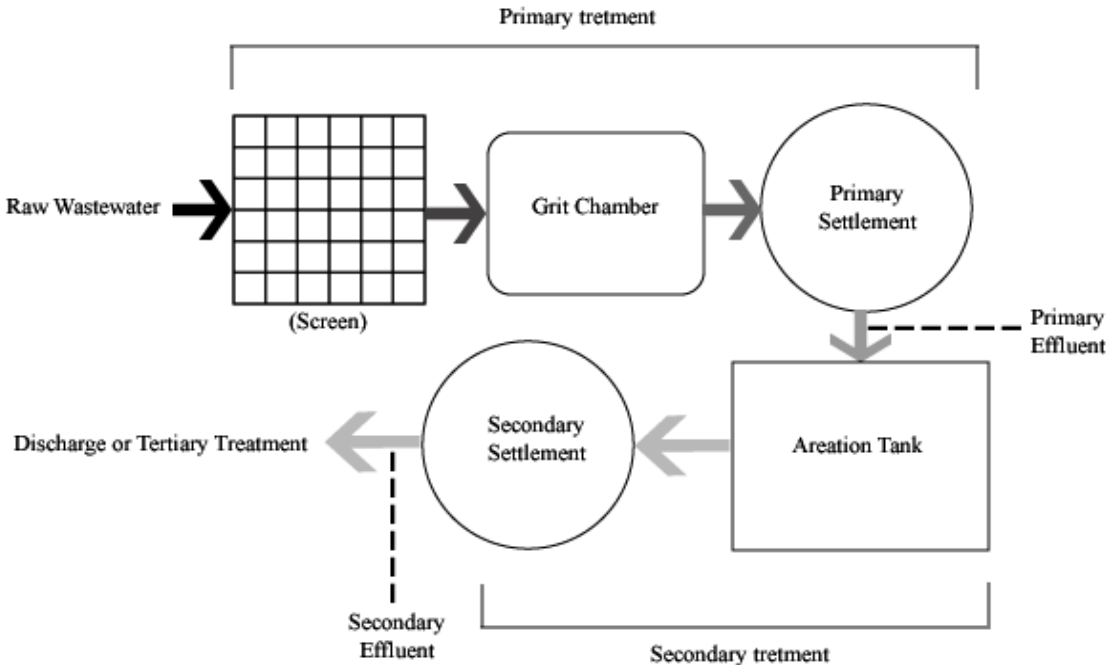

Figure 1-1. Simplified schematic review of a conventional municipal wastewater plant (Shi, 2005)

The primary treatment of municipal wastewater is designed to remove floating solids and suspended solids from raw sewage by physical and/or chemical process. It includes the processes of screening large floating objects by a "screen", removing grit (cinders, sand and small stones) by a "grit chamber" and settling minute suspended solids by a

"sedimentation tank" (primary settlement). The solids settled by the primary treatment are called raw primary biosolids and should be landfilled or incinerated. The water discharged after primary treatment is called primary effluent or primarily treated wastewater (USEPA, 1998).

After the primary treatment, the wastewater still contains high amount of organic matters and should be eliminated by secondary treatment. It is a process generally involving biological treatment, which employs microorganisms and a secondary settlement. After the sewage leaves the primary settlement tank in the primary treatment, it is pumped into an "aeration tank", where it is mixed with air and sludge loaded with bacteria and allowed to remain for several hours. During this time, the bacteria break down the organic matter into harmless degradation-products. From the aeration tank, the partially treated sewage flows to another sedimentation tank (secondary settlement) for removal of excess bacteria (USEPA, 1998).

However, neither the primary treatment nor the secondary treatment has sufficient ability to remove nitrogen and phosphorus from the wastewater. The secondary effluent still contains significant amounts of nitrogen and phosphorus.

1.2 MWTPs & Nitrogen and Phosphorus Pollution

1.2.1 Contribution of MWTPs Effluent to Nitrogen and Phosphorus

Researchers investigating nutrient pollution from nonpoint sources have discovered that nutrient loads were often more strongly influenced by wastewater treatment plants effluent than by nonpoint sources (Carey & Migliaccio, 2009).

European Environment Agency pointed out that most of the dissolved phosphorus loading of inland surface waters is attributable to discharges from point sources, especially municipal wastewater treatment plants and industrial effluent. In Europe, nutrient discharges from municipal wastewater treatment plants are in general higher than from any other point source. Results from large inland and marine catchments show that municipal wastewater constitutes about 75 % of the point source discharges of both nitrogen and phosphorus. Industrial sources constitute about 17 % and other point sources are relatively insignificant (EEA, 2005). In the United States, wastewater

treatment plant effluent is also regarded as the anthropogenically nonpoint derived source of Nr (reactive form of nitrogen, such as ammonium and nitrate) (Driscoll et al., 2003).

1.2.2 Nitrogen and Phosphorus Pollution

1.2.2.1 Freshwater and Marine Eutrophication

Excessive nitrogen and phosphorus loading is a major ongoing threat to water quality and lead to increased rates of eutrophication nowadays. Large discharges of input from wastewater treatment plant may result in the permanent eutrophication of a water system. Eutrophication has been identified as a major environmental issue in both freshwater and marine waters in Europe's environment (EEA, 1999).

Phosphorus has been identified as a key grow-limiting nutrient for algae in lakes and reservoirs (Schindler, 1977). Issues associated with freshwater eutrophication include increased algal biomass, decreased water transparency, low dissolved oxygen (DO) levels, increased fish mortality and more frequent incidence of toxic phytoplankton. Therefore, eutrophication related water quality impairment could have very substantial negative economic effects, for example, higher treatment costs and health hazards due to algal toxins for drinking water (Smith, 2003).

In contrast with freshwater ecosystems, where eutrophication is caused largely by excess inputs of phosphorus, in coastal ecosystems, nitrogen is the most critical element (Ryther and Dunstan, 1971). Coastal eutrophication can cause excessive production of algal biomass, blooms of harmful or toxic algal species (red tides), loss of important estuarine habitat, massive fish and shrimp kills, change in marine biodiversity and species composition, increase in sedimentation of organic particles, and depletion of dissolved oxygen (Driscoll et al., 2003, Smith, 2003).

1.2.2.2 Other Pollutions

Acidification caused by the elevated inputs of reactive nitrogen has also been concerned as the adverse environmental pollution in freshwater ecosystems in the

Northeast of the USA, surface water acidification resulting from HNO_3 has been characterized as a seasonal and episodic phenomenon associated with high stream flows and is typically most severe during spring snowmelt. This causes pronounced decreases in pH, in addition, acidic episodes induced by NO_3^- also mobilize inorganic monomeric Al in the soil, which is toxic to fish (Driscoll et al., 2003).

Another environmental concern over nitrogen in aquatic system is the possible health risks associated with the consumption of drinking water containing high concentrations of nitrate. Nitrate itself does not pose a health threat; however, it is readily reduced to nitrite (NO_2^-) by nitrate reductase. Nitrite poses two distinct health risks, being potentially carcinogenic and causing methaemoglobinaemia (blue-baby syndrome) (McEldowney et al., 1993).

In addition, wastewater discharges to receiving waters characterized by alkaline pH values could exacerbate NH_3-N toxicity and threaten the viability of various fish species (Passell et al., 2007).

1.2.2.3 Phosphorus Crisis

Furthermore, at present, commercial phosphorous production is based almost exclusively on phosphate rock-primarily calcium phosphate in various forms (Morse et al, 1998). The remaining accessible reserves of clean phosphate rock might run out in 50 years, and the world would move from an oil-based to a phosphate-based economy (summarized by Gilbert, 2009). Anthropogenic impacts have intensified release of P such as discharges of urban and industry wastewater (Smil, 2000). Therefore, moving phosphorus towards sustainability is quite urgent. One of possible methods is to recycle the phosphorus from wastewater treatment discharge.

1.2.3 Regulations Concerning Nitrogen and Phosphorus Discharge from MWTP

It is true nowadays to recognize that nitrogen and phosphorus associated problems are one of the major concerns among different environmental issues in the society. Environmental laws are given general applicability and their enforcement has

been increasingly stricter. This tendency will continue because of pressure from the public and various concerned bodies and agencies.

The German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU, 2001) reported that a total of 10.5 billion m^3 of municipal wastewater were treated in the public wastewater treatment plants in 2001, among it, approximately 5% were treated only with mechanical and biological treatment without targeted nutrient removal. Thus, millions of tons of nitrogen and phosphorus have been discharged into the environment. The increasing world population and urbanization will lead to greater water demand of wastewater in the future. Therefore, more effective nitrogen and phosphorus management is important for the sustainability of water resources.

The German BMU (2004) sets up the limitation of nitrogen and phosphorus discharged from domestic wastewater treatment plants, which is 13-18 mg l^{-1} and 1-2 mg 1^{-1} respectively (Table 1-1). While, the European environment agency requires a higher standard (total nitrogen, 15 mg l^{-1}) for wastewater discharged in sensitive areas because such areas need more stringent treatment (EEA, 1991; Table 1-2). In developing countries, for instance in China, regulations (CEPA, 2002; Table 1-3) concerning nitrogen and phosphorus from municipal wastewater treatment plants are also becoming more and more stricter because frequently occurred eutrophication has strongly influenced human health and drinking water supply. For instance, over-exploration and over-utilization for Lake Taihu have caused degradation of the lake ecosystem and deterioration of water quality, hence affected the drinking water supply for several cities, such as Shanghai, Suzhou, Wuxi and Huzhou. From 1981 to 1998, total nitrogen, total phosphorus and COD_{Mn} increased by 2.6, 2.7 and 1.5 times, respectively. Municipal sewage accounts for **25% of the total nitrogen**, **70% of total phosphorus** and 27% of COD pollutant composition based on the data from 1998 (Qin et al., 2007).

Table 1-1. Requirements for wastewater at the point of discharge (BMU, 2004)

	400 up to 4,000 kg d^{-1} BOD_5 (raw)	>4,000 kg d^{-1} BOD_5 (raw)
Total Nitrogen [(1)] mg l^{-1}	18	13
Total phosphorus (P_{tot}) mg l^{-1}	2	1

(1) Total Nitrogen as the sum of ammonia, nitrite and nitrate nitrogen (N_{tot}) mg/l

Table 1-2. Regulation concerning discharge from urban wastewater treatment plants carried out in sensitive areas which are subject to eutrophication (EEA, 1991)

	10,000-100,000 p.e.[(2)] (600-6,000 kg d^{-1} BOD_5)	More than 100,000 p.e. (Greater than 600-6,000 kg d^{-1} BOD_5)
Total Nitrogen [(1)] mg l^{-1}	15	10
Total phosphorus mg l^{-1}	2	1

(1) Total nitrogen means the sum of Kjeldahl-Nitrogen (organic-N+NH_3), nitrate (NO_3)-nitrogen and nitrite (NO_2)-nitrogen

(2) p.e. population equivalent: see glossary

Table 1-3. Requirements for municipal wastewater discharge (GB 18918-2002; CEPA, 2002)

		A	B
Total Nitrogen [(1)] mg l^{-1}		20	-
Total phosphorus (P_{tot}) mg l^{-1}	Build before 31. Dec.2005	1.5	3
	Build after 01.Jan.2006	1	3

A suitable for integrated drinking water supply source, fish protection and swimming area

B suitable for industry water supply area, agricultural water utilization and amenity

Therefore, as part of the "European Water Framework Directive", the effluent demands of, among others, nitrogen and phosphorus will become stricter (EC, 2000). National Dutch legislation, the Vierde Nota Water Huishouding (NWH) introduced in 1998 "maximum tolerable risk concentrations" (MTR). These include requirements for nutrients: P (tot) < 0.15 mg $^{-1}$ and N (tot) < 2.2 mg l^{-1}. Investigation into possible

effluent polishing techniques is required in order to reach these objectives have started recently in the Netherlands (Miska et al., 2006).

1.3 Nitrogen and Phosphorus Removal Technologies

This situation clearly calls for a tertiary treatment step that will remove nitrogen and phosphorus from wastewater. Thus, advanced wastewater treatment (tertiary wastewater treatment) has been considered to be integrated into wastewater treatment facilities and is capable of removing nitrogen and phosphorus from secondary effluents. The methods of tertiary treatment range from physical-chemical separation techniques to biological treatment.

1.3.1 Nitrogen Removal Technology

The biotic nitrogen removal (BNR) is widely used in wastewater treatment plants. It comprises of two steps : (1) nitrification, where NH_4^+-N and organic nitrogen is converted to NO_3^--N by autotrophic bacteria such as *Nitrosomonas* and *Nitrobacter* within the aerobic zone; (2) denitrification, where reduction of NO_3^--N to gaseous nitrogen by heterotrophic bacteria such as *Pseudomonas*, *Alcaligenes*, *Flavobacterium* etc. takes place in an anoxic environment. As the majority of denitrifying bacteria are heterotrophs, oxidizable organic substrates e.g. methanol, glucose, acetic acid and industrial waste such as sulphite waste liquor should be added to support the reaction.

There are different plant configurations using suspended or attached growth processes to achieve sufficient nitrogen removal, such as activated sludge, packed bead reactors and fluidized bed (McEldourney et al., 1993 & Carey and Migliaccio, 2009).

1.3.2 Phosphorus Removal Technology

Development of technologies for phosphorus removal started in the 1950s in response to the issue of eutrophication and the need to reduce the levels of phosphorus entering surface waters. The technologies to remove and recover phosphorus from wastewater include chemical precipitation, which remains the leading technology today, bacteria phosphorus removal, crystallization, novel chemical precipitation approaches

and other wastewater and sludge-based methods like ion exchange, magnetic attraction and adsorbents (Morse et al., 1998).

The chemical precipitation method comprises the addition of iron and/or aluminium salt to wastewater, causing precipitation of an insoluble metal phosphate that is settled out by sedimentation. This method has been firmly established in many countries around the world. However, it requires high cost reagents and produces up to 150% volume more sludge. Moreover, the sludge formed chemical precipitation is neither a good fertilizer nor a suitable raw material (ash from its incineration has too much iron or aluminium) (Smil, 2000).

Bacterial phosphorus removal is achieved by introducing an anaerobic zone, which induces the release of phosphorus from the wastewater. In the following aerobic zone, the luxury uptake and storage of phosphorus occurs. Special bacteria, called polyphosphate accumulating organisms (PAOs), which can enrich and accumulate large quantities of phosphorus within their cells (up to 20% of their mass), mediate this process. This technology requires more complex plant configurations and operating regimes. In practice, the removal is variable; the achievement of a low and consistent effluent standard may require complementary (simultaneous) chemical precipitation (Smil, 2000, Morse et al., 1998).

1.4 Wastewater Treatment with Microalgae

The above-discussed nitrogen and phosphorus removal methods normally consume significant amounts of energy, chemicals and carbon source, and therefore are cost intensive. Furthermore, chemical based treatments often lead to the contamination of the sludge by-product (Tchobanoglous et al., 2003, Hoffmann, 1998). For those reasons, more researches and further methods have been studied toward development and application of a more economical nitrogen and phosphors removal process.

Wastewater treatment with microalgae is a more environmental sound approach to reduce nitrogen and phosphorus from wastewater and has been studied for almost 50 years with one of the first description by Oswald (1957). Microalgae can assimilate a significant amount of nutrients because they require high amounts of nitrogen and phosphorus for proteins (45-60% microalgae dry weight), nucleic acids and

phospholipids synthesis. De la Noüe (1992) summarized the advantages of using microalgae for wastewater treatment in comparison with conventional methods: firstly, nutrients can be removed more efficiently; secondly, secondary pollution in the by – product caused by chemical additives is avoided, so it does not generate additional pollution; thirdly, when the biomass is harvested, it generates recycling of nutrients; and fourthly, the system is less expensive.

1.4.1 Wastewater Treatment with Suspended Microalgae

Most researches have focused on suspended microalgae growing in suspension termed high-rate algal pond. The result of such effort is that some commercial technologies and processes are available in the market such as the Advanced Integrated Wastewater Pond Systems (AIWPS) technology (Figure 1-2) commercialized by Oswald and Green, LLC, in the United States (Olguín, 2003). One of the main limitations of this technology is it is difficult to harvest or separate the suspended microalgal biomass from the treated water discharge. None of the harvesting approaches have proved to be simple, inexpensive and suitable enough for a large-scale outdoor treatment (Hoffmann, 1998).

Figure 1-2. An Advanced Integrated Wastewater Pond Facility at Delhi, California, the USA (http://ponce.sdsu.edu/aiwps.html)

In high-rate algal pond, harvesting or removal of microalgae (e.g. by flocculation) is a major cost factor, especially where a limit on suspended solids is imposed by law

(Mallick, 2002). In addition, the succession of microalgae in these open ponds cannot be controlled well, further complicating the harvesting process.

1.4.2 Wastewater Treatment with Immobilized Microalgae

It is therefore desirable to seek ways to circumvent the removal of microalgae from suspensions. In recent years, there has been an increased interest of using immobilized microalgae to treat wastewater as this technology can avoid excessive harvesting cost. An immobilized cell is defined as a cell that by natural or artificial means is prevented from moving independent of its neighbours to all parts of the aqueous phase of the system under study (Tampion and Tampion, 1988).

Chevalier and de la Noüe (1985) were apparently the first to immobilize microalgae (*Scenedesmus obliquus*) in carrageenan beads for wastewater treatment, and gel entrapment in natural polysaccharide matrixes (carrageenan, agar, alginate) has since then been widely used in experimental studies to remove nitrogen and phosphorus from wastewater. This method is based on the confinement of the cells in a three-dimensional gel lattice. However, one of the drawbacks of this method is that it is difficult to perform the procedure of immobilization and thus could not be scaled up.

Another frequently used immobilization technique is passively immobilizing organisms into a synthetic or natural polymeric matrix, e.g. polyurethane foam (Garbisu et al., 1991), polyvinyl foam (Urrutia et al., 1995) and Loofa sponge (Liu et al., 1998). This technique is based on the fact that many microalgae have a natural tendency to attach to surfaces and grow on them. The immobilization method can be prepared by submerging the foams (or sponge) into the microalgae suspension with aeration for a certain period (Travieso et al., 1996). Generally, this process is easily reversible and contamination of effluents with unstuck cells is unavoidable (Moreno-Garrido, 2008).

1.5 Wastewater Treatment with Microalgae Immobilized on Twin-Layer System

1.5.1 Twin-Layer System

In the present study, a novel cell-immobilization technique, the twin-layer system, is introduced to remove nutrients from wastewater. The twin-layer technology was originally developed for algal biosensors (Podola and Melkonian, 2003), but was later applied to microalgal cultivation in general (Nowack et al., 2005). In the twin-layer system, microalgae are immobilized by self-adhesion on a wet, microporous and ultrathin substrate (the substrate layer). Subtending the substrate layer, a second layer, consisting of a macroporous fibrous tissue (the source layer), provides the growth medium. Twin-layers effectively separate microalgae from the bulk of their growth medium, yet allow diffusion of nutrients. In an essentially open system, neither the microalgae can contaminate the growth medium nor can any contaminating xenobionts reach the microalgal growth layer from the source layer because of the microporous substrate layer.

The immobilization process is relatively simple and cost-efficient compared with gel entrapment methods. Therefore, further development and scale-up of the twin-layer system is necessary for the application of biological and environmental technology. This requirement highlights three research emphases: (1) selection of proper material for the substrate and the source layer; (2) design and construction of twin-layer system; (3) if the specific microalgae could be integrated into the twin-layer system.

1.5.2 Microalgae

The choice of microalgae to be used in wastewater treatment is determined by their robustness against wastewater and by their efficiency to grow in and to take up nutrients from wastewater (Olgu ń, 2003). In well-oxygenated high-rate ponds, climax cultures that are not readily grazed consist mainly of coccoid green algae often displaying long processes such as *Chlorella*, *Scenedesmus* and *Micractinium* (Oswald 1988). These algae are therefore often employed in experimental studies of wastewater treatment using immobilized cells (Chevalier and de la No üe, 1985, Gonz ález et al., 1997, Hernandez et al., 2006, Lau et al., 1997, Mart ínez et al., 2000). In the preliminary

stage of the present research, two microalgae, *Chlorella vulgaris* and *Scenedesmus rubescens* have also been examined to remove nitrogen and phosphorus from synthetic wastewater using a Bench-scale twin-layer system.

Other microalgae have been applied in nitrogen and phosphorus removal studies including *Anabaena, Phormidium, Spirulina, Dunaliella* and *Chlamydomonas* etc. (Mallick 2002). However, no studies have been carried out by far to select suitable strains for municipal wastewater treatment. It is therefore especially interesting to screen microalgae for nitrogen and phosphorus removal. The Culture collection of Algae at the University of Cologne (CCAC) stocks more than 2000 unialga including strains originally isolated from eutrophication ponds and wastewater treatment plants. The microalgal strains from such locations should be investigated.

1.5.3 Previous Study with Twin-Layer System

In 2005, a series of experiments were conducted to investigate the removal of nitrogen and phosphorus from synthetic and municipal wastewater (Grosskläwerk Köln-Stammheim, Stadtentwässerungsbetriebe Köln AöR; Cologne, Germany) by two green microalgae (*Chlorella vulgaris* and *Scenedesmus rubescens*) immobilized on twin-layer system. The experimental results showed that phosphorus, ammonium and nitrate in the synthetic wastewater were assimilated by the algae and incorporated into their biomass. Both algae, after having been starved of phosphorus and nitrogen for 3 days, took up nitrate efficiently from municipal wastewater. Within 4 days of exposure, nitrate was almost completely removed from the wastewater by both algae (Shi, 2005).

1.6 Objective of This Research

Based on the results from the previous research, the twin-layer system seems to have potential for tertiary wastewater treatment with the target of nitrogen and phosphorus removal. Therefore, it is necessary to develop and construct a prototype treatment module and evaluate it in comparison with traditional technologies.

1.6.1 General Objective

The ultimate objective of this research is to develop a twin-layer immobilized microalgae system in bench and large scale for tertiary municipal wastewater treatment with the target of nitrogen and phosphorus removal.

1.6.2 Specific Objectives

1.6.2.1 Synthetic Wastewater Treatment

- To evaluate the growth of *Chlorella vulgaris* and *Scenedesmus rubescens* immobilized on twin-layer system
- To examine phosphate-P, nitrate-N and ammonium-N removal from synthetic wastewater by *Chlorella vulgaris* and *Scenedesmus rubescens* immobilized on twin-layer system

1.6.2.2 Twin Layers Selection

- To investigate different ultrathin layer and fibrous tissue available in the market: e.g. membrane filter, non-woven fabrics and woven fabrics etc.
- To select suitable materials for the substrate layer of twin-layer
- To choose proper materials for the source layer of twin-layer

1.6.2.3 Microalgae Selection

- To select microalgal strains from CCAC for municipal wastewater treatment based on their robustness again wastewater and growth rate
- To isolate microalgae from sewage river for municipal wastewater treatment
- To study the growth of the selected microalgae on twin-layer system

- To conduct experiment in consecutive cycles and examine phosphate-P, nitrate-N and ammonium-N removal from medium by the selected microalgae immobilized on twin-layer

1.6.2.4 Municipal Wastewater Treatment

- To design and construct a large-scale twin-layer system in greenhouse
- To collaborate with Frechen MWTP and understand the treatment process
- To study the growth kinetics of the selected microalgae on twin-layer supplied by municipal wastewater from different treatment processes of Frechen MWTP
- To monitor the system and study the kinetics of phosphate-P, nitrate-N, ammonium-N and Chemical Oxygen Demand (COD) elimination
- To study the light/dark cycle on phosphate-P, nitrate-N and ammonium-N removal

1.6.2.5 Integration of Twin-Layer System into MWTP

- To investigate wastewater nitrogen and phosphorus removal technologies and operational configurations
- To provide different scenarios on integrating twin-layer system into existing technologies

1.6.2.6 Others

- To evaluate if some microalgae can remove organic substances in addition to phosphorus and nitrogen from synthetic secondary wastewater
- To examine if some microalgae could preferentially take up nitrate as the nitrogen source

2. MATERIALS AND METHODS

2.1 Microalgae

There were totally 25 microalgae used in this study. The details of the name of strains, isolation origins, culture collection numbers, original growth culture medium, class and the reason of selections are listed in Table 2-1. Among them, 23 microalgae (No. 1-23, Table 2-1) were used in the experiment of screening suitable microalgae for municipal wastewater treatment. Two others were applied in the study to test if they could preferentially take up nitrate as the nitrogen source (No. 24, 25, Table 2-1).

All the algae are unialga and supplied by CCAC (Culture collection of Algae at the University of Cologne) or from other microalgal collections (Table 2-1). Among them, 6 strains were isolated from sewage fields: *Chlamydomonas terricola*, *Euglena anabaena*, *Euglena deses*, *Pediastrum boryanum*, *Stigeoclonium* sp. and *Trachelomonas* sp..5 strains were obtained from eutrophic ponds: *Chlorella vulgaris*, *Euglena adhaerens*, *Monomorphina pseudonordstedtii*, *Pediastrum duplex* and *Synura uvella*. The remaining 11 strains were chosen because they have been applied in the previous studies of microalgal biotechnology or microalgal wastewater treatment (Vymazal, 1988, Mallick, 2002, Olgu ń, 2003, Metzger and Largeau, 2005, Hoffmann, 1998, Vilchez et al., 1997, Mulbry and Wilkie, 2001, Craggs et al., 1996, Kebede-Westhead et al., 2003, Órpez et al., 2009). These algae are *Botryococcus terribilis*, *Chlorella protothecoides*, *Euglena gracilis*, *Microthamnion kuetzingianum*, *Navicula* sp., *Nitzschia communis*, *Oedogonium stellatum*, *Oscillatoria* sp., *Scenedesmus rubescens* (M2069), *Scenedesmus rubescens* (M2630) and *Synedra* sp.. In addition, one *Scenedesmus* sp. (No.17) was collected from Huayuan Sewage River, Tianjin, P.R China. The sewage collected from the sewage river was enriched in Waris-H medium and incubated in Petri-dish for 7 days at 24 ℃ with illumination about 20-40 μE m^{-2} s^{-1} under a light/ dark cycle of 14/10 h. Afterwards the single cell of microalgae was isolated by micropipette.

Two microalgae described above, *C. vulgaris* and *S. rubescens* (M2630) (Figure 2-1), were used in the bench-scale treatment with continuous mode. Two microalgae described above were used to test if microalgae can improve wastewater treatment by

reducing organic substances in addition to inorganic phosphorus and nitrogen. One is *Euglena gracilis*, and the other is *Chlorella protothecoides.* After detailed selection (to be discussed in 3.2), *S. rubescens* (M2630) was applied throughout this study as a model microalga to test the function of twin-layer, growth patterns and wastewater treatment ability. In addition, two *Haematococcus* strains, *Haematococcus pluvialis* (M 0761/1) and *Haematococcus pluvialis* (M 2072), were used in the study to test if they could preferentially take up nitrate as the nitrogen source. Appropriate growth conditions were used for each strain. Stock microalgae cultures were cultivated as suspension cultures without aeration in Erlenmeyer flasks containing 50 ml different culture medium or grown on agar plate at 14 ℃ with illumination about 20-40 μE m^{-2} s^{-1} under a light/ dark cycle of 14/10 h.

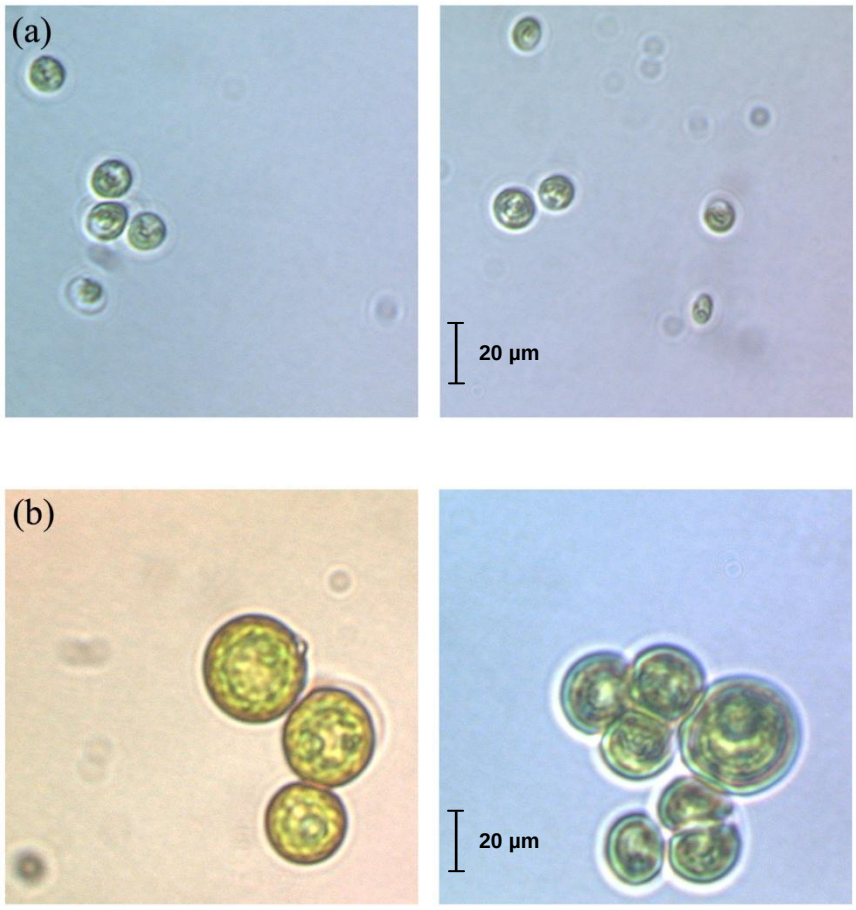

Figure 2-1. Microscopic photos of (a) *C. vulgaris* and (b) *S. rubescens* (M2630)

Table 2-1. Numbers, strain names, original growth culture medium, class and isolation origins of the microalgae used in this study

No.	Strain	Culture medium	Class	Origin and why the strains have been chosen
1	M2311 *Botryococcus terribilis* (ASW 05049)	Waris-H	Chlorophyceae	Literature Austria, Vienna; Danubian backwater (Alte Donau); Freshwater
2	M1259 *Chlamydomonas terricola* (CCAC 0041)	Waris-H	Chlorophyceae	Literature/Sewage field Germany, Münster; squeezed sample from a sewage field; freshwater
3	M0484 *Chlorella vulgaris* (SAG 211-116 x)	Waris-H	Chlorophyceae	Literature/Eutrophic pond The Netherlands, eutrophic pond near Delft
4	M1520 *Chlorella protothecoides* (UTEX 255x)	BBM/agar	Chlorophyceae	Literature The Netherlands, from wound Ulm
5	M2274 *Euglena adhaerens* (ASW 08013)	Soil-water + 1/2 wheat grain	Euglenophyceae	Eutrophic pond Finland, eutrophic pond, freshwater
6	M1250 *Euglena anabaena* (CCAC 0036)	Waris-H:BSM =10:1	Euglenophyceae	Sewage field Germany, Münster; squeezed sample from a sewage field; Freshwater
7	M1251 *Euglena deses*	Waris-H	Euglenophyceae	Sewage field Germany, Münster; squeezed sample from a sewage field; Freshwater
8	M 0902 *Euglena gracilis* (SAG 1224-5/25 x)	Euglena medium	Euglenophyceae	Literature
9	M1683 *Monomorphina pseudonordstedtii* (MK 41)	Waris-H	Euglenophyceae	Eutrophic Germany, Bonn; Rheinauen, south-eastern shores of main stream (shallow, eutrophic; sample from reed sediment)

No.	Strain	Culture medium	Class	Origin and why the strains have been chosen
10	M1948/1 *Microthamnion kuetzingianum* (CCAC 0087)	Waris-H/agar	Trebouxiophyceae	Literature
11	M1772 *Navicula sp.*	Waris-H+Si	Bacillariophyceae	Literature Germany, Cologne, Frülinger See, Lake 4
12	M1762 *Nitzschia communis*	Waris-H+Si	Bacillariophyceae	Literature Germany, Cologne, Botanical garden of the Botany Department
13	M2231 *Oedogonium stellatum*	Waris-H+3V	Chlorophyceae	Literature Germany, Möhrendorf near Erlangen; carp pond; freshwater; Isolated from a soil sample
14	M2010 *Oscillatoria sp.*	BBM+Vitamins	Cyanabacteria	Literature Germany, Cologne; Botany Department, greenhouse floor; freshwater
15	M1252 *Pediastrum boryanum* (CCAC 0037)	BBM+Vitamins /agar	Chlorophyceae	Literature Germany, Münster; squeezed material from a sewage field; freshwater
16	M1253 *Pediastrum duplex*	Waris-H	Chlorophyceae	Literature Germany, Münster; sample drawn from castle moat; freshwater
17	*Scenedesmus sp.*	Waris-H	Chlorophyceae	Self isolation China, Tianjin; Huanyuan sewage river near Huayuan Xiaoqu
18	M2069 *Scenedesmus rubescens*	BBM/agar	Chlorophyceae	Literature Germany, Cologne, Botany Department, greenhouse; 4313 PP-vlies (Sandler company) with Bio Solar culture tubes, continuously moistened; freshwater

No.	Strain	Culture medium	Class	Origin and why the strains have been chosen
19	M2630 *Scenedesmus rubescens*	ASP:BSM=1:1	Chlorophyceae	Literature France, St.Luaire near Dinard (Bretagne) Isolated from a sample of Rivularia bullosa (Cyanobacteria), grow on Lichina confinis (lichen); collected in supralittoral; grow also in freshwater media
20	M1904 *Stigeoclonium sp.* (DO 779)	Waris-H:BSM =100:1	Chlorophyceae	Literature/Sewage field England, Durham; Hollingside Stream, below sewage treatment plant; site 0376-65
21	M1826 *Synedra sp.*	Waris-H+Si	Bacillariophyceae	Literature Germany, Cologne, Frülinger See, lake 4
22	M1159 *Synura uvella* (ASW 02006)	Waris-H	Synurophyceae	Eutrophic pond Finland; eutrophic pond; freshwater
23	M1249 *Trachelomonas sp.*	Waris-H:BSM =10:1	Euglenophyceae	Sewage field Germany, Münster; squeezed material from a sewage field; freshwater
24	M 0761/1 *Haematococcus pluvialis* (CCAC 0051)	Waris-H / agar	Chlorophyceae	Literature Germany, Lohmar near Cologne; reservoir for rain-water in Melkonian's garden in Jexmühle. Freshwater.
25	M 2072 *Haematococcus pluvialis*	Waris-H + 3V ; Waris-H / agar	Chlorophyceae	Literature Germany, Cologne, Botany Department, 1st floor, "Vlies-Sammelkörper", Freshwater.

2.2 Culture Medium and Synthetic Wastewater

Five different culture media/modified culture media and one synthetic wastewater were used in the study. They were: Waris-H medium, modified Waris-H medium, BG-11 medium, modified BG-11 medium and synthetic secondary wastewater according to German standard (DIN 38412-26).

2.2.1 Waris-H Medium

Stock solutions 1-8 and $Na_2SiO_3 \times 9H_2O$ (Table 2-2) were prepared and stocked refrigerately at 4 ℃. Soil extract was prepared by mixing 10g of garden-soil with 120 ml bidistilled water and boiled for 10 minutes. Afterwards it was centrifuged for 10 minutes with low speed, and the supernatant was filtered through a series of membrane filters from 1.2 µm, 0.8 µm, 0.45 µm and 0.2 µm pore size. The remaining filtrate was adjusted to 100 ml with bidistilled water. Aliquots of 10 ml were stored frozen at -28 ℃. The soil should not be recently fertilized and should not contain too much humus.

Waris-H medium was prepared by adding 1ml stock solutions 1-8 and 10 ml thawed soil extract to 1 l demineralized water, and adjusting the pH to 7.0 and autoclave. Waris-H with triplicate vitamins was prepared as Waris-H medium indicated above, however, add 3 ml l^{-1} of vitamins (Table 2-2). Waris-H with silicate is prepared as Waris-H medium indicated above, however, add additional 5ml l^{-1} $Na_2SiO_3 \times 9H_2O$ (Table 2-2).

2.2.2 Modified Waris-H Medium

The composition of the modified Waris-H medium (Table 2-3) was based on the recipe of the Waris-H medium modified to contain about 3 mg l^{-1} phosphate-P as K_2HPO_4, 3 mg l^{-1} nitrate-N as $NaNO_3$ and 20 mg l^{-1} ammonium-N as NH_4Cl. These values were chosen because they were similar to the composition of secondary effluents of some wastewater treatment plants reported before (Nuñez et al., 2001, Martínez et al., 2000, Voltolina et al., 2005). The pH value of the synthetic wastewater was adjusted to 7.0.

Table 2-2. Recipe of Waris-H medium

No.	Stock solution	Concentration in culture medium	Addition per 1 litre stock solution	Addition per litre medium
1	KNO_3	1.00 mM	100 g	1 ml
2	$MgSO_4 \times 7H_2O$	81.1 µM	20 g	1 ml
3	$(NH_4)_2HPO_4$	0.15 mM	20 g	1 ml
4	$Ca(NO_3)_2 \times 4H_2O$	0.42 mM	100 g	1 ml
5	HEPES	1.00 mM	238.31 g	1 ml
6	**P-II-Metals**			1 ml
	EDTA (Titriplex III)	8.06 µM	3.00 g	
	H_3BO_3	18.43 µM	1.14 g	
	$MnCl_2 \times 4H_2O$	0.73 µM	144.00 mg	
	$ZnSO_4 \times 6H_2O$	73.00 nM	21.00 mg	
	$CoCl_2 \times 6H_2O$	16.80 nM	4.00 mg	
7	**Fe-EDTA**			1 ml
	EDTA(Titriplex II)	17.86 µM	5.22 g	
	$FeSO_4 \times 7H_2O$	17.90 µM	4.98 g	
	1 N KOH		54 ml	
8	**Vitamins**			1 ml
	Vitamin B12	0.15 nM	0.20mg	
	Biotin	4.10 nM	1.00 mg	
	Thiamine-HCl	0.30 µM	100.00 mg	
	Niacin amide	0.80 nM	0.1 mg	
9	Soil extract			10 ml
10	$Na_2SiO_3 \times 9H_2O$		28.42 g	5 ml

Table 2-3. Recipe of modified Waris-H medium

No.	Stock solution	Concentration in culture medium	Addition per 1 litre stock solution	Addition per 1 litre medium	Concentration in culture medium
1	**NH_4Cl (N)**	1.4 mM	3.822 g	20 ml	**76.43 mg/l (20 mg/l)**
2	**$K_2HPO_4 \times 3H_2O$ (P)**	0.1 mM	1.103 g	20 ml	**22.06 mg/l (3 mg/l)**
3	**KNO_3 (N)**	0.2 mM	1.082 g	20 ml	**21.64 mg/l (3 mg/l)**
4	$MgSO_4 \times 7H_2O$	81.1 µM	20 g	1 ml	
5	$CaCl_2 \times 2H_2O$	0.45 mM	62.3 g	1 ml	
6	HEPES	0.42 mM	238.31 g	1 ml	
7	**P-II-Metals**	1.00 mM		1 ml	
	EDTA (Titriplex III)	8.06 µM	3.00 g		
	H_3BO_3	18.43 µM	1.14 g		
	$MnCl_2 \times 4H_2O$	0.73 µM	144.00 mg		
	$ZnSO_4 \times 6H_2O$	73.00 nM	21.00 mg		
	$CoCl_2 \times 6H_2O$	16.80 nM	4.00 mg		
8	**Fe-EDTA**			1 ml	
	EDTA(Titriplex II)	17.86 µM	5.22 g		
	$FeSO_4 \times 7H_2O$	17.90 µM	4.98 g		
	1 N KOH		54 ml		
9	**Vitamins**			1 ml	
	Vitamin B12	0.15 nM	0.20mg		
	Biotin	4.10 nM	1.00 mg		
	Thiamine-HCl	0.30 µM	100.00 mg		
	Niacinamide	0.80 nM	0.1 mg		
10	Soil extract			10 ml	
11	$Na_2SiO_3 \times 9H_2O$		28.42 g	5 ml	

2.2.3 BG-11 Medium

The BG-11 medium was developed by Stanier et al.(1971). The components in the medium are (mg l^{-1}): $NaNO_3$, 1500; K_2HPO_4, 40; $MgSO_4 \times 7H_2O$, 75; $CaCl_2 \times 2H_2O$, 36; Citric acid, 6; $Fe_3(NH_4)$-Citrate, 6; Na_2-EDTA (Titriplex III), 1; Na_2CO_3, 20; Thiamine, 0.1; Trace element (μg l^{-1}): H_3BO_3, 2.86; $MnCl_2 \times 4\ H_2O$, 1.81; $ZnSO_4 \times 7H_2O$, 0.22 ; $Na_2MoSO_4 \times 2\ H_2O$, 0.39; $CuSO_4 \times 5\ H_2O$, 79.00; $Co(NO_3)_2$, 49.40. This medium is also buffed by 5 mM HEPES and has a pH about 7.3 to 7.4.

2.2.4 Modified BG-11 Medium

The modification of BG-11 medium is similar to modified Waris-H medium which was adjusted to have 3 mg l^{-1} phosphate-P as K_2HPO_4, 3 mg l^{-1} nitrate-N as $NaNO_3$ and 20 mg l^{-1} ammonium-N as NH_4Cl. Other components in the medium are (mg l^{-1}): $MgSO_4 \times 7H_2O$, 75; $CaCl_2 \times 2H_2O$, 36; Citric acid, 6; $Fe_3(NH_4)$-Citrate, 6; Na_2-EDTA (Titriplex III), 1; Na_2CO_3, 20; Thiamine, 0.1; Trace element (μg l^{-1}): H_3BO_3, 2.86; $MnCl_2 \times 4\ H_2O$, 1.81; $ZnSO_4 \times 7H_2O$, 0.22 ; $Na_2MoSO_4 \times 2\ H_2O$, 0.39; $CuSO_4 \times 5\ H_2O$, 79.00; $Co(NO_3)_2$, 49.40. This medium is also buffed by 5 mM HEPES and has a pH about 7.3 to 7.4.

2.2.5 Synthetic Secondary Wastewater

"German standards methods for the examination of water, waste water and sludge" provide a protocol, that is, DIN 38412-26, to simulate municipal wastewater for biological experiments. It contains peptone, meat extract, urea, NaCl, $CaCl_2 \times 2H_2O$, $MgSO_4 \times 7H_2O$ and K_2HPO_4 (Table 2-4). In the current study, the composition of DIN 38412-26 has been modified (Table 2-5) to have 20% organic matters (peptone, meat extract and urea), and to add 3 mg l^{-1} phosphate-P as K_2HPO_4, 3 mg l^{-1} nitrate-N as $NaNO_3$ and 20 mg l^{-1} ammonium-N as NH_4Cl. The remaining components, NaCl, $CaCl_2 \times 2H_2O$, $MgSO_4 \times 7H_2O$, are the same as described in DIN 38412-26. The prepared medium has been measured to have a COD concentration of about 100 mg O_2 l^{-1}. Stock solutions (Table 2-5) I and V were prepared and stored at -28℃. Stock solutions II, III and IV were stored refrigerately at 4℃. This synthetic wastewater was prepared by adding 4ml l^{-1} stock solution I and 20ml l^{-1} stock solution II-V. The pH value of the synthetic wastewater was adjusted to 7.0. This medium is called synthetic secondary wastewater in this study.

Table 2-4. Composition of the synthetic raw wastewater (DIN 38412-26)

No.	Stock solution	Addition per 1 litre stock solution	Addition per litre wastewater	Final concentration in wastewater
I	Peptone	8 g	20 ml	160 mg l^{-1}
	Meat extract	5.5 g		110 mg l^{-1}
	Urea	1.5 g		30 mg l^{-1}
II	NaCl	350 mg	20 ml	7 mg l^{-1}
	$CaCl_2$. $2H_2O$	200 mg		4 mg l^{-1}
	$MgSO_4.7H_2O$	100 mg		2 mg l^{-1}
III	K_2HPO_4	1.4 g	30 ml	28 mg l^{-1}

Table 2-5. Composition of the synthetic secondary wastewater (modified from DIN 38412-26)

No.	Stock solution	Addition per 1 litre stock solution	Addition per litre wastewater	Final concentration in wastewater
I	Peptone	8 g	4 ml	32 mg l^{-1}
	Meat extract	5.5 g		22 mg l^{-1}
	Urea	1.5 g		6 mg l^{-1}
II	NaCl	350 mg	20 ml	7 mg l^{-1}
	$CaCl_2$. $2H_2O$	200 mg		4 mg l^{-1}
	$MgSO_4.7H_2O$	100 mg		2 mg l^{-1}
III	$NaNO_3$ ($NaNO_3$-N)	0.911 g	20 ml	18.21 mg l^{-1} (3 mg l^{-1})
IV	NH_4Cl (NH_4Cl-N)	3.821 g	20 ml	76.43 mg l^{-1} (20 mg l^{-1})
V	K_2HPO_4 (K_2HPO_4-P)	842 mg	20 ml	16.84 mg l^{-1} (3 mg l^{-1})

2.3 Municipal Wastewater of Frechen MWTP

2.3.1 Erftverband

Frechen MWTP is located in Frechen, Cologne, Germany. It belongs to a non-profit organization named Erftverband, which operates 1,902 km^2 catchment areas of the river Erft and ensures the sustainability development of the area. The catchment contains numerous tributaries and bodies of water along with the 104 km long river (Figure 2-2). Sewage treatment, as the most important water protection measure, therefore, constitutes one of the core activities of the Erftverband, to ensure the existence of an intact and viable environment. The Erftverband operates over 40 high performance sewage plants that purify the municipal sewage produced by approximately 750,000 residents as well as the trade and industry located in the Erft region. This corresponds to a capacity to serve more than one million inhabitants (http://www.erftverband.de/).

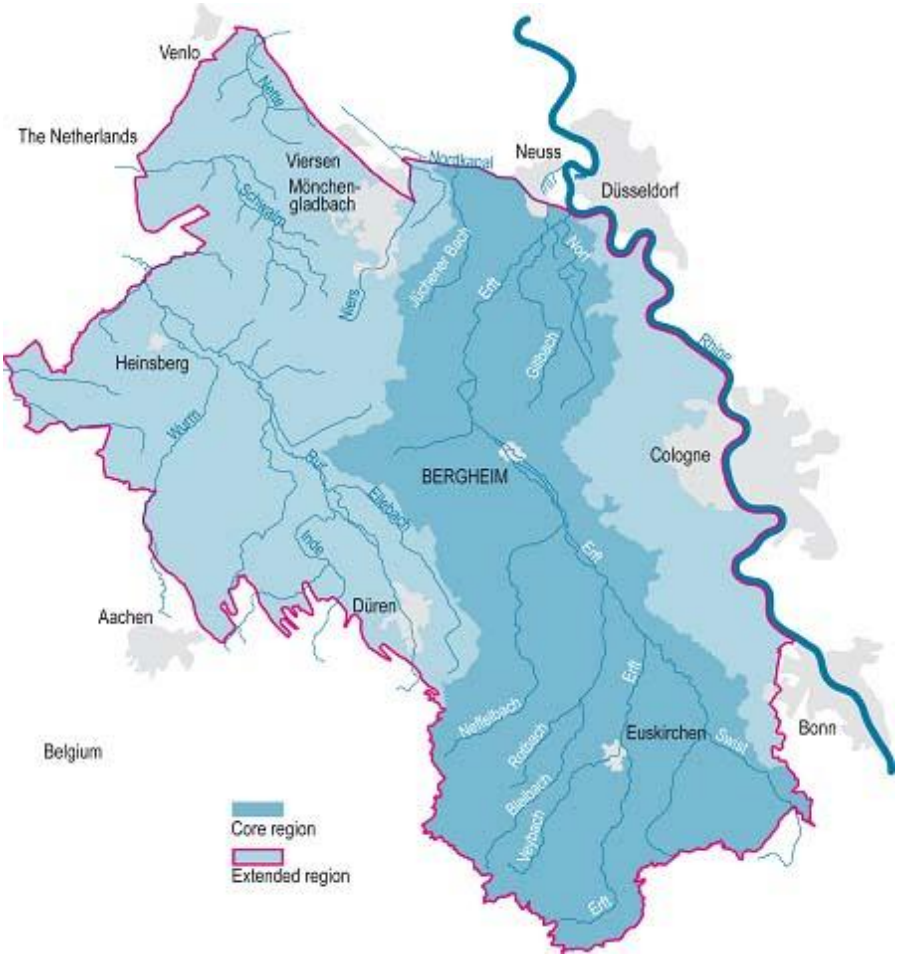

Figure 2-2. Scheme of Erft river catchment

2.3.2 Frechen MWTP (Kläranlage Frechen)

Frechen MWTP is one of biggest plants operated by Erftverband and serves for more than 10,000 inhabitants in the region. It comprises various stages and different techniques. In the primary treatment process, coarse-grained ingredients are mechanically filtered out of the sewage with sieves. Sand and gravel are deposited in a sand trap. Grease and undesirable floating matter are eliminated from the water in a grease trap (Figure 2-3, 1). The wastewater flows to the primary settlement tank (Figure 2-3, 2) while material which easily settles is removed. Next in line is biological and chemical treatment with the target of reducing the organic substances (BOD, COD etc.), nitrogen and phosphorus (Figure 2-3, 3-6). The detail scheme of the biological and chemical treatment is shown in Figure 2-4.

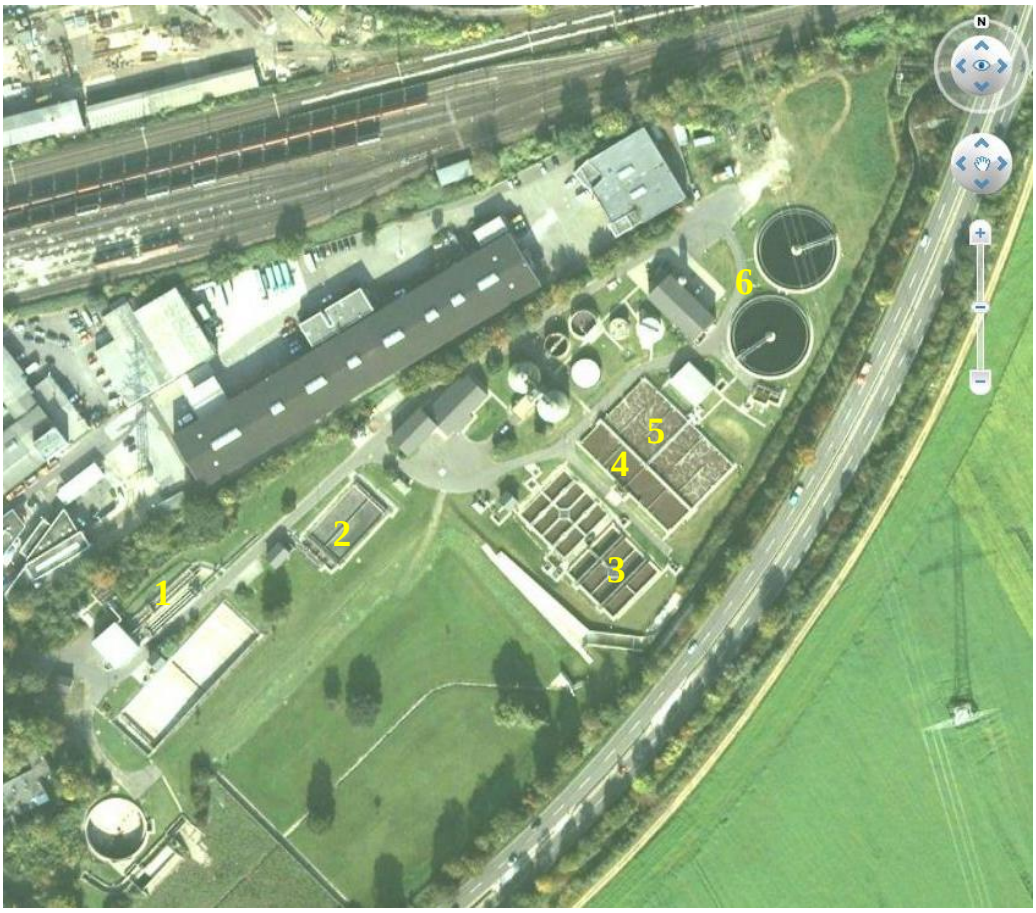

Figure 2-3. Overview of Frechen municipal wastewater treatment plant with (1) grease trap, (2) primary settlement tank, (3) biological phosphorus tank, (4) denitrification tank, (5) nitrification tank and (6) secondary settlement tanks

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The wastewater flows firstly to the **Bio-Phosphorus Tank** (Figure 2-4), where the recycled activated-sludge from the secondary sedimentation tanks flows back to. In this process, bacteria release inorganic phosphorus from stored polyphosphates under anaerobic conditions. Afterwards, the wastewater is pumped to the **Denitrification Tank** where conversion of nitrate (NO_3^-) to nitrogen gas (N_2) happens. This process is mediated by bacteria and has to proceed under anoxic condition. The following step is **Nitrification**, in the presence of oxygen (aerobic condition), bacteria such as *Nitrosomonas* and *Nitrobacter* breaks ammonia (NH_4^+) down to nitrate (NO_3^-). Simultaneously, the organic substance (BOD, COD) is reduced, and phosphorus accumulating bacteria store excess amounts of phosphorus as polyphosphates in their cells. The wastewater then enters into a **Distribution Work** where the wastewater is either recycled to the Denitrification Tank for nitrate reduction or flows to the **Secondary Settlement Tank**. Before the wastewater flows to the Secondary Settlement Tank, aluminium or iron salt is added in order to precipitate phosphate and further improve P removal efficiency. In the secondary Settlement Tank, wastewater and sludge settle for hours and separate. The purified sewage runs through a **Discharge Measurement Well** where the important variables of the wastewater were measured. Afterwards the wastewater is reintroduced into **Frechener River.**

As we have discussed in section 1.3.1, in the **Denitrification** process, the bacteria require carbon food source for energy and conversion of nitrogen. The primary treated wastewater contains sufficient carbonaceous material for the metabolization. Therefore, adding additional carbon source normally has to be performed by “Nitrification → Denitrification” process is not necessary in this plant.

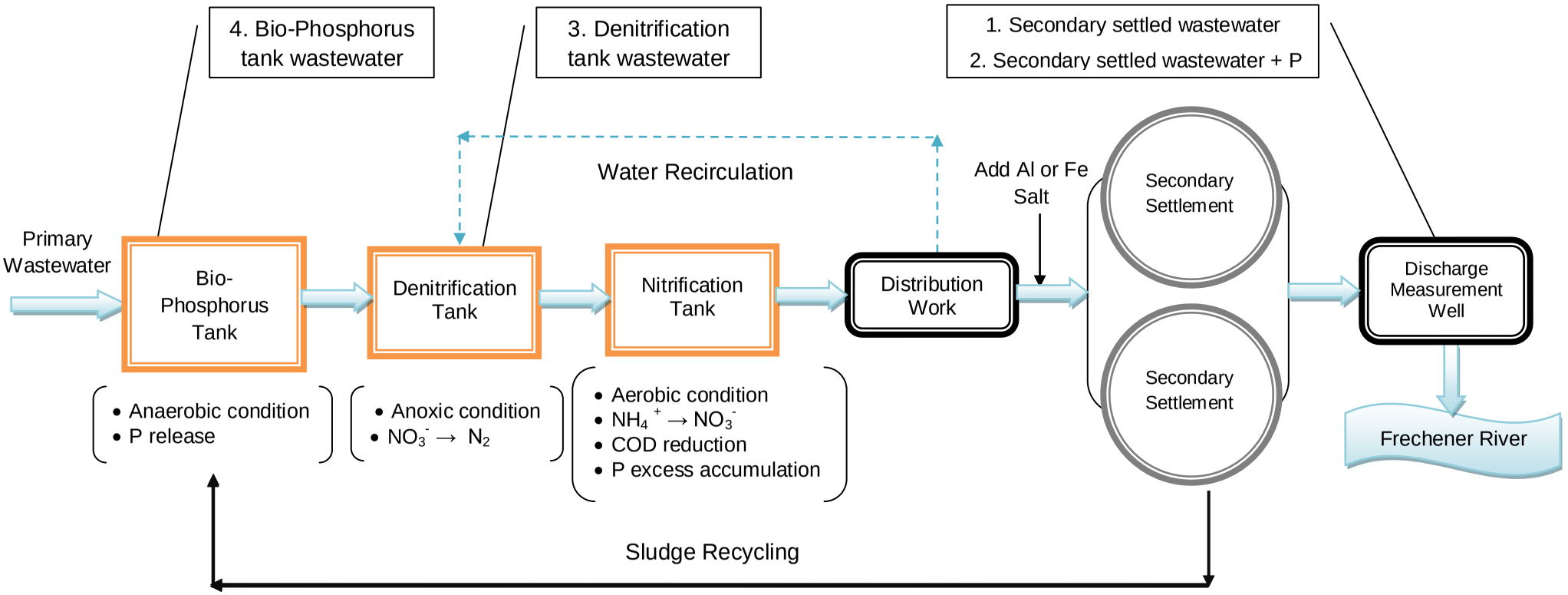


Figure 2-4. Scheme of the biological and chemical treatment processes of Frechen MWTP and the wastewater used in large-scale wastewater treatment: (1) secondary settled wastewater, (2) secondary settled wastewater with additional phosphorus, (3) denitrification tank wastewater and (4) bio-phosphorus tank wastewater

2.3.3 Municipal Wastewater

The wastewater used in this study was obtained from three different positions located during/after chemical and biological treatments from Frechen MWTP. Four different types of wastewater/modified wastewater were applied to the microalgae on twin layers: (1) the wastewater after secondary settlement (Figure 2-4, 1), (2) such wastewater with additional phosphorus (Figure 2-4, 2), (3) the wastewater in the denitrification tank (Figure 2-4, 3), and (4) the wastewater in the Bio-phosphorus tank (Figure 2-4, 4). The wastewater was taken from April 2009 to June 2009, collected in 20 l plastic containers, immediately transported to the laboratory and stored at 4℃ in dark for maximum 3 days before applying to the microalgae. Before each application, the wastewater was kept under room temperature for a while, and the initial conditions were determined with regard to the pH and concentrations of phosphate, ammonium, nitrate and COD. Table 2-6 shows the baseline wastewater composition.

Table 2-6. Physical and chemical variables of the municipal wastewater used in the large-scale wastewater treatment

Physic-chemical variables	Secondary settled wastewater	Secondary settled wastewater with additional P	Denitrification tank wastewater	Bio-P tank wastewater
pH	7.5-8.8	8.2-8-5	7.9-8.3	7.4-7.8
Phosphate-P (mg l^{-1})	0.31 - 0.90	1.65 - 2.36	1.41 - 2.64	2.72 - 4.58
Ammonium-N (mg l^{-1})	0 - 0.31	0 - 0.12	1.22 - 2.97	7.76 - 13.91
Nitrate-N (mg l^{-1})	5.95 - 9.19	4.44 - 7.16	0.31 - 0.97	0.10 - 0.17
COD (mg O_2 l^{-1})	12 - 33	19 - 50	18 - 26	25 - 93

2.3.3.1 Secondary Settled Wastewater

The wastewater after secondary settlement process is generally called secondary settled wastewater. In this study, this type of sewage was collected from the discharge measurement well (Figure 2-5, left) which is located after two consecutive secondary settlement tanks by a pump (Figure 2-5, right). This wastewater is ready to discharge into the

natural body and characterized by slight alkaline (7.5-8.8), low COD (12-33 mg O_2 l^{-1}), low phosphate (0.3-0.9 mg l^{-1}), low ammonium (0-0.3 mg l^{-1}) but relatively high nitrate (5.9-9.2 mg l^{-1}) amount.

Figure 2-5. Discharge measurement well (left) located after secondary settlement tanks and water pump used for collection (right)

2.3.3.2 Secondary Settled Wastewater with Additional Phosphorus

Secondary settled wastewater with additional phosphorus was prepared by adding 1.5 mg l^{-1} phosphorus (KH_2PO_4-P) to the secondary settled wastewater. The physical and chemical parameters of this medium are similar with the secondary settled wastewater except higher phosphorus amount (Table 2-6).

2.3.3.3 Denitrification Tank Wastewater

The sewage in the denitrification tank (Figure 2-6) is a mixture of the effluent from Bio-P tank and the recycling water (2Q) from aerobic active sludge/nitrification tank. It contained significant amount of sludge and was pre-treated through transferring into a round, 40 l, sedimentation tank, allowing sediments to settle for 2 hours. Afterwards, pre-treated water (supernatant water) was transferred to other containers by a peristaltic pump and immediately used for physicochemical character measurement and microalgal treatment experiment. The supernate applied to the microalgae has the initial COD, phosphate, ammonium and nitrate concentration of 18-26 mg O_2 l^{-1}, 1.41-2.64 mg l^{-1}, 1.22-2.97 mg l^{-1} and 0.31-0.97 mg l^{-1}, respectively.

Figure 2-6. Denitrification tank (left) and wastewater sampling (right)

2.3.3.4 Biological Phosphorus Tank Wastewater

Biological phosphorus tank (Figure 2-7) is located after primary treatment. The sewage in this tank contains high levels of COD 25-93 mg O_2 l^{-1}. The application of sludge causes high sedimentation in the sewage. Therefore, this sewage has to be pre-treated and settled for 2 hours as well. Other parameters are slight alkaline (7.5-8.8), high phosphate (2.72-4.58 mg l^{-1}), and high ammonium (7.76-13.91 mg l^{-1}), but low nitrate (0.1-0.17 mg l^{-1}).

Figure 2-7. Bio-phosphorus tank (left) and wastewater sampling (right)

2.4 Microalgal Cultivation in Suspension

2.4.1 Growth in 100 ml Flask

To produce sufficient experimental materials, about 100 µl stock microalgal cultures listed in Table 2-1 were transferred to 50 ml of culture medium in 100 ml Erlenmeyer flasks. The cultures were stored in microalgal culture room at 23±2 ℃ for 14 days using a combination of fluorescent white light ("Universal White 58 W" and "Warm White 36 W"; Osram, Munich, Germany) for illumination at an irradiance of 20-40 µE m^{-2} s^{-1} and a light/ dark period of 14/10 h. Double cultures for strain No 1-23 (Table 2-7) were prepared in order to obtain sufficient amount of cultures for the screening experiment. Most microalgae were grown in Waris-H medium except *Navicula* sp., *N. communis* and *Synedra* sp. in Waris-H + Si medium, and *O. stellatum* and *Oscillatoria* sp. in Waris-H + 3V medium (Table 2-7).

2.4.2 Growth in 500 ml and 10 l Flask, and in 20 l Bag

After the stock microalgal cultures had been inoculated and grown in the 100 ml Erlenmeyer flasks for 14 days, in order to obtain sufficient biomass for the subsequent growth and wastewater treatment experiment, *C. vulgaris*, *S. rubescens* (M2069), *C. protothecoides*, *E. gracilis*, *S. rubescens* (M2630), *H. pluvialis* (M0761/1) and *H. pluvialis* (M2072) were transferred to the 500 ml aerated Erlenmeyer flasks containing 400 ml Waris-H culture medium and grew for 4 weeks before applying or further transferring. The cultivation conditions were same as described in the previous section. *S. rubescens* (M2630) was applied throughout this study as a model microalga to investigate the growth kinetics of microalgae on twin-layer system and nitrogen, phosphorus removal ability. *C. vulgaris* and *S. rubescens* (M2630) were used in the bench-scale treatment to evaluate nitrogen and phosphorus removal efficiency. Therefore, bigger population of them were required. This was achieved by further transferring to 10 l aerated flasks or to 20 l aerated bags (FLEXBOY, Stedim Bag Technology). The same culturing conditions were used as described for 50 ml and 400 ml medium.

When a sufficient cell density was reached, algal biomass was harvested by the rotating centrifuge at 3000 rpm (Sorvall, RC5C; Thermo Electron; Langenselbold, Germany) for 10 minutes or by the continuous centrifuge (Heraeus, 17 RS; Germany) at 4000 rpm.

Table 2-7. Culture medium used to grow microalgae in suspensions

No	Stain	Culture medium
1	*Botryococcus terribilis*	Waris-H
2	*Chlamydomonas terricola*	Waris-H
3	*Chlorella vulgaris*	Waris-H
4	*Chlorella protothecoides*	Waris-H
5	*Euglena adhaerens*	Waris-H
6	*Euglena anabaena*	Waris-H
7	*Euglena deses*	Waris-H
8	*Euglena gracilis*	Waris-H
9	*Monomorphina pseudonordstedtii*	Waris-H
10	*Microthamnion kuetzingianum*	Waris-H
11	*Navicula sp.*	Waris-H+Si
12	*Nitzschia communis*	Waris-H+Si
13	*Oedogonium stellatum*	Waris-H+3V
14	*Oscillatoria sp.*	Waris-H+3V
15	*Pediastrum boryanum*	Waris-H
16	*Pediastrum duplex*	Waris-H
17	*Scenedesmus sp.*	Waris-H
18	*Scenedesmus rubescens* (M2069)	Waris-H
19	*Scenedesmus rubescens* (M2630)	Waris-H
20	*Stigeoclonium sp.*	Waris-H
21	*Synedra sp.*	Waris-H+Si
22	*Synura uvella*	Waris-H
23	*Trachelomonas sp.*	Waris-H
24	*Haematococcus pluvialis* (M 0761/1)	Waris-H
25	*Haematococcus pluvialis* (M2072)	Waris-H

2.5 Microalgal Immobilization

After centrifuging, the immobilization procedure was performed by homogenously distributing a concentrated cell suspension on the substrate layer using a paining brush for bench-scale twin-layer system or a painting roller for large-scale twin-layer system.

2.6 Twin Layers

Twin-layer system consists of two self-adhesion layers, a microporous and ultrathin substrate (the substrate layer) and a macroporous fibrous tissue (the source layer).

2.6.1 Substrate Layer

In this study, the raw materials have been tested including three types of membrane filters (Protran reinforced nitrocellulose membrane, PTFE filter and nylon filter cloth), non-woven polyester filter and printing paper (Table 2-8).

2.6.2 Source Layer

Different macroporous fibrous materials were tested as source layer including ultrathin sheets e.g. glass fibre lamina, non-woven polyester lamina, and mesh grid materials e.g. polyester mesh, PTFE coated glass fabrics mesh, PVC mesh, coated glass fibre mesh, polypropylene mesh, glass grid reinforced laminate (Table 2-9).

2.7 Experimental Set-Up

2.7.1 The Basics of Twin-Layer System

The initiative of using twin-layer system is based on the characteristic that many microalgae have a natural tendency to attach to surfaces and grow on them. Using the twin-layer system microalgal cultures could maintain and grow effectively in an immobilized state. In brief, the twin layer consists of two different porous sheets, the substrate layer and the source layer. The substrate layer is a wet, microporous and ultrathin sheet, on which microalgae are immobilized, while the source layer subtending the substrate layer is a macroporous fibrous tissue, providing growth medium. The system could be horizontally (Nowack et al., 2005) or vertically (Shi et al., 2007) oriented and constitutes an ultrathin photobioreactor utilising artificial illumination or natural sunlight. Nutrients diffuse from the

source layer through the substrate layer to the immobilized algae. The immobilized algae take up light, carbon dioxide and oxygen from the surface of the algal layer exposed to the ambient atmosphere. The functional unit of twin-layer system is shown in Figure 2-8.

Table 2-8. Materials tested as substrate layer

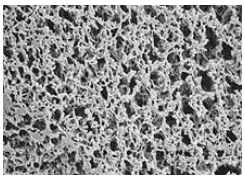
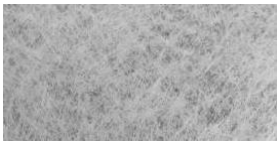
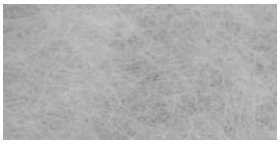
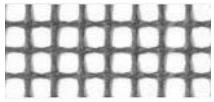
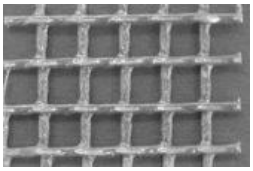
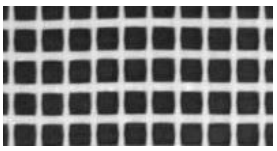
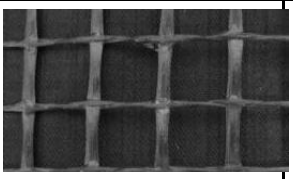
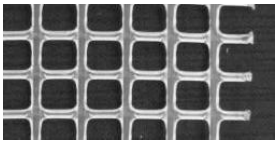
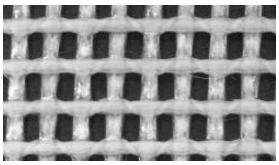
Material	Photo	Pore size (μm)	Supplier
Protran reinforced nitrocellulose membrane (Optitran BA-S 85)		0.45	Schleicher &Schuell Microscience GmbH, Dassel, Germany
PTFE filter (492 P)		---	DeWAL Industries, USA
Non-Woven Polyester filter (2007 K 627061)		---	Freudenberg, Germany
Nylon filter cloth (0.27×37.1)		1.0	Shenghe Chengxin Membrane technology, Beijing, China
Printing Paper (45 g m^{-2})		---	DuMont Schauberg printing plant, Köln, Germany

Table 2-9. Materials tested as source layer

Material	Photo	Pore size (mm ×mm)	Supplier
Glass Fibre Lamina (80 g m^{-2})		---	Isola AS, Eidanger, Norway
Non-Woven Polyester Lamina (AF 955 PE)		---	Freudenberg, Germany
Polyester Mesh (PE4000)		4 ×4	MAPELLI Italy
PTFE Coated Glass Fabrics Mesh (7350)		4 ×4	TACONIC Ireland
PVC Mesh (Type 105)		4 ×4	Novanet Kunststoff GmbH Germany
Coated glass fibre mesh (8560)		4 ×4	Corus Catnic GmbH Germany
Coated glass fibre mesh (110 g m^{-2}, blue)		10 ×10	HUNAN HAOFA Fiberglass Co.LTD, China
Polypropylene mesh (XN 0260)		4 ×6	InterNet USA
Glass grid reinforced laminate (IG VP 271/540)		3 ×4	Isosport GmbH Austria

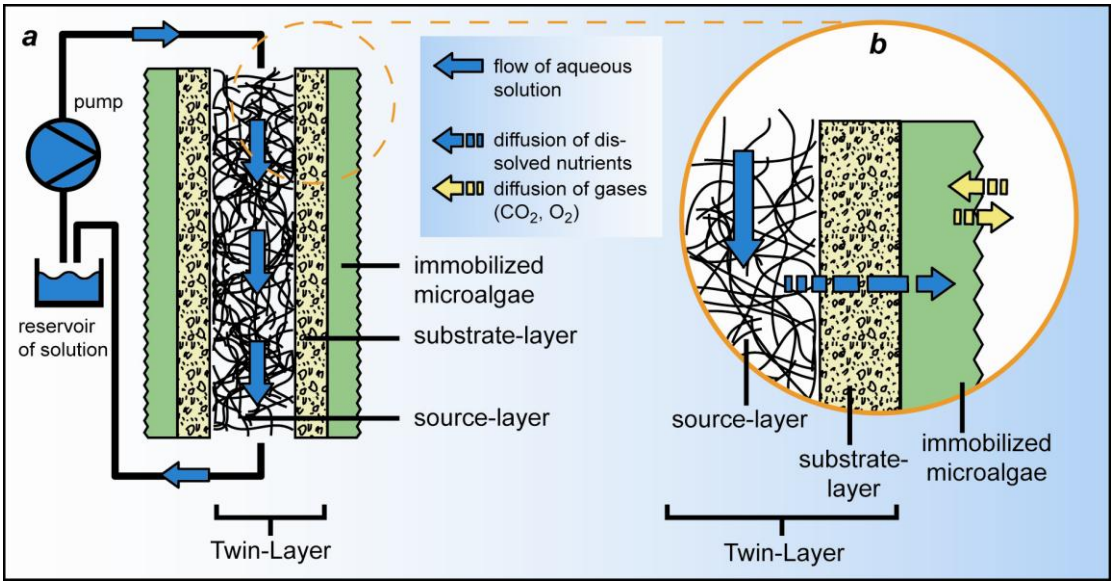


Figure 2-8. Functional unit of twin-layer wastewater treatment system (Shi et al., 2007)

2.7.2 Bench-Scale Twin-Layer System

To integrate the twin layers into a cultivation/wastewater treatment system, twin layers are mounted on a board made of polyvinylchloride (PVC) and are placed vertically inside a transparent PMMA tube (Ø 120 mm; Gehr, Mannheim, Germany). The board holds the twin-layers and serves as the lid of the tube as well. By a peristaltic pump, culture medium/wastewater is applied to the top of the source layer at the PVC board with a flow speed of 3.1-3.4 l m^{-2} h^{-1}. A continuous and gravity driven flow through the source layer is established. After passing the twin layers, the liquid is collected in a darkened glass bottle. Nutrients diffuse from the source layer through the substrate layer to the immobilized algae. The immobilized algae take up light, carbon dioxide and oxygen from the surface of the algal layer exposed to the ambient atmosphere. The basic configuration of the vertically oriented twin-layer system is illustrated in Figure 2-9.

In this study, two types of bench-scale twin-layer systems were applied. The basic configurations of both systems are same except the length of the PMMA tubes. One type is 1 meter, and another type is 0.5 meter. This leaded to the differences of the area of twin layers, the microalgae applied on the substrate layer and the required culture medium/wastewater volume (Table 2-10). Figure 2-10 and Figure 2-11 show photographs of the 1 meter and the 0.5 meter vertically oriented bench-scale twin-layer systems.

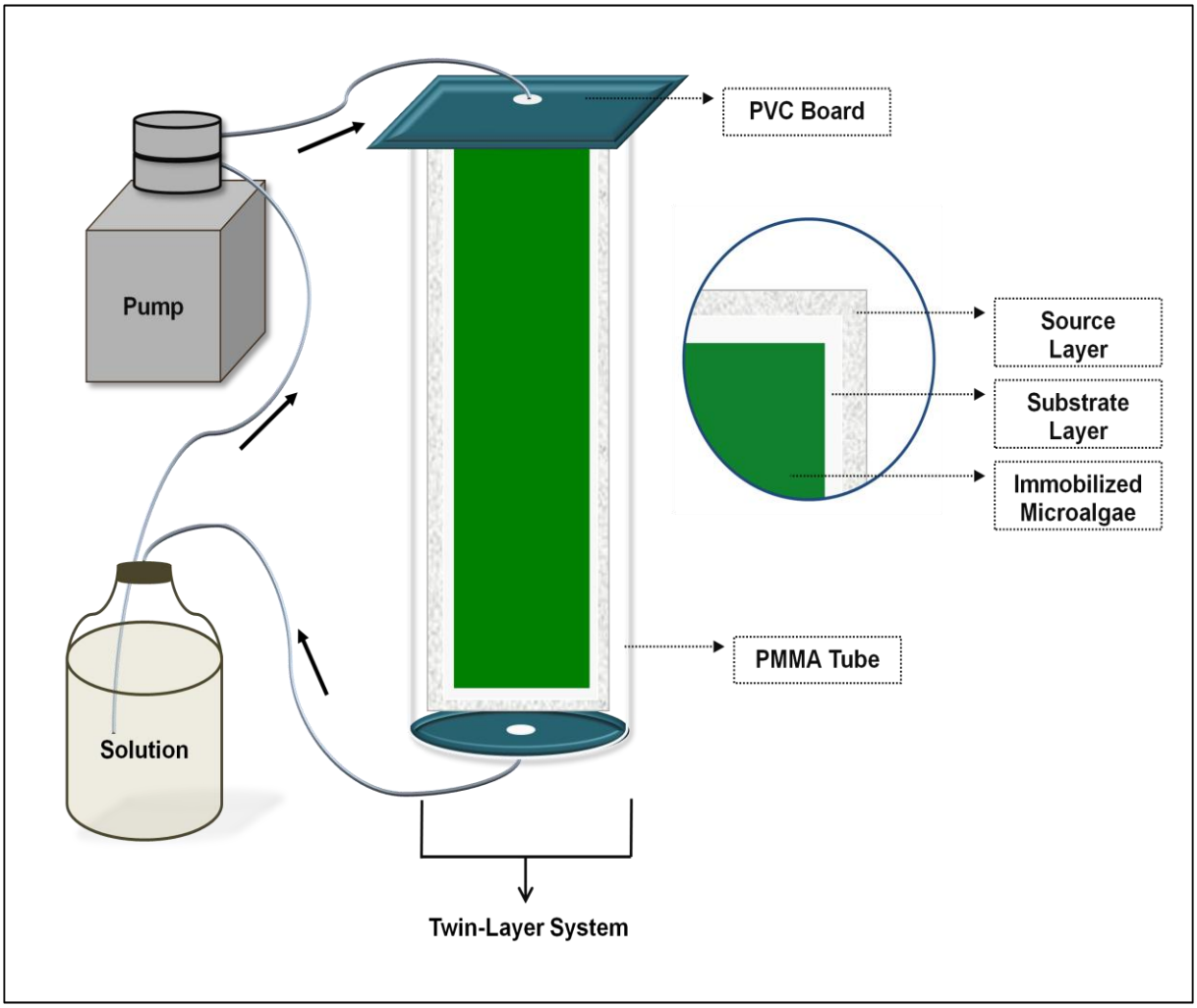


Figure 2-9. Configuration of vertical oriented bench-scale twin-layer system

Table 2-10. Application parameters of two types bench-scale twin-layer systems

PMMA tube length (m)	Substrate layer (cm×cm)	Source layer (cm×cm)	Microalgae (cm×cm)	Liquid volume (l)	Flow speed (l m^{-2} h^{-1})
1	10×95	10×84	9×88	2	3.1-3.4
0.5	10×47.5	10×45.5	9×44.5	1	3.1-3.4

Figure 2-10. Bench-scale twin-layer system with 1 meter PMMA tube, from left to right, control (tube 1, 2), *Chlorella vulgaris* (tube 3-5) and *Scenedesmus rubescens* (tube 6-8)

Figure 2-11. Bench-scale twin-layer system with 0.5 meter PMMA tube, from left to right *Euglena gracilis* (tube 1-3) and *Chlorella protothecoides* (tube 4-6)

2.7.3 Large-Scale Twin-Layer System

A schematic representation of the large-scale twin-layer system with its accessories is presented in Figure 2-12. The large-scale twin-layer system includes three modules of twin-layer mounted vertically on a metal rack (Figure 2-12, a). Culture medium or wastewater is pumped from a container to the filtration device (Cartridge filter nominal, pore size 10 µm, Millipore, USA) and afterwards to the influent pipeline. The water medium distributes to the source layers of three twin-layer modules. Nutrients diffuse from the source layer through the substrate layer to the immobilized algae. The effluent is collected at the bottom of the substrate layer and flows back to the container (Figure 2-12, d). The system operates with a continuous flow rate of 3.8 l m^{-2} h^{-1}. The top of the twin-layer is designed to use ten even-distributed irrigation drippings embedded in the influent pipeline to supply a homogenous water flow on the water channel located beneath (Figure 2-12, b). Once the culture medium or wastewater in the channel was full, it overflows through the weirs and supplies to the source layer. As the substrate layer, a nylon filter (Shenghe Chengxin Membrane Technology; Beijing, China), on which the microalgae were immobilized, is used. Microalgae are supplied with water medium by the source layer, which consists of a reinforced glass fibre mesh (Isosport, Austria) (Figure 2-12, c). Table 2-11 summarizes some operational parameters of the large-scale wastewater treatment experiment.

Table 2-11. Application parameters of large-scale twin-layer system

Substrate layer (cm×cm)	Source layer (cm×cm)	Microalgae (cm×cm)	Liquid volume (l)	Flow speed (l m^{-2} h^{-1})
100×100	100×100	90×95	55	3.8

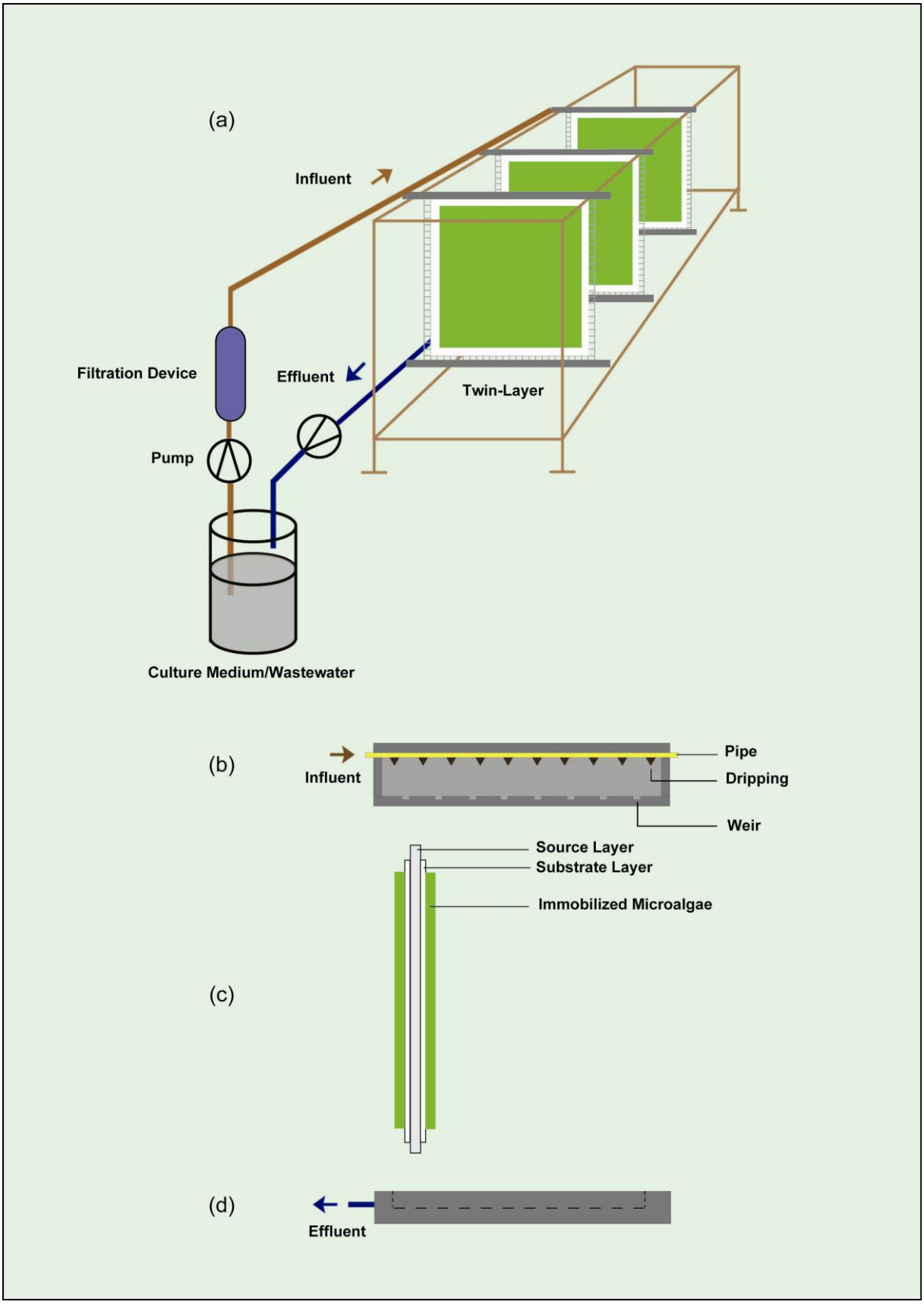


Figure 2-12. Schematic representation of the large-scale twin-layer system with its accessories, (a) the whole system (b) influent supply (c) twin layers and microalgae and (d) effluent collection

2.8 Experimental Design

2.8.1 Selection of Twin Layers

The general selection criteria for both substrate and source layers were hydrophilicity, easiness of immobilization, chemical tolerance, endurance, price and if the material cause any contamination or secondary pollution. The selection criteria of the substrate layer also included if microalgae could penetrate through it. In this study, Protran reinforced nitrocellulose membrane, PTFE filter, nylon filter cloth, non-woven polyester filter and paper were selected as candidates for the substrate layer. Two microalgae, *C. vulgaris* and *S. rubescens* (M2630) were inoculated on those materials by filtration in order to find out whether the microalgae could penetrate through the materials. The selection criteria of the source layer also included the water distribution on the subtended substrate layer. The materials tested as source layer included glass fibre lamina, non-woven polyester lamina, polyester mesh, PTFE coated glass fabrics mesh, PVC mesh, coated glass fibre mesh, polypropylene mesh and glass grid reinforced laminate. These materials combined with printing paper (as substrate layer) were installed on the bench-scale twin-layer system. Blue ink was applied to the system to check the water distribution.

2.8.2 Microalgal Growth on Twin-Layer System

The growth experiments were performed in the bench-scale twin-layer system with 0.5 m PMMA tube (Figure 2-11). Each experiment was performed in triplicate. The systems were stored in microalgal culture room at 14±1 ℃ for about 21-25 days under illumination at irradiance of 30 μE m^{-2} s^{-1} and a light/ dark period of 14/10 h. *S. rubescens* (M2630) was cultivated in suspension in 500 ml Erlenmeyer flask, centrifuged and immobilized. The substrate and source layers applied are: (1) non-woven polyester filter and non-woven polyester lamina, (2) nylon filter cloth and coated glass fibre mesh (10×10mm) and (3) nylon filter cloth and coated glass fibre mesh (4×4mm). 1 litre BG-11 culture medium was continuously supplied to the algae. Every 7 days the culture medium was replaced by fresh medium. Microalgae increments were determined by scratching five 1cm^2 algal samples from the substrate layer and analyzing for Chlorophyll *a*.

2.8.3 Screening Microalgae for Wastewater Treatment

2.8.3.1 Microalgal Growth in Suspension

The first selection was based on the growth of 23 microalgae (No 1-23, Table 2-1) in 50 ml culture media as suspension. The growth conditions and culture media used in the experiment have been discussed in section 2.4.1.

2.8.3.2 Screening with Continuous Mode

After the first step, 20 microalgae were chosen for further investigation. Double cultures were prepared and cultivated as described in section 2.4.1. The microalgae were immobilized by homogenously filtrating suspension cultures on nylon filters with an inoculation diameter of 12 mm, corresponding to the inoculums density of 0.75 µg chlorophyll *a* cm^{-2}. The filtration process is illustrated in Figure 2-13 (a). The nylon filters with immobilized microalgae were afterwards transferred into Petri-dishes with triple-layer glass fibres soaked with 5 ml water medium. The water media used were Waris-H medium and synthetic secondary wastewater. All treatments were conducted by quadruple. The screening experiment was carried out in a microalgal culture room at 23±2 ℃ for 20 days using a combination of fluorescent white light ("Universal White 58 W" and "Warm White 36 W"; Osram, Munich, Germany) for illumination at an irradiance of 20-40 µE m^{-2} s^{-1} and a light/ dark period of 14/10 h. During the application, the effective quantum yield of photosynthetic energy conversion, Yield=ΔF/Fm', the so-called Genty-parameter were measured at day 0,1,2,3,4,5,9,10,13,15,16,17,18 and 20 by a MINI-PAM photosynthesis yield analyzer (WALZ; Effeltrich, Germany). After 20 days, the microalgae were measured for Chlorophyll *a* content.

2.8.3.3 Screening with Semi-Continuous Mode

In the previous step, fluorescence yield measurement gave a general image of the adaptation of 20 microalgae in the synthetic wastewater and Waris-H medium. Therefore, 13 microalgae with better performance were chosen for detailed further investigation. The differences between the semi-continuous mode and continuous mode is that in the semi-continuous mode, every three days the nylon filters were transferred into Petri-dishes with

fresh medium soaked glass fibres in order to avoid nutrients limitation. Double cultures were prepared and cultivated as described before (2.4.1). The microalgae were immobilized by homogenously filtrating suspension cultures on nylon filters with an inoculation diameter of both 12 mm and 40 mm, corresponding to the inoculums density of 0.87 µg chlorophyll *a* cm^{-2} and 0.5 g dry weight m^{-2}, respectively. The filtration processes are illustrated in Figure 2-13, (a) & (b). The former cultures were used for chlorophyll *a* measurement, and the latter ones were examined for dry weight increment. The nylon filters with immobilized microalgae were afterwards transferred into Petri-dishes with triple-layer glass fibre soaked with 5 ml water medium. Modified Waris-H, synthetic secondary wastewater and BG-11 were used for Chlorophyll *a* comparison. Synthetic secondary wastewater was applied for dry weight increment. All treatments were cultured by quadruple or triplicate (Table 2-12).

Batch experiments were carried out in a microalgal culture room at 23±2 ℃ for 20 days under the same conditions as described in the last section. At the end of the experiment, biomass increments of the microalgae on nylon filters, with respect to chlorophyll *a* (d=12 mm) or dry weight (d=40 mm), were determined.

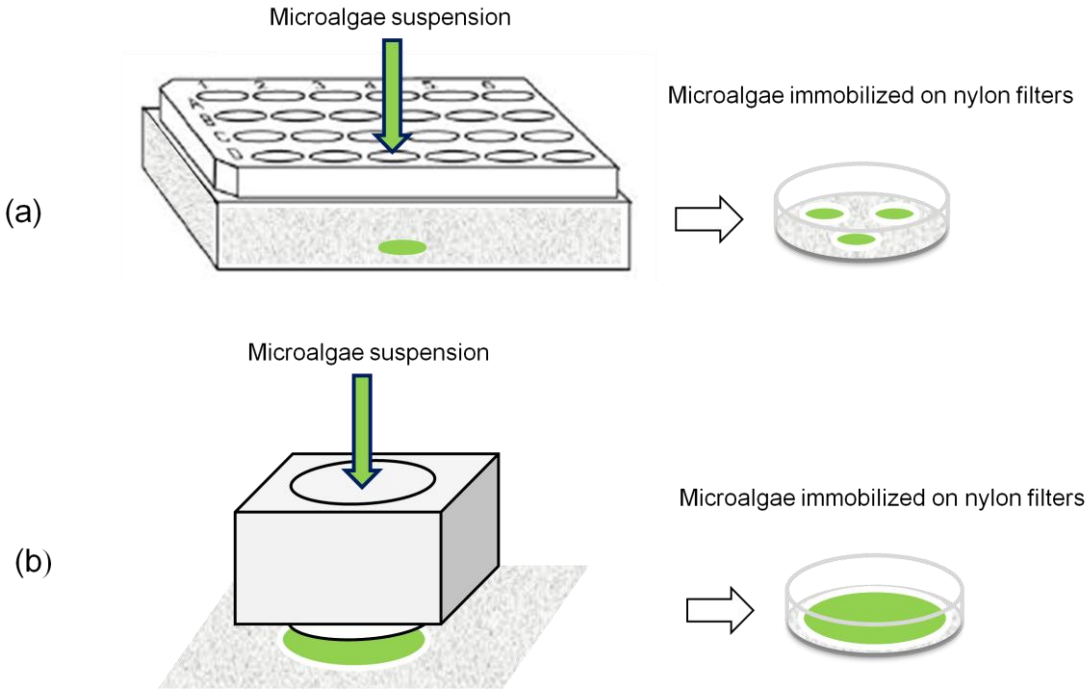


Figure 2-13. Microalgal filtration processes: (a) inoculums d=12 mm for continuous and semi-continuous mode screening and (b) inoculums d=40 mm for semi-continuous mode screening

Table 2-12. Number of replicates for microalgal screening

	Modified Waris-H medium	Synthetic wastewater	BG-11
d=12 mm (Chl *a*)	4	4	4
d=40 mm (DW)	---	3	---

2.8.4 Bench-Scale Treatment: Continuous Mode

The bench-scale treatment experiment was carried out in April under ambient illumination of about 20-120 µE m^{-2} s^{-1} in a greenhouse (N 50°55' and W 6°55') in the Botanical Garden, University of Cologne, Cologne, Germany (Figure 2-14, 1). An evaporation-based cooling system (CoolingPad; Büttner Klimatechnik, Unterschwaningen, Germany) was operated to prevent temperature inside the greenhouse to rise above 30 ℃.

Figure 2-14. Greenhouses in the botanical garden for (1) bench-scale treatment with continuous mode and (2) large-scale wastewater treatment

S. rubescens (M2630) and *C. vulgaris* were applied for the uptake of nutrients. The starting density of the immobilized algae were 1.3±0.1 and 3.5±0.4 µg Chlorophyll *a* cm^{-2} (n=3 ± SD), respectively. 2 litre modified BG-11 medium was supplied to the microalgae to examine nutrients uptake by the two algae.

Bench-scale twin-layer system was used for the treatment. The system consisted of eight independent tubes mounted in a rack (Figure 2-9 & Figure 2-10). Both algae were only immobilized on one side of the substrate layer facing north (20 ° to the west). After cell immobilization, the tubes were exposed to modified BG-11 for 9 days with *C. vulgaris* and *S. rubescens*, respectively. The modified BG-11 had the initial phosphate-P, nitrate-N and ammonium-N concentration of about 3, 3 and 20 mg l^{-1}, respectively. The modified BG-11 medium was buffered by 5mM HEPES to avoid great pH fluctuation which might cause damage to the microalgae. As substrate layer, a Protran reinforced nitrocellulose membrane (Optitran BA-S 85; Schleicher & Schuell Microscience GmbH, Dassel, Germany), on which the microalgae were immobilized, was used. Microalgae were supplied with culture medium by the source layer, which consisted of a glass fibre fleece (80 g m^{-2}; Isola AS, Eidanger, Norway). Two tubes were prepared as controls using only nitrocellulose membranes and glass fibres and without microalgae.

The modified BG-11 medium in the collection bottles was mixed carefully. 40 ml medium was sampled daily for further phosphate-P, nitrate-N and ammonium-N analysis. At the end of the experiment, the microalgae were harvested by carefully scratching from the substrate layer. Dry weight, chlorophyll *a* and total internal phosphorus of the microalgae were determined.

2.8.5 Bench-Scale Treatment: Semi-continuous Mode

This experiment was conducted using bench-scale twin-layer system with 0.5m PMMA tube (Figure 2-9 & Figure 2-11) in a microalgal culture room at 14±1 ℃ under illumination at irradiance of 30 µE m^{-2} s^{-1} and a light/ dark period of 14/10 h. *S. rubescens* (M2630) was previously cultivated in suspension in 10 l flask, centrifuged and subsequently immobilized on the nylon filter. The starting density of *S. rubescens* was 1.7±0.2 µg Chlorophyll *a* cm^{-2} (n=3 ± SD). Coated glass fibre mesh (10mm×10mm) served as the source layer. 1 litre modified BG-11 medium was supplied to the microalgae, which had the initial

phosphate-P, nitrate-N and ammonium-N concentration of about 3, 3 and 20 mg l^{-1}, respectively. Three tubes with *S. rubescens* were used, and two tubes were prepared as controls using only nylon filter and coated glass fibre mesh. The treatment was operated as three cyclic treatments, and each cycle lasted 4 days. At the end of each treatment cycle, the medium was replaced by fresh one.

The modified BG-11 medium in the collection bottles was mixed carefully. 20 ml medium was sampled daily for further phosphate-P, nitrate-N and ammonium-N analysis. At the end of the experiment, the microalgae were harvested by carefully scratching from the substrate layer for chlorophyll *a* determination.

2.8.6 Large-Scale Wastewater Treatment

Large-scale twin-layer system (Figure 2-12) was employed in this study. The wastewater treatments were carried out from April to July in the same botanical garden as described above, but in a different greenhouse (Figure 2-14, 2), which offered automatic ventilation and heating system to ensure the temperature would not be too high or too low. The recorded ambient illumination inside the greenhouse in the light period during the experiment was 22-220 µE m^{-2} s^{-1}.

S. rubescens (M2630) was preselected from the screening experiments and cultivated in two 20 l bags (2.4.2). The starting density of the immobilized algae was about 2 g dry weight m^{-2} or 1.7 µg Chlorophyll *a* cm^{-2}. After cell immobilization, the microalgae were adapted and grown on the twin-layer system for 22 days with Waris-H culture medium. Afterwards the system was washed thoroughly with demineralised water, decanted and immediately supplied for municipal wastewater treatment.

The municipal wastewater was collected from Frechen MWTP. The details of the plant and treatment processes have been discussed in section 2.3.2. Four different types of wastewater were supplied to the algae continuously: (1) the secondary settled wastewater, (2) such wastewater with 1.5 mg l^{-1} additional phosphorus, (3) the wastewater of denitrification tank, and (4) the wastewater of Bio-phosphorus tank. The system was operated by continuously recycling 55 litre wastewater with a flow rate of 3.8 litre h^{-1} m^{-2}. Each type of wastewater was supplied to the system for eight consecutive days. The first and second wastewater was changed daily (one day/cycle), and the third and fourth wastewater was replaced every two

days (two days/cycle). At the end of each treatment cycle, the wastewater was completely decanted and 55 litre fresh wastewater was renewed to start a new treatment cycle.

This large-scale system included three modules of twin-layer mounted in a rack. Nylon filter and reinforced glass fibre mesh were used as the substrate layer and the source layer. In this experiment, the substrate layers, on which the microalgae were immobilized, were applied on both sides of the source layers. The control was designed using the same system but without microalgae supplied by Bio-P wastewater for two days.

The municipal wastewater in the container was mixed carefully before sampling. About 80 ml wastewater were sampled daily throughout the experiments for further COD, PO_4^{3-}-P, NH_4^+-N and NO_3^--N determination. At day 12 and 22 during the period of adaptation with Waris-H medium and at the end of the supply of each type of municipal wastewater (day 30, 38. 46, 54), five 5×5 cm^2 microalgae on each side of the substrate layer were sampled randomly (selected by Excel RANDBETWEEN). Dry weight and chlorophyll *a* of *S. rubescens* were determined.

2.8.7 Reduction of Organic Substances in Addition to Nitrogen and Phosphorus

The bench-scale twin-layer system with 0.5 m PMMA tube (Figure 2-11) was employed in this experiment. *C. protothecoides* and *E. gracilis* were firstly grown in suspension in 500 ml Erlenmeyer flasks, centrifuged and immobilized on nylon filters. Coated glass fibre mesh (10mm×10mm) was served as the source layer. Three tubes were applied for immobilized *C. protothecoides* and *E. gracilis*, respectively. Both algae were preadapted on twin-layer system with 1 litre Waris-H medium for 7 days and afterwards exposed to 1 litre synthetic secondary wastewater for 5 days. The experiment was carried out in the 24℃ culture room with illumination of about 20-40 μE m^{-2} s^{-1} under a light/ dark cycle of 14/10 h. Two tubes were prepared as controls using only nylon filter and coated glass fibre mesh but without algae.

Synthetic wastewater in the bottle was mixed carefully. About 40 ml wastewater were sampled daily throughout the treatment period for further COD, PO_4^{3-}-P, NH_4^+-N and NO_3^--N determination. Finally, the microalgae were harvested by carefully scratching from the substrate layer for chlorophyll *a* and dry weight determination.

2.8.8 Preferential Uptake of Nitrate as Nitrogen Source

Two *Haematococcus*, *H. pluvialis* (M 0761/1) and *H. pluvialis* (M 2072), were used in this experiment to investigate if they could preferentially take up nitrate as nitrogen source. *S. rubescens* (M 2630) was used as a control organism because the previous result indicated a pronounced lag phase of nitrate removal by this alga. Three algae were washed carefully with distilled water three times, and afterwards about 8.9×10^6 cell l^{-1} cells (measured by haemocytometer) were inoculated in 300 ml modified Waris-H medium in 500 ml aerated Erlenmeyer flask. The experiment was carried out in 24℃ culture room with illumination about 20-40 μE m^{-2} s^{-1} under a light/ dark cycle of 14/10 h. Three algae were cultivated in triplicates. 5 ml water samples were taken at day 0, 4 and 8, and immediately determined for nitrate.

2.9 Sampling, Analytical Methods and Statistical Analysis

2.9.1 Water Sampling

The modified culture medium/municipal wastewater in the collection bottles/container was always mixed carefully and sampled throughout the experiments. Depending on the amount needed for subsequent analyses, different amount of culture medium or municipal wastewater was collected (refer to each experimental design). All samples were stored in sterile polypropylene (PP) tubes at -28℃ for a maximum of 28 days before determination.

2.9.2 Hach Lange Cuvette Tests

Four laboratory analysis cuvette tests supplied by Hach Lange (HACH LANGE GmbH; Düsseldorf, Germany) were used for preliminary phosphate, ammonium and nitrate determination, and for COD analysis. A spectrophotometer (UV-2450, UV-VIS Spectrophotometer, SHIMADZU; Kyoto, Japan) was used for extinction determination.

2.9.2.1 Phosphorus LCK 349

Principle: Phosphate ions react with molybdate and antimony ions in an acidic solution to form an antimonyl phosphomolybdate complex, which is reduced by ascorbic acid to phosphomolybdenum blue. The measurement was taken under a wavelength of 880 nm.

Table 2-13. Preparation of phosphorus calibration curve

PO_4^{3-}-P (mg l^{-1})	0	0.3	0.5	1.0	1.2	1.5
Extinction	0	0.188	0.379	0.592	0.721	0.853

Formula 2-1

$y=0.593x$

Where,

y =Extinction

x=PO_4^{3-}-P concentration (mg l^{-1})

2.9.2.2 Ammonium LCK 305

Principle: Ammonium ions react at pH 12.6 with hypochlorite ions and salicylate ions in the presence of sodium nitroprusside as a catalyst to form indophenol blue. The final absorbance is reached after a reaction time of 15 min and then remains constant for a further 15 min. The measurement was taken under a wavelength of 694 nm.

Table 2-14. Preparation of ammonium calibration curve

NH_4^+-N (mg l^{-1})	0	1	3	5	7	10
Extinction	0	0.152	0.483	0.777	1.108	1.575

Formula 2-2

$y=0.157\ x$

Where,

y=Extinction

x= NH_4^+-N concentration (mg l^{-1})

2.9.2.3 Nitrate LCK 339

Principle: Nitrate ions in solutions containing sulphuric and phosphoric acids react with 2.6-dimethylphenol to form 4-nitro-2.6-dimethylphenol pink. The measurement was taken under a wavelength of 370 nm.

Table 2-15. Preparation of nitrate calibration curve

NO_3^--N (mg l^{-1})	0	0.5	1	3	5	10	12
Extinction	0	0.057	0.075	0.104	0.226	0.544	0.680

Formula 2-3

y=0.054x

Where,

y=Extinction

x=NO_3^--N concentration (mg l^{-1})

2.9.2.4 COD LCK 614

Principle: Oxidizable substances react with sulphuric acid potassium dichromate solution in the presence of silver sulphate as a catalyst (digested under 148℃ for 2 hours). Chloride is masked by mercury sulphate. The reduction in the yellow coloration of Cr^{6+} is evaluated. Undigested distilled water mixed with reagent was prepared as blank. The measurement is taken under a wavelength of 420nm. Potassium hydrogen phthalate (KHP) has a theoretical COD of 1.176 mg O_2 mg^{-1}. A serial concentration of KHP was prepared and produced the COD calibration curve. All the preparation and transferring of KHP solution was done under sterile conditions (APHA, AWWA and WEF, 1998)

Table 2-16. Preparation of COD calibration curve

KHP(mg l^{-1})	0	42.5	85.0	127.6	170.1	212.6	255.1
O_2 (mg l^{-1})	0	50	100	150	200	250	300
Difference of Extinction	0	0.008	0.028	0.092	0.122	0.181	0.196

Formula 2-4

y=0.003 x

Where

y=Extinction

x=COD (mg O_2 l^{-1})

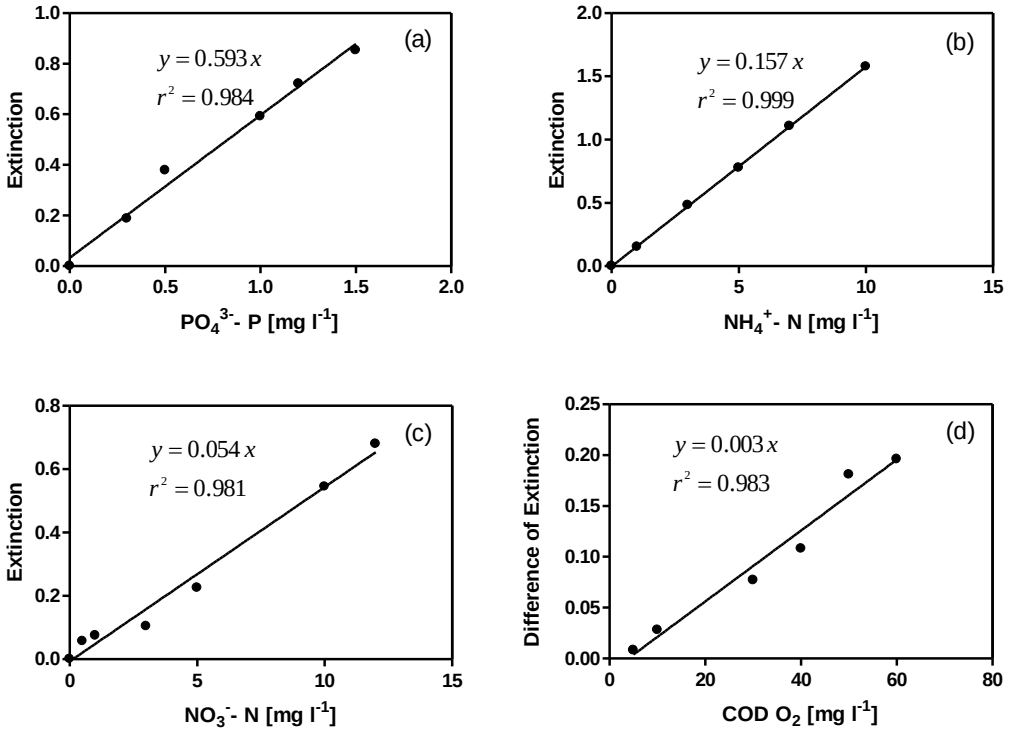


Figure 2-15. Calibration curves of cuvette tests of (a) PO_4^{3-}-P, (b) NH_4^+-N, (c) NO_3^--N and (d) COD

2.9.3 Standard Measurement

In the final measurement, phosphate-P was measured spectrophotometrically with the ammonium molybdate method according to the CEN (1996). Total internal phosphorus of microalgae was determined by digesting the sample in sulphuric acid and potassium peroxysulphate at 121 ℃ for 30 minutes, and afterwards determined as phosphate-P (CEN, 1996). Nitrate-N was measured by a colorimetric method using brucine following USEPA (1979). Ammonium was determined by the phenate method according to APHA, AWWA and WEF (1998). The pH of the wastewater was measured *in situ* with MultiLab P5 (WTW, Germany).

2.9.4 Chlorophyll *a* and Dry Weight

In order to determine chlorophyll *a* and dry weight of the immobilized microalgae, the cells were harvested from the substrate layers by scratching. Chlorophyll *a* was extracted with dimethyl sulfoxide (Hiscox and Israelstam, 1979) and incubated in darkness for 2 hours. To

remove non-dissolved particles, extracts were cleared by centrifugation. Chlorophyll *a* was determined spectrophotometrically according to Jeffrey and Humphrey (1975) using a UV-2450 spectrophotometer (UV-2450, UV-VIS Spectrophotometer, SHIMADZU; Kyoto, Japan). Chlorophyll a was calculated as the following equation:

Chlorophyll *a* ($\mu g\ ml^{-1}$) = $11.93\ E_{664}$-$1.93\ E_{647}$

(E represents the extinction measured spectrophotometrically)

The extraction of five microalgae, *P. boryanum* (M1252), *P. duplex* (M1253), *Scenedesmus* sp., *S. rubescens* (M2069) and *S. rubescens* (M2630) were incomplete when directly adding dimethyl sulfoxide. Therefore, they were mixed with liquid nitrogen and fine sand, ground in a mortar with pestle for 10 minutes before adding dimethyl sulfoxide for extraction.

The harvested algal biomass was dried at 105 ℃ for 24 hours, cooled down in the desiccator for 20 min and measured gravimetrically for dry weight.

2.9.5 Statistical Test

GraphPad Prism (version 5.00) for Windows (GraphPad software, San Diego California, USA) was used to analyze all the parameters statistically. PO_4^{3-}-P, NO_3^--N, NH_4^+-N, total internal phosphorus, chlorophyll *a* and dry weight were analyzed and will be expressed by mean value accompanying with standard deviation (concentration ± SD).

2.9.6 Microscopic Obeservation

The microscopic observation was performed using a Microscope Olympus at 100, 200 and 400x magnifications (Olympus CKX41, Olympus Optical Culture Miscroscope Co.GmbH; Hamburg, Germany). The photos were taken using a Microscope Digital Camera Olympus (Olympus DP 20 5E) connected to the microscope.

3. RESULTS

3.1 Selection of Twin Layers

Three tested membrane filters showed high qualification when applied as substrate layer. Nitrocellulose membrane, PTFE filter and nylon filter cloth were hydrophilic, stable in the water medium and caused no secondary pollution either on the substrate layer or in the water medium. Moreover, microalgae were easily attached to and grew well on these materials. However, both nitrocellulose membrane and PTFE filter are quite expensive, about 100 € m^{-2}, which limited further large-scale application of them. Nylon filter cloth showed higher endurance than the other two membrane filters and could be reused many times. The price of the nylon filter cloth is moderate, about 23€ m^{-2}.

Non-woven polyester (PE) filter was originally hydrophobic. By spaying ethanol and afterwards washing thoroughly with distilled water, the hydrophilicity of this material could be temporarily established. Water medium had to be immediately applied to it to sustain the hydrophilicity of the material. Microalgae could also grow on printing paper although it was fragile. However, applying freshwater medium or wastewater on printing paper could cause secondary pollution such as fungi *Aspergillus* sp. and *Saccharomyces* sp. on it. The details of the testing results of different substrate layers are listed in Table 3-1.

Two types of materials were tested as source layers: lamina materials and grid mesh materials (Table 3-2). In the first type of the materials, glass fibre lamina was hydrophilic. Water could evenly distribute to the subtended substrate layer. However, fibre filaments of glass fibre lamina could hurt skin and caused secondary pollution in the water medium, which was difficult to be removed. Non-woven polyester lamina had similar properties as the non-woven polyester filter tested as the substrate layer. When the hydrophilicity of this material was established, water medium could distribute evenly on the subtended printing paper (substrate layer). However, the hydrophilicity of the polyester was very difficult to be established.

Grid mesh materials were the second type of the materials. Water distribution on the subtended substrate layer of the grid mesh materials depended on if the surface structure of the gird mesh material. The surface of Polyester, PVC and polypropylene mesh were not straight, especially when they were more than 10 cm. The surface of PTFE coated glass fabrics mesh and coated glass fibre mesh were moderately straight. Two types of coated glass

fibre mesh (GFM), 10×10 mm and 4×4 mm, were further examined in this study. Both of them were relatively economical, 0.3€ m^{-2} and 0.5€ m^{-2}, respectively. It turned out that the surface of glass grid reinforced laminate was very straight and it was also hydrophilic. Such properties made it became the most competitive candidate as the source layer. The price of it was more expensive than coated glass fibre mesh, about 8.2€ m^{-2}. But it had the advantage of high stability, therefore it could last much longer time.

3.2 Microalgal Growth on Twin-Layer System

The combination and utilization of different twin-layer materials experienced four different stages. Firstly, nitrocellulose membrane and glass fibre lamina were used as substrate and source layer; secondly, non-woven polyester (PE) filter and non-woven polyester (PE) lamina were used; thirdly, nylon filter cloth and coated glass fibre mesh were applied; and finally, nylon filter cloth and glass grid reinforced laminate were employed.

The time course effect of Chl *a* of *S. rubescens* (M2630) on twin-layer system is depicted in Figure 3-1. The experimental time was 21 days for PE filter/PE lamina and 25 days for nylon/glass fibre meshes. The immobilized cells exhibited a linear growth pattern during the experiment, and no lag or adaptation phase was observed. The linear regression coefficiencies (r^2) of PE filter/PE lamina, nylon/GFM (10×10 mm) and nylon /GFM (4×4mm) are summarized in Table 3-3. There was a significant difference between growth rates on PE and GFM materials, whereas no differences were observed between GFM with the mesh size of 10×10 mm and 4×4mm. Chl *a* productivity in the linear phase can be calculated as *Chlap = d Chla/dt* (Table 3-3). Using PE material, the *Chlap* was highest, 2.62 μg cm^{-2} day^{-1}, during the experiment, while, both GFM gave lower productivity, about 1.43 and 1.51 μg cm^{-2} day^{-1}, respectively.

Table 3-1. Summary of the properties of the selected substrate layers

Material	If microalgae can penetrate through?		Hydrophilic?	Easiness of immobilization	Chemical tolerance	Endurance	Price (€ m^{-2})	Any secondary pollution?	Others
	S. rubescens **(M2630)**	***C. vulgaris*** **(M0484)**							
Protran reinforced nitrocellulose membrane	No	No	Yes	Easy	High	Moderate	100	No	Autoclavable
PTFE filter	No	Yes	Yes	Easy	High	Moderate	100	No	Autoclavable
Nylon filter cloth	No	No	Yes	Easy	High	High	23	No	Autoclavable
Non-woven polyester filter	No	Yes	No	Moderate	Moderate	High	2	No	Autoclavable
Printing paper	No	No	Yes	Moderate	Moderate	Low	0.03	*Aspergillus* sp. *Saccharomyces* sp.	---

Table 3-2. Summary of the properties of the selected source layers

Material	Hydrophilic?	Water Flow Distribution	Other Properties	Price (€ m^{-2})
Glass fibre lamina	Yes	Good	Fibre filaments (Secondary Pollution) Unstable	1.5
Non-woven polyester lamina	No	Good (when hydrophilic)	Robust	2
Polyester mesh	No	Bad	Robust Temperature resistance up to 130 ℃ Not straight	10
PTFE coated glass fabrics mesh	No	Moderate	Robust Temperature -70 ℃ – 260 ℃ Moderate straight	20
PVC	No	Bad	Robust Not straight	4
Coated glass fibre mesh (10mm ×10mm)	No	Moderate	Stable Moderate straight	0.3
Coated glass fibre mesh (4mm ×4mm)	No	Moderate	Stable Moderate straight	0.5
Polypropylene mesh	No	Bad	Vicat softening point 152 ℃ Stable Not straight	4
Glass grid reinforced laminate	Yes	Good	Stable Straight	8.2

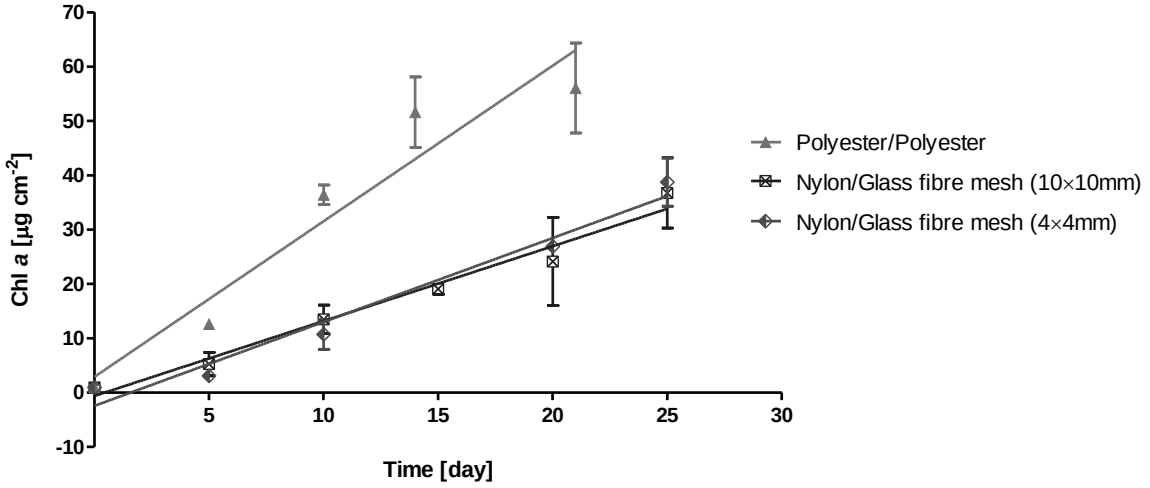


Figure 3-1. Growth (Chl *a* ± SD, n=3; µg cm^{-2}) curves of immobilized *S. rubescens* on different twin-layer materials with BG-11 medium

Table 3-3. Results of linear regression analyses performed on the growth (Chl *a*) curves of *S. rubescens* grown on different twin-layer materials

	r^2	*Chlap* (µg cm^{-2} day^{-1})	*p*
PE filter/PE lamina	0.9261	2.62	---
Nylon/GFM (10×10mm)	0.9753	1.43	<0.0001
Nylon/GFM (4×4mm)	0.9711	1.51	<0.0001

3.3 Screening Microalgae for Wastewater Treatment

3.3.1 Microalgal Growth in Suspension

After growing 23 microalgae in 50 ml suspension for 14 days, vision observations of the increment of the algae were recorded as excellent growth, good growth, growth and no obvious growth. The results are summarized in Table 3-4. No obvious growth was found for the following algae: *E. adhaerens*, *E. deses*, *Synedra* sp. and *S. uvella*. Therefore they were discarded from the subsequent selection.

The other 19 microalgae showed reasonable growth and were investigated further. The strains were: *B. terribilis*, *C. terricola*, *C. vulgaris*, *C. protothecoides*, *E. anabaena*, *E. gracilis*, *M. pseudonordstedtii*, *M. kuetzingianum*, *Navicula* sp., *N. communis*, *O. stellatum*, *Oscillatoria*

sp., *P. boryanum*, *P. duplex*, *Senedesmus* sp., *S. rubescens* (M2069), *S. rubescens* (M2630), *Stigeoclonium* sp. and *Trachelomonas* sp..

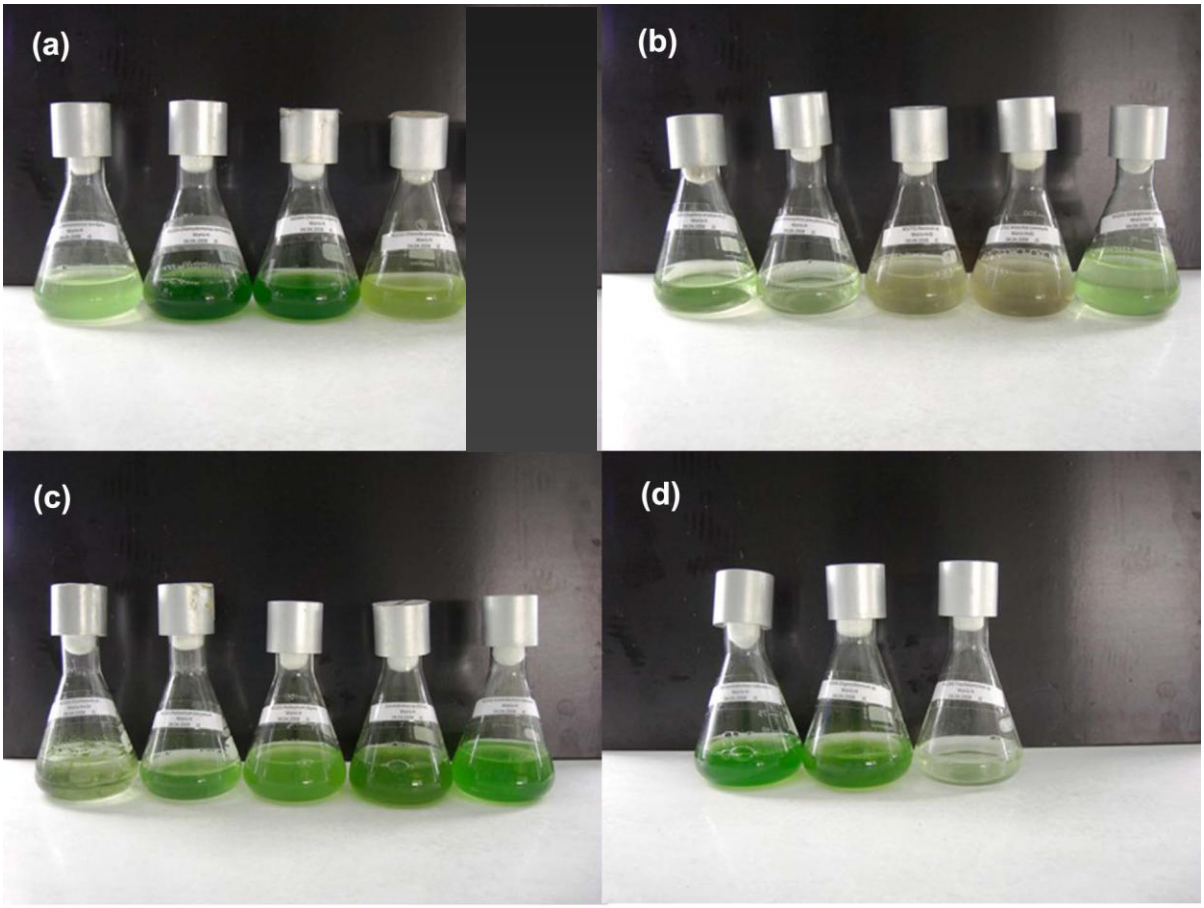


Figure 3-2. Photographs of microalgae grew in 50 ml Waris-H/W+Si/W+3V medium **after 14 days of (a) (from left to right)** ***Botryococcus terribilis, Chlamydomonas terricola, Chlorella vulgaris, Chlorella protothecoides*****; (b)** ***Euglena anabaena, Monomorphina pseudonordstedtii, Navicula*** **sp.,** ***Nitzschia communis, Oedogonium stellatum*****; (c)** ***Oscillatoria*** **sp.,** ***Pediastrum boryanum, Pediastrum duplex,*** **Senedesmus sp.(China) ,** ***Scenedesmus rubescens*** **(M2069) and (d)** ***Scenedesmus rubescens*** **(M2630),** ***Stigeoclonium*** **sp. and** ***Trachelomonas*** **sp.**[1]

Among those 19 strains, *C. terricola*, *C. vulgaris*, *P. boryanum*, *P. duplex*, *Senedesmus* sp., *S. rubescens* (M2069), *S. rubescens* (M2630) and *Stigeoclonium* sp. exhibited excellent growth in the culture medium, as being illustrated in Figure 3-2. And *B. terribilis*, *C. protothecoides*, *E anabaena*, *M. kuetzingianum*, *Navicula* sp., *N. communis*, *O. stellatum*, *Oscillatoria* sp., *P. boryanum* and *P. duplex* showed good growth (Figure 3-2).

[1] No photosgraphs were taken for *E. adhaerens*, *E. deses*, *Synedra* sp., *S. uvella*. *E. gracilis* and *M. kuetzingianum*

Table 3-4. Vision observations of the growth of microalgae in suspension after 14 days, +++ represents excellent growth, ++ represents good growth, + represents growth, and - represents no obvious growth

No.	Stain	Growth	No.	Stain	Growth
1	*Botryococcus terribilis*	++	13	*Oedogonium stellatum*	++
2	*Chlamydomonas terricola*	+++	14	*Oscillatoria* sp.	++
3	*Chlorella vulgaris*	+++	15	*Pediastrum boryanum*	++
4	*Chlorella protothecoides*	++	16	*Pediastrum duplex*	++
5	*Euglena adhaerens*	-	17	*Scenedesmus* sp. (China)	+++
6	*Euglena anabaena*	++	18	*Scenedesmus rubescens* M2069	+++
7	*Euglena deses*	-	19	*Scenedesmus rubescens* M2630	+++
8	*Euglena gracilis*	+++	20	*Stigeoclonium* sp.	+++
9	*Monomorphina pseudonordstedtii*	++	21	*Synedra* sp.	-
10	*Microthamnion kuetzingianum*	++	22	*Synura uvella*	-
11	*Navicula* sp.	++	23	*Trachelomonas* sp.	+
12	*Nitzschia communis*	++			

3.3.2 Screening with Continuous Mode

3.3.2.1 Chlorophyll Fluorescence

Nylon filters prepared with 19 microalgae at a density of 0.75 μg Chl *a* cm^{-2} were continuously supplied by glass fibre saturated with 5 ml Waris-H/Waris-H+Si/Waris-H+3V medium or synthetic secondary wastewater for 20 days. During the application, the effective quantum yield of photosynthetic energy conversion, Yield=ΔF/Fm', the so-called Genty-parameter were recorded at day 0,1,2,3,4,5,9,10,13,15,16,17,18 and 20 (Figure 3-3). The recorded Yield (ΔF/Fm') can be differentiated into the following five categories.

(1) During 20 days cultivation, ΔF/Fm' started at around 0.8 and remained at a constant level between 0.6 to 0.8 for nylon filters supplied by both Waris-H medium and synthetic secondary wastewater. Only a slightly decrease of ΔF/Fm' could be observed. The stains with this tendency were *B. terribilis*, *C. terricola*, *E. gracilis*, *M. kuetzingianum*, P. *boryanum*, *Senedesmus* sp. and *Stigeoclonium* sp..

(2) ΔF/Fm' also started at around 0.8, however, the tendency of ΔF/Fm' exhibited faster decline and ended at about 0.4 to 0.6. The algae with this tendency were *C. vulgaris*, *C. protothecoides*, *E. anabaena*, *O. stellatum*, *P. duplex*, *S. rubescens* (M2069) and *S. rubescens* (M2630). Interestingly, there was a significant difference between ΔF/Fm' of *C. vulgaris* supplied by synthetic secondary wastewater and by Waris-H medium ($p<0.05$). During 20 days, the values of ΔF/Fm' of the former were always lower than the latter's.

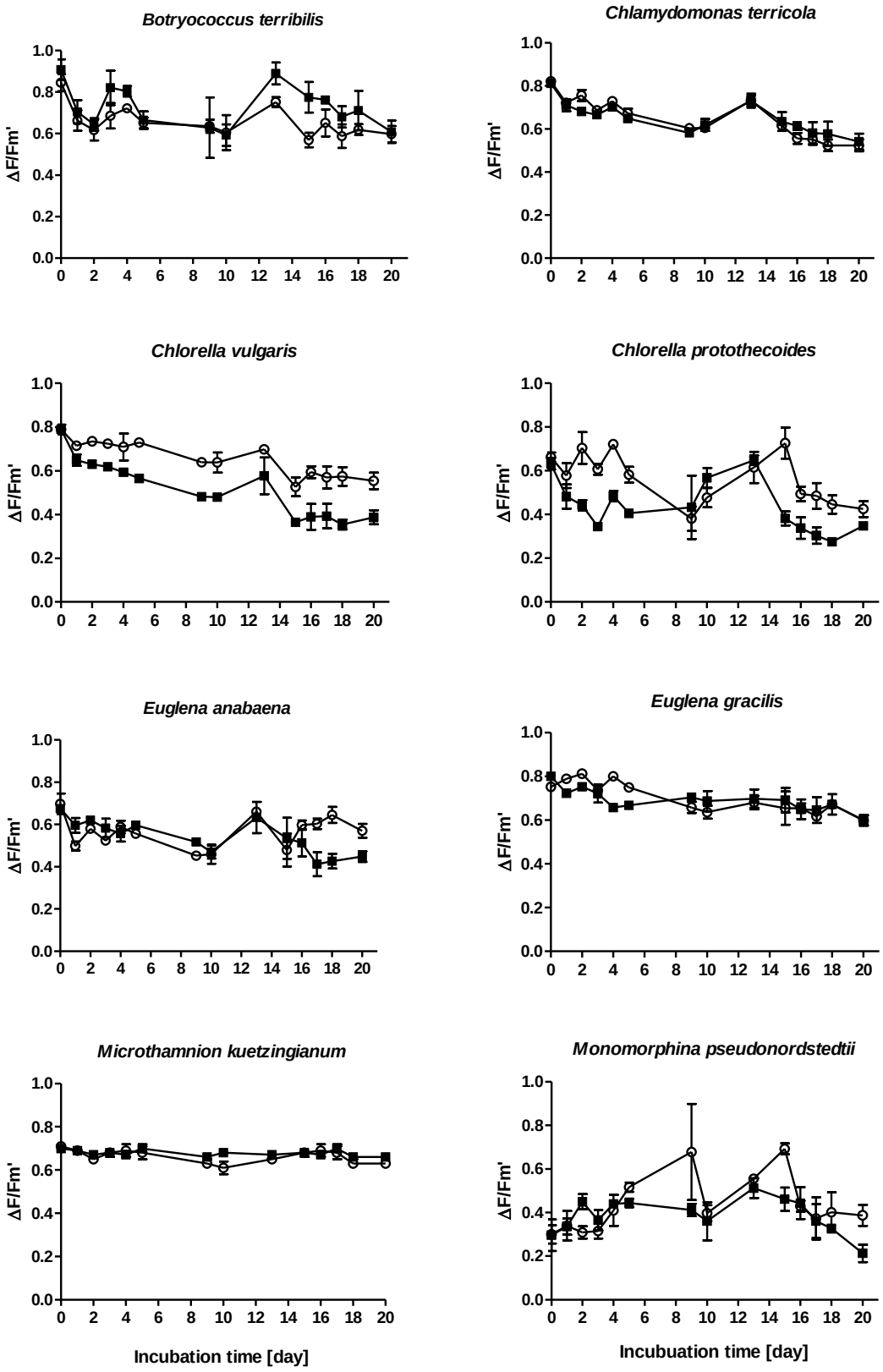
(3) The value of ΔF/Fm' fluctuated during 20 days in the range of 0.2 to 0.6 with both Waris-H and synthetic wastewater. The algae which showed this tendency were *M. pseudonordstedtii* and *Trachelomonas* sp..

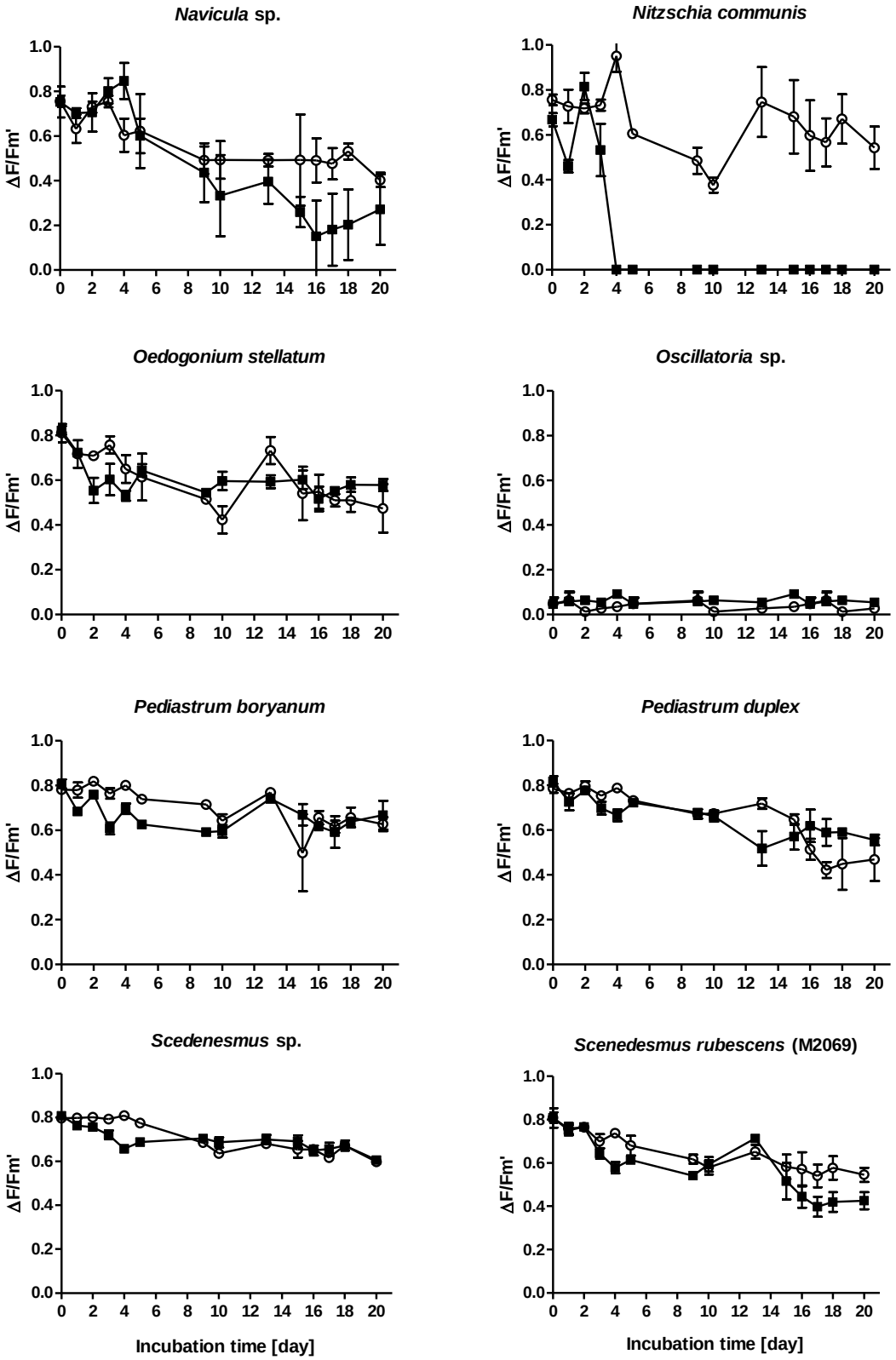
(4) A significant decrease of ΔF/Fm' for microalgae supplied by synthetic secondary wastewater compared with Waris-H culture medium was observed. The reduction was already monitored at day four for *N. communis* supplied by synthetic wastewater. Afterwards, no ΔF/Fm' was recorded. In contrast, ΔF/Fm' for Waris-H medium remained at a constant level between 0.4 and 0.8. The similar phenomenon was also found for *Navicula* sp. supplied by synthetic wastewater after day 10 (about 0.26 ± 0.14, mean $\pm$ SD).

(5) Extreme low ΔF/Fm' for both Waris-H medium and synthetic secondary wastewater was observed. The value kept below 0.1 for *Oscillatoria* sp..

3.3.2.2 Chlorophyll *a*

At day 20, microalgae were harvested. Chl *a* of each replicates were extracted and determined. Chl *a* productivities (μg cm^{-2} day^{-1}) of the 19 algae were summarized in Table 3-5. Six algae, *B. terribilis*, *M. pseudonordstedtii*, *Navicula* sp., *N. communis*, *Oscillatoria* sp. and *Trachelomonas* sp., exhibited low growth rate with respect to *Chlap* with synthetic secondary wastewater. About 0.06, 0.07, 0.04, 0.01, 0.08 and 0.15 μg Chl *a* cm^{-2} day^{-1} were obtained from them, respectively. Among those six strains, *M. pseudonordstedtii*, *Navicula* sp., *N. communis*, *Oscillatoria* sp. and *Trachelomonas* sp. showed both low ΔF/Fm' (Figure 3-3) and low Chl *a* productivity (Table 3-5) supplied by synthetic secondary wastewater. And they were excluded from further investigation. Although ΔF/Fm' of *B. terribilis* during 20 days kept relatively high (between 0.6 to 0.8), the low Chl *a* productivity, 0.06 μg cm^{-2} day^{-1}, would limit the large-scale application of this algae. Therefore, it was also eliminated from the subsequent selection.


Botryococcus terribilis
Chlamydomonas terricola
Chlorella vulgaris
Chlorella protothecoides
Euglena anabaena
Euglena gracilis
Microthamnion kuetzingianum
Monomorphina pseudonordstedtii
ΔF/Fm'
Incubation time [day]
Incubuation time [day]


Navicula sp.
Nitzschia communis
Oedogonium stellatum
Oscillatoria sp.
Pediastrum boryanum
Pediastrum duplex
Scedenesmus sp.
Scenedesmus rubescens (M2069)
ΔF/Fm'
Incubation time [day]

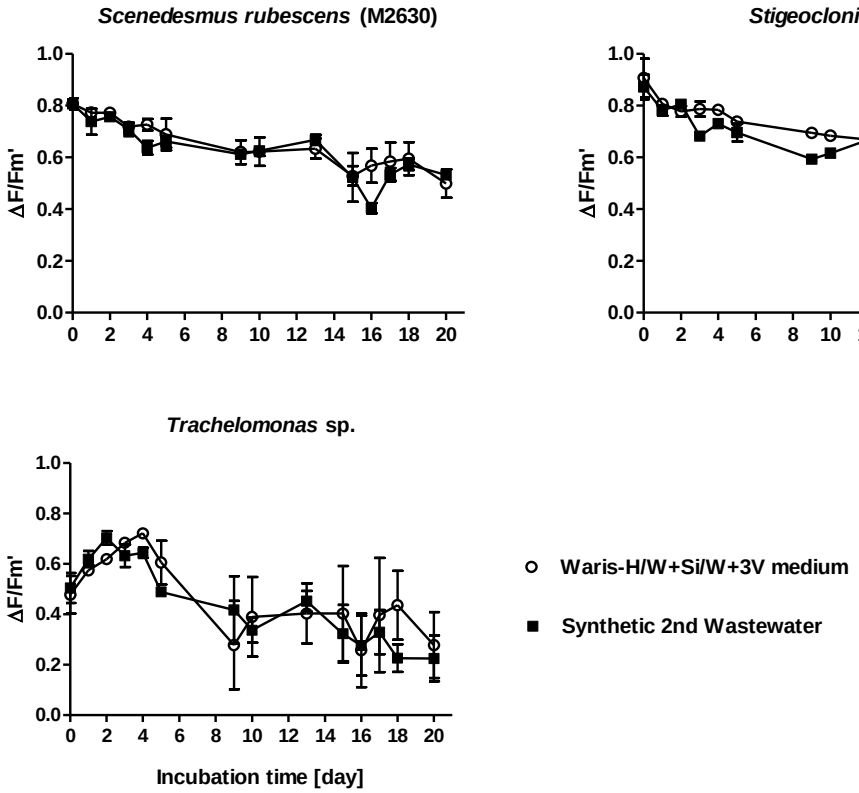


Figure 3-3. ΔF/Fm'± SD (n=4) of immobilized microalgae on nylon filters supplied by glass fibre saturated with (○) Waris-H/W+Si/W+3V medium or (■) synthetic secondary wastewater during 20 days

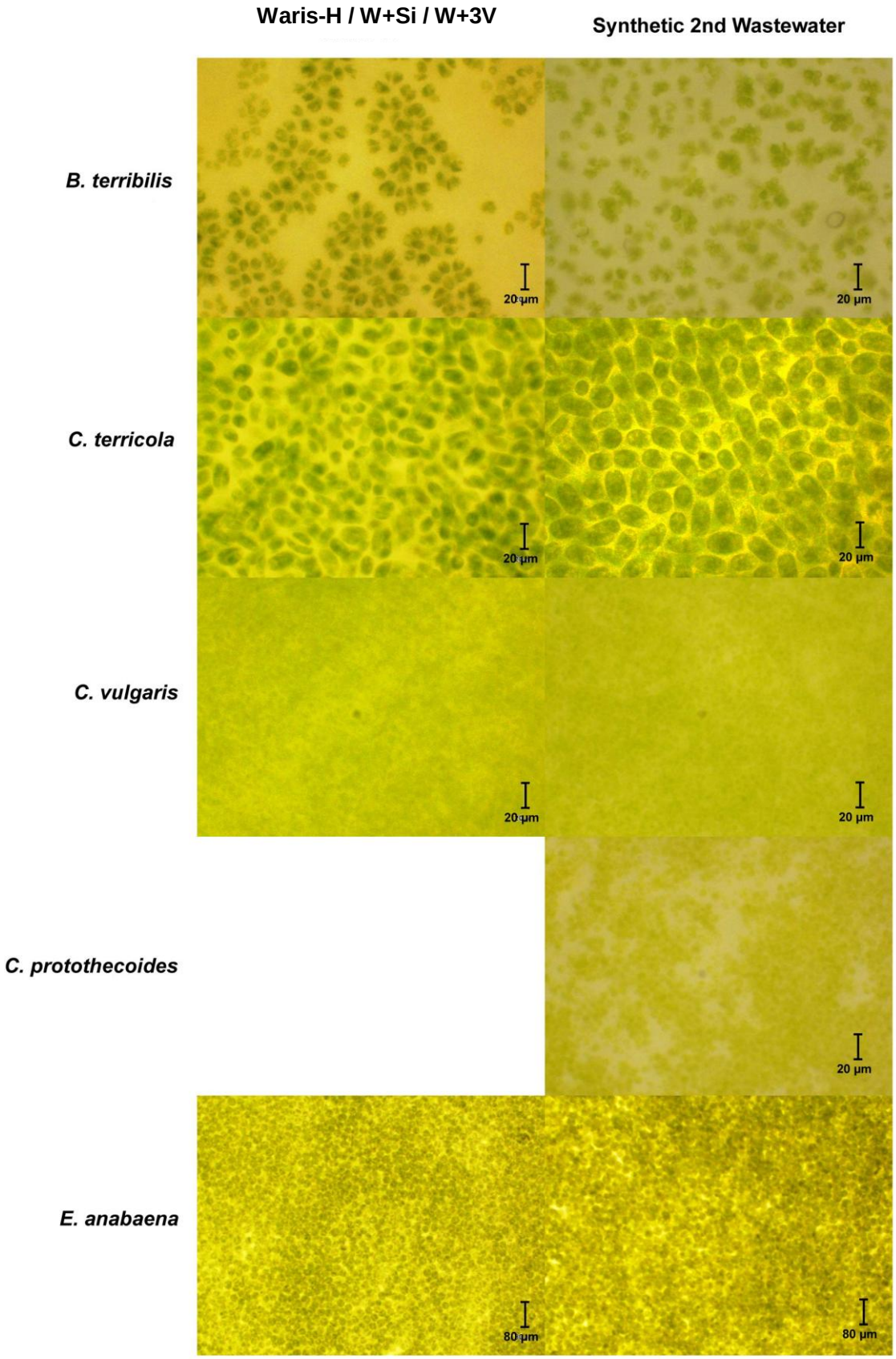
3.3.2.3 Observation of Immobilized Microalgae on Nylon Filters

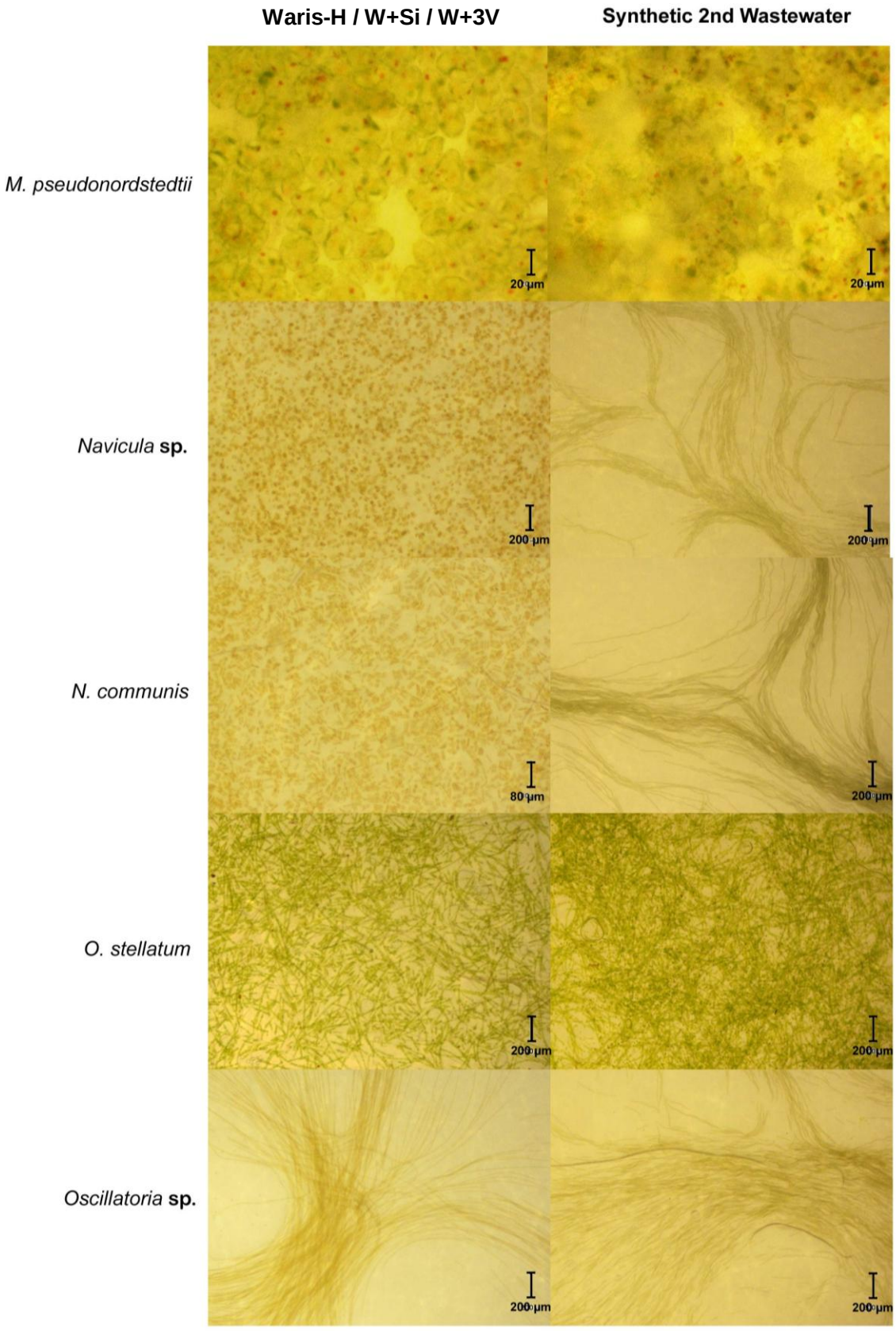
Figure 3-4 shows the microscopic observations of the cells of microalgae grown on nylon filters supplied by Waris-H medium or synthetic secondary wastewater after cultivation of 20 days. The immobilized cells were greenish or brownish depending on the species. More aggregated cells in a single colony and clearer colony structure were found for *B. terribilis* and *P. boryanum* supplied by Waris-H medium than by synthetic wastewater. The observed cell densities of *B. terribilis* and *M. pseudonordstedtii* were low in both medium. But no obvious difference could be found between two types of medium. Application of two types of medium caused morphological difference for *C. terricola*: the size of each cell was 20 µm in length under light microscopy supplied by synthetic wastewater in comparison with 15 µm by Waris-H medium. Cell density of *O. stellatum* in synthetic wastewater was obviously higher than in Waris-H, which has also been confirmed by the Chl *a* productivity, 0.70 and 0.24 µg cm^{-2} day^{-1} for synthetic wastewater and Waris-H medium, respectively. Observations by light microscope also indicated that there were no cell formations for either *Navicula* sp. or *N.*

communis supplied by synthetic wastewater after 20 days. Only the filaments of dead cells could be found. In contrast, clear cell formation and relatively dense culture could be observed for both algae grown on nylon filters supplied by Waris-H medium. The low ΔF/Fm' value (Figure 3-3) and low Chl *a* productivity (Table 3-5) supplied by synthetic wastewater also verified this observation. *Oscillatoria* sp. could grow neither with Waris-H medium nor with synthetic wastewater, and only dead filaments were found on the nylon filter. The growths of other microalgae immobilized on nylon filters were regular. No obvious difference between Waris-H medium and synthetic secondary wastewater could be defined by the observations.

Table 3-5. Chlorophyll *a* productivity (μg cm^{-2} day^{-1}) of microalgae grown on nylon filters for 20 days

		Waris-H/W+Si/W+3V	Synthetic 2nd wastewater
1	*Botryococcus terribilis*	0.14	0.06
2	*Chlamydomonas terricola*	0.80	1.14
3	*Chlorella vulgaris*	1.85	0.67
4	*Chlorella protothecoides*	0.26	0.32
5	*Euglena anabaena*	1.36	0.93
6	*Euglena gracilis*	0.24	0.45
7	*Monomorphina pseudonordstedtii*	0.13	0.07
8	*Microthamnion kuetzingianum*	0.48	0.35
9	*Navicula* sp.	0.17	0.04
10	*Nitzschia communis*	0.20	0.01
11	*Oedogonium stellatum*	0.24	0.70
12	*Oscillatoria* sp.	0.03	0.08
13	*Pediastrum boryanum*	1.67	1.02
14	*Pediastrum duplex*	0.69	0.96
15	*Scenedesmus* sp.	0.63	0.66
16	*Scenedesmus rubescens* M2069	1.31	0.72
17	*Scenedesmus rubescens* M2630	1.26	0.44
18	*Stigeoclonium* sp.	1.91	0.91
19	*Trachelomonas* sp.	0.18	0.15


Waris-H / W+Si / W+3V
Synthetic 2nd Wastewater
B. terribilis
C. terricola
C. vulgaris
C. protothecoides
E. anabaena
20 µm
80 µm


Waris-H / W+Si / W+3V
Synthetic 2nd Wastewater
M. pseudonordstedtii
Navicula sp.
N. communis
O. stellatum
Oscillatoria sp.
20 µm
200 µm
80 µm

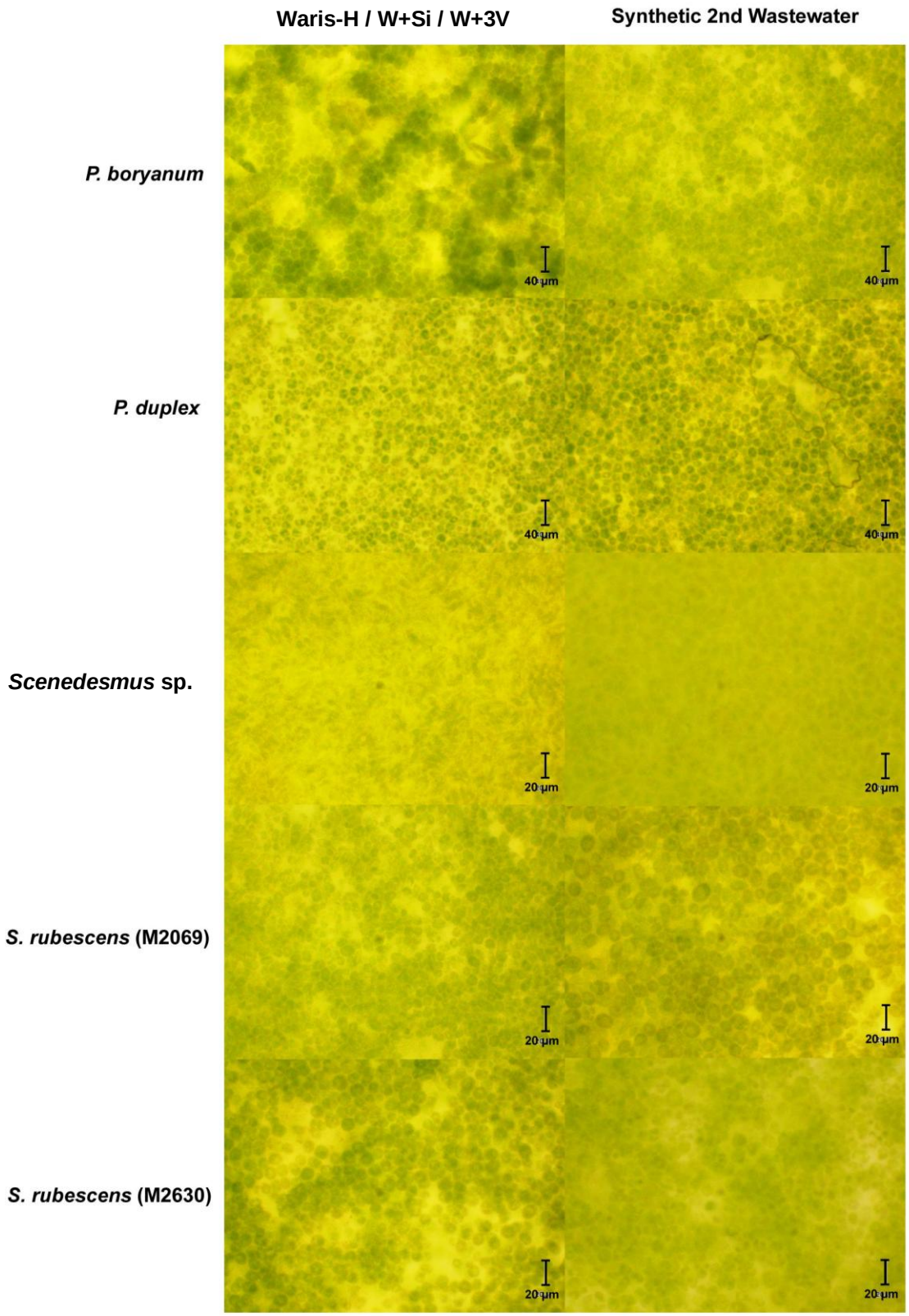


Figure 3-4. Light microscopic photos of microalgae immobilized on nylon filters supplied by Waris-H/W+Si/W+3V medium or synthetic secondary wastewater

3.3.3 Screening with Semi-Continuous Mode

The 13 microalgae showed better ΔF/Fm' values, higher Chl *a* productivities and good microscopic observations supplied by synthetic secondary wastewater throughout the cultivation period in the last experiment were retained for the screening with semi-continuous mode. The strains were *C. terricola*, *C. vulgaris*, *C. protothecoides*, *E. anabaena*, *E. gracilis*, *M. kuetzingianum*, *O. stellatum*, *P. boryanum*, *P. duplex*, *Senedesmus* sp., *S. rubescens* (M2069), *S.rubescens* (M2630) and *Stigeoclonium* sp..

3.3.3.1 Chlorophyll *a*

All the 13 cultures were prepared with the starting inoculums density of 0.87 μg Chl *a* cm^{-2} on nylon filters and incubated for 20 days. Synthetic secondary wastewater, modified Waris-H medium and BG-11 medium were supplied to the algae by saturating the glass fibre underneath the nylon filters. Every three days the nylon filters were transferred to fresh medium. The Chl *a* final yields of the 13 microalgae with modified Waris-H medium, synthetic secondary wastewater and BG-11 culture medium were compared in Figure 3-5. *C. terricola* could grow well and yielded similar Chl *a* in three media. Four algae, *C. protothecoides*, *E. anabaena*, *E. gracilis* and *S. rubescens* (M2630) could grow better in synthetic secondary wastewater than in the other media. Especially *E. anabaena*, whose Chl *a* productivity (μg cm^{-2} day^{-1}) in the synthetic secondary wastewater was about two times and three times higher than in the Waris-H (0.50) and in the BG-11(0.36) medium. This could also be observed in the photos taken at day 20 (Figure 3-6). The cells density of *E. anabaena* on the nylon filter with synthetic wastewater was denser than with Waris-H and BG-11 medium. Chl *a* yields of *C. vulgaris*, *M. kuetzingianum* and *Stigeoclonium* sp. were highest in Waris-H medium. Three algae, *P. boryanum*, *Scenedesmus* sp. and *S. rubescens* (M2069), grew best in BG-11 medium. It could also be observed from Figure 3-6 that *E. gracilis* was mobile on the nylon filter, and cells moved to the edge of nylon filter and polluted the glass fibre underneath. The green filaments of *Stigeoclonium* sp. and *O. stellatum* also grew cross the immobilized edge, but, comparing with *E. gracilis*, to a much less extent. Final Chl *a* yields of the 13 algae from highest to lowest is exhibited in Figure 3-7 (a). Chl *a* productivity is summarized in Table 3-6.

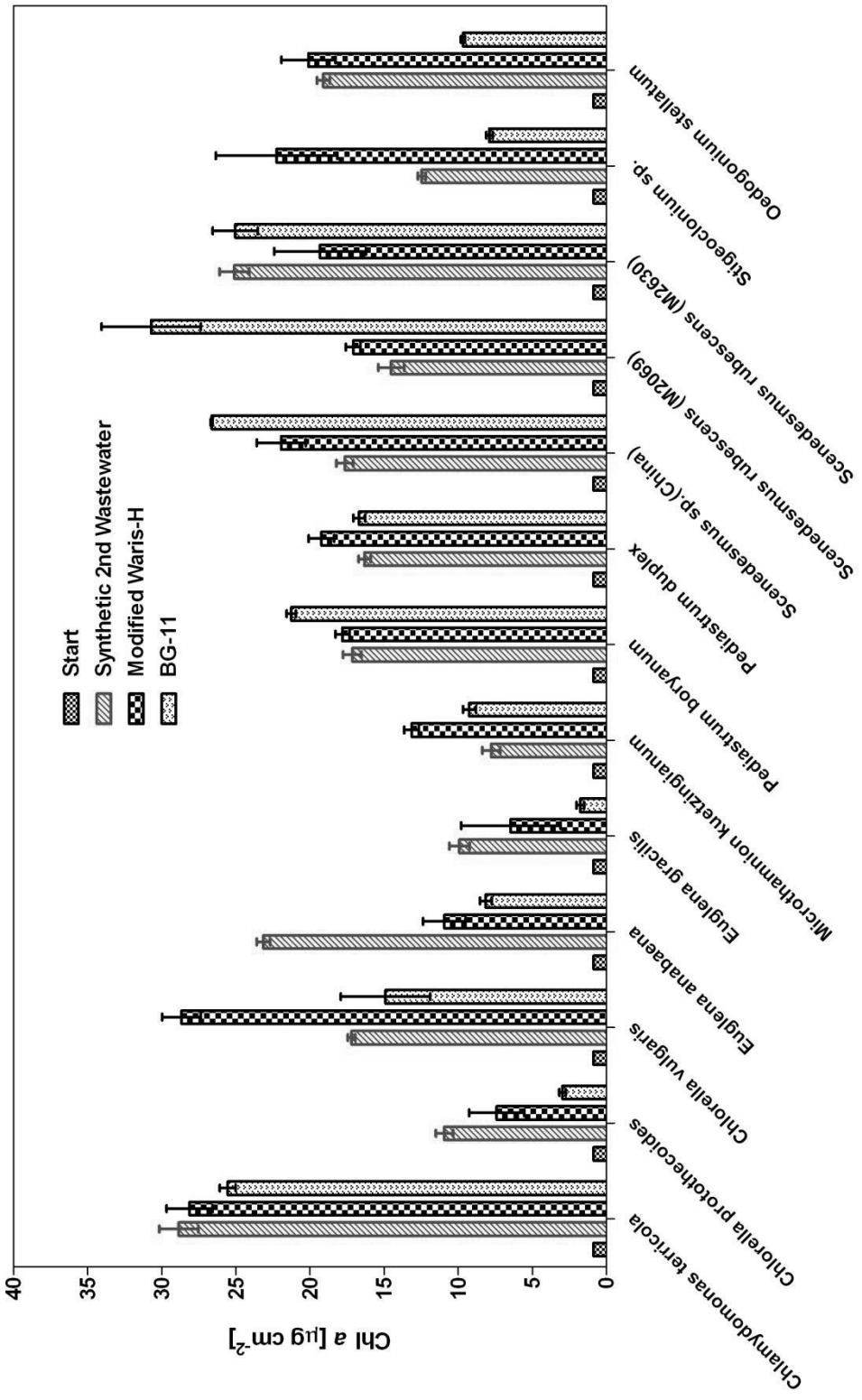


Figure 3-5. Chlorophyll *a* (±SD, n=4, µg cm^{-2}) of immobilized microalgae on nylon filters with different media

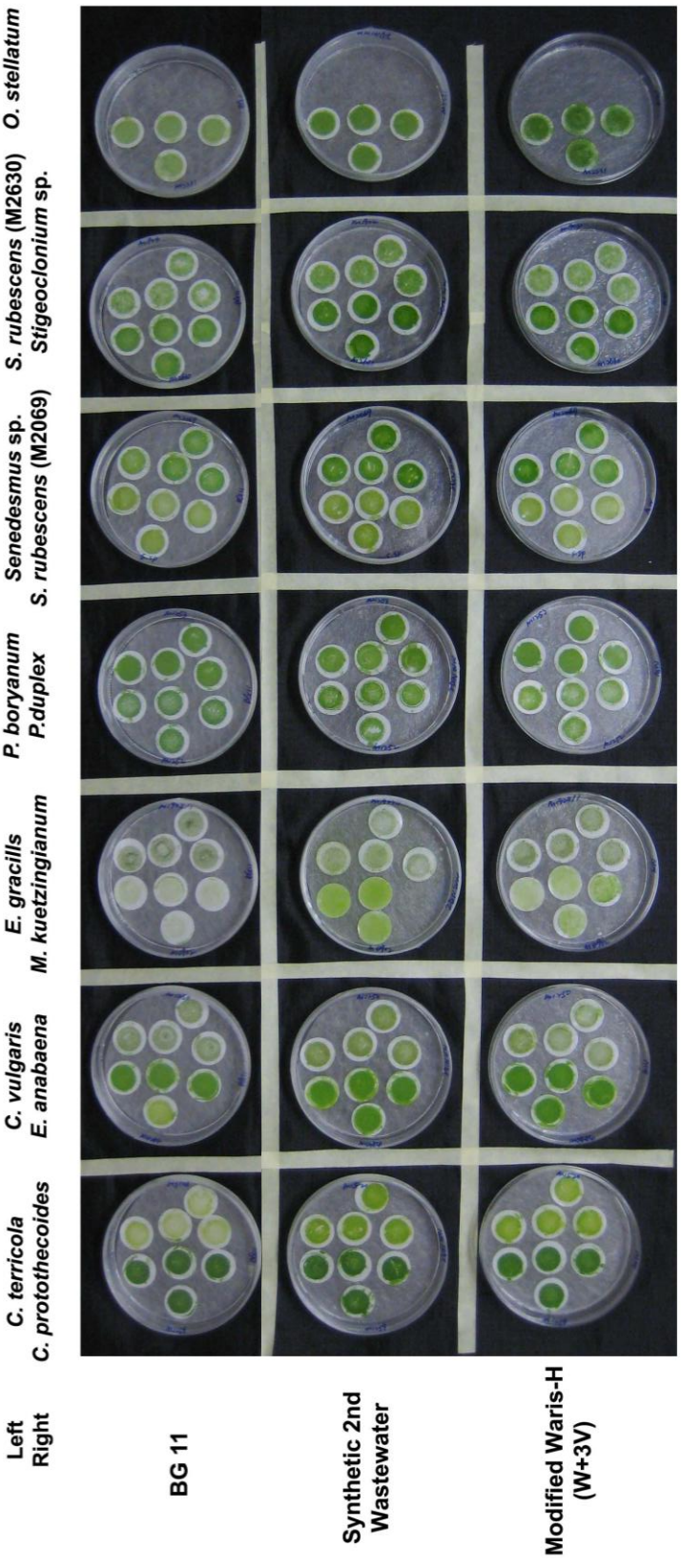


Figure 3-6. Microalgae immobilized on nylon filters supplied by different media for chlorophyll *a* comparison (day 20)

3.3.3.2 Dry Weight

The 13 cultures were also prepared with the starting inoculums density of 0.5 g dry weight m^{-2}. And each culture with three replicates supplied by synthetic secondary wastewater. Every three days the nylon filters were transferred to fresh media. The dry weight increments after 20 days were determined. Dry weight final yields of 13 microalgae from highest to lowest is illustrated in Figure 3-7 (b). Dry weight productivity can be calculated in a similar method with *Chlap*, as $DWp = d\,DW/dt$, (g m^{-2} day^{-1}). The results of *DWp* obtained from 13 microalgae are summarized in Table 3-6. Five microalgae produced both high *Chlap* (>0.80 μg cm^{-2} day^{-1}) and high *DWp* (>0.20 g m^{-2} day^{-1}), which were, *C. terricola*, *E. anabaena*, *P. boryanum*, *Senedesmus* sp. and *S. rubescens* (M2630).

Table 3-6. Chlorophyll *a* productivity (μg cm^{-2} day^{-1}) and dry weight productivity (g m^{-2} day^{-1}) of 13 microalgae supplied by synthetic secondary wastewater in 20 days

		Chlap (μg cm^{-2} day^{-1})	*DWp* (g m^{-2} day^{-1})
1	***Chlamydomonas terricola***	**1.40**	**0.24**
2	*Chlorella protothecoides*	0.50	0.13
3	*Chlorella vulgaris*	0.55	0.07
4	***Euglena anabaena***	**1.11**	**0.22**
5	*Euglena gracilis*	0.45	0.15
6	*Microthamnion kuetzingianum*	0.34	0.19
7	***Pediastrum boryanum***	**0.81**	**0.21**
8	*Pediastrum duplex*	0.77	0.18
9	***Scenedesmus* sp. (China)**	**0.84**	**0.22**
10	*Scenedesmus rubescens* (M2069)	0.68	0.19
11	***Scenedesmus rubescens* (M2630)**	**1.21**	**0.31**
12	*Stigeoclonium* sp.	0.58	0.26
13	*Oedogonium stellatum*	0.91	0.17

Two algae offered a little bit lower *Chlap* and *DWp*, *P. duplex* (0.77, 0.18) and *S. rubescens* (M2069), (0.68, 019). Although the *Chlap* of *M. kuetzingianum* and *Stigeoclonium* sp. were only 0.34 and 0.58 µg cm^{-2} day^{-1}, respectively, the *DWp* for both of them were relatively high, 0.19 and 0.26 g m^{-2} day^{-1}, respectively. *O. stellatum* produced high *Chlap*, 0.91 µg cm^{-2} day^{-1}, and moderate *DWp*, 0.17 g m^{-2} day^{-1}.

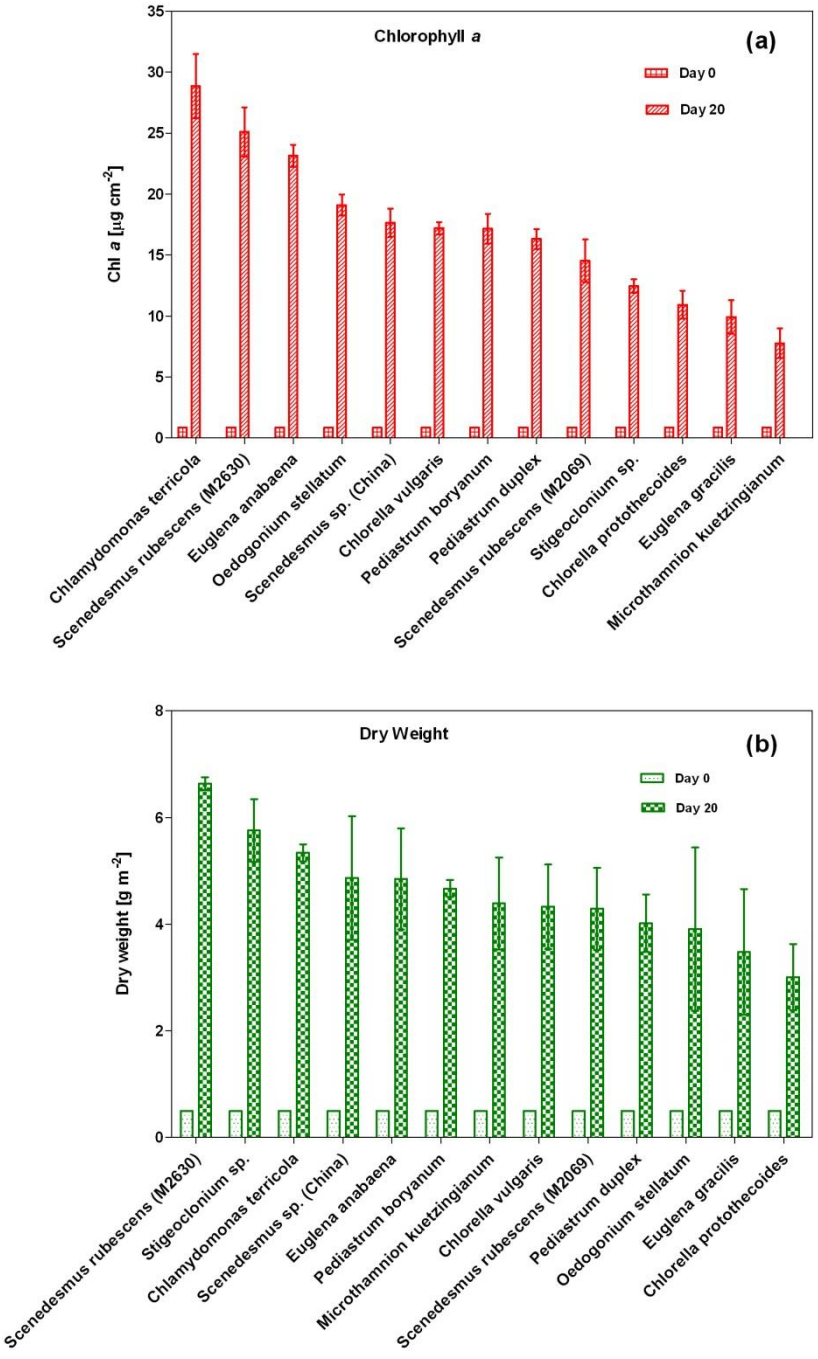


Figure 3-7. Final Chlorophyll *a* (±SD, n=4, µg cm^{-2}) and dry weight (±SD, n=3, g m^{-2}) of immobilized microalgae on nylon filters cultivated with synthetic secondary wastewater for 20 days

3.4 Bench-Scale Treatment: Continuous Mode

The temperature during the bench-scale treatment in the greenhouse was between 15 and 30 ℃. The ambient illumination was about 20-120 μE m^{-2} s^{-1}. And the pH of the modified BG-11 medium kept between 7.1 and 7.5 for control and two algae *Chlorella vulgaris* and *S. rubescens* (M2630). Both algae grew well on the bench-scale twin-layer system with nitrocellulose membrane and glass fibre, which served as substrate layer and source layer, respectively. The Chl *a* content of *C. vulgaris* increased from 3.5±0.4 μg cm^{-2} to 11.8±1.4 μg cm^{-2} in 9 days, and Chl *a* of *S. rubescens* rose from 1.3±0.1 μg cm^{-2} to 12.3±0.5 μg cm^{-2} during the same time interval, which corresponds to Chl *a* productivity (*Chlap*) of 0.92 and 1.22 μg cm^{-2} day^{-1} for *C. vulgaris* and *S. rubescens*, respectively (Table 3-7).

The removal of phosphate from modified BG-11 medium during 9 days is shown in Figure 3-8. Phosphate was efficiently removed from the medium by both algae within two days from a starting concentration of about 3 mg l^{-1} to < 0.3 mg l^{-1} (Figure 3-8, a). The phosphate uptake efficiency (calculated as $Pe_i = (P_0-P_i)/P_0 \times 100\%$, where P_0 and P_i were the concentrations of phosphate at the beginning and day *i* of the treatment) was about 89% and 90% after 2 days for *C. vulgaris* and *S. rubescens* respectively. In the control, the phosphate concentration decreased only slightly over the experimental time period.

In the same experiment, removal of ammonium from modified BG-11 medium was also determined (Figure 3-8, b). The removal efficiency of ammonium-N (*AMe*) could be calculated as $AMe_i = (AM_0-AM_i)/AM_0 \times 100\%$, where AM_0 and AM_i were the concentrations of ammonium at the beginning and day *i* of the treatment. As expected, ammonium was removed less rapidly by the algae than phosphate. For both algae, ammonium levels were lowest (about 1 mg l^{-1} NH_4-N) at the end of the experiment. Ammonium removal efficiency was 78% and 69% in the first 3 days, and at day 9, 94% and 96% of the initial ammonium concentration had been removed by *C. vulgaris* and *S. rubescens* respectively. In the control, ammonium levels dropped slowly (presumably related to NH_3 formation and release into the atmosphere).

Finally, nitrate was also removed from modified BG-11 medium by the two algae (Figure 3-8, c). However, a pronounced lag phase of nitrate removal was observed, the duration of which also differed between the two algal strains: for *C. vulgaris*, the lag phase was about two days, for *S. rubescens* about 4 days. After the lag phase, *C. vulgaris* reduced nitrate levels gradually to about 0.2 mg l^{-1} within 3 days, whereas for *S. rubescens*, removal of nitrate to the same low

level occurred more rapidly, i.e. within one day (Figure 3-8, c). In the control, the nitrate concentrations increased slowly (from 2.9 mg l^{-1} to 3.5 mg l^{-1}) during the experimental period.

The internal phosphorus content of both algae at day 0 and day 9 were determined. The internal phosphorus increments were 4.27 and 4.95 mg P for *C. vulgaris* and *S. rubescens* respectively. Phosphorus depletions from modified BG-11 medium could be calculated as 5.56 and 5.25 mg for two algae respectively. And the reduction could only slightly contribute to the uptake by non-algal reasons indicated by the control (0.92 mg). The differences of P reduction between the treatment with algae and control were the actual P uptake exclusively by algae. It could be calculated as 4.64 and 4.33 mg P, or 0.52 and 0.48 mg P day^{-1} for *C. vulgaris* and *S. rubescens* respectively. The deviation was 8.6% and 12.5% comparing with the measured internal phosphorus increments.

The internal nitrogen contents of microalgae were not determined, but the total nitrogen depletion from water medium could be calculated as 45.1 mg and 48.2 mg for *C. vulgaris* and *S. rubescens*, respectively, in which 13 mg was depleted due to non-algal reasons indicated by control. The differences between total N depletion between the treatment with algae and control were the actual total N uptake exclusively by algae, which could also be regarded as internal N increment. Therefore, the internal N increment of *C. vulgaris* and *S. rubescens* could be approximately calculated as 32.1 and 35.2 mg N or 3.6 and 3.9 mg P day^{-1}, respectively. The internal N/P ratio for *C. vulgaris* and *S. rubescens* was 7.5 (32.1/4.27) and 7.1 (35.2/4.95), respectively.

Table 3-7. Chlorophyll *a* productivity (μg cm^{-2} day^{-1}), phosphorus and nitrogen balance of bench-scale treatment with continuous mode

	Chlap (μg cm^{-2} day^{-1})	Microalgal internal phosphorus increment (mg)	P depletion from medium (mg)	Total N (NH_4^+-N and NO_3^-- N) depletion from medium (mg)
Control	---	---	0.92	13
C. vulgaris	0.92	4.27	5.56	45.1
S. rubescens	1.22	4.95	5.25	48.2

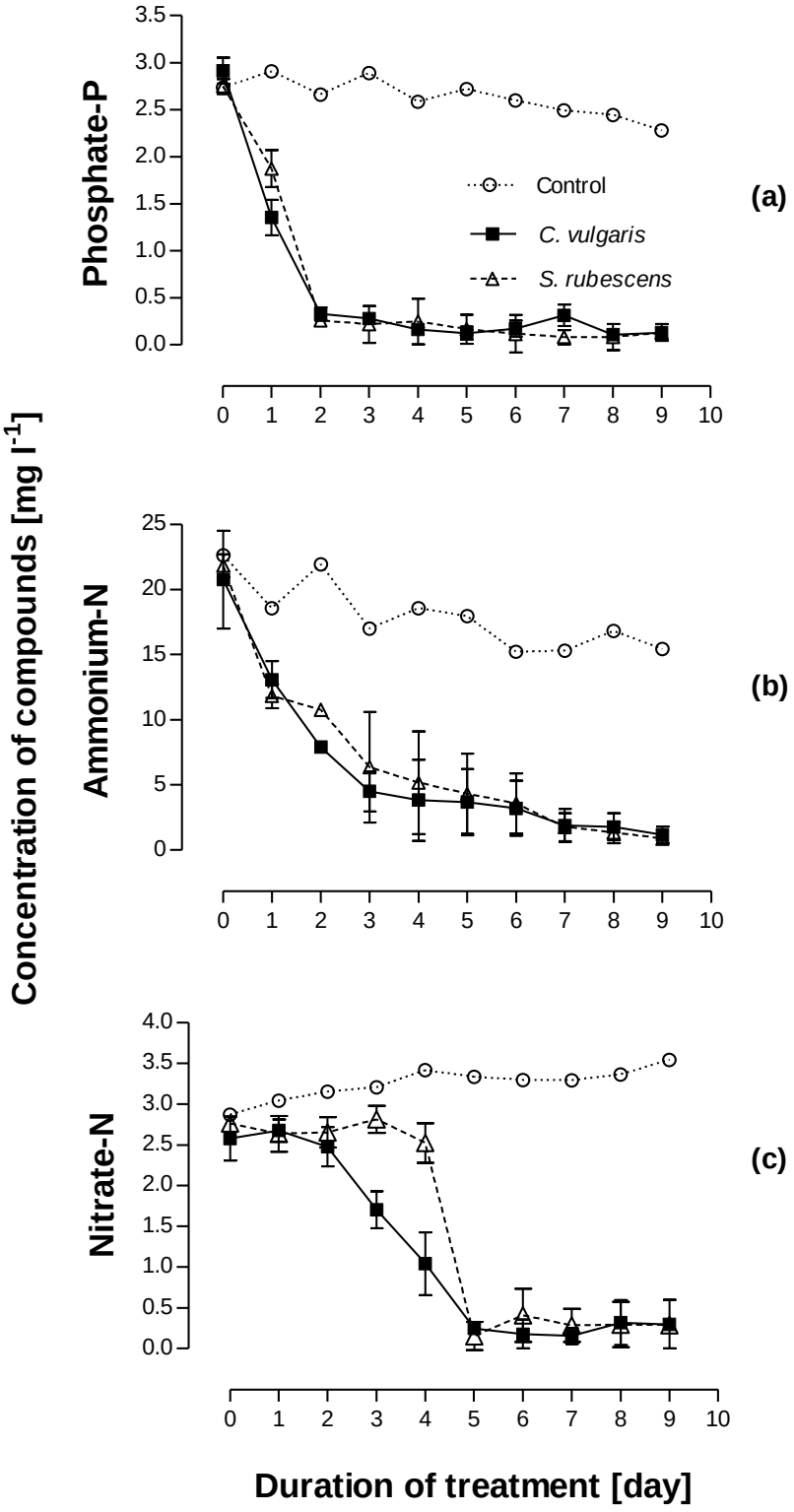


Figure 3-8. Residual concentration of Phosphate-P (a), Ammonium-N (b) and Nitrate-N (c) from modified BG-11 medium by *C. vulgaris* and *S. rubescens* and control on bench-scale twin-layer system

3.5 Bench-Scale Treatment: Semi-Continuous Mode

S. rubescens grew well with modified BG-11 medium on the bench-scale twin-layer system in three consecutive cycles. The chlorophyll *a* content of *S. rubescens* increased from the beginning to the end in each treatment cycle. About 1.7 ± 0.2, 4.2 ± 1.2, 6.9 ± 1.2 and 8.4 ± 1.4 μg Chl *a* cm^{-2} were recorded at the beginning, end of the first, second and third cycle, respectively. The overall Chl *a* productivity (*Chlap*) throughout the experiment was 0.56 μg cm^{-2} day^{-1} for *S. rubescens*, which was lower than 1.22 μg cm^{-2} day^{-1} in the last experiment with the same algae.

The residual phosphate in the medium plotted time (day) is shown in Figure 3-9 (a). Phosphate was removed continuously from modified BG-11 medium by *S. rubescens* within the three cycles from a starting concentration of about 3 mg l^{-1} to about 1 mg l^{-1} at the end of each cycle. The removal efficiencies of phosphate at the end of each cycle are summarized in Table 3-8. The percentage of removal of phosphate-P (*Pe*) by *S. rubescens* was 48.9%, 61.1% and 44.6% during the first, second and third cycle, respectively. While, the removal of phosphate-P by control was marked in the first cycle (13.6%), reduced gradually in the following two cycles, and ended with a minor reduction of 0.6% in the third cycle.

The simultaneous ammonium removal is shown in Figure 3-9 (b). Ammonium was reduced from about 18 mg l^{-1} to 7.9 ± 0.1, 5.5 ± 0.8 and 10.9 ± 2.4 mg l^{-1} at the end of first, second and third cycle, respectively. Considering the removal efficiency of ammonium-N (*AMe*), it increased slightly from 57.9% in the first cycle to 67.4% in the second cycle and dropped back to 39.8% in the third cycle (Table 3-8). In the blank control, ammonium was reduced less than *S. rubescens* throughout the experimental cycles. Residual ammonium in the control decreased gradually in the first cycle; showed a lag phase, then decreased sharply in the second cycle; and deceased rapidly, then increase again to the same level as the starting concentration in the third cycle (Figure 3-9, b).

Figure 3-9 (c) shows that, in the first and second treatment cycles, removal of nitrate from medium by *S. rubescens* were very similar with the control treatment within three days. In these two cycles, nitrate in the medium decreased only in the last day (day 4 and day 8, respectively) of the cycles. In the third cycle of the experiment, there was no significant difference ($p<0.05$) between the means of control and microalgae. The pronounced lag phase of nitrate removal was similar with the results reported in the last experiment by *C. vulgaris*

and *S. rubescens*, where a lag phase of about two days and four days were recorded. In the control, the nitrate concentrations also increased slowly from about 3 mg l^{-1} to > 4 mg l^{-1} during the experimental period.

Table 3-8. Phosphate and ammonium removal efficiencies at the end of each treatment cycle of the bench-scale treatment with semi-continuous mode

	Phosphate-P			Ammonium-N		
Cycles	1st	2nd	3rd	1st	2nd	3rd
S. rubescens	48.9	61.1	44.6	57.9	67.4	39.8
Control	13.6	3.8	0.6	36.9	26.3	0

Phosphorus and total nitrogen (ammonium + nitrate) depletion from modified BG-11 medium at the end of each treatment cycle is summarized in Table 3-9. The difference between *S. rubescens* and control showed the actual uptake of P and N exclusively by algae. The average uptake of P and N was 1.22 mg P and 7.66 mg N per cycle respectively, or 0.30 mg P day^{-1} and 1.9 mg N day^{-1} respectively. The N/P ratio could be calculated as 6.3, which was lower than 7.1 in the last experiment.

Table 3-9. Phosphorus and total nitrogen balance (mg) of the bench-scale treatment with semi-continuous mode

	P depletion from medium (mg)			Total N depletion from medium (mg)		
	S. rubescens	Control	*S. rubescens*-control	*S. rubescens*	Control	*S. rubescens*-control
1st	1.33	0.37	0.96	11.4	5.97	5.43
2nd	1.72	0.11	1.61	12.62	3.36	9.26
3rd	1.12	0.01	1.11	8.12	-0.87 (N↑)	8.99
Mean	---	---	1.22	---	---	7.66

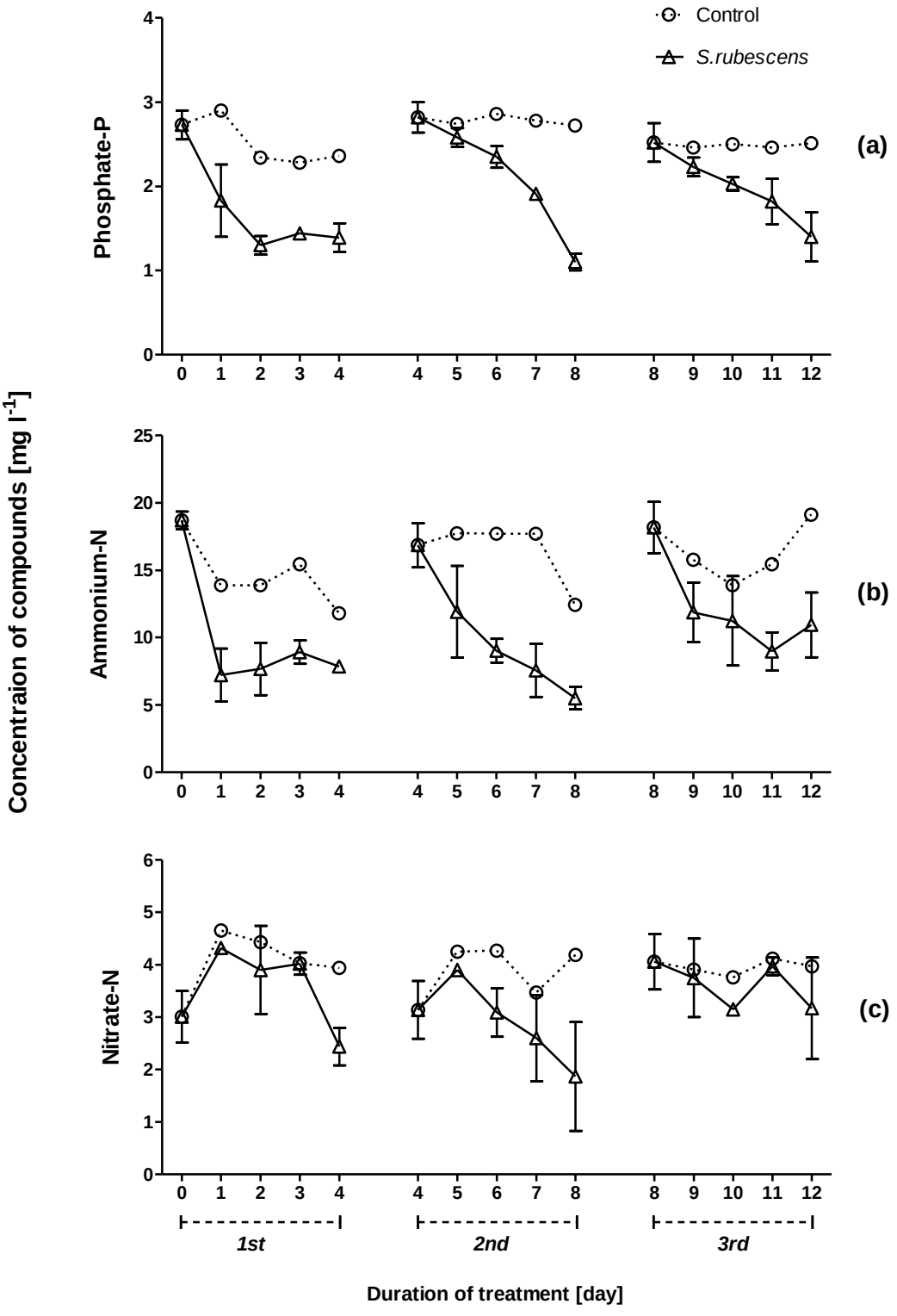


Figure 3-9. Residual concentrations of Phosphate-P (a), Ammonium-N (b) and Nitrate-N (c) in modified BG-11 medium by *S. rubescens* and control on bench-scale twin-layer system in three consecutive cycles

3.6 Large-Scale Wastewater Treatment

3.6.1 Experimental Conditions

3.6.1.1 pH

The pH values of Waris-H medium, original and treated municipal wastewater were measured daily *in situ* throughout the experiment. Waris-H medium was well buffered, and pH was between 7.0 and 7.5 during the cultivation period (day 0 to day 22). Municipal wastewater (from Frechen Kläranlage) treatment with secondary settled wastewater and secondary wastewater with additional phosphorus was conducted for eight consecutive cycles, each lasted one day. Recorded pH during the treatment is exhibited in Figure 3-10. The pH of the secondary settled wastewater was between 7.5 and 8.8 after one day's treatment with *Senedesmus rubescens* (M2630) on twin-layer system. And the pH always increased slightly (8.6-9.0). The similar rise was also found with secondary settled wastewater with additional phosphorus, where the pH increased from the beginning at 8.2-8.5 to 8.4-9.1 after one day's treatment. The treatment with the wastewater of denitrification tank and Bio-P tank was conducted for four consecutive cycles, each lasted two days. pH of the wastewater from denitrification tank was also alkaline, about 7.9 to 8.3. After one day's treatment with twin-layer system, the pH value increased to 8.5-9.5. After two days' treatment, the pH did not increased further (only very slight+0.1 in the first and second cycle) and kept 8.6-9.1. The tendency of pH in the wastewater from Bio-P tank showed similar patterns in four treatment cycles. It was slightly alkaline at the beginning (7.4-7.8), increased to 8.5-9.3 after day 1, and however, reduced to 8.2-8.5 after day 2.

3.6.1.2 Temperature

Temperature inside the greenhouse, above the surface of immobilized *S. rubescens*, was recorded between 18 and 32 ℃ during the experiment. Temperature of the water medium (Waris-H medium and municipal wastewater) was between 15 and 27 ℃ during the experiment.

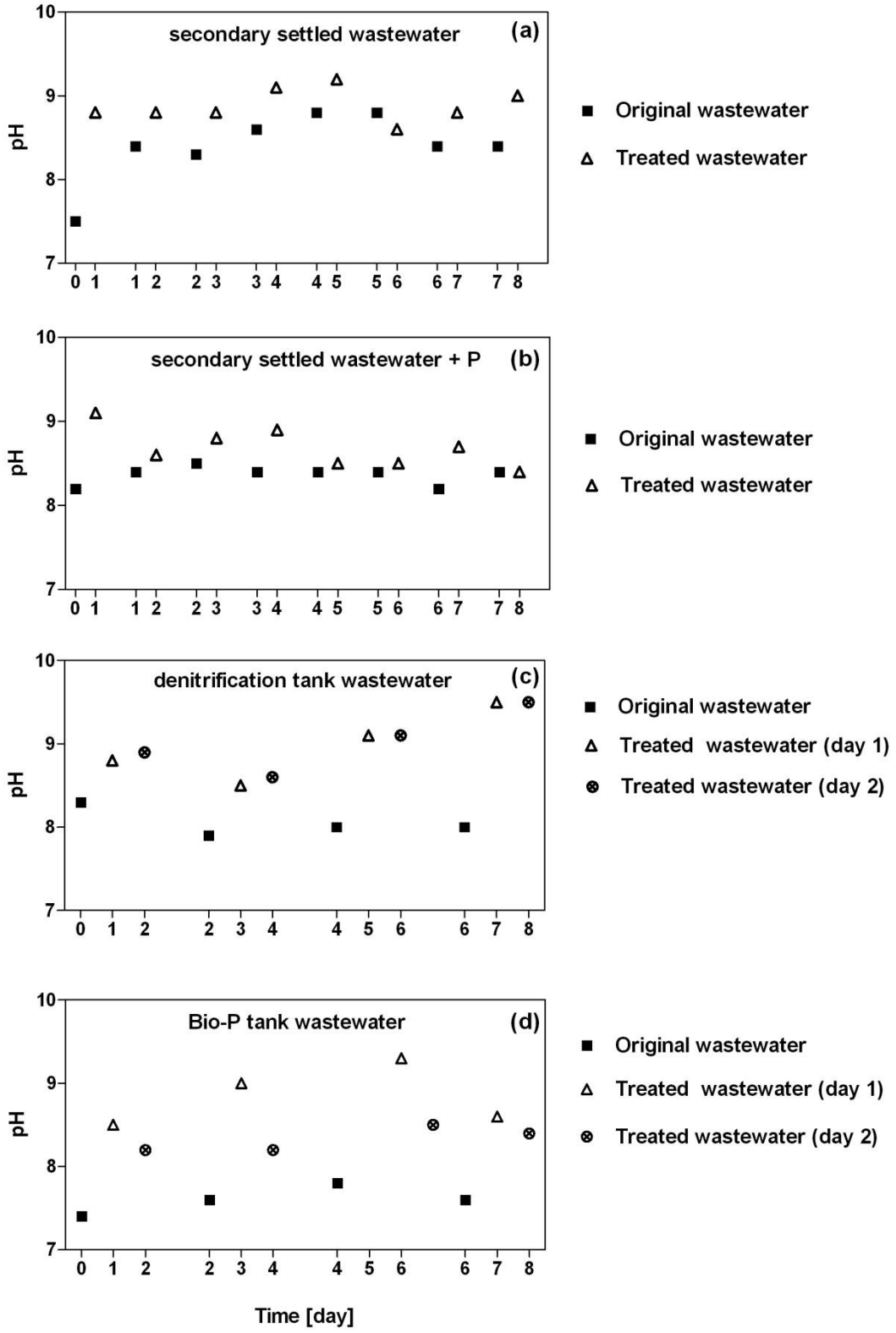


Figure 3-10. pH of the original and treated wastewater in large-scale wastewater treatment, (a)secondary settled wastewater, (b) secondary settled wastewater with additional phosphorus, (c) denitrification tank wastewater and (d) Bio-P tank wastewater

3.6.1.3 Illumination

Illumination irradiance above the surface of immobilized microalgae on each twin-layer module was recorded in the daytime throughout the experiment. In general, the recorded illumination was between 22-220 μE m^{-2} s^{-1} during the daytime. The surface facing west (front side) obtained higher light intensity than the surface facing east (back side) on the same module of twin layer. And the west side of the first twin-layer module gained highest light intensity (110-220 μE m^{-2} s^{-1}). The second module was in the second place (30-150 μE m^{-2} s^{-1}). And the third module was in the last place (30-60 μE m^{-2} s^{-1}). The difference of illumination irradiance was not obvious for the three twin-layer modules facing east (Table 3-10).

Table 3-10. Illumination irradiance (μE m^{-2} s^{-1}) above the three twin-layer modules in large-scale wastewater treatment

	Module 1	Module 2	Module 3
West (Front side)	110-220	30-150	30-60
East (Back side)	22-42	17-35	30-40

3.6.2 Microalgal Growth

Figure 3-11 shows the growth curves of *S. rubescens* with respect to dry weight and Chl *a* obtained from large-scale wastewater treatment experiment. During the treatment time of 54 days, dry weight of *S. rubescens* exhibited a linear growth pattern (Figure 3-11, a). It increased from 2 to 25.5±4.3 g m^{-2} (n=3) on the front side (west) of twin layers and from 2 to 15.8±1.5 g m^{-2} (n=3) on the back side (east) of twin layers when cultivated with Waris-H medium for 22 days. Afterwards, the twin-layer system was exposed to the municipal wastewater collected from different treatment processes of Frechen MWTP for 32 days. The dry weight of *S. rubescens* continuously increased to 73.7±3.5 on the front side and to 45.8±5.0 g m^{-2} on the back side of twin layer. The overall dry weight productivity (*DWp*) was 1.05 in average, and 1.3 and 0.8 g m^{-2} day^{-1} for the front side and back side, respectively. When supplying Waris-H medium, the *DWp* of the front side and the back side was 1.07 and 0.63 g m^{-2} day^{-1} respectively, and when wastewater was applied, the *DWp* was 1.51 and 0.94 g m^{-2} day^{-1} respectively (Table 3-11).

It could also be observed from Figure 3-11 that, from supplying Waris-H medium to applying secondary settled wastewater with additional phosphorus, the *DWp* was even. The even growth was interrupted when the wastewater of denitrification tank was applied, i.e. *DWp* decreased. Afterwards, the wastewater of Bio-P tank was applied, and the *DWp* increased considerably in 8 days (Figure 3-11, a).

The time course effect of Chl *a* also exhibited a linear pattern (Figure 3-12, b). It increased from 1.4 to 19.0±4.8 g m^{-2} on the front side and from 1.4 to 12.3±2.5 g m^{-2} on the back side of twin layers when cultivated with Waris-H medium for 22 days. Afterwards, Chl *a* of *S. rubescens* increased to 46.4±3.5 and 28.9±5.6 g m^{-2} on the front side and the backside respectively of twin layers at the end of the experiment (day 54). However, the linear correlation coeffeciencies (r^2) of Chl *a* on both sides of twin layers (0.8791 and 0.9034) were lower than those calculated from dry weight curves (0.9708 and 0.9805), (Table 3-11). The reason is when supplying the wastewater of denitrification tank, the Chl *a* showed sharp decrease in 8 days. However, when the wastewater from Bio-P tank was applied, the Chl *a* increased rapidly again. This phenomenon could be observed on both sides of twin layers (Figure 3-11, b).

The overall Chl *a* productivity (*Chlap*) was 0.83 and 0.51 µg cm^{-2} day^{-1} for the front side and back side respectively. When supplying Waris-H medium, the *Chlap* of the front side and the back side was 0.80 and 0.49 µg cm^{-2} day^{-1} respectively. When wastewater was applied, the *Chlap* was 0.86 and 0.51 µg cm^{-2} day^{-1} respectively (Table 3-11).

Moreover, statistical test was performed for the dry weight of the front sides of the three modules of twin-layer. One-way ANOVA showed there was no significant difference among the means of dry weight (p<0.05), although the light intensity above the immobilized microalgae was different (Table 3-10).

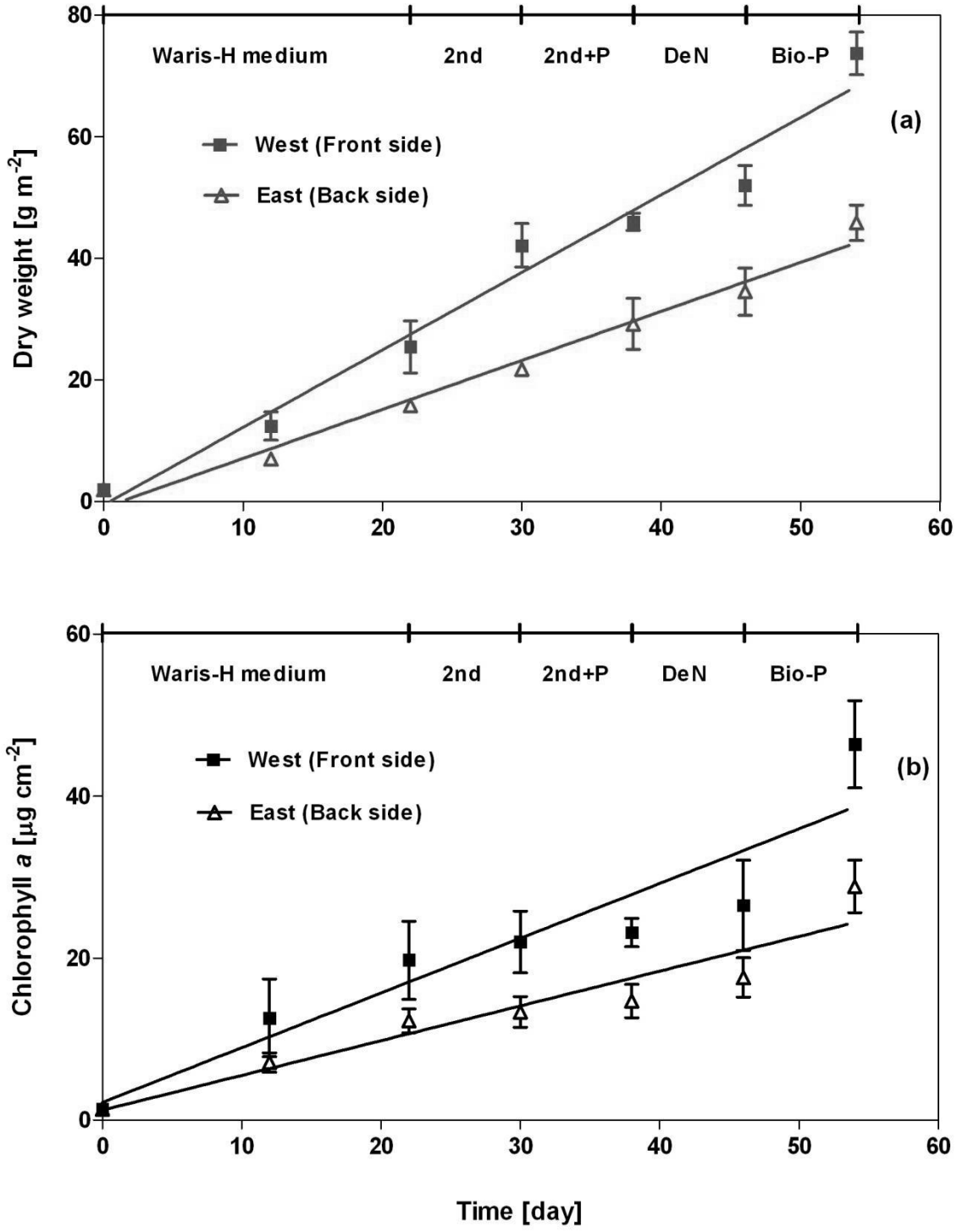


Figure 3-11. Growth curves of *S. rubescens* on large-scale twin-layer system with (a) dry weight (±SD, n=3; g m^{-2}) and (b) chlorophyll *a* (±SD, n=3; µg cm^{-2}). The water medium supplied to the microalgae was Waris-H medium, secondary settled wastewater (2nd), secondary settled wastewater with additional phosphorus (2nd+P), denitrification tank wastewater (DeN) and Bio-P tank wastewater (Bio-P)

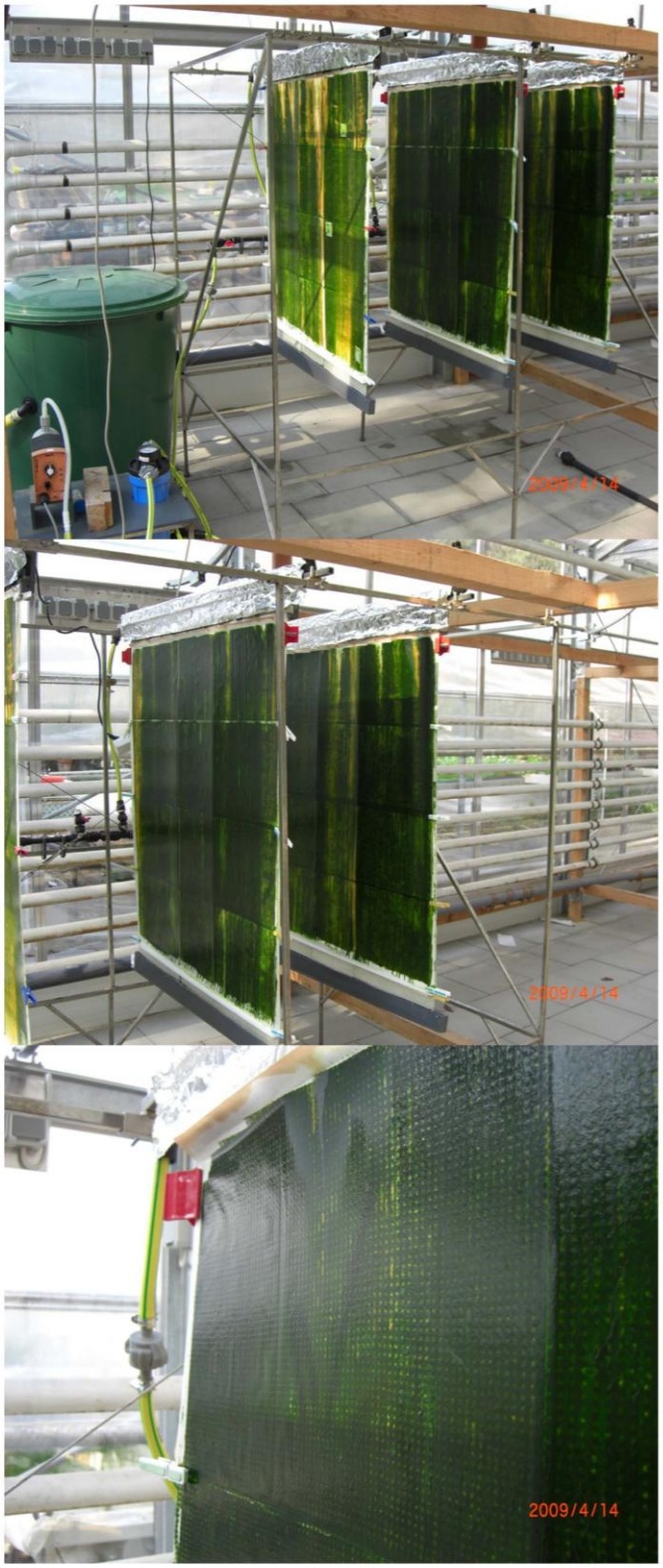


Figure 3-12. Photographs of *Scenedesmus rubescens* grown on large-scale twin-layer system in greenhouse, (a) the overview of the system, (b) second and third module, and (c) *S. rubescens* grown on the second module

Table 3-11. Results of linear regression analyses of dry weight and Chl *a* curves of *S. rubescens* grown on large-scale twin-layer system

	Dry weight				Chlorophyll *a*			
	r^2	*DWp* (g m^{-2} day^{-1})			r^2	*Chlap* (µg cm^{-2} day^{-1})		
		Overall	Waris-H	Wastewater		Overall	Waris-H	Wastewater
Front	0.9708	1.3	1.07	1.51	0.8791	0.83	0.80	0.86
Back	0.9805	0.8	0.63	0.94	0.9034	0.51	0.49	0.51
Mean		1.05	0.85	1.23		0.67	0.65	0.69

3.6.3 Municipal Wastewater Treatment

3.6.3.1 Control

There was only one large-scale twin-layer system available in the greenhouse. Therefore, one control experiment was conducted for two days with the wastewater of Bio-P tank using twin-layer system with nylon filter and glass fibre mesh but without *S. rubescens*. Phosphate-P at day 0 was 2.53 mg l^{-1} and kept stable after one day's treatment. However, it decreased to 2.07 mg l^{-1} at the end of day 2. In the same experiment, removal of ammonium-N from wastewater was also determined. Ammonium levels dropped in one day from 10.63 to 6.97 mg l^{-1} (presumably related to NH_3 formation and release into the atmosphere) and increased to 7.62 mg l^{-1} at the end of day 2. Finally, nitrate-N was not significantly removed from wastewater by control, and the nitrate concentrations decreased slightly from 3.67 mg l^{-1} to 3.48 mg l^{-1} during the experiment (Figure 3-13).

During the experiment period, pH was also measured *in situ*. It increased from 7.7 slowly to 8.3 at the end of day one, and dropped a little bit in the second day to 8.2. The tendency of pH was similar comparing with the treatment with *S. rubescens*, where an increase of pH was reported in one day from 7.4-7.8 to 8.5-9.3, and afterwards decreased to 8.2-8.5.

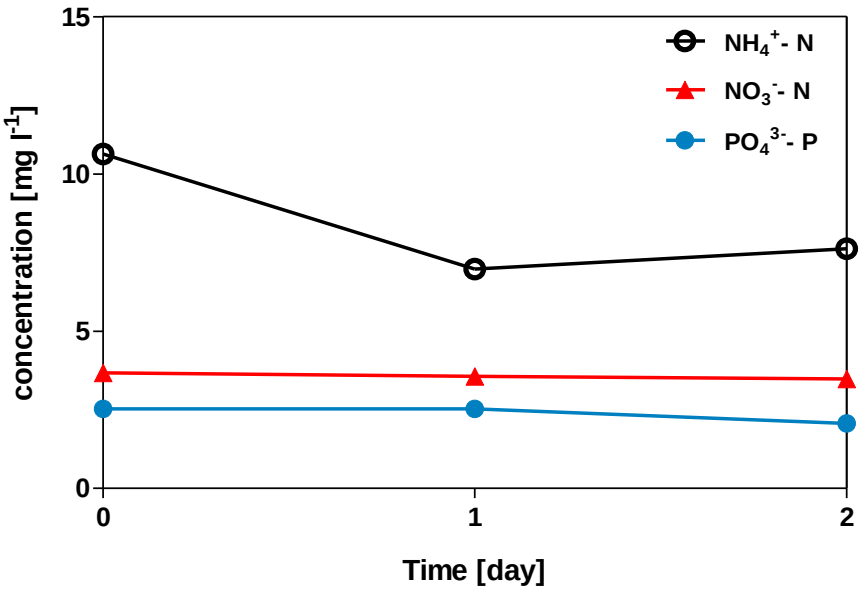


Figure 3-13. Phosphate-P, ammonium-N and nitrate-N concentrations (mg l^{-1}) in control of large-scale wastewater treatment

3.6.3.2 Secondary Settled Wastewater

As a result of the nature of the municipal wastewater, there were variations in the concentration of phosphate, ammonium and nitrate in the wastewater used for the experiments. The secondary settled wastewater was characterized by low COD (12-33 mg O_2 l^{-1}), low phosphate (0.3-0.9 mg l^{-1}), low ammonium (0-0.3 mg l^{-1}) but relatively high nitrate (5.9-9.2 mg l^{-1}) amount.

The removal of phosphate from wastewater during 8 days is shown in Figure 3-14 (a). The curves of residual phosphate over time reflected a pronounced fall in each treat cycle in the phosphate concentration. Residual phosphate in the wastewater decreased from 0.3-0.9 mg l^{-1} to 0.05-0.22 mg l^{-1} after one day's treatment with the twin-layer system. The percentage of phosphate removal *Pe* was always more than 63% but in no case reached 100% elimination (Table 3-12). 55 litre wastewater was supplied to the twin-layer system every day. Therefore, total phosphate-P reduction with the twin-layer system could be calculated as 199 mg P in this treatment.

In the same experiment, removal of nitrate from secondary settled wastewater was also determined (Figure 3-14, b). This type of wastewater was collected after nitrification process

and secondary sedimentation. Therefore, the amount of nitrate was high (5.9-9.2 mg l^{-1}). At the end of the treatment cycles, nitrate concentrations in the wastewater were between 0.5 and 2.6 mg l^{-1}. The removal efficiency of nitrate could be calculated as $NOe_i = (NO_0 - NO_i)/NO_0 \times 100\%$, where NO_0 and NO_i were the concentrations of nitrate at the beginning and day *i* of the treatment. *NOe* in most cycles were more than 75%, except day 7, where the *NOe* was 62% (Table 3-12).

Finally, ammonium was also removed from wastewater by twin-layer system (Figure 3-14, c). The amount of ammonium was rather low in this type of wastewater. In the first four cycles, ammonium was only minor (<0.03 mg l^{-1}) in the wastewater. After the treatment, NH_4^+- N was undetectable. In the last four cycles, most ammonium was removed from the wastewater.

Total nitrogen (NH_4^+-N and NO_3^--N) reduction with twin-layer system in eight consecutive cycles can be calculated as 2787 mg N.

Table 3-12. Removal efficiencies of phosphate and nitrate from secondary settled wastewater

Cycles	1st	2nd	3rd	4th	5th	6th	7th	8th
Pe (%)	82	76	70	72	63	71	70	83
NOe (8%)	93	82	79	90	76	91	62	91

Table 3-13 summaries the concentration of COD in the secondary settled wastewater during eight cycles. In most treatment cycles, COD increased, to a small extent, in the wastewater, with only one exception at day three, where the COD amount decreased from 32.3 to 23.8 mg O_2 l^{-1}.

Table 3-13. COD in the secondary settled wastewater

Cycles	1st	2nd	3rd	4th	5th	6th	7th	8th
COD original (mg O_2 l^{-1})	32.5	21.6	32.2	22.2	24.4	14.1	12.2	24.1
COD treated (mg O_2 l^{-1})	40.0	21.9	23.8	31.3	30.3	24.4	15.6	29.4

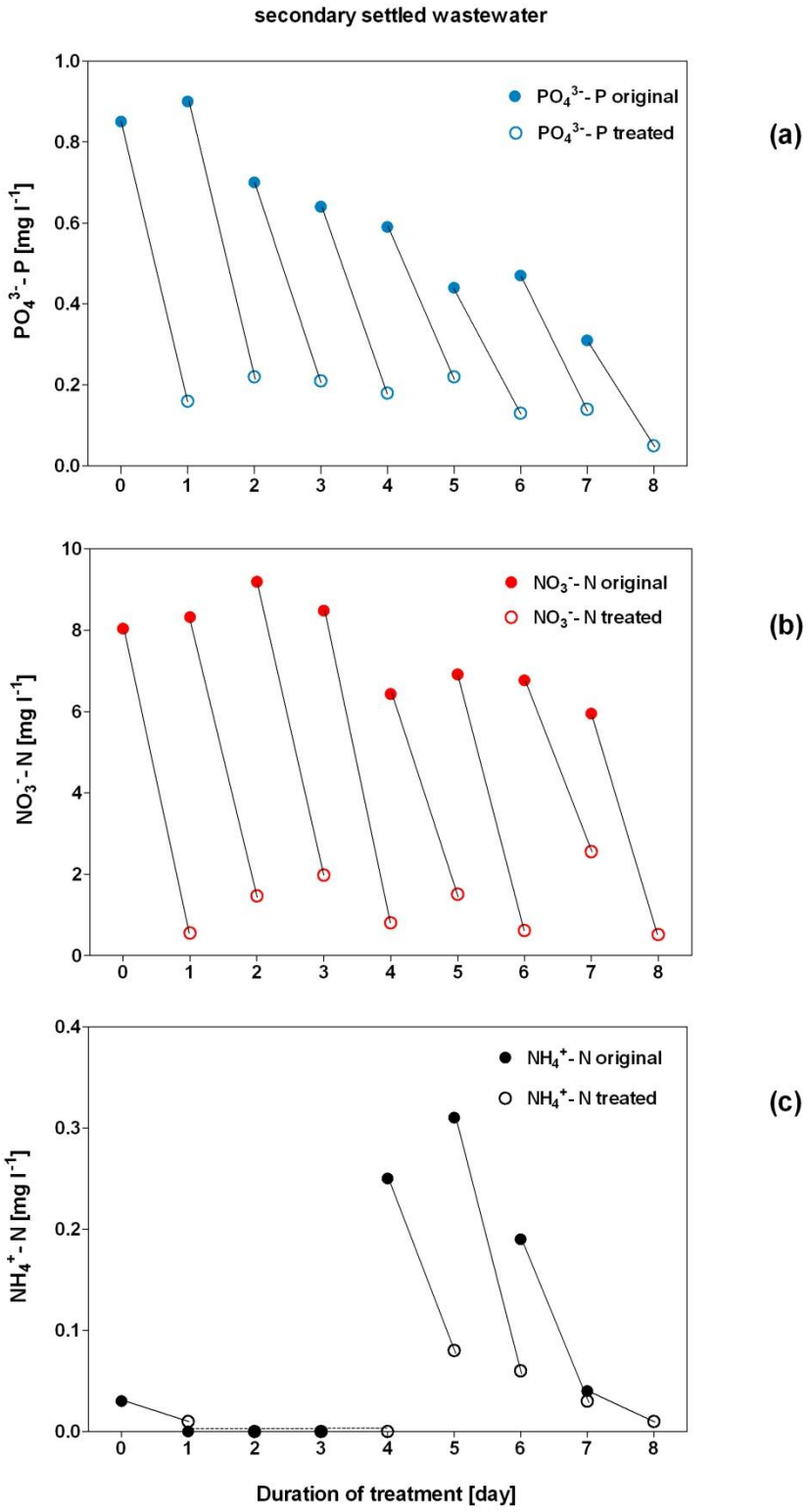


Figure 3-14. Residual concentrations of Phosphate-P (a), Nitrate-N (b) and Ammonium-N (c) in the secondary settled wastewater in eight consecutive cycles, each lasted one day; ● represents the concentrations in the original wastewater, and ○ represents the concentrations in the treated wastewater

3.6.3.3 Secondary Settled Wastewater with Additional Phosphorus

Secondary settled wastewater with additional phosphorus was prepared by adding 1.5 mg l^{-1} phosphorus (KH_2PO_4-P) to the secondary settled wastewater. The physical and chemical parameters of this wastewater are similar with secondary settled wastewater except higher phosphorus amount (1.7-2.4 mg l^{-1}).

Figure 3-15 (a) shows the residual phosphate concentrations in the wastewater. Phosphate decreased significantly within all the treatment cycles from 1.7-2.4 mg l^{-1} to < 1 mg l^{-1} (0.4-0.8). With respect to the removal efficiencies, over 55% and up to 81% phosphate was removed by the twin-layer system within one day (Table 3-14). In this experiment, 55 litre wastewater was supplied to the twin-layer system every day. Therefore, total phosphate reduction with twin-layer system could be calculated as 624 mg P, which was much higher than 198.5 mg P in the last experiment with secondary settled wastewater. Higher phosphate reduction was due to the additional P supply in this experiment.

Nitrate consumption pattern was similar with the last experiment although the concentration of nitrate in the original wastewater collected from WWTP in this experiment was lower (4.4-7.2 mg l^{-1}). In all the treatment cycles, nitrate decreased significantly from 4.4-7.2 mg l^{-1} to 0.4-1.7 mg l^{-1} after one day (Figure 3-15 b). The removal efficiency of nitrate (*NOe*) was between 70% and 94% during the experiment period (Table 3-14).

Ammonium concentrations were rather low (<0.05 mg l^{-1}) both in the original municipal wastewater and in the treated wastewater. It was difficult to ensure the quality of measurement due to the measurement limitation. Therefore, the results would not be reported here. Total nitrogen (NH_4^+- N and NO_3^-- N) reduction with twin-layer system for eight consecutive cycles could be calculated as 2136 mg N, which was lower than 2787 mg N in the last experiment with secondary settled wastewater. It was due to the lower NO_3^-- N concentration in the original wastewater in this batch.

Table 3-15 summaries the concentration of COD in the secondary settled wastewater with additional phosphorus during eight cycles. The COD variation was difficult to be summarized with a pattern, in some cases, COD increased (cycle 1 and 8) or kept stable (cycle 2, 3 and 4) in the wastewater, while in the other cases, COD decreased in the wastewater (cycle 4, 6 and 7).

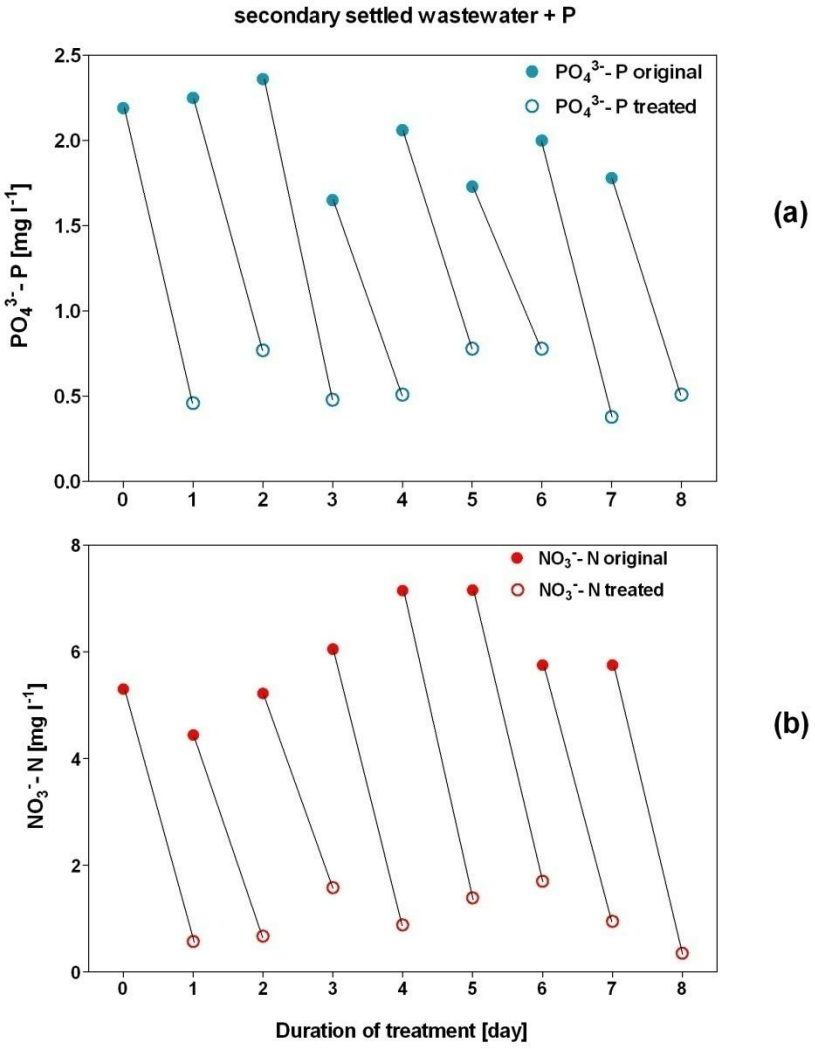


Figure 3-15. Residual concentrations of Phosphate-P (a) and Nitrate-N (b) in the secondary settled wastewater with addition phosphate in eight consecutive cycles, each lasted one day; ● represents the concentrations in the original wastewater, and ○ represents the concentrations in the treated wastewater

Table 3-14. Removal efficiencies of phosphate and nitrate from the secondary settled wastewater with additional phosphorus

Cycles	1st	2nd	3rd	4th	5th	6th	7th	8th
Pe (%)	79	66	80	69	62	55	81	71
NOe (%)	89	85	70	85	81	76	83	94

Table 3-15. COD in the secondary settled wastewater with additional phosphorus

Cycles	1st	2nd	3rd	4th	5th	6th	7th	8th
COD original (mg O_2 l^{-1})	20.0	21.9	20.9	31.3	50.0	45.6	41.9	18.8
COD treated (mg O_2 l^{-1})	27.8	21.3	20.9	30.9	45.9	26.6	23.4	25.0

3.6.3.4 Denitrification Tank Wastewater

The wastewater of denitrification tank applied to the microalgae had the initial COD, phosphate-P, ammonium-N and nitrate-N concentration of 18-26 mg O_2 l^{-1}, 1.41-2.64 mg l^{-1}, 1.22-2.97 mg l^{-1} and 0.31-0.97 mg l^{-1}, respectively. Phosphate concentration of this type of wastewater was similar with the one used in the last experiment. However, the nitrogen amount was rather low, which led to a low N/P ratio for this wastewater (0.6-2.3).

Figure 3-16 (a) shows the ability of immobilized *S. rubescens* in removing PO_4^{3-}- P from DeN wastewater in four consecutive cycles. As expected, PO_4^{3-}- P was efficiently removed from the medium by *S. rubescens* in four cycles. Continuous reduction of PO_4^{3-}- P was observed in two days' treatment. About 59-75% *Pe* was achieved in one day and further removal was recorded as 78-89% at the end of day two (Table 3-16). Total phosphorus reduction was 365 mg P in this treatment, which was much lower than 199 and 624 mg P recorded by the last two types of wastewater.

In the same treatment, nitrate was also reduced by the twin-layer system in four cycles continuously. Nitrate decreased from 0.31-0.97 mg l^{-1} to 0.10-0.17 mg l^{-1} after one day, and further reduced to 0.08-0.11 mg l^{-1} at the end of day two (Figure 3-16, b). Although the treatments were conducted for two days, ammonium was almost completely depleted in the first day of the treatment cycles (Figure 3-16, c). And it kept stable in the second day. Total nitrogen reduction by the twin-layer system was 469 mg in this experiment, which was much lower than 2787 mg N for secondary settled wastewater and 2136 mg N for secondary settled wastewater with additional P.

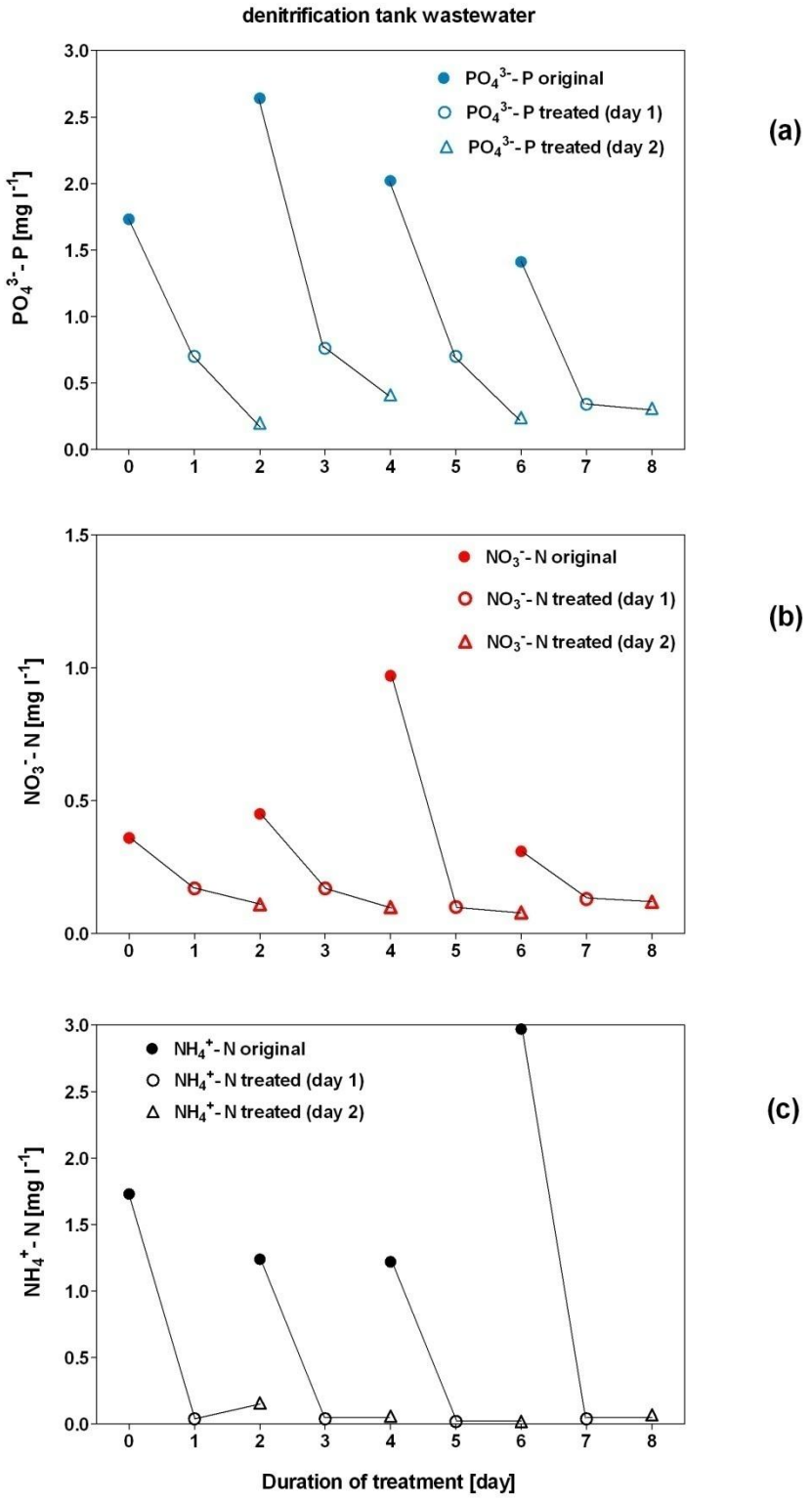


Figure 3-16. Residual concentrations of Phosphate-P (a), Nitrate-N (b) and Ammonium-N (c) in the denitrification tank wastewater in four consecutive cycles, each lasted two days; ● represents the concentrations in the original wastewater, ○ represents the concentrations in the treated wastewater at day 1 and Δ represents the concentrations in the treated wastewater at day 2

Finally, COD concentrations during the experiment were summarized in Table 3-17. COD kept comparably stable from the starting concentration of 18.1-26.3 mg O_2 l^{-1} to 16.3-21.3 mg O_2 l^{-1} in day one and afterwards increased to 18.8-39.4 mg O_2 l^{-1} in day two.

Table 3-16. Removal efficiencies of phosphate, nitrate and ammonium from the denitrification tank wastewater

Cycles	1st		2nd		3rd		4th	
Day	1	2	3	4	5	6	7	8
Pe (%)	59	89	71	84	65	88	76	78
NOe (%)	53	69	62	78	89	91	58	62
AMe (%)	98	91	97	95	98	98	99	98

Table 3-17. COD in the denitrification tank wastewater

Cycles	1st	2nd	3rd	4th
COD original (mg O_2 l^{-1})	26.3	18.1	19.4	21.3
COD day 1 (mg O_2 l^{-1})	21.3	21.3	21.9	16.3
COD day 2 (mg O_2 l^{-1})	39.4	39.4	18.8	34.4

3.6.3.5 Biological Phosphorus Tank Wastewater

The removal of phosphate from the wastewater of Bio-P tank during 8 days, in four consecutive cycles, is shown in Figure 3-17. Phosphate was efficiently removed from the wastewater by *S. rubescens* within one day from a starting concentration of 2.7-4.6 mg l^{-1} to < 2 mg l^{-1} (Figure 3-17, a). Afterwards, phosphate was further reduced to < 1 mg l^{-1} at the end of day two. The *Pe* was about 55-86% at day one and 58-94% at day two for *S. rubescens* (Table 3-18). In the control, the same type wastewater has been applied. The concentration of phosphate-P plotted time in the control is also presented in Figure 3-17 (a). Although the concentrations of phosphate in control were lower than the ones applied in the treatment with *S. rubescens* (due to the natural of municipal wastewater), it could be observed that in control phosphate decreased only slightly over the experimental period comparing with applying

microalgae. Total phosphorus reduction was 636 mg P in this treatment, which was highest among all the treatments with different wastewater.

In the same experiment, removal of ammonium from Bio-P wastewater was also determined (Figure 3-17, b). Ammonium was removed rapidly by *S. rubescens* within one day from 7.8-13.9 mg l^{-1} to < 0.4 mg l^{-1}, and further reduced to <0.1 mg l^{-1} at day 2. Ammonium removal efficiencies were more than 96% in all the cases (Table 3-18). In the control, ammonium levels dropped much less than the treatment with *S. rubescens*. And only about 3 mg l^{-1} ammonium was removed from wastewater in two days.

Nitrate content was rather low (<0.2 mg l^{-1}) in this type of wastewater, but some tendencies of nitrate in the wastewater still could be observed. Nitrate concentrations decreased in first cycle, kept stable in the second cycle, and however, increased about 0.2-0.3 mg l^{-1} in the first days of the third and forth cycles, but decreased in the second days.

Total nitrogen reduction by twin-layer system was 2434 mg in this experiment, which was in the second place among all the treatments with different wastewater.

Finally, COD concentrations during the experiment were summarized in Table 3-19. In the first and second cycles, COD kept comparably stable in day one (around 90 mg O_2 l^{-1}) and reduced to 35.9 and 42.2 mg O_2 l^{-1} in day two for the first and second cycles, respectively. In the third and forth cycles, COD kept stable in the wastewater.

Table 3-18. Removal efficiencies of phosphate and ammonium from the Bio-P tank wastewater

Cycles	1st		2nd		3rd		4th	
Day	1	2	3	4	5	6	7	8
Pe (%)	86	94	57	71	58	74	55	58
AMe (%)	100	100	98	99	96	99	96	99

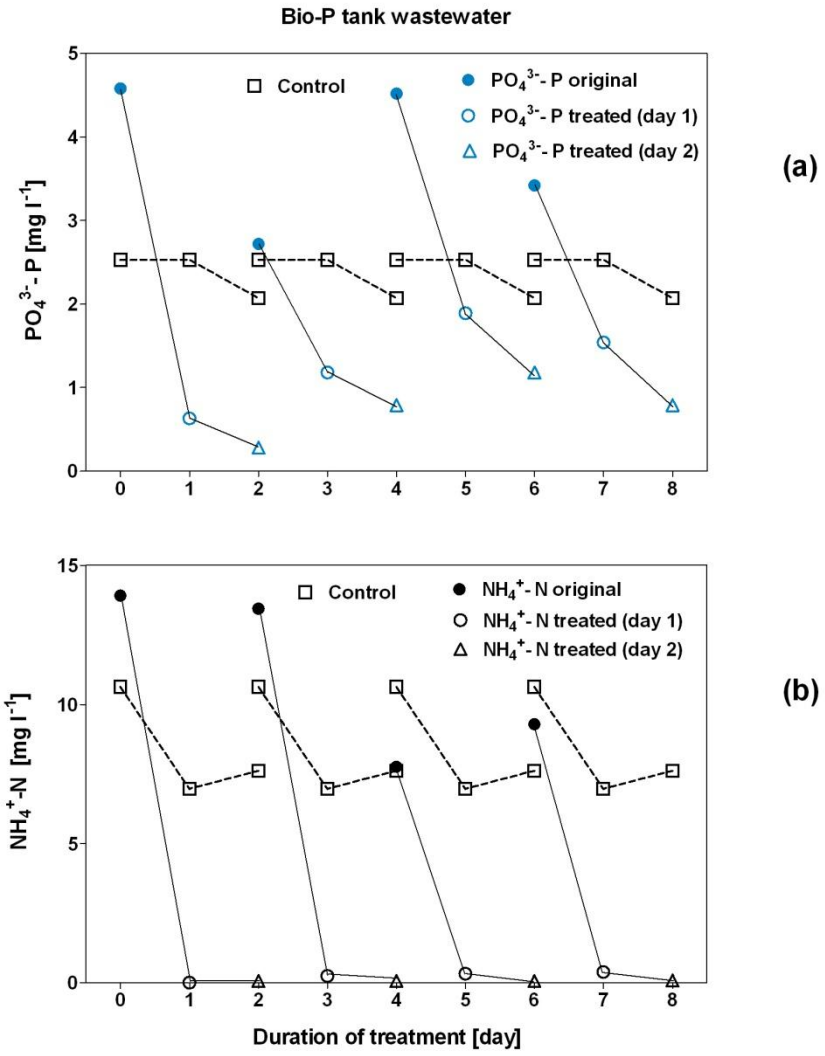


Figure 3-17. Residual concentrations of Phosphate-P (a) and Ammonium-N (b) in the Bio-P tank wastewater in four consecutive cycles, each lasted two days; ● represents the concentrations in the original wastewater, ○ represents the concentrations in the treated wastewater at day 1 and Δ represents the concentrations in the treated wastewater at day 2

Table 3-19. COD in the wastewater of Bio-P tank

Cycles	1st	2nd	3rd	4th
COD original (mg O_2 l^{-1})	93.8	96.9	28.1	25.0
COD day 1 (mg O_2 l^{-1})	92.2	93.8	26.3	26.9
COD day 2 (mg O_2 l^{-1})	35.9	42.2	25.0	24.4

3.6.3.6 Effect of Light/Dark Cycle on Nitrogen and Phosphorus Removal

Bio-P tank wastewater treatment was conducted for four consecutive cycles, and each cycle lasted two days. In the last cycle, wastewater was taken continuously at the beginning of the treatment and after 0,6,17,24,29,42 and 48 hours. This experiment was conducted in late May, and the light period in the greenhouse was approximately between 7:30 to 19:30 (http://www.sonnenuntergang.de). Figure 3-18 shows the effect of light/dark on phosphorus and ammonium removal by *S. rubescens* immobilized on twin-layer system. Phosphorus uptake by the algae was obvious light dependent as being indicated by zero uptakes from 17:00 to 23:00 in both days. Phosphorus concentrations kept at around 3.4 mg l^{-1} between 17:00 and 23:00 in day one and 1.5 mg l^{-1} in the same time intervals in day two. Although no sample was taken in the dark period after 23:00, this tendency was clear. During the first light period, phosphorus was reduced rapidly to 1.5 mg l^{-1} at 17:00. In the following dark period, no phosphorus reduction was observed. However, phosphorus uptake could be re-established when the microalgae was illuminated again. Phosphorus decreased further to 0.79 mg l^{-1} at 17:00 (day two).

Ammonium depletion from the wastewater exhibited a different pattern compared with phosphorus. No illumination dependence of ammonium uptake by microalgae was observed (Figure 3-18, b). Ammonium concentration in the wastewater decreased continuously during 48 hours with sharp reduction at the beginning from 9.3 to 5.2 mg l^{-1} from 17:00 and 23:00 (dark) and further decreased from 5.2 to 0.4 mg l^{-1} from 23:00 to 17:00 (dark and light). In the second day, ammonium was continuously reduced regardless of illumination to 0.08mg l^{-1}.

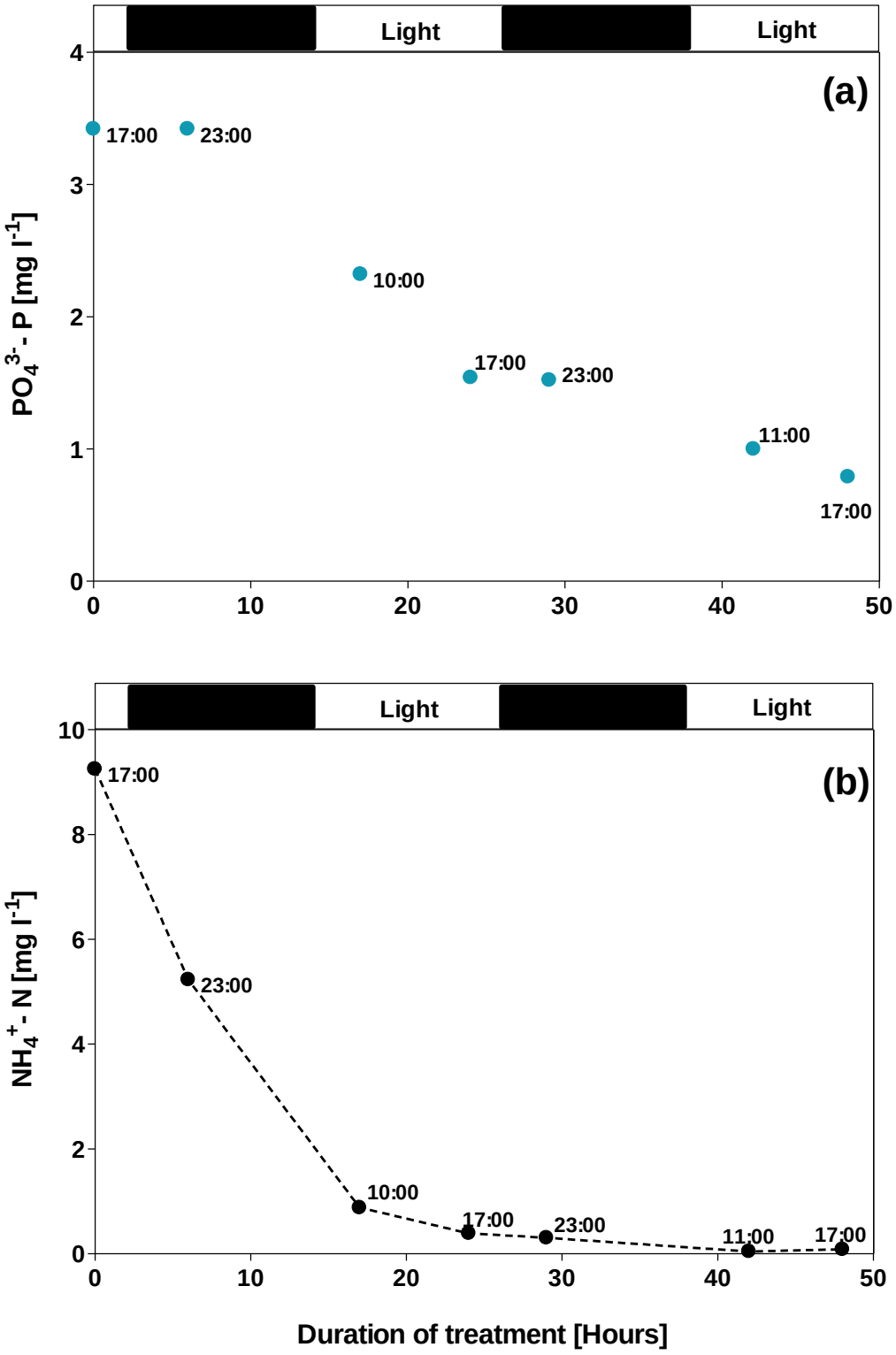


Figure 3-18. Effect of light/dark cycle on the (a) Phosphate-P and (b) Ammonium-N removal by *S. rubescens* immobilized on twin-layer system, a residence time of 48 hours was used

3.6.4 Reduction of Organic Substances in Addition to Nitrogen and Phosphorus

Both *Chlorella protothecoides* and *Euglena gracilis* could grow on the bench-scale twin-layer system with nylon filter and GFM (10×10 mm) served as substrate and source layer respectively. During the period of pre-cultivation with Waris-H for 7 days and synthetic wastewater treatment for 6 days, dry weight of *C. protothecoides* and *E. gracilis* increased from 1.1 to 9.4 g m^{-2} and from 1.7 to 8.0 g m^{-2}, respectively. Moreover, *Chlap* during 13 days were 0.63 and 0.73 µg cm^{-2} day^{-1} for two algae, which were higher than *Chlap* achieved by cultivating them on the horizontal twin-layer system in Petri-dishes during screening experiments with synthetic secondary wastewater or Waris-H medium.

The residual phosphate-P in the medium plotted time (day) is shown in Figure 3-19 (a). Phosphate was removed continuously from synthetic wastewater by *C. protothecoides* and *E. gracilis* from a starting concentration of about 4.4-4.6 mg l^{-1} to < 0.2 mg l^{-1} at the end of day 6. One of the three replicates of *C. protothecoides* was not handled very well and fallen down from the source layer during the experiment. Therefore, the statistical test could not be performed to find out if the means of both algae were significant different. However, it is still possible to observe from Figure 3-19 (a) that the residual phosphate in the synthetic wastewater over time for two algae was similar, while, the concentration of phosphate for control was constant for the beginning and end of the treatment.

The simultaneous ammonium removal is shown in Figure 3-19, b. Ammonium was reduced from about 20-24 mg l^{-1} to < 10 mg l^{-1} at the end of the treatment. Although ammonium in the wastewater at day four by *C. protothecoides* was the same as control, this might due to the error by the measure. In the blank control, ammonium was reduced less than both algae throughout the experiment. Residual ammonium in the control decreased 20% from day 0 to three and then rose to the same level as the starting time (Figure 3-19, b).

The results of residual COD in the synthetic wastewater did not indicate positive uptake by either *C. protothecoides* or *E. gracilis*, as the tendency of COD for *E. gracilis* was similar to the control and great fluctuation of COD for *C. protothecoides* was observed (Figure 3-19, c).

Table 3-20. Dry weight and chlorophyll *a* of *C. protothecoides* and *E. gracilis* grew with synthetic secondary wastewater

	Dry weight			Chlorophyll *a*		
	Start ($g\ m^{-2}$)	End ($g\ m^{-2}$)	*DWp* ($g\ m^{-2}\ day^{-1}$)	Start ($\mu g\ cm^{-2}$)	End ($\mu g\ cm^{-2}$)	*Chlap* ($\mu g\ cm^{-2}\ day^{-1}$)
Chlorella protothecoides	1.1	9.4	0.63	4.0	12.2	0.63
Euglena gracilis	1.7	8.0	0.56	4.1	13.6	0.73

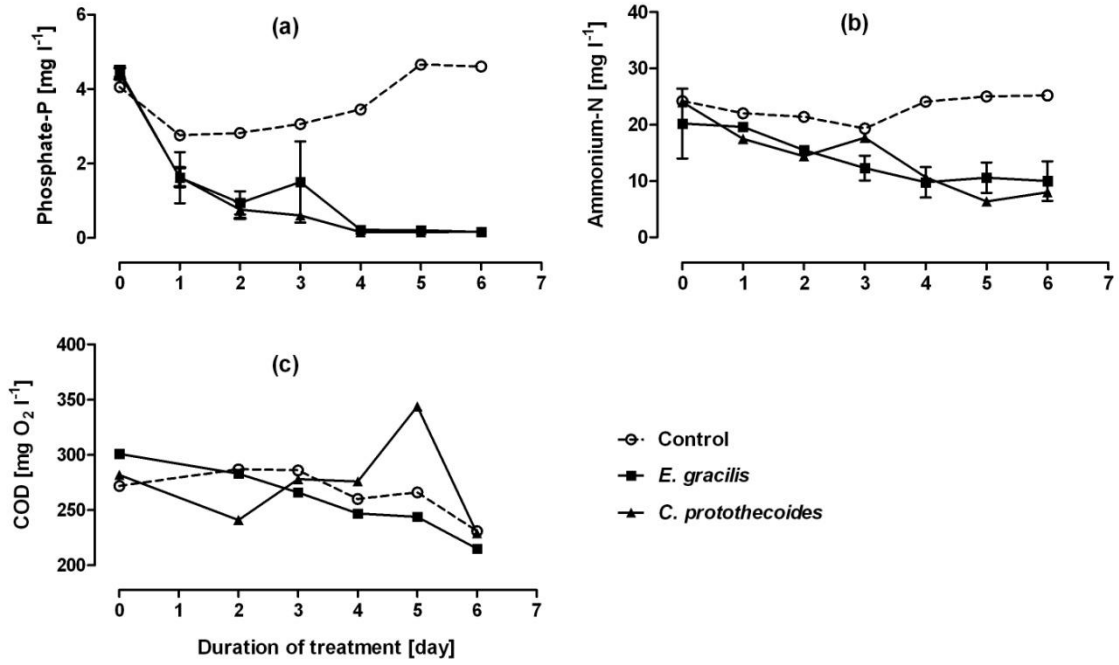


Figure 3-19. Residual concentrations of (a) Phosphate-P, (b) Ammonium-N and (c) COD in the synthetic secondary wastewater by *E. gracilis* and *C. protothecoides*, and control

3.6.5 Preferential Uptake of Nitrate as Nitrogen Source

Haematococcus pluvialis (M 0761/1), *Haematococcus pluvialis* (M 2072) and *Senedesmus rubescens* (M 2630) were washed carefully with distilled water and inoculated in 300 ml modified Waris-H medium. Figure 3-20 shows the residual concentrations of nitrate in modified Waris-H medium during the treatment. Clear lag phases of nitrate uptake were observed for three microalgae from day 0 to day 4, and nitrate was not reduced during this period in the medium. However, nitrate decreased obviously by *S. rubescens* from day 4 to

day 8. About 1.5 mg l^{-1} nitrate was reduced by *S. rubescens*. Both *Haematococcus* took up less nitrate compared with *S. rubescens*.

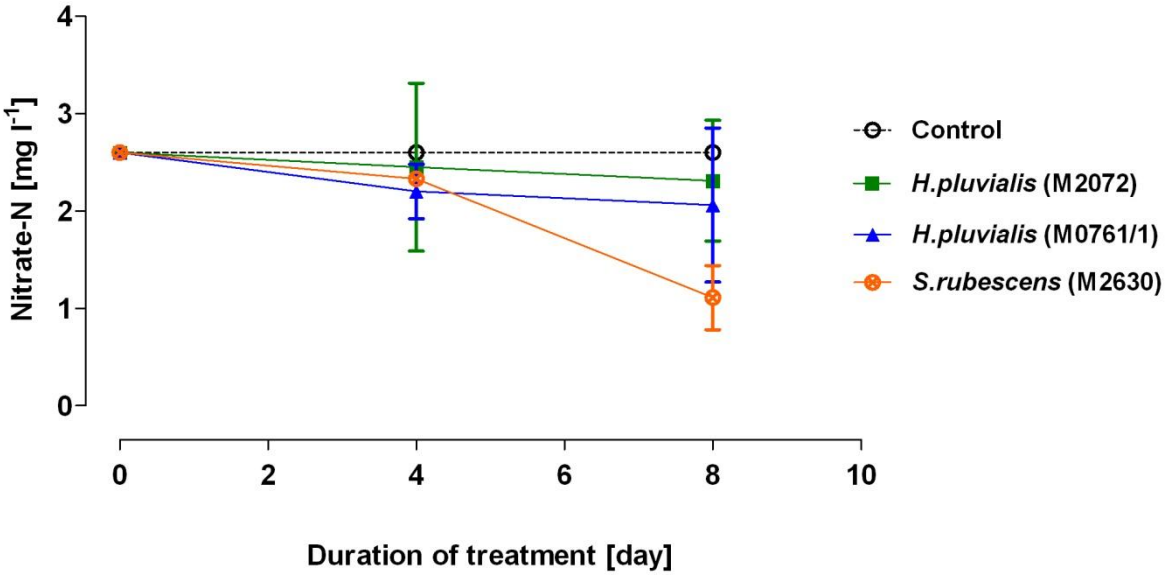


Figure 3-20. Residual concentrations of Nitrate-N in the modified Waris-H medium of *H. pluvialis* (M 0761/1), *H. pluvialis* (M 2072) and *S. rubescens*, and control

4. DISCUSSION

4.1 Microalgae for Nitrogen and Phosphorus Removal

Nitrogen and phosphorus removal from wastewater could be increased by numerous factors including selection of the most appropriate species. I considered that it is the most important factor for municipal wastewater treatment using microalgae. However, few studies have addressed this issue and no research has been done so far to screen microalgae for municipal wastewater treatment. On the contrary, most researches on nitrogen and phosphorus removal by immobilized algae have used a small number of algal strains especially *Chlorella vulgaris* (Mallick, 2002). Therefore, microalgae screening was conducted in this research to select the appropriate species, especially microalgae's ability to grow on twin-layer system with municipal wastewater, because one of the key issues for cost-effective nutrient removal using algae is the algae growth rate as it controls directly and indirectly the nitrogen and phosphorus removal efficiency (Olgu ń, 2003).

It is reasonable to assume that microalgae that naturally develop in wastewater and eutrophic environment, which are well adapted to the chemical composition and nutrient enrichment condition, should be more robust in wastewater and have higher phosphorus and nitrogen removal ability. For instance, *Scenedesmus intermedium* and *Nannochloris sp.* isolated from pig manure showed higher phosphorus and nitrogen removal rates than commercial species (Jiménez-Pérez et al., 2004). Lau et al. (1996) reported physiological acclimation for commercial cells in wastewater prior to their utilisation improved their nutrient removal efficiency. Moreover, many researches have been done to use single or multi microalgae for municipal, industry wastewater and manure treatment, and microalgal bioengineering e.g. *Botryococcus*, *Chlamydomonas*, *Chlorella*, *Euglena*, *Microthamnion*, *Navicula*, *Nitzschia*, *Scenedesmus*, *Stigeoclonium* and *Synedra*. (Vymazal, 1988, Mallick, 2002, Olgu ń, 2003, Metzger and Largeau, 2005, Hoffmann, 1998, Vilchez et al., 1997, Mulbry and Wilkie, 2001, Craggs et al., 1996, Kebede-Westhead et al., 2003, Órpez et al., 2009). In my research group, a small-scale twin-layer system, Phycomat (Nowack et al., 2005), has been applied to cultivate different microalgae. The microalgae grown in passive immobilization on the small-scale twin-layer system should also be able to grow on larger scale ones. Base on the considerations discussed above, the microalgae (to the genera level) chosen for screening experiments included: (1) the ones isolated from sewage treatment plant or sewage discharge

place; (2) the ones obtained from eutrophic environment; (3) the ones have been frequently used in the field of sewage treatment and microalgal biotechnology; (4) the ones have ability to grow on Phycomat. Therefore, 22 microalgae were selected from CCAC (Culture collection of Algae at the University of Cologne) and one *Scenedesmus* sp. was isolated from a sewage river in Tianjin, P.R China for the screening experiments. The subsequent microalgal screening included three steps.

Firstly, microalgal was grown in suspension. This is necessary for large scale application in the future because microalgae have to grow initially in suspension and afterwards be immobilized. Four microalgae, *Euglena adhaerens*, *Euglena deses*, *Synedra* sp. and *Synura uvella*, did not show obvious growth in suspension and thus excluded from selection.

Secondly, chlorophyll fluorescence measurement of the immobilized microalgae on nylon filters. The chlorophyll fluorescence is the measurement used mainly in the study of photosynthetic apparatus but nowadays is being frequently used as the tools to assess the general physiological state of photoautotrophic organisms since photosynthesis is often affected when plants experiment adverse conditions such as nutrient deficiency, extreme values in temperature, water deficiency or light stress (Krause and Weis, 1991). In this study, chlorophyll fluorescence was measured consistently for the microalgae immobilized on nylon filters with synthetic secondary wastewater and Waris-H (Waris-H+3V/Waris-H+Si) medium. The effective quantum yield of photosynthetic energy conversion, Yield=ΔF/Fm', the so-called Genty-parameter were calculated automatically by the fluorescence meter and used as the indicator to assess the general physiological state of the immobilized microalgae. It was clear that *Oscillatoria* sp. was not suitable to be immobilized on twin-layer system because ΔF/Fm' for both Waris-H+3V medium and synthetic secondary wastewater was always below 0.1. Low Chlorophyll *a* productivities (<0.1 μg cm^{-2} day^{-1}) for both media also confirmed this conclusion. Two diatoms, *N. communis* and *Navicula* sp. could grow on the nylon filters with Waris-H+Si medium, however, not with synthetic secondary wastewater. The synthetic wastewater might be deficient of silica, which is essential for the cell wall formation of diatoms. Microscopic observations also confirmed that there were no cell formation of *N. communis* and *Navicula* sp. in synthetic wastewater. Both ΔF/Fm' and Chlorophyll *a* productivities of two Euglenophyceae, *M. pseudonordstedtii* and *Trachelomonas* sp., in Waris-H medium and synthetic wastewater were low in comparison with the remainder strains. Therefore, they were also excluded from further screening. My conclusion here is that

the microalgae showed low ΔF/Fm' during 20 days' incubation were also low in Chlorophyll *a* productivity, and those strains were not suitable for wastewater treatment. Microalgae *B. terribilis* exhibited high physiological ability as indicated by ΔF/Fm' (0.8) throughout the incubation period, but the productivity of this strain in wastewater was low (0.06 μg cm^{-2} day^{-1}), which is obvious not favourable for large scale wastewater treatment. But this strain can be recommended in other fields of microalgal bioengineering. The remaining 13 strains were used afterwards for detailed screening.

In the final step, in order to avoid nutrients limitation on the growth of immobilized algae, the nylon filters were transferred to the fresh medium every three days. In addition, the growth experiments with synthetic wastewater were conducted in parallels employing both dry weight and Chlorophyll *a* as growth indictors, which lead to more accurate results. The results showed all the selected 13 strains could grow in synthetic secondary wastewater on nylon filters, however, with different growth rates. Five microalgae demonstrated both high dry weigh and Chlorophyll *a* productivity during 20 days' cultivation with synthetic secondary wastewater (Figure 3-7). I recommend using them in the future for wastewater treatment. Among them, four were isolated originally from the sewage field, and one was collected in supralittoral near the sea, where is enrich of nutrients. This again confirmed the advantage of using microalgae adapted in wastewater/nutrient enrich environment for wastewater treatment. *S. rubescens* (M2630) produced the highest dry weight and second highest Chlorophyll *a* in the synthetic wastewater (Figure 3-7), and was employed later for bench-scale and large-scale wastewater treatment. The large-scale wastewater treatment indicated that *S. rubescens* (M2630) could grow well on twin-layer system in four types of municipal wastewater collected from Frechen MWTP, and phosphorus, nitrate and ammonium were successfully removed from wastewater within short retention times. I see prospect of applying it for municipal wastewater treatment and for brackish/aquaculture wastewater treatment because it originally grew in supralittoral near the sea.

Among the five microalgae selected, four were isolated in Germany and France, where the temperature was moderate, and the other strain was collected in midsummer from a sewage river in Tianjin, China under high temperature (40 ℃ midday) and high isolation condition. In practice, this particular strain does not require high temperature for growth, and could normally grow at 24 ℃ under 20-40 μmol m^{-2} s^{-1} in immobilized stage. It could be applied in the future in tropical area for wastewater treatment.

In this study, *C. vulgaris*, the most frequent used specie for wastewater treatment, produced less dry weight in comparison with the other 12 strains. Of course, this might be attributed to our strain was different from *C. vulgaris* used in other researches, but, it still, to some extent, implied the importance of screening microalgae before the uptake experiment.

Table 4-1. Five microalgae strains show high dry weigh and Chlorophyll *a* productivity during screening experiment

Microalgae	Class	Origin
Scenedesmus rubescens (M2630)	Chlorophyceae	France, St.Luaire near Dinard (Bretagne) Isolated from a sample of *Rivularia bullosa* (Cyanobacteria), grow on *Lichina confinis* (lichen); collected in supralittoral; grow also in freshwater media
Chlamydomonas terricola	Chlorophyceae	Germany, Münster; squeezed sample from a sewage field; freshwater
Scenedesmus sp.	Chlorophyceae	China, Tianjin; Huayuan sewage river near Huayuan Xiaoqu; freshwater
Euglena anabaena	Euglenophyceae	Germany, Münster; squeezed sample from a sewage field; freshwater
Pediastrum boryanum	Chlorophyceae	Germany, Münster; squeezed material from a sewage field; freshwater

Moreover, Chl *a* productivity of *E. anabaena* in synthetic wastewater (1.11 µg cm^{-2} day^{-1}) was about two times higher than in Waris-H (0.50 µg cm^{-2} day^{-1}) and three times BG-11 (0.36 µg cm^{-2} day^{-1}). It implied that this alga might be able to take up organic substances in the wastewater for heterotrophic growth. Discovery of the heterotrophic microalgae is very important for the development of microalgal wastewater engineering because if microalgae could reduce organic substances in addition to phosphorus and nitrogen, the whole complex biological treatment processes of wastewater treatment plant will be simplified and integrated into one process.

Finally, Chl *a* productivity of *S. rubescens* (M2069) in BG-11 medium (1.49 µg cm^{-2} day^{-1}) was much higher than in modified Waris-H medium (0.81 µg cm^{-2} day^{-1}). This is attributed to high nitrate-N concentration in BG-11 medium favoured the growth of *Scenedesmus rubescens* (M2069). Comparably, ammonium-N is the major inorganic nitrogen source in

modified Waris-H medium, while nitrate was much lower. This strain could be possibly used to remove high nitrate content from nitrate enriched groundwater.

Table 4-2. Nitrogen and phosphorus sources in synthetic secondary wastewater, Modified Waris-H medium and BG-11 medium

	Synthetic 2nd Wastewater	Modified Waris-H	BG-11
Ammonium-N (mg l^{-1})	20	20	---
Nitrate-N (mg l^{-1})	3	3	210
Phosphate-N (mg l^{-1})	3	3	7
COD (mg O_2 l^{-1})	96	---	---

4.2 Twin-Layer System

4.2.1 Twin Layers

Due to the progresses in this research, the combination and utilisation of the substrate layer and the source layer experienced four different stages. Firstly, nitrocellulose membrane and glass fibre lamina were used as substrate and source layer, respectively; secondly, non-woven polyester (PE) filter and non-woven polyester (PE) lamina; thirdly, nylon filter cloth and coated glass fibre mesh; and finally, nylon filter cloth and glass grid reinforced laminate.

In the bench-scale treatment with continuous mode, nitrocellulose membrane and glass fibre lamina were applied. Both *C. vulgaris* and *S. rubescens* could grow on them and remove phosphorus and nitrogen from modified BG-11 medium, however, nitrocellulose membrane was quite expensive (100 € m^{-2}), which limited further large scale application. In addition, glass filaments of the glass fibre lamina could cause secondary pollution in the wastewater.

Jöbgen et al., (2005) applied non-woven polypropylene as substrata to colonize periphyton, which competed successfully with phytoplankton. As a result, about 275 g of phosphorus was removed together with the substrata from the lake after six month exposure. Therefore, cheaper, non-filamentary non-woven polymers were searched from different manufactures as twin-layer materials. Finally, we decided to use non-woven polyester (PE) filter and non-woven polyester (PE) lamina as substitutes for nitrocellulose membrane and glass fibre lamina. Two researches (master thesis) were afterwards conducted with them in bench-scale

twin-layer system to remove zinc from mining wastewater (Lin, 2006) and to removal nutrients from artificial aquaculture wastewater (Wang, 2006). The results indicated that two *Stichococcus bacillaris* (Lin, 2006), and three marine algae *Navicula erifuga*, *Phaeodactylum tricornutum* and *Porhyridium purpureum* (Wang, 2006) could grow on non-woven polyesters. Therefore, I decided to use the materials as well and conducted microalgal growth (3.2) and wastewater treatment experiments (results are not presented in this thesis). Although *S. rubescens* grew well on polyesters, however, the hydrophobic property of polyester complicated the immobilization process because ethanol spray must be applied thoroughly to establish hydrophilicity temporarily. Afterwards, the materials must be washed with plenty of distilled water and supplied immediately with water to keep the hydrophilicity. Furthermore, I found out that the non-woven structure of polyester filter (substrate layer) was uneven, *C. vulgaris* (about 10 µm) could penetrate through it and contaminated the source layer. This is obvious again the purpose of immobilization. Simultaneously, another large-scale project (50 m^2 growth area) was carried out in my research group to cultivated *Symbiodinium* sp. on the same polyesters. Unfortunately, the project was not successful due to the complexity of hydrophilicity process, the penetration of *Symbiodinium* sp. (about 10 µm) through polyester filter (substrate layer) and more important, the blocking of the polyester lamina (source layer) caused by biofilm after approximately two weeks of immobilization.

Therefore, we switched to the mesh grid materials as substrate layer to solve the problem of blocking and find out some membrane filter materials as source layer simultaneously. Different grid mesh materials were investigated afterwards, e.g. polyester mesh, PVC mesh, polypropylene mesh, coated glass fibre mesh (GFM) and glass grid reinforced laminate. The water distribution on the subtended substrate layer is critical for the application of twin-layer system. Polyester mesh, PVC mesh and polypropylene mesh failed in the selection because the surface structure of them were not straight, and water distribution were not even on the subtended substrate layer. Coated glass fibre mesh (GFM) and glass grid reinforced laminate was much better than the polymer materials. Especially, the glass grid reinforced laminate is stable, straight and hydrophilic. Membrane filters include various natural and synthetic materials like nitrocellulose membrane in the former category, and PTFE (Polyetrafluroethylene), PVDF (Polyvinylidene fluoride), PSU (Polysulfone), PESU (Polyethersulfone), PPSU (Polyphenylsulfone), glass fibre and nylon in the latter. Market research showed that PTFE, PVDF, PSU, PESU, PPSU were quite expensive for wastewater treatment (about 100 € m^{-2}). Nylon membrane filter is much cheaper (23€ m^{-2}) and has wide

chemical compatibility range and naturally hydrophilic property. The exclusive impregnation process results in the nylon filter having uniform pore sizes and consistent flow rates for continuously reliable performance. The flow rates of nylon membrane filters are approximately equal to those of standard cellulosic membranes (http://www.osmolabstore.com). The application of nylon filter in the field of wastewater engineering ranges from clarification of microorganism biomass, particle analysis to large particulate filtration. The nylon filter cloth used in this study has wide chemical compatibility range (weak acids, weak alkaline, alcohols, esters, oil, hydrocarbons, halohydrocarbon and organic oxygenate) and is unaffected by autoclaving and by continuous high temperature up to 60 ℃ (product instruction, Shenghe Chengxin Membrane technology, Beijing, China).

Accordingly, *S. rubescens* were grown on nylon filter/coated glass fibre mesh (4×4 mm and 10×10 mm) and the results were positive. No cross contamination and blocking problem was observed during 25 days' cultivation on bench-scale twin-layer system. Escate Ramos (2008) also grew marine algae *Symbiodinium* sp. on printing paper/coated glass fibre mesh (4×4 mm). The experiment was also successful. During the cultivation of 29 days, water distribution through GFM to the printing paper was good, and *Symbiodinium* sp. grew well on the printing paper. Our experiments confirmed that mesh grid materials could be used as substrate layers, and nylon filter and printing paper could be used as source layers. The choice of the kind of materials should be applied depends on the requirements of cultivation, for example, printing paper is only suitable for marine species, and coated glass fibre mesh is not very stable and is only used within short period, etc. Therefore the stable glass grid reinforced laminate and the nylon filter cloth were chosen as source layer and substrate layer respectively in the large-scale wastewater treatment experiment. The experiment was conducted for 54 days in the greenhouse, and Waris-H medium and four different types of municipal wastewater were applied to the twin-layer system. *S. rubescens* was easily immobilized on nylon filters and grew well. The observations throughout the experiment showed that the both nylon filter and the glass grid reinforced laminate were stable, hydrophilic and compatible in either Waris-H medium or municipal wastewater.

4.2.2 Twin-Layer System Design and Further Improvement

In the current study, the large-scale twin-layer system included three modules of twin-layer mounted vertically on a metal rack. The top of the twin-layer was designed to use ten

even-distributed irrigation drippings embed in the influent pipeline to supply a homogenous water flow to the water channel located beneath. Once the water in the water channel was full, it overflowed through the weirs and supplied water to the source layer. During the experiment, three irrigation drippings were blocked by the particular matter and exchanged. But the application of weirs turns out to be a good idea to have even distribution of water flow on the source layer.

The experiment showed the large-scale twin-layer system must be operated at hydraulic loading approximately 3.8 l m^{-2} h^{-1} in order to supply sufficient water to the microalgae. However, if the water flow was too high (>4.5 m^{-2} h^{-1}), the microalgae could also be washed out from the substrate layer. This is obviously against the immobilization. In the bench-scale experiment with nitrocellulose membrane and glass fibre, the hydraulic loading was about 3.1-3.4 l m^{-2} h^{-1}, which was lower than large-scale experiment using nylon and glass grid reinforced laminate. Escate Ramos (2008) operated twin-layer system with printing paper and GFM (4×4 mm) at hydraulic loading between 2-2.5 m^{-2} h^{-1} to keep sufficient water supply. So the hydraulic loading supplied to the algae is dependent on the twin-layer material, especially the source layer because water holding capacities of different source layers are quite differ. In conclusion, the hydraulic loading should always be adjusted before applying new twin-layer materials.

Moreover, no influence of light intensity on the growth of microalgae was found in two treatment experiments in the greenhouse (March-June) in this study. Travieso et al. (1996) summarized that increasing light intensity can increase the microalgae activity and removal of nutrients from wastewater. Natural illumination recorded in the greenhouse during the experiments were higher (22-220 μE m^{-2} s^{-1}) than in the culture room (20-40 μE m^{-2} s^{-1}). In addition, using natural illumination is of cause more realistic and cheaper than using artificial illumination. But few researches related to microalgal nutrients removal have been conducted in greenhouse. In winter, the illumination intensity is weaker and light period is shorter, longer treatment period or supplement of artificial illumination might be required to sustain microalgal growth and nutrients removal efficiency. This is still an open area need to be investigated in the future.

In the large-scale experiment, the distance between each twin-layer module was about 50 cm, which was adequate to supply sufficient illumination to the microalgae, which proved again

that a similar distance of 55 cm found by Escate Ramos (2008) is appropriate between each twin-layer module to grow marine specie *Symbiodinium* sp.

In this study, rubber scraper was used for harvesting, which is easy to perform. Escate Ramos (2008) used an electric spray gun to harvest *Symbiodinium* sp. from twin-layer system. It was also efficient (1 min m^{-2}). Applying both methods, the final harvested microalgae could be collected at the bottom part of the substrate layer.

In conclusion, the proto type of large-scale twin-layer system was successfully designed, installed and operated in this study. And several operational parameters have been established through different studies in my research group. In the future, more professional engineering design must be cooperated to make the twin-layer system industrialized.

4.2.3 Immobilization Techniques Comparison

Immobilization is the process which limits the free migration of cells by aggregating them to a solid support or entrapping them into a fibrous or porous material or a membrane. Immobilization techniques can be primarily divided into two groups: “passive” and “active” immobilization (Moreno-Garrido, 2008).

Normally, the passive immobilization process applies natural or synthetic adsorbent materials, e.g. loofa sponge (Akhtar et al., 2004) in the former category, polyurethane (Garbisu et al., 1991) and polyvinyl (Urrutia et al., 1995) form in the latter category, by absorption/adsorption in/on preformed foams or by entrapment in the prepolymer followed by polymerisation (Urrutia et al., 1995). The usage of preformed foams for cell adsorption avoids exposure to residual toxic products employed in the polymerisation of foams, while synthetic polymers in general have several advantages including lower cost and simplicity of operation. However, the immobilization processes are easily reversible and contamination of effluents with unstuck cells is unavoidable (Moreno-Garrido, 2008).

Entrapment of microalgae in phycocolloids such as alginate or carrageenan beads belongs to “active” immobilization, and is the most frequently used immobilization technique in wastewater treatment experiments (summarized by Moreno-Garrido, 2008; Mallick, 2002). However, leakage of microalgae from the beads after cell proliferation is considered to be a major problem, as microalgae are released from the beads when the maximum holding

capacity is exceeded. This obviously contradicted the purpose of immobilization. Although massive leakage could be delayed by incorporating a rehardening process and extended the life of operation by one more cycle for 9 days before a similar leakage problem, the algal growth eventually saturated the holding capacity of the bead, upon which leakage was again unavoidable (Lau et al., 1998). In addition, the polysaccharide-based gels are unstable in the presence of phosphate, which is usually present in wastewater or added to stimulate algal growth in the effluent.

Twin-Layer system is actually also a "passive" immobilization system. It utilises the characteristic of many microalgae having a natural tendency to attach to surfaces and grow on them. In the twin-layer system, microalgae self-adhere to the substrate layer and are effectively separated from the flow of water by the microporous nature of the substrate layer. It has advantages comparing with the traditional "passive" immobilization method using polymers and "active" immobilization method using phycocolloids. Mallick (2002) summarized the properties of an ideal matrix for algal immobilization as follows: non-toxicity, phototransparency, stability in growth medium, retention of biomass and resistance to disruption by cell growth. Our studies showed that the twin-layer system fulfils these requirements in an ideal way. Twin-layers are non-toxic and stable in wastewater and represent an open cultivation system using sunlight, thus being both cost-efficient and easy to operate. Most importantly, microalgae frequently used in wastewater treatment or isolated from sewage treatment plant/discharge, such as strains of *Chlamydomonas*, *Scenedesmus*, *Euglena* and *Pediastrum* grew well on twin-layer system (small-scale) in the synthetic secondary wastewater. In the large-scale wastewater treatment during 54 days, *S. rubescens* showed consistent growth in four different types of municipal wastewater and remained immobilized during the experimental period without leakage of cells into the wastewater.

The immobilization process of twin-layer system is relatively simple comparing with entrapment of microalgae in phycocolloids beads. Painting roller, painting brush (in this study) or sprayer (Escate Ramos, 2008) can be applied to immobilize microalgae homogeneously on the substrate layer.

Although twin-layer system can be recognized as an open system, but it is quite different from traditional open pond systems. In conventional open pond cultivation systems, harvesting of microalgae has to be performed by centrifugation. It is energy consuming and cost intensive. Furthermore, the shear stress could cause consequently loss of important cell content. Other

harvesting technology such as flocculation and decantation need more space and additional equipment. Furthermore, open systems such as ponds are subject to contamination from a variety of organisms such as alien algae, bacteria, yeast, viruses and invertebrates like rectifiers, and it is a major problem in the culture of freshwater (Grobbelaar, 2003). We have cultivated different microalgae, *Scenedesmus*, *Symbiodinium*, *Haematococcus*, *Navicula*, *Nitzschia* and *Phaeodactylum* etc. (Escate Ramos, 2008, Naumann, 2003, Koenigs, 2005) on twin-layer system in greenhouse, little contamination by other algae or predators was observed to compete with the immobilized algae. However, it is necessary to place yellow sticky board traps close to the twin-layer modules to attract flying insects in the greenhouse. Finally, twin-layer system constitutes vertical-oriented ultrathin photobioreactor modules and it occupies much less space comparing with open pond cultivation techniques.

To summarize, twin-Layer system has several advantages over other immobilization technologies. Firstly, twin-layer system is an ultra-thin photobioreactor and microalgae can take carbon dioxide and light directly and effectively from the environment, which favour the growth of microalgae. Secondly, microalgae and water are separated. The leakage problem usually happen in other immobilization techniques is avoided. Thirdly, the immobilization process is much simpler than other techniques.

4.2.4 Other Applications of Twin-Layer System

One of the most promising areas using twin-layer system is reducing environmental pollutions through biosorption and biodegradation of many harmful compounds. In this study, twin-layer system is proved to be very efficient in nitrogen and phosphorus removal. The previous studies in my research group also showed that the system was efficient in zinc removal (Lin, 2006). It is still an open area for various researches in screening microalgae for bioremediation such as heavy metal (nickel and plumbum etc.) and organic pollutions (phenol and mineral oil etc.).

We see more beneficial of using twin-layer system in the future for production of pharmaceuticals and reagents, biosensors, chemicals, food and beverages, cosmetic and aquaculture. For example, three economic microalgae, *Navicula erifuga*, *Nitzschia laevis* and *Phaeodactylum tricornutum* had been successfully cultivated on twin-layer system and

produce eicosapentaenoic acid (EPA) (Koenig, 2005), which is an effective treatment to lower inflammation and beneficial in mental conditions.

4.3 Microalgal Growth in Wastewater Treatment

4.3.1 Microalgal Growth in Bench-Scale treatment

At the beginning of the study, *C. vulgaris* and *S. rubescens* was applied to remove nitrogen and phosphorus from modified BG-11 culture medium which was supplied continuously in 9 days (continuous mode). The results showed that both strains could grow and took up nitrogen and phosphorus in modified BG-11. In the second experiment, three cycles (semi-continuous mode) were applied, each lasted 4 days. The Chlorophyll a content of *S. rubescens* increased continuously in each cycle. This established the foundation for the subsequent large-scale experiment.

Chl *a* productivities of *S. rubescens* (M2630) in different wastewater is summarized in Table 4-3. *Chlap* in the bench-scale treatment with continuous mode was higher than that with semi-continuous mode. It is well known that temperature is a crucial environmental parameter for the growth of microalgae. The latter experiment was conducted in the 14℃ culture room, while former experiment was in greenhouse, where the temperature was much higher. Moreover, different source layers applied might also be the reason of diverse microalgal growth rate. Although microalgae could grow better when lamina materials (glass fibre/PE lamina) applied as the source layer, the blocking of the lamina caused by biofilm after approximately two weeks of immobilization could not be avoided. Therefore, the source layer must be mesh grid materials to avoid the serious blocking problem in order to sustain continuous operation of the system. In the large-scale treatment with glass grid reinforced laminate, which provided better water distribution to the substrate layer, a comparable higher *Chlap* (front side) was achieved.

Table 4-3. Chlorophyll *a* productivities of *Scenedesmus rubescens* (M2630) in wastewater treatment experiments

	Chlap ($\mu g\ cm^{-2}\ day^{-1}$)	Medium	Twin-layer material	T (℃)	Illumination
Bench: continuous	1.22	Modified BG-11	Nitrocellulose membrane/glass fibre	<30	20-120
Bench: Semi-continuous	0.56	Modified BG-11	Nylon/GFM (10×10mm)	14±1	30
Large	0.86	Waris-H/Wastewater	Nylon/glass grid reinforced laminate	18-32	22-220

4.3.2 Microalgal Growth in Large-Scale Wastewater Treatment

The results clearly showed that *S. rubescens* could grow on large-scale twin-layer system with Waris-H medium and four different types of municipal wastewater. During 54 days, dry weight of *S. rubescens* increased 37 times and 23 times for the front side and back side of the twin layer, respectively. The increment of Chlorophyll *a* was less than dry weight, with 33 times for the front side and 21 times for the back side. An interesting phenomenon was observed that the biomass productivity (dry weight) was not limited, at least, to a less extent, whereas the pigment productivity (Chl *a*) was limited when nitrogen was insufficient in the wastewater.

It could be observed from the Chl *a* curve (Figure 3-11, b), when the wastewater from denitrification tank was applied, the even linear growth was obviously interrupted, i.e. *Chlap* decreased. Afterwards, Bio-P wastewater was applied, Chl *a* increased rapidly during 8 days. This phenomenon could be observed on both sides of twin layers (Figure 3-11, b). The denitrification wastewater was characterized by low ammonium-N (1.22-2.97 mg l^{-1}) and low nitrate (0.31-0.97 mg l^{-1}), which leaded to the deficiency of nitrogen. It has been commended that nitrogen limitation greatly reduced the synthesis of chloroplastic proteins, and among the pigments, Chl *a* decreases, whereas carotinoids increase (Geider et al., 1998, Falkowski et al., 1989). The denitrification wastewater was essentially lack of nitrogen, and it had been replaced only every two days, which further enhanced nitrogen limitation to the algae, so *Chlap* decreased. There was also a decrease of dry weight productivity when supplying the

denitrification wastewater, but much less than Chl *a*. This could be explained by during nitrogen limitation, algal cells have a surplus of carbon metabolites that often accumulate as lipids (Roessler, 1990). For example, analyses of 18 freshwater and 11 marine algal species showed in most cases an increased lipid content at nitrogen limitation, often two to three times higher than cultures with replete nitrogen (Shifrin and Chisholm, 1981). Another explanation is that the surplus carbon was accumulated as carbohydrate in the cells. It has been confirmed that several algae (*Scenedesmus* sp. and *Spirulina* sp.) are able to produce high concentrations (about 70%) of carbohydrate polymers without a decrease in overall productivity under nitrogen-limited conditions (summarized by Becker; 1994). For example, during nitrogen starvation for two days, % carbohydrate of three marine phytoplankters (*Isochrysis galbana, Chaetoceros calcitrans and Thalassiosira pseudonana*) increased, whereas % lipid remained relatively constant and % protein decreased (Harrison et al., 1990): Ahlgren and Hyenstrand (2003) also summarized that carbon content was stable in both the green alga *Scenedesmus quadricauda* and the cyanobacterium *Synechococcus* sp., under nitrogen limited conditions. Detailed researches on the physiological activity and chemical composition of *S. rubescens* under different nutrients conditions should be conducted in the future.

In this experiment although the illumination irradiance above the west (front) sides of twin-layers were differ from 110-220 to 30-60 μE m^{-2} s^{-1} , no significant difference was found among the dry weight throughout the experiment. This indicated there was no light limitation on the growth of microalgae. The distance between each module was about 50 cm, which was adequate to supply sufficient illumination to microalgae. However, the growth rates of the west (front) sides were always better than the east (back) sides (Figure 3-11). The difference was caused mainly by the design of the large-scale twin-layer system, leading to insufficient nutrients supply on the backside, instead of light limitation. The colour of *S. rubescens* on the east (back) side was reddish instead of greenish on the west (front) side, as *Scenedesmus* was capable of synthesizing secondary carotenoids upon nutrient limitation (Droop, 1954, Kessler et al., 1997). On the top of the twin-layer, a water channel was design to collect water. Once the water medium in the channel was full, it overflowed through the weirs, supplied water to the source layer and distributed to the immobilized algae on the substrate layer by water diffusion. In this experiment, the water always flowed to the west (front) sides of the source layer, which was about 3 mm (thick). So the microalgae on the west (front) sides had priority to take up nutrients, while, the microalgae on the east (back) sides had insufficient nutrients

supply. Escate Ramos (2008) grew marine microalgae *Symbiodinium* sp. on the large-scale twin-layer system, with printing paper and coasted glass fibre mesh (4×4 mm) severed as substrate layer and source layer. The results showed that there was no significant difference of dry weight between the west and the east side during 29 days. The reason is the coasted glass fibre mesh (4×4 mm) was thin (<0.5 mm), so there was no difference of water diffusion on two sides. Water supplying to the source layer need to be designed better in the future.

The growth curve of *S. rubescens* demonstrated that no stationary phase appeared after 54 days. In the future *S. rubescens* should be cultivated for longer period to find out the kinetic of the growth and the operating time for wastewater treatment before harvesting the cells.

4.3.3 Microalgal Linear Growth on Twin-Layer System

In this study, we observed the growth of *S. rubescens* with respect to dry weight and chlorophyll *a* always exhibited a linear growth pattern. In the microalgal growth experiment, *S. rubescens* was cultivated on different twin-layer materials, PE/PE, nylon/coated glass fibre mesh (4×4 mm and 10×10 mm), Chlorophyll *a* was linear (Figure 3-1). In the large-scale wastewater treatment, *S. rubescens* was cultivated with Waris-H and different types of wastewater, the growth were also linear, regardless the position. In our research group, the growths of different microalgae on twin-layer system have been extensively studied, and the linear growth patterns were found cultivating different species, e.g. two *Stichococcus bacillaris* species grown on non-woven polyester filters for 15 days (Lin, 2006), *Haematococcus pluvialis* grown on membrane filter for 50 days (Naumann, 2003) and *Symbiodinium* sp. cultivated on printing paper for 29 days (Escate Ramos, 2008). We can draw the conclusion that the linear growth pattern was irrelevant to the water medium applied, nutrients availability, twin-layer materials and cultivation conditions such as temperature and illumination intensity. Especially the linear growth pattern was not attributed to the nutrients limitation, which is always considered as one of the reasons for the linear growth of microorganisms.

Beer-Lambert's law is the linear relationship between absorbance of light and concentration of an absorbing material. It has been used to in the field of microalgal biotechnology to formulate mathematical model for microalgal flat-plat bioreactor (Formula 4-1) (Kim et al., 2002, Lee, 1999)

Formula 4-1. Application of Beer-Lambert's law for flat-plate bioreactor

$$\text{Log}\ (I_0/I) = \alpha \cdot X \cdot x$$

Where,

I_0 incident light intensity (at surface) (W/m^2)

I light intensity at depth x (W/m^2)

α specific light absorption coefficient ($cm^2/\mu m^3$ cell)

X cell concentration (μm^3 cell/ml)

x distance from the illumination surface (cm)

The twin-layer system is actually a flat bioreactor as well, however, ultra thin, in comparison with the flat-plat bioreactor made by glass. In twin-layer system, light energy penetrates into the immobilized microalgae from the illuminating surface, and the intensity of the light energy decreases as the light travels into the culture, due to absorption by the light-harvesting pigments of algae and scattering by algal cells (Figure 4-1).

Kim et al.(2002) successfully explained the reason of linear growth of microalgae on flat-plate photobioreactor, and formulate a simple monodimensional model for linear growth rate of microalgae in flat-plat photobioreactors based on Beer-Lambert's law and the specific growth rate of microalgae. Linear growth rate (LGR) of microalgae on flat-plat photobioreactor was proposed as formula 4-2.

In formula 4-2, the K and I_0 are the operating parameters, μ_m, α, ε, Is and I_C are the inherent properties of the strain and L represents the geometry of a flat-plate photobioreactor. It is clear that the linear growth rate is inverse proportional to L, the light path of reactor and direct proportional to I_0, the incident light intensity on the surface of photobioreactor. Accordingly, many types of photobioreactors were made by glass in order to have higher I_0, in the same time, to have shorter thickness (L). Therefore, relatively higher linear growth rate of microalgae can be achieved for the photobioreactor. In twin-layer system, microalgae immobilized on the substrate layer and get light (I_0) directly without any hindrance e.g. glass, in addition, the ultra thin property of the immobilized microalgae on twin-layer offers low light path (L). In conclusion, twin-layer system is the optimal photobioreactor by far to achieve high linear grow rate.

The different mathematical models for linear growth of microalgae flat-plat photobioreactors were established on the suspension cultures (Kim et al., 2002, Lee, 1999), in the future, the model for the linear growth rate of immobilized microalgae on twin-layer system should also be formulated.

Formula 4-2. Linear growth rate of microalgae on flat-plat photobioreactor

$$LGR = K \cdot \frac{\mu_m}{\alpha \cdot L} Log(I_0 \cdot I_s^{(\varepsilon-1)} \cdot I_c^{-\varepsilon})$$

Where,

α specific absorption coefficient ($cm^2/\mu m^3$ cell)

ε constant for mean of specific growth rate between x_s and x_c (-)

I_0 incident light intensity (W/m^2)

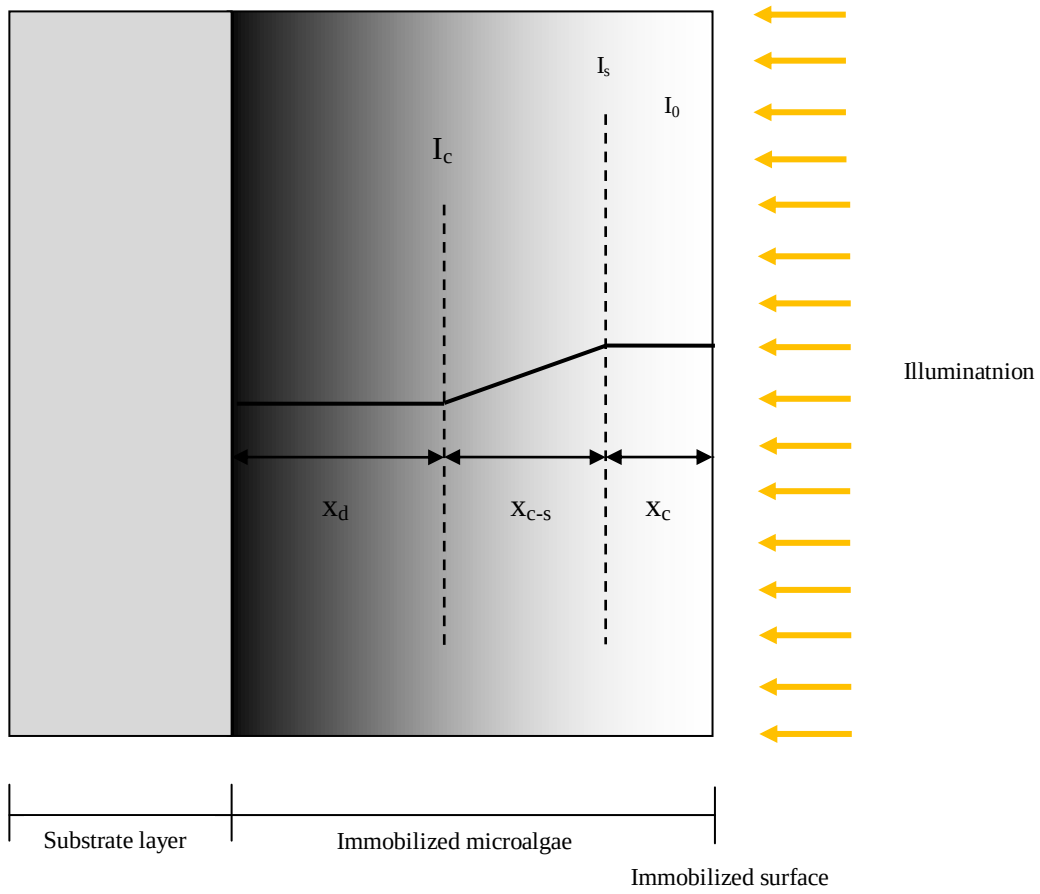
Is compensation light intensity (W/m^2)

I_C compensation light intensity (W/m^2)

K proportionality constant for mixing effect (-)

L light path of reactor (cm)

μ_m maximal specific growth rate (h^{-1})



I light intensity at depth x (cm)

I_0 incident light intensity (W/m^2)

I_C compensation light intensity (cm)

I_S saturation light intensity (cm)

I_d photoinhibition light intensity (W/m^2)

x distance from irradiating surface (cm)

x_c length of photic zone (cm)

x_{c-s} distance between compensation point and saturation point (cm)

x_s distance to saturation point from irradiating surface (cm)

x_d dark distance (below compensation point) (cm)

Figure 4-1. Schematic representation of light penetrating through immobilized microalgae on the substrate layer of twin-layer system (kim et al. 2002, modified for twin-layer system)

4.4 Nitrogen and Phosphorus Removal from Wastewater

4.4.1 Bench-Scale Treatment

In bench-scale treatments, microalgae, *C. vulgaris* and *S. rubescens* in the continuous mode, and *S. rubescens* in the semi-continuous mode, grew well with modified BG-11 medium on twin-layer system and remove nutrients from the medium, whereas in controls, nutrients were neither precipitated nor absorbed (or very little) on the twin layers. We conclude that phosphate, ammonium and nitrate were taken up metabolically by microalgae and incorporated into their biomass.

4.4.1.1 Phosphorus Removal

In the bench-scale experiment with continuous mode in greenhouse, our results demonstrated that about 90% of the phosphate was removed from the medium within two days by both microalgal strains on twin-layers (Figure 3-8, a). The rapid phosphate uptake by algae could be explained by "luxury uptake of phosphorus". Under sufficient phosphorus supply, phosphate is accumulated in the algae as acid-labile polyphosphate in large granules, which are metabolized under phosphorus deficiency (summarized by Becker, 1994). Using alginate bead immobilized *C. vulgaris* to treat raw sewage (PO_4^{3-}-P =6±2 mg l^{-1}) can achieve an average 72% phosphate removal efficiency (Travieso et al., 1996). The similar result was also found by Tam et al. (1994), who reported a 70.6% phosphate removal efficiency by *C. vulgaris* immobilized in alginate beads in seven days with a starting PO_4^{3-}-P concentration about 9.58 mg l^{-1} (primarily treated wastewater). The specifics, of course, depend on the type of wastewater, the type of algae and their growth conditions, and most importantly on the relationship between the amount of biomass applied and the hydraulic loading of the wastewater.

In the bench-scale treatment with semi-continuous mode, the important outcome is that the twin-layer system was successfully operated in three continuous cycles: Phosphorus and ammonium were always removed from the medium during all treatment cycles (Figure 3-9, a&b). This experiment was conducted in the culture room at 14±1 ℃ and under illumination irradiance of 30 μE m^{-2} s^{-1}. Both temperature and light intensity were lower than the greenhouse. This leaded to a less *Chlap* of *S. rubescens* (0.56 μg cm^{-1} day^{-1}) and further

correlated to the lower nutrients removal efficiency by the algae. At the end of each treatment cycle, the residual concentrations of phosphate in the medium kept about 1 mg l^{-1}, which was higher than the residual phosphate (< 0.2 mg l^{-1}) in the last experiment during the same time interval, however, the residual concentration of phosphate-P still meet the wastewater discharge regulation forced by different government (1.2.3). In the first cycle, depletion of phosphate from medium was the sum results of algal uptake and nylon/glass grid reinforced laminate adsorption, upon the saturation of adsorption, the adsorption by twin-layer material was much less in the next two cycles.

The most important mechanism to remove phosphate from the medium is direct cellular assimilation by the immobilized microalgal cultures. The adsorption of phosphate by the twin-layer materials i.e. nitrocellulose membrane/glass fibre and nylon/GFM is minor, which is evidential from the control experiments. While, using alginate beads, phosphorus depletion was contributed mainly to the blank control bead (Lau et al., 1998). The adsorption of phosphate, noted by the continuous mode experiment, contributed only slightly (13.6%) to the removal of phosphate from the medium. In the experiment with semi-continuous mode, consistent decrease (13.8%→3.8%→0.6%; Figure 3-9, a) in the phosphate depletion by control in three consecutive cycles implies that adsorption by the twin-layer materials can reach saturation. The magnitude of phosphorus removal by the layers depended clearly on the surface area available for adsorption. Upon saturation of the binding with phosphate by twin-layer materials, extent of phosphate reduction will become less in subsequent treatment cycles and finally to zero.

Precipitation of phosphate was unlikely in the bench-scale experiments since the modified BG-11 was well buffered with 5mM HEPES to keep pH between 7.1 and 7.5. Precipitation of soluble reactive phosphorus with cations such as Ca^{2+}, Mg^{2+} and Al^{3+} is known to occur at pH between 8.9 and 9.5, depending upon the buffering capacity of the water (Belsare and Belsare, 1987 *in* Craggs, 2001).

Furthermore, the internal phosphorus increase of *C. vulgaris* and *S. rubescens* were measured in the continuous experiment as 4.27 mg P for the former and 4.95 mg P for the latter (Table 3-7). These values were comparable with the differences of P reduction between the treatment with algae and control. It again confirmed that phosphate was assimilated by algae and incorporated into their biomass.

4.4.1.2 Ammonium Removal

In the bench-scale treatment with continuous mode, the depletion of ammonium from the modified BG-11 medium (94% and 96% by *C. vulgaris* and *S. rubescens* respectively, within 9 days; Figure 3-8, b) was comparable to studies conducted by Martínez et al. (2000), who described elimination of ammonium (between 79-100%) after 188.25 hours (about 8 days), and González et al. (1997) who reported ammonium removal efficiencies of 90% from agro-industrial wastewater after 216 hours (9 days).

In the semi-continuous treatment with three cycles, the removal efficiency of ammonium increased slightly from 57.9% in the first cycle to 67.4% in the second cycle and dropped back to 39.8% in the third cycle (Table 3-8). In the first and third cycle, increase of ammonium in the control was observed at day 2,3,11 and 12. This might caused by ammonium adsorbed on the nylon was desorbed or released for algal assimilation. A certain portion of the adsorbed ammonium was depleted from the nylon filter leaving the binding sites available again for further ammonium assimilation. In addition, as has been discussed in the last section, less *Chlap* of *S. rubescens* correlated to the lower nutrients removal efficiency by the algae. The residual ammonium concentrations were 7.9, 5.5 and 10.9 mg l^{-1} at the end of each cycle, which were higher than 5.1 mg l^{-1} in the last experiment during same time interval (4 days). However, the concentration at the end of each cycle still met the requirement i.e. < 13 mg l^{-1} of wastewater discharge from MWTP forced by German government.

The depletion of ammonium in the medium could be attributed mostly to microalgae uptake and secondarily to non-biological mechanisms e.g. membrane and glass fibre adsorption. Air-stripping of NH_3 is unlikely in these experiments since when pH in the medium was between 7.0-7.5, the percentage of gas NH_3 is rather low. There are obvious advantages of eliminating ammonium from wastewater using microalgae: (1) it does not generate secondary pollution by generation of NH_3 (dependent on pH); and (2) the microalgal biomass can be harvested and used as a slow-release fertilizers or soil conditioner (de la Noüe et al., 1992, Mulbry et al., 2005, Mallick, 2002).

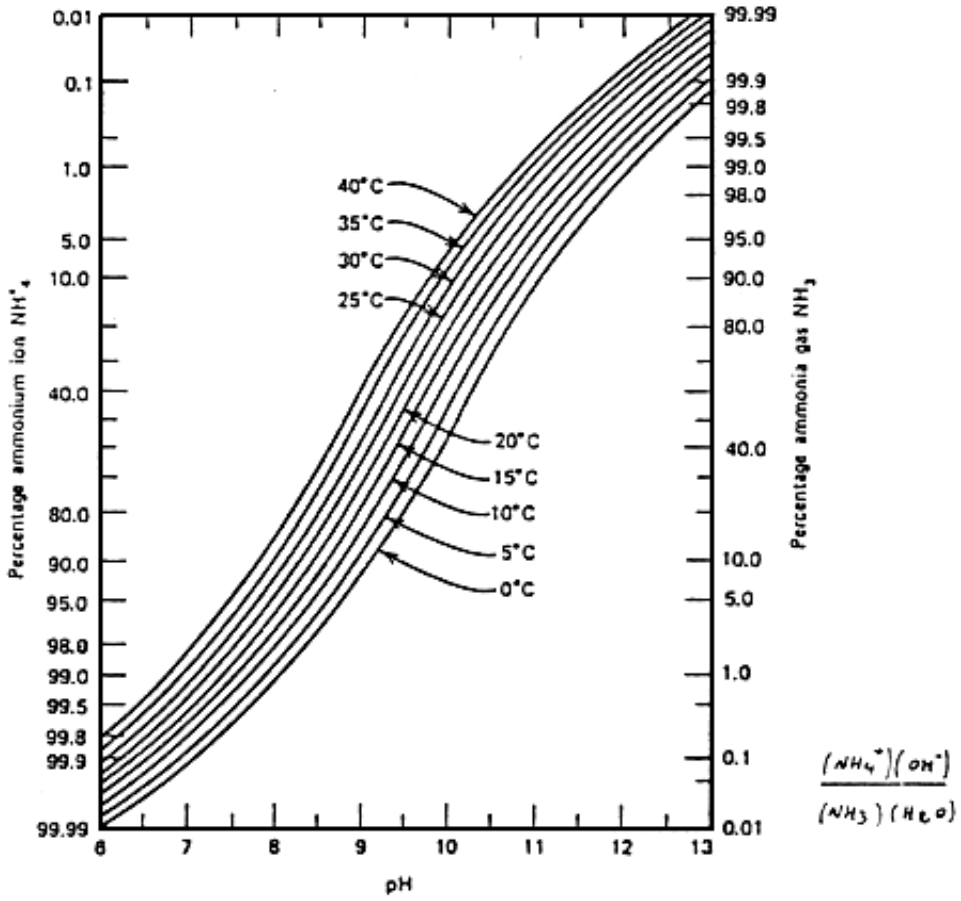


Figure 4-2. Aqueous ammonia equilibrium: effect of pH and temperature (Emerson et al., 1975)

4.4.1.3 Nitrate Removal

The obvious lag phases of nitrate diminishment happened at the beginning of the bench-scale experiment (Figure 3-8, c) can be explained by two nitrogen sources (ammonium and nitrate) have been supplied in the synthetic wastewater. Although microalgae are capable of active uptake of inorganic nitrogen (ammonium, nitrate and nitrite) from external environment, ammonium-nitrogen is preferentially absorbed and nitrate is often not utilised until all the ammonium salts have been consumed (Summarized by Becker, 1994). Since nitrate-nitrogen has to be reduced prior to incorporate into macromolecules and this process consumes reductive power (Syrett, 1981). Therefore, *C. vulgaris* began to assimilate nitrate nitrogen after day 2, when 62% (residual 7.8 mg l^{-1}) ammonium has been removed, and *S. rubescens* absorbed nitrate after day 4, when 75% (residual 5.1 mg l^{-1}) ammonium was eliminated. In the bench-scale experiment with semi-continuous, the lag phase of nitrate uptake by *S. rubescens* between 3-4 days.

4.4.2 Large-Scale Wastewater Treatment

In the large-scale wastewater treatment, *S. rubescens* grew well in Waris-H medium and other four types of municipal wastewater. During the treatment, phosphate, ammonium and nitrate were efficiently removed by *S. rubescens* within short retention time (1 or 2 days). Phosphate and ammonium reduction by non-algal reasons were minor indicated by blank control experiment with Bio-P wastewater. We conclude that phosphate, ammonium and nitrate were taken up metabolically by *S. rubescens* and incorporated into their biomass. However, no organic substances (measured by COD) were reduced by *S. rubescens*.

The nutrients removal by immobilized *S. rubescens* was clearly affected by the nutrients supplied in the wastewater. Lower concentrations of phosphate and ammonium in the denitrification wastewater negatively impacted the growth of algae. The total reductions of phosphate and nitrogen were also lower than those of other wastewater (Table 4-4). Growth of *S. rubescens* was recovered later when supplying P and N enriched Bio-P wastewater (Figure 3-11). It means that the algae can be supplied with more P and N in order to keep optimal growth rate and nutrients uptake.

Table 4-4. Total nitrogen and phosphorus reduction with different wastewater

	N (mg)	P (mg)
Secondary settled wastewater	2787.3	198.5
Secondary settled wastewater + P	2135.5	624.0
Denitrification tank wastewater	468.7	364.7
Bio-P tank wastewater	2434.4	636.2

Nitrogen uptake changes pH of the water medium. The pH decreases when ammonium is used as the sole nitrogen source. Assimilation of nitrate ions tends to raise the pH. The pH during the wastewater treatment always exhibited a slightly increase. However, the similar increase was also found in the blank control experiment. Therefore, the change of pH in the wastewater was caused by non-algal reasons. Nevertheless, the pH of the wastewater has to be adjusted before discharging into natural water bodies when applying microalgal mediated twin-layer system.

4.4.2.1 Secondary Settled Wastewater

Immobilized *S. rubescens* was able to take up phosphate from the wastewater continuously within 8 cycles (Figure 3-14, a). The residual phosphate in the wastewater was between 0.05 and 0.22 mg l^{-1}. The removal efficiencies were between 55%-81%, however, never reached 100%. This might be attributed to the outflow of the wastewater storage tank, which is connected to the top of the twin-layer modules, is about 10 cm above the bottom. It means about 5 litres wastewater was not provided to the microalgae for their assimilation. The wastewater still contained nutrients. However, wastewater was always mixed well before sampling. The similar problem for phosphate, ammonium and nitrate reduction could be observed when the other types of wastewater were applied (Figure 3-15, 17 & 18). The removal efficiency would have been improved if the wastewater in the storage tank had been mixed well. In addition, I think the precipitation of phosphate and the adsorption of phosphate by twin-layer materials were insignificant, because cations such as Ca^{2+}, Mg^{2+} and Al^{3+} are not sufficient in the wastewater to cause precipitation. In the bench-scale treatment with semi-continuous mode, I have found out the magnitude of phosphate adsorption depended clearly on the available surface area. Upon saturation of the adsorption with phosphate by twin-layer materials, extent of phosphate reduction became less and less in the subsequent treatment cycles and finally almost to zero (12 days). Before wastewater treatment, the twin-layer system had been operated for 22 days with Waris-H medium. We can presume that the adsorption of phosphate by twin-layer materials had terminated before starting the treatment. Phosphate reduction was mainly due to direct cellular assimilation by the immobilized microalgal cultures.

Ammonium concentrations in this wastewater were rather low. Therefore no inhibition of ammonium on nitrate uptake could be observed (Figure 3-13, b&c). At the end of each treatment cycle, most nitrate (62%-93%) was removed from the wastewater. The residual nitrate in the wastewater was in most cases < 2 mg l^{-1} with only one exception at cycle 7, where the nitrate concentration was 2.56 mg l^{-1}. The configuration of Frechen wastewater treatment plant has been designed as denitrification → nitrification (2.3.2). The wastewater used in this experiment was collected after nitrification process. In this kind of wastewater treatment plant, the final discharge comes from nitrification tank and usually contents high nitrate concentration from 4 to 7 mg l^{-1} (Tchobanoglous et al., 2003). In the secondary settled wastewater of Frechen MWTP, this value was comparable, about 4.4-9.2 mg l^{-1}. In the

wastewater plant operated like Frechen MWTP, further reduction of phosphorus and nitrate level was very difficult. However, as part of the "European Water Framework Directive", the effluent demands of, among others, nitrogen and phosphorus might become stricter. Using immobilized algae on twin-layer system, the "Maximum Tolerable Risk Values" (Table 1-4) will be met.

4.4.2.2 Secondary Settled Wastewater with Additional Phosphorus

This type of wastewater was prepared by adding 1.5 mg l^{-1} phosphate-P to the secondary settled wastewater. The physical and chemical parameters of this wastewater were similar with secondary settled wastewater except higher phosphorus amount. In Frechen MWTP and most other wastewater treatment plants, part of the phosphate reductions are achieved by chemical (Al, Fe) precipitation, yet is cost intensive. For that reason, additional phosphate was put into the secondary wastewater in order to test if the immobilized microalgae could reduce higher amount of phosphate and therefore reduce the chemical additives for precipitation. The percentages of phosphate removal were between 55%-81% at the end of treatment cycles (Table 3-14). The residual phosphate concentrations were < 1mg l^{-1} (Figure 3-15, a), which met Germany, Chinese and European Union's requirements of wastewater discharge from MWTPs (Table 1-3). As has been discussed in the last section, phosphate reduction was mainly caused by microalgal assimilation, and other non-biological reasons such as precipitation and nylon/glass grid reinforced laminate adsorption was not significant.

Nitrogen constitute in this type of wastewater was similar with the secondary wastewater used in last experiment. Ammonium concentrations were rather low, and nitrogen was between 4.4-7.2 mg l^{-1}. At the end of each treatment cycle, most nitrate (70%-94%) was removed from the wastewater. The residual nitrate in the wastewater was < 2 mg l^{-1}. The conclusion can be draw that using immobilized *S. rubescens* on twin-layer system, phosphate and nitrate can be efficiently removed from secondary wastewater within one day without any chemical additives.

4.4.2.3 Denitrification Tank Wastewater

The sewage in the denitrification tank is the mixture of the effluent from Bio-P tank and the recycling water (2Q) from aerobic active sludge/nitrification tank. The supernate applied to the microalgae has the initial COD, phosphate, ammonium and nitrate concentration of 18-26 mg O_2 l^{-1}, 1.41-2.64 mg l^{-1}, 1.22-2.97 mg l^{-1} and 0.31-0.97 mg l^{-1}, respectively. In this experiment, phosphate was reduced gradually to <0.4 mg l^{-1} within two days. Both ammonium and nitrate were presented in this type of wastewater. However, no inhibition of nitrate uptake was observed in each treatment cycle. Both nitrate and ammonium were removed rapidly in each cycle. This was attributed to the low ammonium concentration presented in the wastewater. Ammonium was possibly taken up by *S. rubescens* rapidly within some hours and afterwards nitrate could be assimilated. Furthermore, nitrogen content in this type of wastewater was obviously too low to support the growth of *S. rubescens* as indicated by the growth curves (Figure 3-11).

4.4.2.4 Biological Phosphorus Tank Wastewater

Bio-P tank is located after primary sedimentation. And this type of wastewater is comparable with primary wastewater and characterized by high phosphate (2.72-4.58 mg l^{-1}), high ammonium (7.76-13.91 mg l^{-1}) and very low nitrate (0.1-0.17 mg l^{-1}) concentrations. During four consecutive treatment cycles, phosphate was continuously and efficiently removed from wastewater by *S. rubescens* (Figure 3-17, a). It could also be observed that phosphate was removed more rapidly in the first cycle than the subsequent ones. About 86% phosphate was eliminated within one day in the first cycle, followed by 55%-57% in the following three cycles (Table 3-18). This can be attributed to the algae has been starved of phosphorus (only 0.34 mg l^{-1} P in the last day) for one day before starting the first cycle (Figure 3-16). Phosphorus removal efficiency was indeed enhanced by starvation as reported by Hernandez et al. (2006) using microalga *Chlorella* sp. co-immobilized with bacteria *Azospirillum brasilense*. Regardless of starvation, phosphate level reduced to < 2 mg l^{-1} within one day, and further decreased to <1 mg l^{-1} (except cycle 3, 1.18 mg l^{-1}) in the second day, in four treatment cycles.

Removal of ammonium was found to be a faster process than that observed for phosphate. Almost all the ammonium was reduced by *S. rubescens* within one day in all treatment cycles.

In the second day, ammonium concentration kept stable in the wastewater (Figure 3-17, b). The depletion of ammonium from the Bio-P wastewater can be attributed mostly to microalgae uptake and secondarily to air-stripping of gas NH_3 caused by the enhanced pH in the medium (Figure 3-10). However, air-stripping of NH_3 was not the major reason of ammonium depletion from the wastewater as indicated by control experiment, that only 3 mg l^{-1} ammonium was reduced within two days by control (Figure 3-13). Nitrogen assimilation by microalgae using twin-layer system is higher than using suspension. The results from Nuñez et al. (2001) indicated that only between 25% and 33% of the total nitrogen missing from the medium was actually recycled into proteins by the *Scenedesmus obliquus* in suspension with aeration.

Although COD reduction was observed on the second days in the first and second cycle, it kept stable in the third and forth cycles as had also been recorded in the last three types of wastewater. I doubt about whether the reductions of COD in this experiment were caused by the algae, or bacteria in the Bio-P wastewater, or other non-biological reasons. 327 also found out that the removal of COD and TON (Total Organic Nitrogen) was mainly due to the metabolism of the indigenous bacteria in the wastewater instead of microalgae *Chlorella vulgaris*. The reduction of organic substances from wastewater needs to be investigated in the future.

According to Germany regulations, the total phosphorus and nitrogen concentrations discharged from urban wastewater treatment plants (>4,000 kg d^{-1} BOD_5) should be less than 1 mg l^{-1} phosphorus and 13 mg l^{-1} total inorganic nitrogen (Table 1-1). Considering phosphate and ammonium are the most abundant phosphorous and nitrogen form in the Bio-P wastewater, these requirements were met within 2 days of exposure of the algae to the Bio-P wastewater in all treatment cycles. Compared with other wastewater treatment technologies, the residence time in twin-layer system is lower than in HRAP (High-rate algal pond) technologies (2–6 days summarized by Hoffmann, 1998 and 4-10 days commended by Olguín, 2003), but higher than when using chemical methods (0.3–0.5 days). The actual residence time, of course, depends both on the initial concentration of total nitrogen and phosphorus in the wastewater and on the amount of algal biomass employed.

4.4.2.5 Effect of Light/Dark Cycle on Nitrogen and Phosphorus Removal

It was clear that phosphate uptake by microalgae *S. rubescens* from the Bio-P wastewater was light dependent. During the light period, residual phosphate concentration in the wastewater decreased sharply, whereas during the dark period, no phosphate reduction from the wastewater was observed (Figure 3-18, a). Algae use three different processes to transform P into high energy organic compounds: (1) photophosphorylation, (2) phosphorylation at the substrate level and (3) oxidative phosphorylation. In the first process, light energy is transformed and incorporated into ATP. In the second and third processes, the energy comes either from the oxidation of the respiration substrates or from the electron transport system of the mitochondria (Mart ńez et al., 1999). The reason of residual phosphate concentration kept stable during the dark period is attributed to the main part of phosphate uptake is finally incorporated into the polyphosphate pool via photophosphorylation by the energy providing from photosynthesis. In the dark, hydrolysis (storage) of these polyphosphate happens which leads to an increase in the amount of intracellular phosphate (Falkner et al., 1980). It means microalgae take up phosphate in the day time and stored them in the night. That's why the depletion of phosphate from Bio-P wastewater was only detected in the light period, but not in the dark period. Another explanation is that the influence of light upon P uptake was a function of cellular metabolic status. In a comprehensive research conducted by Chisholm and Stross (1976), P sufficient batch cultures of *Euglena gracilis*, synchronized to a 14: 10 light/dark cycle exhibited an almost complete inhibition of P uptake in the dark. Since the Bio-P wastewater used in this study was enriched of phosphorus. And this experiment was the final cycle, the inhibition of P uptake in the dark is also reasonable. Nevertheless, we can conclude both light and dark period are important for phosphorus assimilation by microalgae.

Unlike the tendency of phosphate uptake, ammonium reduction from the Bio-P wastewater was absolutely not light dependent (Figure 3-18, b). During the first 6 hours (Light/Dark, 2.5/3.5), ammonium dropped rapidly from a staring concentration of 9.3 mg l^{-1} to 5.2 mg l^{-1} following by more reduction in the next 11 hours (L/D, 8.5/2.5) to 0.9 mg l^{-1}. It has been proved that ammonia is converted to organic nitrogen compounds and its assimilation takes place at the expense of endogenous carbohydrate reserves (summarized by Becker, 1994). So the reduction of ammonium from the wastewater was not light dependant and take place during the whole day.

Nitrate content in the Bio-P wastewater was very low (<0.2 mg l^{-1}), and high ammonium (9.3 mg l^{-1}) in the wastewater could also cause inhibition of nitrate uptake. Nevertheless, the effect of light on the efficiency of nitrate removal by polyvinyl-adsorbed cells of *Scenedesmus obliquus* was conducted by Urrutia et al. (1995). It was clear from the result that nitrate uptake was light dependent, just like higher plants. Nitrate removal efficiency decreased promptly when lights were turned off. However, the initial efficiency could be rapidly established when the reactor was illuminated again (Figure 4-3). The inhibition of nitrate uptake in dark is again related to energy used for assimilation. Nitrate assimilation is a energy consuming process since nitrate-nitrogen has to be reduced prior to incorporating into macromolecules. And this process consumes reductive power (Syrett, 1981).

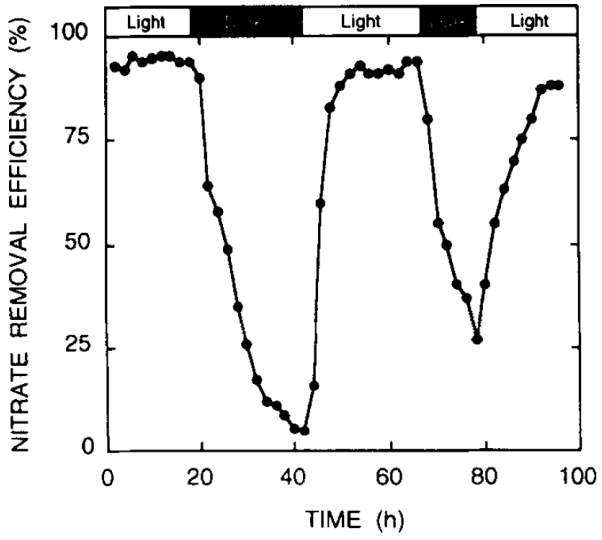


Figure 4-3. Effect of light on the efficiency of nitrate removal by polyvinyl-adsorbed cells of *Scenedesmus obliquus* packed in a continuous-flow reactor (Urrutia et al., 1995)

4.4.3 Scenarios on Integration of Twin-Layer System into MWTP

The configuration of Frechen MWTP named A^2O (Anaerobic-anoxic-aerobic) which is used most often in the developed countries because of the relative ease of retrofit to existing plants which had no nitrogen and phosphorus treatment processes. In such kind of plants, phosphorus and nitrogen can be integrated with the removal of organic substances with the target to decrease chemical oxygen demand (COD) mediated by heterotrophic bacteria. However, the discharged effluent comes from aerobic tank (nitrification) and still contents significant amount of nitrate, in case of Frechen, 4.4-9.2 mg l^{-1}. Since the discharge

requirement will become stricter in the near future (1.2.3), microalgal twin-layer system can be added after the secondary sedimentation process and further reduce nitrogen and phosphorus from wastewater **(Scenario 1)**. Furthermore, in practice phosphorus removal is variable in the plants and the achievement of a low and consistent effluent standard require complementary chemical (simultaneous) precipitation. Chemical phosphorus precipitation increases sludge mass by 50% and volume by up to 150%, and even if there were no heavy metals in the sludge, it is not a suitable fertilizer, as the excess Fe or Al can remove dissolved phosphate (Smil, 2000). It means the possibility of recycling phosphorus from the sludge is very limited. And the cost to process the sludge is intensive. So it offers another opportunity to integrate microalgal mediated twin-layer system after the secondary sedimentation process to reduce the expense caused by chemical phosphate precipitation and sludge treatment, and to reduce more nitrogen from wastewater **(Scenario 2)**. Moreover, in many wastewater treatment plants in the world, only solid, particles (primary treatment) and BOD/COD (secondary treatment) is reduced from wastewater. No sophisticated ammonium (the main nitrogen source in this kind of wastewater) and phosphorus removal facilities are installed. Wastewater discharges directly into natural water bodies which has caused many environmental and sanitary problems (1.2.2). Microalgal mediated twin-layer system offers a superior solution to reduce both nitrogen and phosphorus from this kind of secondary wastewater **(Scenario 3)**. Based on the discussion above, the different scenarios can be summarized as follows:

Scenario 1 Further reduction of nitrogen and phosphorus from wastewater before discharging

Scenario 2 Saving expense of chemical precipitation and sludge treatment

Scenario 3 Integration into traditional secondary treatment plants to reduce high ammonium and phosphorus contents

Based on the results from the large-scale wastewater treatment, the detail constructional and operational parameters of three scenarios are presented in Table 4-5. The target volume of the wastewater is 100 m^3 per day. The calculations of phosphate and ammonium removal rate are based on the results obtained from “Effect of light/dark cycle on nitrogen and phosphorus removal” experiment with Bio-P wastewater. And nitrate removal rate is based on the average reduction of nitrate when supplying secondary settled wastewater and secondary settled wastewater with additional P. However, 12 hour is considered as the treatment time instead of

24h hour applied in the experiment because it has been confirmed that nitrate uptake is light dependent (4.4.2.5). Therefore, phosphate, ammonium and nitrate removal rate by *S. rubescens* mediated twin-layer system can be calculated as approximately 2.13 mg P m^{-2} h^{-1}, 5.31 mg N m^{-2} h^{-1} and 4.99 mg N m^{-2} h^{-1}, respectively. The influent concentrations used for the estimation are the average measurement results of different wastewater collected from Frechen. One module of twin-layer is supposed to have the surface area of 4 m^2 (2m×2m). And the distance between each module is 0.5m. The power (kW) of the pump to elevate water is calculated by the formula of "hydraulic pump power" and "shaft pump power"[2]. We assume that the differential head from the wastewater to the top of the twin-layer system is 2.3 meter and the efficiency of the pump is 0.6. Finally, the diurnal light/dark cycle is assumed to be 12/12 h.

Table 4-5. Constructional and operational parameters of three scenarios on integrating twin-layer system into wastewater treatment plants; the light/dark cycle is 12/12 h, the hydraulic retention time (HRT) is 24 h, the target treated volume is 100 m^3 and the water flow rate is 3.8 litre m^{-2} h^{-1}

Scenario	Influent concentration (mg l^{-1})	Effluent concentration (mg l^{-1})	Surface area of twin layers (m^2)	Ground area (m^2)	Total water flow rate (m^3 h^{-1})	Pump power (kW)	Total pump energy consumption (kWh)
1	PO_4^{3-}-P=0.6 NO_3^--N=6.7	PO_4^{3-}-P=0 NO_3^--N=2.2	7,515	939	28.6	0.48	11.5
2	PO_4^{3-}-P=2.0 NO_3^--N=6.7	PO_4^{3-}-P=1.0 NO_3^--N=4.3	3,912	489	14.5	0.24	5.8
3	PO_4^{3-}-P=3.8 NH_4^+-N=11.1	PO_4^{3-}-P=1.0 NH_4^+-N=4.1	10,954	1369	41.6	0.69	16.6

Scenario 1: Within short hydraulic retention time (1 day), all the phosphorus and most nitrate can be removed from the secondary settled wastewater. The residual concentration of phosphorus and nitrogen will be lower than the "maximum tolerable risk concentrations" i.e. P < 0.15 mg^{-1} and N < 2.2 mg l^{-1} (1.2.3). And ground area of approximately 939 m^2 will be required to install twin-layer modules to process 100 m^3 wastewater.

[2] http://www.engineeringtoolbox.com/pumps-power-d_505.html

Scenario 2: With the purpose of reducing phosphorus to 1 mg l^{-1}, the minimal electricity consumption for the pump is about 5.8 kWh to process each 100 m^3 wastewater per day. Considering the electricity price for industries in Germany is 8.53 ct/kWh, the energy cost will be 0.49 € to process each 100 m^3 per day, which is higher than 0.31-0.43 € of the operating cost using precipitation method reported by kinebas et al. (2007). However, using twin-layer system, the sludge treatment is not necessary. And the microalgal biomass contained valuable nutrients which can be easily harvested and recycled. These will compensate the expense caused by the electricity consumption. More researches need to be conducted for the cost effectiveness of twin-layer system in comparison with chemical precipitation in the future.

Scenario 3: In order to remove phosphorus and ammonium from 100 m^3 primary wastewater to meet the discharge requirement, i.e. 1 mg l^{-1} P, 13 mg l^{-1} N, minimal ground area of 1369 m^2 is required to install twin-layer modules and at least 16.6 kWh electricity is consumed to operate the pumps daily. In a conventional activated sludge treatment plant (without nutrients removal), approximately 458-1000 kWh of electricity are required to process each 1000 m^3 wastewater. Small plants with lower capacity e.g. 100 m^3 per day, usually consume higher electricity to treat each m^3 wastewater. Plants that have biological treatment e.g. A^2O for nutrients removal use on the order of 30-50 percent more electricity for aeration, pumping, and solids processing than conventional activated sludge treatment (Tchobanoglous et al., 2003). It means the extra electricity consumption to remove nutrients is elevated by 30-50 kWh to process each 100 m^3 wastewater, which is higher than 16.6 kWh using twin-layer system. In addition, according to German standard (ATV-DVWK-AG, 2004), about 127 litre wastewater is discharged by one person daily. Therefore, the ground area of about 1.7 m^2 is required for one person to remove sufficient phosphorus and ammonium from the discharged wastewater.

The previous research on twin-layer system showed that the maximum holding capacity of microalgae on twin-layer system was up to 100 g DW m^{-2} or higher. Considering a linear growth of *S. rubescens* and the average dry weight productivity of 1.05 g m^{-2} day^{-1} recorded in the large-scale wastewater treatment, the twin-layer system can be operated for 95 days before harvesting.

4.4.4 Wastewater Treatment Processes Comparison

The A^2O (Anaerobic-anoxic-aerobic) technology is primarily an activated sludge process and applied very often in wastewater treatment plants with the target of organic substance, phosphorus and nitrogen reduction. A constructed wetland is an alternative that uses living plants and microorganisms in association with plant roots to remove contaminants and nutrients from wastewater. It has been applied in small and medium sized communities in different European countries (EC, 2001). Different operational parameters and treatment effects of twin-layer system (results from Bio-P), activated sludge (A^2O) and a typical constructed wetland named horizontal reed bed filters is compared in Table 4-6. Nitrogen and phosphorus removal efficiencies of twin-layer system are higher than A^2O and wetland technology. The constructional and operational costs of twin-layer system and constructed wetland are generally lower than A^2O. In twin-layer system, the microalgal biomass which contains abundant nutrients could be easily harvested and recycled, while it is not easy and effective to harvest the plants from the wetlands because they contain less % P (0.15-1.05% in helophytes, McJannet et al., 1995) than microalgae (1- 1.2% in microalgae Reynolds, 2008). The sludge formed from A^2O is not a good resource of nutrient recycling. And the sludge treatment is cost intensive. Both A^2O and wetland can remove organic substances from wastewater. By far, it is not achieved by microalgal mediated twin-layer system. More efforts should be focus on selection of microalgae and co-immobilization of microalgae with microalgae/other microorganisms to reduce organic substances.

Table 4-6. Comparison of twin-layer system, activated sludge (A^2O) and constructed wetland (if not specified, the information about activated sludge and constructed wetland were taken from EC (2001))

	Twin-layer system	Activated sludge (A^2O)	Constructed wetland (Horizontal reed bed filters)
Construction	• Greenhouse • Twin-layer photobioreactors • Ground area about 1.7 m^2/person	• Tank • Relatively high capital cost	• A lot of ground area is need, 10 m^2/person • Topography: 1 meter between the feeding point of the plant and downstream point
Organic substances reduction	No (to be investigated in the future)	Yes 70-90%	Yes 70-90%
N and P reduction	• N 100%, • P 58-94%	• N 70-80% • P 80% (chemical precipitation supplementary)	• N 5-10% (poor nitrification, good denitrification) • P < 5% (Stottmeister et al., 2003)
Energy consumption	moderate	High (50 % for aeration) (Tchobanoglous et al., 2003)	Low
Maintenance	Qualified personnel needed for maintenance and regular monitoring	High skilled personnel and regular monitoring	No highly-qualified personnel needed for maintenance
Heavy metal Reduction	Yes	No	Yes (with hyper-accumulate plant), (Stottmeister et al., 2003)
Biosolid treatment	• Produce valuable microalgal biomass that could be potentially recycles • Harvesting is relatively easy	• High sludge production • Sludge treatment is cost intensive	• Effect of harvesting the plant biomass is insignificant (Stottmeister et al., 2003) • Cutting reed is not necessary

4.5 Further development

4.5.1 Reduction of Organic Substances in Addition to Nitrogen and Phosphorus

The results from both "reduction of organic substance" and "large-scale wastewater treatment" in this study showed that there were no biodegradation of organic substances (measured as COD) by three microalgae *C. protothecoides*, *E. gracilis* (Figure 3-19) and *S. rubescens* although the screening experiment showed that they could grow better in the synthetic wastewater than the culture medium (Figure 3-5). Therefore this question is still uncertain and should be further investigated. De la Noüe et al. (1992) also pointed out some efforts on microalgal wastewater engineering should be directed to mixotrophy and heterothrophy because most effluents contain organic substances. And it is necessary to research on the ability of various species to growth on organic effluents with less demand for light.

Khoja and Whitton (1975) reported that *Tolypothrix tenuis*, was capable of dark heterotrophic growth if ammonium is available as a nitrogen source; five strains, *Phormidium luridum*, *Phormidium* sp., *Plectonema boryanum*, *P. boryanum* and *P. calothricoides* are capable of dark heterotrophic growth with nitrate as nitrogen source. These algae might be interesting to be examined for organic substances removal. Furthermore, several strategies have been proposed for cultivation of phototrophic microorganisms by genetic engineering, for example, an obligate photoautotrophic microalga *Phaeodactylum tricornutum* could grow heterotrophically when a single gene (*Glut1*) that encoded the glucose transporter protein (Glut1) was introduced into this algae (Zaslavskaia et al., 2001).

From the application point of view, further research should focus on screening photoheterotrophic microorganisms which could reduce BOD, COD in addition to phosphorus and nitrogen in the wastewater. More advantages of twin-layer system can be predicated when co-immobilizing nitrogen, phosphorus assimilated algae and organic substances absorbed algae (or other microorganisms) together. It will possibly make the configuration of wastewater treatment plants much simpler and save the extensive operational cost.

4.5.2 Preferential Uptake of Nitrate as Nitrogen Source

Although Proctor (1957) reported the preferential assimilation of nitrate by *H. pluvialis*, the results in this experiment did not support it. In the bench-scale experiment, nitrate uptake by *S. rubescens* was clearly inhibited for four days when ammonium (20 mg l^{-1}) presented in the medium (Figure 3-8). Same lag period of four days was found when *H. pluvialis* (M 0761/1), *H. pluvialis* (M 2072) and *S. rubescens* (M 2630) were cultivated in suspension with the same ammonium and nitrate concentrations (Figure 3-20). Only after the lag period, nitrate was assimilated by three algae.

In some types of municipal wastewater, both high ammonium (39.8 mg l^{-1}) and high nitrate (11.83 mg l^{-1}) concentrations existed (Voltolina et al., 2005). Although it has been proved for many algae that ammonium is preferentially absorbed and nitrate is often not utilised until all the ammonium salts have been consumed (summarized by Becker, 1994). In order to reduce both nitrogen pollutants simultaneously, I decide to find some microalgae which can preferentially assimilate nitrate, co-immobilizing with other algae. In practice, the municipal wastewater from Frechen MWTP is enriched with either nitrate (secondary settled wastewater) or ammonium (Bio-P wastewater), or low in both ammonium and nitrate (DeN wastewater). The large-scale experiment has proved that both nitrate and ammonium were successfully removed from all types of wastewater. So the selection of microalgae which can preferentially take up nitrate algae is not necessary for plants operated in similar pattern with Frechen MWTP. However, this is still a potential area for further investigation.

4.5.3 Co-Immobilization and Synergistic Relationship

Some researches have been conducted that co-immobilized microalgae *Chlorella* with active sludge loaded with bacteria to reduce nitrogen, phosphorus and organic substances from wastewater, using the symbiotic interactions between microalgae and bacteria. Microalgae enhance the removal of nutrients and supply O_2 to heterotrophic aerobic bacteria to reduce organic pollutants by using in turn the CO_2 released from bacterial respiration (Wang and Huang, 2005, Gutzeit et al., 2005). A more promising approach for the utilisation of twin-layer system seems to be their employment as a source of photosynthetically oxygen producer in combination with other heterotrophic, oxygen requiring microorganisms, e.g.

heterotrophic bacteria, i.e. co-immobilizing nitrogen, phosphorus assimilated algae, and organic substance absorbed bacteria together utilising the synergistic relationship.

Hernandez et al. (2006) reported that co-cultures of *Chlorella sorokiniana* and microalgae growth-promoting bacterium *Azopirillum brasilense* removed ammonium better than cultures containing only *C. sorokiniana*, where 100% removal was achieved by the co-cultures after 96 under extreme incubation conditions (40 ℃, 2500 μmol m^{-2} s^{-1}). In the same research, they found out *C. sorokiniana* growth rates were increased when using co-cultures under high ammonium concentrations (50 mg l^{-1} NH_4Cl-N) (De-Bashan et al., 2008). We see further advantages of the twin-layer system when synergistic effects of different microorganisms are to be exploited in co-immobilization systems. On twin layers different microorganisms such as bacteria and microalgae can be physically separated in a precise manner, so that e.g. the flow of medium first encounters one organism and then the second or vice versa. This could be important, if one organism releases substances that favour the growth of the second organism or in cases of co-metabolization of xenobiotics.

4.5.4 Sustainable Nitrogen and Phosphorus Treatment/Microalgal Recycling

Phosphorus is an important element, making a major contribution to agricultural and industry development. But its release to surface water in agricultural runoff and wastewater has led to the environmental problems of eutrophication. All life forms require phosphorus in the form of phosphate, which has an essential role in RNA and DNA and in cellular metabolism. But it does not have a rapid global cycle akin to the circulations C or N (Gilbert, 2009, Smil, 2000). On the civilization time scale (10^3 years), the grand natural global P cycle appears to be just a one-way flow (Smil, 2000). At present, commercial phosphorous production is based almost exclusively on phosphate rock-primarily calcium phosphate in various forms (Morse et al, 1998). However, some scientists and industry representatives have started to argue that phosphate rock is becoming a strategic material for many countries. The remaining accessible reserves of clean phosphate rock would run out in 50 years. And the world would move from an oil-based to a phosphate-based economy (summarized by Gilbert, 2009). Therefore, moving phosphorus towards sustainability is urgent.

Anthropogenic impacts have intensified release of P such as increase soil erosion and runoff from fields, recycling of crop residues and manure (land application), discharges of urban and

industry wastes, and application of inorganic fertilizer (Smil, 2000). Most of the dissolved phosphorus loading of inland surface waters is attributable to discharges from point sources, especially municipal wastewater treatment plants and industrial effluent (EEA 1999). Van Van Drecht et al. (2009) predicted a rapid increase in global sewage emissions from 6.4 Tg of N and 1.3 Tg of P per year in 2000 to 12.0-15.5 Tg of N and 2.4-3.1 Tg of P per year in 2050, combining effect of increasing population, urbanization, and the development of sewage systems.

In wastewater treatment plants with nutrients treatment facility, chemical phosphorus precipitation is usually employed, however, it increases sludge mass by 50% and volume by up to 150%, and even if there were no heavy metals in the sludge, it is not a suitable fertilizer, as the excess Fe or Al can remove dissolved phosphate (Smil, 2000). Unlike the chemical method using Fe or Al additives, microalgal wastewater treatment offers an elegant solution to wastewater tertiary treatment due to the ability of microalgae to use inorganic phosphorus and nitrogen for their growth leading to an efficient recycling of nutrients without contamination. In this study, we conclude that phosphate, ammonium and nitrate are taken up metabolically from municipal wastewater by the microalgae immobilized on twin-layer system and incorporated into their biomass. The harvesting procedure of microalgae was also quite simply. This offers a sustainable method for solving eutrophication, wastewater treatment and recycling nutrient (most importantly, phosphorus) from wastewater. Some studies on recycling manure sewage nutrients produced by immobilized algae as a slow release fertilizer has been conducted and proved that algal biomass was equal to fertilizer in supplying N and P to cucumber and corn seedlings (Mulbry et al., 2005). Of course, more intensive researches have to be conducted in the future on recycling twin-layer microalgal biomass produced during wastewater treatment. In case of applying microalgae as fertilizer, the ash-free heavy metal content in the biomass needs to be considered.

5. CONCLUSION

Excessive nitrogen and phosphorus loading from municipal wastewater treatment plants is an ongoing threat to water quality, which leads to more stringent environmental regulations in different countries. An innovative microalgal immobilization technology, the twin-layer system, was investigated in this study to remove nitrogen and phosphorus from municipal wastewater. In the twin-layer system, microalgae are immobilized by self-adhesion on a wet, microporous, ultrathin substrate (the substrate layer). Subtending the substrate layer, a second layer, consisting of a macroporous fibrous material (the source layer), provides the growth medium. Twin-layer effectively separate microalgae from the bulk of their growth medium, yet allow diffusion of nutrients.

In this study, different types of laminar and grid mesh materials were tested as the substrate layer and the source layer of the twin-layer system, considering the hydrophilicity, water distribution, easiness of immobilization, chemical tolerance, endurance and price etc. It concluds that nylon filter cloth and glass grid reinforced lamina were the optimal substrate and source layer respectively. They were applied in the large-scale experiment to process real municipal wastewater from Frechen wastewater treatment plant.

Simultaneously, screening of microalgae for municipal wastewater treatment was carried out with small-scale twin-layer system in horizontal direction. Among 23 preselected microalgae, immobilized *Scenedesmus rubescens*, *Chlamydomonas terricola*, *Scenedesmus* sp., *Euglena anabaena* and *Pediastrum boryanum* exhibited both higher dry weigh productivity (>0.20 g m^{-2} day^{-1}) and higher Chlorophyll *a* productivity (>0.80 µg cm^{-2} day^{-1}) during the cultivation of 20 days supplied by synthetic secondary wastewater. Therefore, they are recommended, in general, to be applied for wastewater treatment in the future.

The growth of microalgae on twin-layer system always exhibited a linear pattern regardless the twin-layer materials and medium applied. The twin-layer system can be regarded as a flat-plat photobioreactor, however, ultra thin, in comparison with the flat-plat bioreactor made by glass. The previous researches on flat-plat bioreactor found out the linear growth rate (LGR) of microalgae was inverse proportional to the light path of the reactor (L) and direct proportional to the incident light intensity (I_0). In the twin-layer system, microalgae immobilized on the substrate layer utilising natural sunlight and get I_0 directly without any hindrance e.g. glass. In addition, the ultra thin property of the immobilized microalgae on the

twin-layer offers low L. So the twin-layer system is the optimal photobioreactor by far to achieve high linear grow rate. In the future, the mathematic model for the linear growth rate of immobilized microalgae on twin-layer system should be formulated.

In the bench-scale treatment with one cycle, *Chlorella vulgaris* and *S. rubescens* removed about 90% phosphorus within 2 days and about 95% ammonium within 9 days from the modified BG-11 medium. These compare well with the studies using other immobilization methods. In the treatment with semi-continuous mode, the important outcome is that the twin-layer system was successfully operated in three continuous cycles. At the end of each treatment cycle (4 days/cycle), the residual concentrations of phosphate-P and ammonium-N kept about 1 mg l^{-1} and < 11 mg l^{-1}, respectively, which met the wastewater discharge regulation forced by different authorities. However, due to the inhibition of ammonium on nitrate assimilation, nitrate uptake always delayed for 2-4 days until most of the ammonium was used. The attempt to apply *Haematococcus pluvialis* as nitrate preferential uptake microalgae was also not succeed. Other microalgae should be tested in the future.

In the large-scale experiment, *S. rubescens* grew well on the twin-layer system. Average dry weight productivity of 1.05 g m^{-2} day^{-1} and Chlorophyll *a* productivity of 0.69 µg cm^{-2} day^{-1} were obtained respectively throughout the experiment. The growth rate of the microalgae on the backside was always lower than on the front side of the twin-layer system because the water contact of the backside was worse than the front. In the future, the water supplying components of twin-layer system need to be designed better. The municipal wastewater which supplied to the microalgae named the secondary settled wastewater, the secondary settled wastewater with additional phosphors, the denitrification tank wastewater and the biological phosphorus (Bio-P) tank wastewater. Immobilized *S. rubescens* was able to take up both phosphorus and nitrogen efficiently and continuously in all treatment cycles supplied by the four types of wastewater. (1) The residual phosphate-P and nitrate-N in the secondary settled wastewater was <0.22 mg l^{-1} and < 2 mg l^{-1} respectively after one day. (2) The residual phosphate-P concentrations were < 1mg l^{-1} in all the eight treatment cycles within one day in the secondary wastewater with additional phosphorus. (3) Both ammonium and nitrate were low in the denitrification tank wastewater, therefore both of them were reduced by the algae rapidly within one day, and no limitation of ammonium on nitrate uptake was observed. Phosphate-P was reduced gradually to <0.4 mg l^{-1} within two days (start with 14-2.6 mg l^{-1}). The low nitrogen source in this type of wastewater limited the growth of *S. rubescens*

indicated by Chlorophyll a and dry weight. (4) Bio-P tank wastewater was enriched of both phosphate and ammonium. Phosphate was reduced gradually by the algae in two days from 2.7-4.6 mg l^{-1} to < 2 mg l^{-1} at day 1 to < 1 mg l^{-1} at day 2. Ammonium was also removed rapidly by *S. rubescens* from 7.8-13.9 mg l^{-1} to < 0.4 mg l^{-1} within one day, and further reduced to <0.1 mg l^{-1} at day 2. Furthermore, the results obtained from the experiment with continuous sampling for 48 hours (plus literature review) indicated that phosphate and nitrate uptake by *S. rubescens* was light dependent, whereas ammonium was not. The growth of microalgae and nutrients depletion from the wastewater indicated that nutrients were taken up metabolically by microalgae and incorporated into their biomass. However, no organic substances (measured by COD) were reduced by *S. rubescens* in this experiment. Moreover, the results obtained from two algae, *Euglena gracilis* and *Chlorella protothecoides*, showed there was no assimilation of organic substances by both algae. From an application point of view, further research should focus on screening of photoheterotrophic microalgae which could reduce organic substances in addition to phosphorus and nitrogen in the wastewater.

Based on the results obtained from the large-scale experiment, three scenarios on integration of twin-layer system into municipal wastewater treatment plant with 100 m^3 capacity daily were posed. In scenario 1, within short hydraulic retention time (1 day), all the phosphorus and most nitrate (residual concentration=2.2 mg l^{-1}) can be removed from the secondary settled wastewater. In scenario 2, with the purpose of reducing phosphorus to 1 mg l^{-1}, the minimal electricity consumption for the pumps is about 5.8 kWh daily. In scenario 3, in order to remove phosphorus and ammonium from primary wastewater to meet the discharge requirement, i.e. 1 mg l^{-1} P, 13 mg l^{-1} N, minimal ground area of 1369 m^2 is required to install twin-layer modules and at least 16.6 kWh electricity is consumed daily to operate pumps.

Comparing the *S. rubescens* mediated twin-layer system with the activated sludge and the constructed wetland, nitrogen and phosphorus removal by twin-layer system is more efficient (removal efficiency, twin-layer 100% N > activated sludge 70-80% N > wetland 5-10% N). The constructional and operational cost of the twin-layer system is moderate. And the microalgal biomass produced can be easily harvested, which contains abundant nutrients, while, it is not cost effective for the other techniques.

To summarize, twin-layer system offers a promising alternative for nitrogen and phosphorus removal since it provides a treatment to remove both nutrients efficiently coupled with the

production of potentially valuable biomass. The basic aspects upon which more work is essential include:

1. Screening of phototrophic microalgae which could reduce organic substances in addition to phosphorus and nitrogen in the wastewater.

2. Employing microalgae as a source of photosynthetically oxygen producer in combination with other heterotrophic, oxygen requiring microorganisms, e.g. heterotrophic bacteria, i.e. co-immobilizing nitrogen, phosphorus assimilated algae and organic substance absorbed bacteria together, utilising the synergistic relationship.

3. Optimizing the configuration of the twin-layer system to achieve most favourable growth of microalgae on both sides.

4. Cultivating *S. rubescens* for longer period to find out the kinetic of the growth and the operating time for wastewater treatment before harvesting the cells.

5. Formulating mathematic model for the linear growth rate of immobilized microalgae on twin-layer system.

6. Selection of microalgae/other microorganisms which can preferentially take up nitrate as nitrogen source.

7. Designing and constructing an industry-scale twin-layer system.

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APPENDIX

Chlorophyll *a* productivity (μg cm^{-2} day^{-1}) of immobilized microalgae grown on nylon filters with different medium

		Synthetic 2nd wastewater	Modified Waris-H medium	BG-11
1	*Chlamydomonas terricola*	1.40	1.36	1.23
2	*Chlorella protothecoides*	0.50	0.33	0.10
3	*Chlorella vulgaris*	0.55	1.39	0.70
4	*Euglena anabaena*	1.11	0.50	0.36
5	*Euglena gracilis*	0.45	0.28	0.05
6	*Microthamnion kuetzingianum*	0.34	0.61	0.42
7	*Pediastrum boryanum*	0.81	0.85	1.02
8	*Pediastrum duplex*	0.77	0.92	0.79
9	*Scenedesmus* sp. (China)	0.84	1.05	1.29
10	*Scenedesmus rubescens* (M2069)	0.68	0.81	1.49
11	*Scenedesmus rubescens* (M2630)	1.21	0.92	1.21
12	*Stigeoclonium* sp.	0.58	1.07	0.35
13	*Oedogonium stellatum*	0.91	0.96	0.44

GLOSSARY

BOD_5: The biochemical oxygen demand is a measurement of the pollution by organic matter. It is expressed in milligrams of oxygen per day and per p.e. It corresponds to the quantity of oxygen that is needed to oxidise the discharges of polluted effluents produced on average by each inhabitant in a watercourse or by a given agglomeration. This measurement is carried out according to standardised tests after five days of oxidation of the organic matter, hence the term BOD_5.

COD: The chemical oxygen demand or represents the quantity of oxygen consumed, expressed in milligrams per litre, by the chemically oxidizable matter contained in a discharge. According to the standard method, this is the oxidation by an excess of potassium dichromate ($K_2Cr_2O_7$) in a fermenting and acidic medium, of the chemically oxidizable matter contained in a discharge. COD is a valuable parameter indicating the presence of pollution in wastewater. It represents the major part of the organic compounds but also oxidizable mineral salts (sulphides, chlorides, etc.). Industrial wastewater can frequently reach COD values of several grams per litre.

Eutrophication means the enrichment of water by nutrients, especially compounds of nitrogen and/or phosphorus causing an accelerated growth of algae and higher forms of plant life to produce an undesirable disturbance to the balance of organisms present in the water and to the quality of the water concerned;

Population equivalent (p.e.) is a measure of pollution representing the average organic biodegradable load per person per day: it is defined in EU Directive 91/271/EEC as the organic biodegradable load having a five-day biochemical oxygen demand (BOD_5) of 60 g of oxygen per day.

Nutrients are chemical elements which are involved in the construction of living tissue and which are needed by both plant and animal. The most important in terms of bulk are carbon hydrogen and oxygen with other essential ones including nitrogen, potassium, calcium, sulphur and phosphorus.

Source: http://glossary.eea.europa.eu/EEAGlossary/N/nutrient

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DECLARATION

ERKLÄRUNG

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen - , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. M. Melkonian betreut worden.

Köln, den 18.12.2009