# Settleable algal-bacterial culture for municipal wastewater treatment

Der Fakultät Nachhaltigkeit der Leuphana Universität Lüneburg zur Erlangung des Grades Doktorin der Naturwissenschaften

-Dr. rer. nat.-

vorgelegte Dissertation von


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Eingereicht am: 11.09.2012

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Tag der Disputation: 07.12.2012

*Nature does not hurry, yet everything is accomplished…*

- Lao Tzu

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Paper II: Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: Influence of algae and sludge inoculation ratios. *Bioresource Technology* 105, 67-73 (2012).

Paper III: Comparison of nutrient removal capacity and biomass settleability of four high-potential microalgal species. In press. DOI: 10.1016/j.biortech.2012.08.037.

Paper IV: Coupled nutrient removal and biomass production with mixed algal culture: Impact of biotic and abiotic factors. *Bioresource Technology* 118, 469-476 (2012).

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# List of Abbreviations

| | |
|---|---|
| AIWPS | Advanced integrated wastewater pond system |
| ANAMMOX | Anoxic ammonium oxidation process |
| ATP | Adenosine-triphosphate |
| ATS | Algal turf scrubbing |
| BOD | Biochemical oxygen demand |
| CANON | Completely autotrophic nitrogen removal over nitrite process |
| COD | Chemical oxygen demand |
| DAF | Dissolved air flotation |
| DGGE | Denaturing gradient gel electrophoresis |
| DO | Dissolved oxygen |
| DNA | Deoxyribonucleic acid |
| EPS | Extracellular polymeric substances |
| FTU | Formazin Turbidity Unit |
| HRT | Hydraulic retention time |
| mM | Millimolarity, mmol/l |
| NADH | Nicotinamide adenine dinucleotide |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Nicotinamide adenine dinucleotide phosphate hydrogen |
| $NADPH_2$ | Nicotinamide adenine dinucleotide phosphate dihydrogen |
| PAOs | Polyphosphate accumulating organisms |
| PPP | Pentose phosphate pathway |
| PHAs | Poly-β-hydroxyalkanoates |
| R4P | Erythrose-4-phosphate |
| R5P | Ribose-5-phospate |
| rpm | Revolutions per minute |
| SHARON | Single reactor system for high activity ammonium removal over nitrite process |
| TCA | Tricarboxylic acid cycle |
| TKN | Total kjeldahl nitrogen |
| TOC | Total organic carbon |
| TSS | Total suspended solid |
| VFAs | Volatile fatty acids |
| WWTP | Wastewater treatment plant |

# Summary

Algae-bacteria-based biotechnology has received more and more attention in recent years, especially in the subtropical and tropical regions, as an alternative method of conventional multistep wastewater treatment processes. Moreover, the algal biomass generated during wastewater treatment is regarded as a sustainable bioresource which could be used for producing biofuel, agricultural fertilizers or animal feeds. Although this technology is attractive, a number of obstacles need to be solved before large-scale applications. The main purposes of this work are to find more effective biomass harvesting strategies and develop high-effective algal-bacterial systems to improve wastewater treatment performance, biomass generation rate and biomass settleability.

A wastewater-borne algal-bacterial culture, cultivated and trained through alternate mixing and non-mixing strategy, was used to treat pretreated municipal wastewater. After one month cultivation and training, the acclimatized algal-bacterial system showed high carbon and nutrient removal capacity and good settleability within 20 minutes of sedimentation. Algal biomass uptake was the main removal mechanism of nitrogen and phosphorus. The biomass productivity, nitrogen and phosphorus accumulation in biomass during the wastewater treatment process were investigated. The characterization of the microbial consortium composition in the enriched algal-bacterial system provided new insights in this research field.

Aerobic activated sludge which already showed good settleability was used as bacterial inoculum to enhance the wastewater treatment performance and biomass settleability of algal-bacterial culture. The influence of different algae and sludge inoculum ratios on the treatment efficiency and biomass settleability was investigated. There was no significant effect of the inoculation ratios on the chemical oxygen demand (COD) removal. But algae/sludge inoculum ratio of 5 showed the best nitrogen and phosphorus removal efficiencies (91.0 ± 7.0% and 93.5 ± 2.5%, respectively) within 10 days. Furthermore, 16S rDNA gene analysis showed that the bacterial communities were varying with different algae and sludge inoculation ratios and some specific bacteria species were enriched during the operation.

Four commonly used and high-potential microalgae species including one cyanobacteria (*Phormidium* sp.) and three green microalgae species (*Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Scenedesmus rubescens*) were cultivated and trained through alternate mixing and non-mixing strategy for tertiary municipal wastewater treatment. After one month of cultivation, the four microalgae species

were compared in terms of biomass settleability, nutrient removal rates and biomass productivity. The three green microalgae showed good settleability within 1 h sedimentation and had higher biomass generation rates (above 6 $g/m^2/d$). The nutrient removal efficiencies were 99% for the four selected microalgae species but within different retention time, resulting in 3.66 ± 0.17, 6.39 ± 0.20, 4.39 ± 0.06 and 4.31 ± 0.18 mg N/l/d (N removal rate) and 0.56 ± 0.07, 0.89 ± 0.05, 0.76 ± 0.09 and 0.60 ± 0.05 mg P/l/d (P removal rate) for *Phormidium* sp., *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Scenedesmus rubescens*, respectively.

A mixed algal culture composed of three selected high-effective green microalgae (*Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Scenedesmus rubescens*) was used for tertiary municipal wastewater treatment. The key biotic factor (algal inoculum concentration) and abiotic factors such as illumination cycle, mixing velocity and nutrient strength were studied. Based on the nitrogen and phosphorus balance, it was found that assimilation into algal biomass was the main removal mechanism.

# Chapter 1

# Background and aims of the study

## 1.1 Background and aims of the study

The growing of world population and human activities pose a serious threat to the environment due to the release of a big amount of wastewater. To care for our environment and thus for our health, wastewater needs to be treated before being returned to the environment. The algal-bacterial culture as a sustainable and cost-efficient biosystem for municipal, industrial and animal wastewater treatment has experienced increased momentum over the past few years (Aslan and Kapdan, 2006; Borde et al., 2003; de-Bashan et al., 2002; Munoz and Guieysse, 2006; Munoz et al., 2005; Olguin, 2003; Oswald, 1995; Zhang et al., 2011). It is especially favorable in regions with year-round high solar radiation as the removal process is powered by solar energy (Oswald, 2003). Under illuminated conditions, algae uptake nutrients and produce $O_2$ that may be used as an electron acceptor by aerobic bacteria to degrade organic matter. Algae also consume the $CO_2$ released from bacterial respiration to complete the photosynthetic cycle, which contributes to greenhouse gas abatement (Oswald, 1988; Oswald and Gotaas, 1957). In wastewater-fertilized systems, the roles of algae include $O_2$ supply, nutrient uptake and adsorption of heavy metals (Munoz et al., 2006; Nurdogan and Oswald, 1995). Furthermore, the high pH and high dissolved oxygen concentration induced by algal photosynthesis would lead to indirect disinfection (Nurdogan and Oswald, 1995; Schumacher et al., 2003). Thus it is an attractive alternative or supplement to the conventional multistep wastewater treatment process (Ahn, 2006; Oehmen et al., 2007; Yeoman et al., 1988).

In addition, algal-bacterial cultures for wastewater treatment retain useful nitrogen compounds in the biomass instead of releasing a majority of the nitrogen as $N_2$ or $N_2O$ gas with common nitrogen removal methods such as bacterial nitrification/denitrification (Buys et al., 2000; Zeng et al., 2003). Furthermore, the microalgae accumulated during wastewater treatment as an important bioresource can be used as animal feeds, soil amendments and agricultural fertilizers (Benemann et al., 1977; Mulbry et al., 2008; Mulbry et al., 2006; Mulbry et al., 2005). In addition, they can also be used for biodiesel and biogas production to face the global demand of renewable fuel resources in the near future (Rawat et al., 2011). Considering the

above, this algal-bacterial system would realize the integration of wastewater treatment with high added value algal biomass production (Christenson and Sims, 2011). Not only the solar energy inducing algal photosynthesis is nearly free of charge, but also the nutrients in the wastewater are costless and ideally suited for algal mass cultures and the wastewater treatment revenues could partly offset algae production costs.

However, harvesting the algal biomass from the discharge after wastewater treatment in a cost-efficient way remains a major hurdle to the implementation of this technology. Current harvesting methods include physical based, chemical based and immobilized systems, but each of them has their own disadvantages (de-Bashan et al., 2004; Lee et al., 1998; Perez-Martinez et al., 2010; Sukenik and Shelef, 1984). Physical based methods such as filtration and centrifugation are cost-prohibitive for large scale use as the power requirements and investment are high (Shelef et al., 1984; Uduman et al., 2010). Chemical based technology is often performed to promote the flocculation and coagulation by addition of chemical compounds such as aluminum, aluminum chloride, ferric cations and calcium chloride (Imase et al., 2008; Lee et al., 1998; Oh et al., 2001). Such technology needs additional costs for chemicals, leads to the increase of effluent salinity and causes secondary contaminations (Nurdogan and Oswald, 1995). Immobilization system also offers an option for harvesting biomass. There are two kinds of immobilization technology. The first one is through increasing the particle size with natural or synthetic polymers such as chitosan, alginate, carrageenan and cellulose fibres (de-Bashan and Bashan, 2010; Gonzalez and Bashan, 2000), but these polymeric matrices are costly and weak during long-term operation (Chevalier and de la Noue, 1985a; Su et al., 2009). The second one is attached system by promoting the formation of algal biofilm on the carrier, but the photoinhibition and diffusional transport within the biofilm could result in low nutrient removal efficiency (He and Xue, 2010). Therefore, a more effective biomass harvesting strategy such as a settleable algal-bacterial system is required. Besides, Gutzeit et al. (2005) used *Chlorella vulgaris* and activated sludge to cultivate algal-bacterial aggregates in SBR mood for wastewater treatment. These aggregates showed a good settleability. However, whether this good settleability is because of activated sludge is uncertain in previous works.

Usually, the algal species were selected to be used for coupled wastewater treatment and algal bioproducts production according to the nutrients removal rate, biomass productivity and lipid content (Gonzalez et al., 2008b; Subashchandrabose et al., 2011; Zhou et al., 2011). But there is a lack of systematical comparison of these commonly used algal species as most of these researches were performed separately

under different environmental conditions and bioreactor configurations. More importantly, the settleability of an algae-based culture should also be considered as an important algal selection criterion associated with the success of the whole system.

Besides, although the identification and biometry of the dominant algal species have been investigated in previous researches (Godos et al., 2009), the information on the bacterial community involved in these symbiotic cultures is still blank. Because bacteria as an important partner have maintained symbiotic relationship with algae, investigation of the microbial compositions and their functionalities could provide some insights into these synergistic systems.

Therefore, the main objectives of this PhD project were to improve the nutrient removal performance and reduce the algal biomass harvesting costs for municipal wastewater treatment with algal-bacterial culture from the point of views of cultivation strategy, high-potential algae-based culture selection and the investigation of the influence of abiotic and biotic factors. Specific objectives are:

- Cultivation and training of the settleable algae-based culture through alternate mixing and non-mixing strategy
- Developing the novel and settleable algal-bacterial system with different algal and bacterial compositions to improve the treatment performance and biomass settleability
- Selection of high-potential unicellular microalgae species in terms of nutrient removal, biomass productivity and settleability
- Investigation of the influence of abiotic and biotic factors on the treatment performance, biomass generation and settleability
- Investigation of N and P removal mechanisms
- Identification and characterization of the microbial consortium composition in algal-bacterial culture

In order to fulfill the above objectives, the following work-tasks were addressed.

**1. Municipal wastewater treatment and biomass accumulation with a wastewater-born and settleable algal-bacterial culture (*Paper I in Appendices*)**

A settleable algal-bacterial culture, composed of wastewater-borne algae and wastewater bacteria, was cultivated through alternate mixing and non-mixing strategy to treat municipal wastewater. The N and P removal mechanisms were explored for better understanding of the system. Besides, the biomass generation and the processes of N and P accumulation into biomass were monitored. The evolution of the microbial community of this algal-bacterial culture was investigated as a new insight in this

area.

**2. Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: Influence of algae and sludge inoculation ratios (*Paper II in Appendices*)**

Aerobic activated sludge was used as bacterial inoculum to enhance biomass settleability and the wastewater treatment capacity. The influences of the algae and activated sludge inoculation ratios on the removal efficiency and corresponding biomass settleability were explored for further optimization. The nutrient removal mechanisms were studied for better understanding of the symbiotic system with different algae and sludge inoculation ratios. The microbial communities enriched in the reactors with different inoculation ratios were studied.

**3. Comparison of nutrient removal capacity and biomass settleability of four high-potential microalgal species (*Paper III in Appendices*)**

Four commonly used and high-potential microalgal species including one cyanobacteria and three green microalgae, were cultivated and trained through alternate mixing and non-mixing strategy for tertiary wastewater treatment. The four selected microalgae were compared in terms of nutrient removal rate, biomass settleability and biomass generation rate. The N and P balance of four different algae-based cultures were evaluated.

**4. Coupled nutrient removal and biomass production with mixed algal culture: Impact of biotic and abiotic factors (*Paper IV in Appendices*)**

As the mixed algal culture showed better nutrient removal rates compared with the individual unicellular green algal species, a mixed algal culture composed of three green microalgae species selected based on the results of Paper III, was used for tertiary wastewater treatment to optimize the algae-based system. The biotic (algal inoculum concentration) and biotic factors (illumination cycle, mixing velocity and nutrient strength) on nutrient removal, biomass productivity and biomass settleability were explored. The N and P removal mechanisms in certain favorable conditions were evaluated.

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# Chapter 2

# Microalgae

## 2.1 The advantages and potentials of microalgae

In this study, the definition of microalgae includes prokaryotic cyanobacteria (blue-green algae), eukaryotic microalgae (green algae, red algae, golden algae and brown algae). They belong to one of the oldest life-forms of earth, which constitute the basis of the food chain and contribute approximately 40 to 50% of the oxygen to the atmosphere (Andersen, 2005; Brennan and Owende, 2009). Compared with terrestrial crops, microalgae show some advantages: (1) The average productivity of microalgal cultures is 15 g/$m^2$/d being much higher than the current average world crop productivity (0.1 g/$m^2$/d) (Nurdogan and Oswald, 1995); (2) As microalgae grow in aqueous media, no additional water (e.g. irrigation) is needed and the load on freshwater sources is reduced (Dismukes et al., 2008); (3) Under favorable conditions, microalgae are capable of all year round production (Schenk et al., 2008); (4) Herbicides or pesticides are not required during algae cultivation (Rodolfi et al., 2009). Besides, a wide range of applications are explored due to their high protein content and the ability to synthesize an extraordinary variety of metabolites (Table 2.1).

## 2.2 Microalgal metabolism

Microalgae could either be autotrophic or heterotrophic and some species are mixotrophic.

### 2.2.1 Autotrophic metabolism

For autotrophic algae, photosynthesis is their key survival mode, as all microalgae are photoautotrophs. Photosynthesis is the process of transferring light energy into chemical energy and converting $CO_2$ and water into carbohydrates and oxygen, which is quite similar in algae and higher plants (Richmond, 2004). It includes two processes: light (light-dependent) reactions and dark (light-independent) reactions (Fig. 2.1). The photosynthetically active radiation ranges from 400 nm to 750 nm, which represents about 40% of the direct solar irradiance (Richmond, 2004). In the light reactions, light

energy (natural or artificial) is captured by antenna pigments and excites the electrons in photosystem I. The excited electrons are used to reduce nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide phosphate dihydrogen ($NADPH_2$). The lost electron in photosystem I is compensated from photosystem II, which causes the concomitant synthesis of adenosine-triphosphate (ATP). The photosystem I gains the electrons from photosystem II, the electrons lost by photosystem II are further replaced by splitting water ($H_2O \rightarrow 2H^+ + 2e^- + 1/2O_2$) (Falkowski and Raven, 1997). The $NADPH_2$ and ATP generated in light reactions are required and participate in the dark reactions to incorporate $CO_2$ into carbohydrate via a cyclic pathway called the Calvin cycle (Raven et al., 2005). The light reactions are the precondition of dark reactions, as dark reactions rely on the products of the light reactions.

**Table 2.1** Summary of microalgal applications in different areas

| Area | Application/products | reference |
|---|---|---|
| Environment purification | Wastewater treatment | (Hoffmann, 1998; Munoz et al., 2003a; Munoz et al., 2003b; Oswald, 1988; Park and Craggs, 2011) |
| | Waste gas cleaning | (Berthe-corti et al., 1998; Nagase et al., 2001) |
| | Hydrophobic and toxic compounds degradation | (Daugulis, 2001; Deziel et al., 1999) |
| Energy production | Bio-oil | (Kumar et al., 2010; Lin and Lin, 2011) |
| | Biogas: $H_2$ or methane | (Berberoglu and Pilon, 2010; Oswald, 2003) |
| | Bioethanol | (Grima et al., 2003) |
| Agriculture | Fertilizer | (Banerjee et al., 1997; Benemann et al., 1977) |
| | Animal feeds | (Hemaiswarya et al., 2011) |
| Human health: pharmaceuticals and nutraceuticals | Beta-carotene, phycobiliprotein, fatty acids, antioxidant astaxanthin and microalgal tablet or powder | (Metting, 1996; Milledge, 2011; Oswald, 2003) |
| Human food | Noodle, drink | (Liang et al., 2004) |
| Skin care | Cosmetics | (Spolaore et al., 2006) |

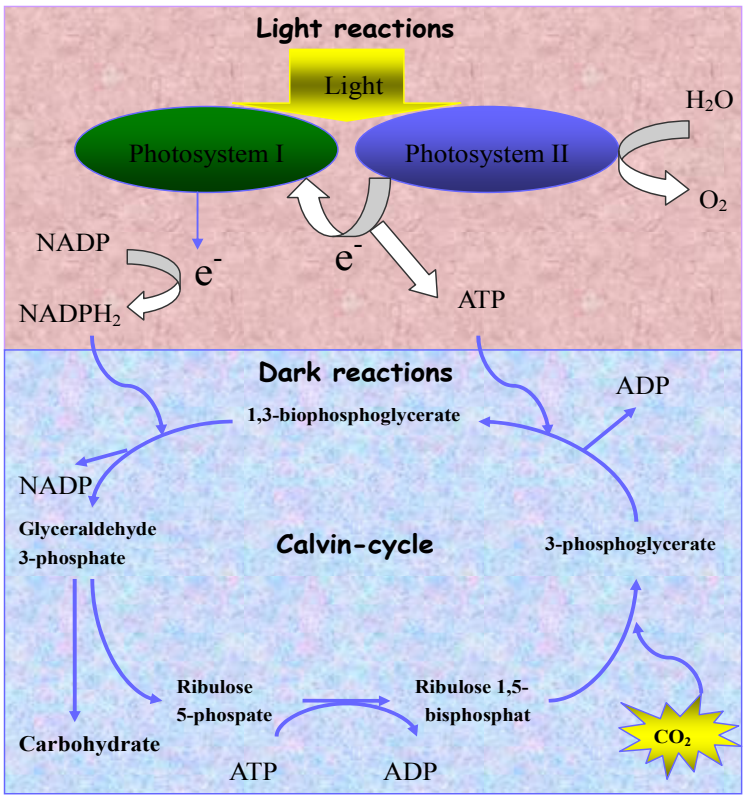


**Figure 2.1** General schematic diagram of algal photosynthesis

The carbohydrates produced by photosynthesis are used as carbon skeletons to form other organic compounds in the algal cells. There are major interactions between N-assimilation and photosynthetic metabolism. N-metabolism (the synthesis of protein and deoxyribonucleic acid (DNA)) is integrated with the generated carbohydrates, accounting for approximately 50% of algal carbon (Vanlerberghe et al., 1990). The energy required for the assimilation of N is also provided through further use of the carbohydrates which were accumulated by photosynthetic processes (Turpin, 1991). Table 2.2 summarizes the N-assimilation process with the main N sources ($NH_4^+$, $NO_3^-$ and $NO_2^-$) in algal cells (Table 1.2). $NO_2^-$ should be transferred into $NH_4^+$ first and then further into the polypeptide synthesis. $NO_3^-$ needs more steps ($NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$) to finish the protein synthesis. After that, a group of proteins of the ammonium transporter family is required to transport the ammonium across the membranes to finish the synthesis of amino acids (Perez-Garcia et al., 2011; Turpin, 1991). Paper III indicates that microalgae prefer to use ammonium first, followed by nitrate and nitrite. These results are consistent with previous studies (Perez-Garcia et al., 2011).

**Table 2.2** N-assimilation processes with different N sources

| N source | Process | Reactions |
|---|---|---|
| $NO_3^-$ | 1. Nitrate reduction | $NO_3^- + 2e^- \rightarrow NO_2^-$ |
| $NO_2^-$ | 2. Nitrite reduction | $NO_2^- + 6e^- \rightarrow NH_4^+$ |
| $NH_4^+$ | 3. Ammonium assimilation | $NH_4^+$ + glucose + 2-oxoglutarate + ATP + $2e^- \rightarrow$ 2glucose |
| | 4. Amino acid interconversion | Glucose + keto acid → amino acid + 2-OG |
| | 5. Protein synthesis | Amino acid + 4ATP → polypeptide |

### 2.2.2 Heterotrophic metabolism

Not all microalgae can grow under heterotrophic conditions and only a few microalgae species are heterotrophic (Lee, 2001). Heterotrophic algae are non-photosynthetic and therefore, require organic matter to replace light as the substrate and energy source for their growth (Kaplan et al., 1986). During respiration, oxygen is supplied through aeration to mineralize organic substrate into $CO_2$ (Griffiths et al., 1960).

For heterotrophic microalgae, glycolysis, tricarboxylic acid cycle (TCA), glyoxylate cycle, the pentose phosphate pathway (PPP), nitrogen assimilation and fatty acid synthesis are the main important metabolisms (Perez-Garcia et al., 2011). Glycolysis is the metabolic pathway that transfers glucose into pyruvate and generates concomitant ATP. The generated pyruvate is then converted into acetate and transferred into the mitochondrion and further to the TCA cycle under aerobic conditions. Through the TCA cycle, acetate in form of acetyl-CoA is further oxidized into $CO_2$, and following along with this process, energy (e.g., ATP) and precursors (e.g., NADH) are generated for further metabolic activity (Lowenstein, 1969). The TCA cycle is the central important chemical reaction for carbohydrate, lipid and protein metabolism. PPP is a glucose oxidation process generating reducing equivalents (nicotinamide adenine dinucleotide phosphate hydrogen, NADPH), ribose-5-phospate (R5P) for nucleotides and nucleic acids synthesis, and erythrose-4-phosphate (R4P) for amino acids synthesis (Kruger and von Schaewen, 2003). Although PPP and glycolysis are the only two ways for glucose breakdown in algal cells, they play different roles and take place under different conditions (Neilson and Lewin, 1974). Glucose is mainly metabolized via PPP under dark conditions as anabolism. And the Glycolysis mainly takes place in light conditions as the glycolytic process (Yang et al., 2000). Glyoxylate cycle is the alternative anaerobic process for TCA to utilize simple carbon compounds as the carbon source when complex carbon sources such as glucose are absent (Kondrashov et al., 2006).

Although it is impossible to precisely predict which specific substrate could be used or preferred by any given microalgae species, glucose is available to the great majority of heterotrophic algae which is most commonly used (Droop, 1974; Neilson and Lewin, 1974). More than 85% of the assimilated glucose is converted to oligo- and polysaccharides. About 1% remains are as free glucose in algal cells and the other are used for catabolism to provide energy.

As for autotrophic microalgae, carbon and nitrogen metabolism are closely linked because the required carbon skeletons for N-assimilation are supplied through respiration (heterotrophic algae) and $CO_2$ fixation (autotrophic algae), whereas the required energy derives from TCA cycle and electron transport chain (Perez-Garcia et al., 2011). Ammonium, nitrate and urea are the main nitrogen sources for algal N-assimilation, as well as yeast extract, peptone, amino acids and purines (Chen and Chen, 2006; Ganuza et al., 2008; Perez-Garcia et al., 2011). It is reported that the preferred nitrogen source of most microalgal species follow a declining order: ammonium > nitrate > nitrite > urea (Perez-Garcia et al., 2011).

Mixotrophic microalgae could grow through both autotrophy and heterotrophy and there is no definite switch between them, except in total darkness (Richmond, 2004).

## 2.3 Microalgal cultivation

Carbon is the basic element for microalgal growth which is supplied in terms of $CO_2$ for autotrophic algae and organic carbon for heterotrophic algae. Besides, several inorganic elements are universally required for algal growth, such as N, P, K, Mg, Ca, S (macronutrients) and Fe, Cu, Mn and Zn (micronutrients). Some algae require Co, Mo, Na, Se, V or vitamins (Acreman, 1994).

Basically two main photoautotrophic microalgal cultivation systems are used so far: open raceway ponds and closed photobioreactors (Chisti, 2007; Munoz and Guieysse, 2006; Rawat et al., 2011). The open raceway ponds are shallow open ponds built in raceway configuration with mixing and circulation paddle wheel (Brennan and Owende, 2009). The $CO_2$ requirement is usually from surface air or submerged aerators installed to enhance $CO_2$ absorption (Terry and Raymond, 1985). Although the theoretical biomass productivity of open raceway ponds is around 50-60 $g/m^2/d$ (Christenson and Sims, 2011), even a range of 10-20 $g/m^2/d$ is difficult to achieve in practice (Shen et al., 2009). Tubular or flat plate photobioreactors made of glass or plastic are the two types of closed systems and only the former type is used at large scale (Brennan and Owende, 2009; Tredici and Zittelli, 1998). They are arranged in a vertical, horizontal, helical or inclined manner (Miron et al., 1999; Molina et al., 2001;

Tredici, 1999). A mechanical pump or airlift system are used to allow $CO_2$ and $O_2$ to be exchanged as well as to provide a mechanical mixing (Eriksen, 2008). The biomass productivities for closed reactors generally range from 20-40 $g/m^2/d$ and are higher than those of open ponds (Shen et al., 2009). The comparisons of open raceway ponds and closed photobioreactors are summarized in Table 2.3.

**Table 2.3** Advantages and disadvantages of open ponds and closed photobioreactors

| Production system | Advantages | Disadvantages |
|---|---|---|
| Open raceway pond | Relatively inexpensive to build and operate | Low biomass productivity |
| | Low energy inputs | Poor mixing and light |
| | Easy to clean | Large area of land required |
| | | High pollution and contamination risks |
| | | Limited to only a few species |
| | | Inefficient use of $CO_2$ |
| Closed reactor | Better pH and temperature control | Expensive to construct and operate |
| | Less evaporation losses | Some degree of wall growth |
| | Better protection against culture contamination | Difficult to clean the walls of reactors |
| | More appropriate for sensitive strains and mono-cultivation | |
| | Low cost harvesting cost due to high cell mass productivities | |
| | Low hydrodynamic stress | |

Heterotrophic microalgae cultivation has been developed by using organic carbon such as glucose instead of $CO_2$ as sole carbon and energy source in fermenters or stirred tank bioreactors (Brennan and Owende, 2009; Chen, 1996). Compared to photoautotrophic cultivation, heterotrophic production systems eliminate the requirement for light, provide a high degree of growth control and lower the harvesting cost due to the higher microalgal cell concentration achieved (Chen and Chen, 2006). However, some problems, such as the limited number of available heterotrophic algae, inability to produce light-induced products and potential contamination by bacteria, are still obstacles in this technology (Chen, 1996).

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# Chapter 3

# Municipal wastewater treatment and microalgae

Municipal wastewater is a combination of wastewater originating from households, institutions, commercial establishments and industrial facilities. It is high in organic matter, nitrogen, phosphorus and may also contain toxic compounds and pathogenic microorganisms thus needing a treatment before being reused or returned to the environment. On the other hand, municipal wastewater could be used as a readily available and cost-effective substrate to fulfill the requirements of microalgal growth (Nandini et al., 2010; Wang et al., 2010). Municipal wastewater treatment with algae-based technology would realize the integration of wastewater treatment and algal biomass production.

## 3.1 Municipal wastewater treatment

Combinations of physical, chemical and biological methods are used to remove the contaminants from municipal wastewater. Several steps are usually needed to achieve the agreeable levels of effluent in the wastewater treatment plant (Fig. 3.1).

### 3.1.1 Primary treatment

Primary treatment is the initial stage in the treatment of municipal wastewater. It is designed to remove floating and settleable solid materials in raw wastewater mainly by physical processes. During the primary treatment process, the raw wastewater flows through a screen firstly to remove large floating objects. After that, it flows into a grit chamber where mineral particles settle to the bottom. With the screen and grit completed, wastewater then enters the last step to remove fine organic particles using further treatment such as sedimentation or gravity settling, sometimes supported by chemical precipitation (Tchobanoglous et al., 2003). The wastewater discharged after primary treatment still contains high amounts of organic carbon and nutrients which need further treatment.

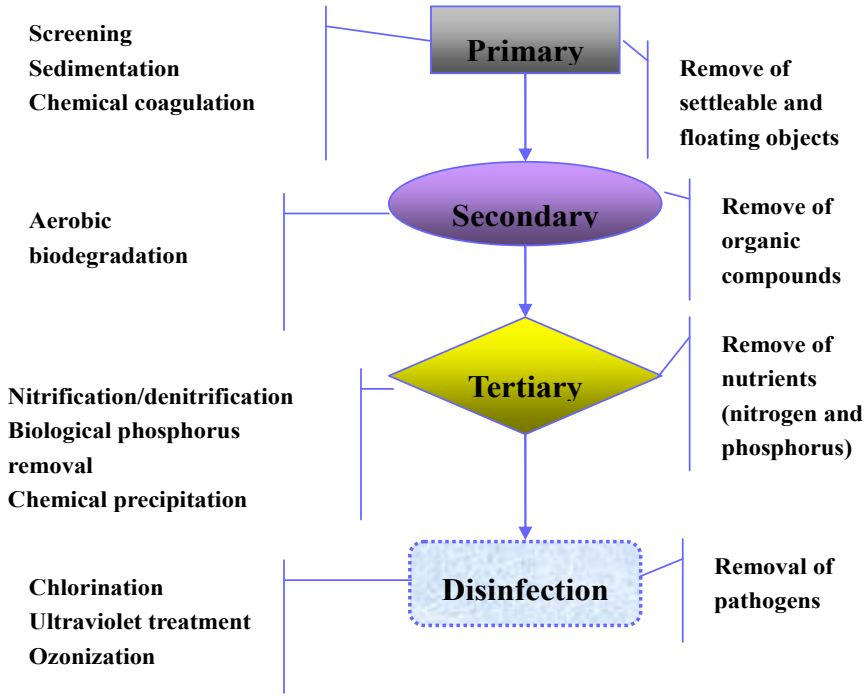


**Fig. 3.1** Schematic review of treatment processes for conventional municipal treatment plants

### 3.1.2 Secondary treatment

The aim of secondary treatment is to deplete dissolved and suspended organic matter. It is a process generally involving biological treatment which could be divided into attached growth based and suspended growth based systems (EPA, 2004). The mechanism of both attached and suspended growth processes is that indigenous, water-borne microorganisms use oxygen and convert most of the organic matter in the wastewater into carbon dioxide. The oxygen is provided by compressed air systems, mechanical agitation or injection of relative pure oxygen.

In attached growth processes, the microorganisms grow on the surface of certain carriers such as stone or plastic media. There are three main attached growth process designs: trickling filters, biotowers and rotating biological contactors.

Suspended growth systems where the microorganisms are suspended in the wastewater under aerobic conditions, include aerobic ponds, variations of activated sludge, oxidation ditches and sequencing batch reactors. 70 to 85% of the biochemical oxygen demand (BOD) entering with the primary effluent is removed in the aeration tank after several hour of degradation by microorganisms acclimated to the wastewater (EPA, 2004). The effluent after secondary treatment should flow to the second clarifier to remove the excess bacteria by settling before discharging or further treatment.

### 3.1.3 Tertiary treatment

Nitrogen and phosphorus, which could not be removed by the conventional secondary biological treatment processes, are the key nutrients that cause eutrophication in aquatic water ecosystems. The tertiary treatment (advanced wastewater treatment) is added in order to further purify the secondary effluents.

- **Nitrogen**

Autotrophic nitrification and heterotrophic denitrification processes as conventional biotic methods are widely used for nitrogen removal in wastewater treatment plants. In the nitrification process, autotrophic nitrifying bacteria convert ammonium to nitrites and further to nitrates using molecular oxygen as the electron acceptors. Denitrification, where nitrates and nitrites are converted to nitrogen gas, is accomplished by heterotrophic bacteria under anoxic conditions (Khin and Annachhatre, 2004). Usually, organic carbon of the raw wastewater is used as an electron donor for denitrification. The related reactions are as follows:

Nitrification: $NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$

$NO_2^- + 0.5O_2 \rightarrow NO_3^-$

Denitrification: $2NO_3^- + 10H^+ + 10e^- \rightarrow N_2 + 2OH^- + 4H_2O$

$2NO_2^- + 6H^+ + 6e^- \rightarrow N_2 + 2OH^- + 2H_2O$

Through the above two processes, nitrogen in wastewater is released into atmosphere in form of nitrogen gas. While, during the denitrification process, the toxic gas ($N_2O$ and NO) are important intermediates ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), which are also the products during conventional nitrogen removal processes (Clauwaert et al., 2007; Zhang et al., 2011). In addition, these two processes need to be separated in time or space as nitrification is carried out by autotrophic bacteria under aerobic condition and denitrification takes place in an environment free of oxygen with heterotrophic bacteria (EPA, 1975). Besides, the consumption of a large amount of oxygen in nitrification process and the requirement of organic carbon to complete denitrification would increase the operation costs and energy inputs. Although some novel nitrogen removal systems, including anoxic ammonium oxidation process (ANAMMOX), single reactor system for high activity ammonium removal over nitrite process (SHARON), combined SHARON and ANAMMOX process and completely autotrophic nitrogen removal over nitrite process (CANON) are developed to overcome the limitations of the nitrification/denitrification process

(Hendrickx et al., 2012; Magri et al., 2007; Third et al., 2001; van Dongen et al., 2001; Vilar et al., 2010). The strict operation conditions such as pH, temperature and initial ammonium concentration are difficult to be maintained, which hinders the wide application (Magri et al., 2007; Third et al., 2001; Vilar et al., 2010).

- **Phosphorus**

The worldwide awareness of the need to control phosphorus emissions is growing with the increasing stringent discharge regulations in many countries. Not only removal but also reuse is important. Municipal wastewater may contain from 4 to 16 mg P/l and the effluent discharge limits range from 0.01 to 2.0 mg P/l depending on the plant location and potential impact on receiving waters (Tchobanoglous et al., 2003). Phosphorus removal can be achieved by chemical precipitation or biological phosphorus removal. Chemical precipitation is used to remove inorganic phosphorus by addition of specific chemicals such as alum, lime or iron salts to promote the coagulation sedimentation process by generation of insoluble metal phosphate. This chemical method can reduce more than 95% of the phosphorus concentration which has been used in many countries around the world (EPA, 2004). But the high volume of sludge (chemical precipitant) produced during this process causes the secondary contamination as these chemical sludge is neither a good fertilizer nor a suitable raw material for further use (Smil, 2000).

A group of microorganisms named polyphosphate accumulating organisms (PAOs) is largely responsible for P removal in the process of biological phosphorus removal (Oehmen et al., 2007). Under anaerobic condition, PAOs are selectively enriched in the bacterial community within the activated sludge and then assimilate fermentation products such as volatile fatty acids (VFAs) intracellularly as carbon polymers with the concomitant release of phosphate by cleavage of polyphosphate (Tchobanoglous et al., 2003). Under the following aerobic conditions, PAOs are able to use the energy produced by oxidation of their stored poly-β-hydroxyalkanoates (PHAs) for their growth and the new biomass with high polyphosphate storage accounts for phosphorus removal (Oehmen et al., 2007). Net P removal from the wastewater is realized through removal of a portion of the activated sludge storing a high amount of polyphosphates. The aerobic process could also be replaced by anoxic operations because some PAOs are able to use nitrate or nitrite instead of oxygen through which both nitrogen and phosphorus are removed (Henze et al., 1997). Although this process is capable of phosphorus removal, the phosphorus removal efficiency is variable and unstable in practice (Oehmen et al., 2007; Wu et al., 2010).

### 3.1.4 Disinfection

Disinfection refers to the partial destruction of microorganisms or pathogens contained in untreated wastewater that cause human diseases (Tchobanoglous et al., 2003). Chlorination, ozonization and ultraviolet radiation treatment are three most common used disinfection methods. Chlorine is an effective way for removing microbial pathogens by destroying cellular material. But as chlorine is a highly toxic gas, any free chlorine remaining in water would lead to dangerous outcomes. Although ozonization is a powerful way to destroy viruses and bacteria, the ozone gas is unstable and costly. UV disinfection is a physical process that retards the survival of the microorganisms by destroying their genetic material. The suspended solids or soluble organic matter in wastewater can react with or absorb the UV radiation thus reducing the disinfection performance.

## 3.2 Municipal wastewater treatment with microalgae

Municipal wastewater treatment with microalgae-based technology offers a cost-efficient and environmental friendly alternative to conventional treatment processes. In this system, microalgae assimilate nutrients in wastewater for their growth, generate oxygen through photosynthesis thus offering a low-cost aerobic environment, absorb heavy metals and indirectly remove pathogens and viruses by increasing pH, temperature and dissolved oxygen concentration during photoautotrophic metabolism (Ansa et al., 2011; Munoz and Guieysse, 2006). So far, algae-based technology is used for pretreated wastewater treatment, tertiary treatment and other advanced treatment (Jarvie et al., 2002; Martinez et al., 2000; Olguin et al., 2002; Wang and Lan, 2011).

### 3.2.1 Pretreated municipal wastewater treatment with algal-bacterial culture

When using algal-bacterial culture for pretreated municipal wastewater treatment, organic matter is oxidized by heterotrophic bacteria into $CO_2$. Under the illuminated condition, microalgae fix the generated $CO_2$, assimilate the nutrients ($NH_4^+$, $NO_3^-$ and $PO_4^{3-}$) in the wastewater and simultaneously produce $O_2$ through their photosynthesis, which is required by aerobic bacteria as electron acceptor (Fig. 3.2). In conventional wastewater treatment plants, the oxygen supply accounts for more than 56% of the total energy cost (Oswald, 1995). While, the energy consumption of microalgae based technology is greatly reduced as microalgae provide a low-cost aerobic condition for

aerobic bacteria. In addition, heterotrophic algae could also contribute to the organic carbon removal in the dark (Abeliovich and Weisman, 1978).

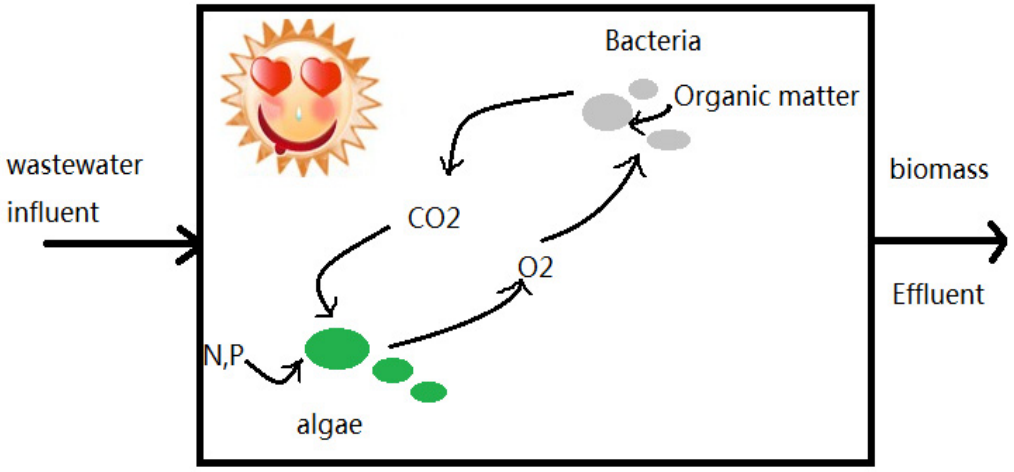


**Fig. 3.2** Symbiotic principle of algal-bacterial culture for municipal wastewater treatment

In the 1950s, Owald et al. (1988) designed large-scale open ponds called high-rate algal pond (HRAP) for algal growth and $O_2$ generation to fulfill the $O_2$ demand of the receiving domestic wastewater, which are by far the most cost-effective systems available for wastewater management and for efficient capture of solar energy (Olguin, 2003). They are usually 2-3 m wide and 10-30 cm deep shallow open ponds, and range from 1000 to 5000 $m^2$. The highest BOD removal rate is up to 35g/$m^2$/d under the optimal operation conditions (Munoz and Guieysse, 2006). And the hydraulic retention time (HRT) for continuous operation ranges between 4-10 days depending on climatic conditions (Rawat et al., 2011). The advanced integrated wastewater pond system (AIWPS) is an adapted traditional pond system developed by Oswald and his co-workers, which incorporates a series of low-cost ponds (Oswald, 2003). A typical AIWPS consists of advanced facultative pond followed by secondary facultative pond, algae settling pond and maturation pond (Green et al., 1995; Tadesse et al., 2004). In Paper I, pretreated municipal wastewater was treated in batch experiment. The removal efficiency of total organic carbon (TOC) was around 75.2%, while the COD removal efficiency was about 98%. It has been reported that the TOC removal rate is normally slower than that of COD (Zhang et al., 2005), because some products of biodegradation have a higher oxidation state of carbon compared to the initial substrate. This is consistent with our results. The organic substance having the lowest COD/TOC ratio is oxalic acid, with a carbon oxidation state of +3.0 (COD/TOC = 0.67). This means that, COD/TOC cannot be less than 0.67. In our results, COD/TOC

measured was as low as 0.2, which is not possible. We did not find a final explanation for this phenomenon. Aziz and Tebbutt (1980) reported that, COD/TOC ratio could be lower than 0.479 in the treated wastewater effluent due to the less accurate COD analysis at low COD concentration. Accordingly, we suppose inaccuracies in the sampling and analysis were being the cause of the deviations.

### 3.2.2 Tertiary treatment with microalgae based technology

Microalgae based technology has also been applied for tertiary treatment especially for further nitrogen and phosphorus removal, through two possible mechanisms: biotic removal through biomass assimilation and abiotic removal induced by the increase in pH through microalgal photosynthesis (Paper III; Li et al., 2010b; McGriff and Mckinney, 1972). Microalgae require high amounts of nitrogen and phosphorus for protein (45-60% of microalgae dry weight), nucleic acids and phospholipids synthesis (Laliberte et al., 1994). The nitrogen assimilation could be increased after pretreatment of microalgae by starvation (Rawat et al., 2011). In addition, as the content of organic carbon in tertiary influent is low, the $CO_2$ for microalgae photosynthesis is mainly in forms of inorganic carbon from the air. It is reported that pH will increase in reactors with high inorganic/organic carbon during the microalgal photosynthesis (Hende et al., 2011). Besides, the algal photosynthetic growth would also cause the increase in pH. The elevated pH in the system would lead to ammonia volatilization and phosphorus precipitation, which contributes to the overall nutrient removal efficiency (Paper IV; Nurdogan and Oswald, 1995; Serodes et al., 1991).

### 3.2.3 Other advanced treatments based on microalgae technology

The microalgal photosynthesis has the potential in elimination of pathogens and viruses by increasing the pH, dissolved oxygen concentration and temperature (caused by the conversion of light energy into heat) in the culture (Ansa et al., 2011; Schumacher et al., 2003). Sunlight inactivation of pathogens and viruses is dependent on and increase with the pH and dissolved oxygen (Van der Steen et al., 2000).

Microalgae based culture has also been used to absorb and remove heavy metals due to the following reasons. (1) High pH induced by microalgal photosynthetic growth promotes the production of heavy metal flocculation and sedimentation by chemical precipitation (Munoz, 2005). (2) As the surface of actively growing microalgae is negatively charged, polyvalent cations such as heavy metals can be strongly adsorbed thus removed from the wastewater (Munoz et al., 2006; Oswald, 2003).

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# Chapter 4

# Microalgae harvesting strategies

Both effective wastewater treatment and algal biomass production for further usage require separation of biomass from water. How to harvest the microalgae remains a major practical limitation for microalgae based wastewater treatment technology due to the small size of the microalgae (typically 3-30 μm diameter) and relatively low cell density in a typical open pond system (Christenson and Sims, 2011; Grima et al., 2003). There is no single best harvesting microalgae method. The suitable technology should be chosen according to algal species, growth medium and end product (Shelef et al., 1984). Current harvesting methods include chemical based methods, physical based methods, biological based methods and immobilization systems.

## 4.1 Physical based methods

Nearly most of microalgae could be harvested by centrifugation. It is a rapid and stable method for harvesting suspended algae, based on density differences. More than 95% of cell harvest efficiency is obtained at 13,000 × g (gravity) and the harvest efficiency is only around 60% at 6,000 × g (Grima et al., 2003). Although this method is effective in harvesting, it is highly energy intensive and not economical for large scale application (Pittman et al., 2011).

Filtration is a common method to separate solid from liquid. A conventional filtration process such as vacuum or pressure filtration is not appropriate for small size microalgae, and only membrane micro-filtration or ultrafiltration are practical (Rawat et al., 2011). But the cost for pumping and membrane replacement due to membrane fouling and clogging is also high (Uduman et al., 2010).

Ultrasonic separation process uses ultrasound together with enhanced sedimentation to harvest microalgae biomass. The maximum harvest efficiency is 92% and the highest concentration factors (up to 20) could be obtained at low biomass concentration and low harvest flows (Bosma et al., 2003).

Gravity sedimentation is a low cost method and usually the first biomass harvesting option for algal wastewater treatment systems due to the low value of the biomass generated in large treated volumes (Brennan and Owende, 2009). But the settling rates are unreliable and slow as oleaginous microalgae cells are suspended in water and do not easily settle by natural gravity force (Zhang and Hu, 2012). Besides, the settling

rates are also dependent on the overflow rates and the shape of the gravity settling equipment (Christenson and Sims, 2011; Nurdogan and Oswald, 1996).

## 4.2 Chemical based methods

The surfaces of microalgae cells are generally negatively charged which prevents their aggregation in cell suspensions (Zhang and Hu, 2012). Addition of chemical flocculants can reduce or neutralise this charge and thus promote the chemical flocculation through increasing the particle size. The algal biomass flocculation reactions are sensitive to the pH, properties of cellular surface, the flocculants concentrations, divalent cations and ionic strength (Oh et al., 2001). Multivalent metal salts such as ferric chloride, aluminium sulphate and ferric sulphate are widely used for charge neutralization in the wastewater industry (Brennan and Owende, 2009). Nurdogan and Oswald (1995) enhanced algal biomass autoflocculation by adding freshly slaked lime to the high-rate algal pond. It was reported that addition of calcium chloride solution could increase the sheath (a polysaccharide gel) produced by microalgae thus promote the microalgal flocculation (Imase et al., 2008). Although the addition of chemical flocculants is efficient and reliable, these cationic metals lead to an increase of heavy metal uptake into the sludge and also increase the effluent salinity causing a secondary pollution (Christenson and Sims, 2011; Munoz, 2005).

An alternative flocculant are organic polymers such as chitosan, which do not cause a secondary contamination (Christenson and Sims, 2011). Chitosan is an edible and nontoxic flocculant, which costs 2 $/kg. In the previous study, more than 90% of biomass removal could be obtained using 15 mg/l chitosan during acetonitrile treatment with algae based technology (Munoz, 2005). Increasing the algae culture pH by addition of hydroxide salts, such as NaOH and KOH, could also enhance the biomass sedimentation through autoflocculation (Lavoie and de la Noue, 1987; Lee et al., 1998; Pittman et al., 2011). The pH adjustment for flocculation has the advantage that it could minimize changes in the culture medium compared with chemically-induced flocculation, but for a large volume of culture, pH adjustment is also expensive.

## 4.3 Biological based methods

Compared with chemical based methods, biological methods are low cost, do not cause secondary pollutants but are not sufficiently reliable (Sukenik and Shelef,

1984).

Biomass autoflocculation is the spontaneous aggregation of particles associated with the increases of culture pH caused by $CO_2$ consumption by algal photosynthetic activity (Sukenik and Shelef, 1984). These alkaline conditions cause supersaturation of calcium and phosphate ions (Christenson and Sims, 2011). Excess of calcium phosphate precipitation could occur with algae cell as a solid support thus promote algal flocculation (Christenson and Sims, 2011; Lavoie and de la Noue, 1987). Autoflocculation is strongly dependent on the proper concentration of $Ca^{2+}$ and $PO_4^{3-}$ in the medium and if their concentrations in the treated effluent are too low, the autoflocculation would not take place (Lavoie and de la Noue, 1987). The previous study showed microalgal autoflocculation was obtained at 0.1 mM-0.2 mM (millimolarity, mmol/l) $PO_4^{3-}$ and 1.5 mM-2.5 mM $Ca^{2+}$ within pH of 8.5-9.0 (Sukenik and Shelef, 1984).

Bioflocculation is a microalgae sedimentation process induced by the extracellular polymeric substances (EPS) secreted by algae (Lee et al., 2009). The separation efficiency is related to the quantity of EPS and the aging of culture (Lavoie and de la Noue, 1987). The maximum EPS productions are obtained at the end of the growth phase, possibly as a result of increased cell density (Christenson and Sims, 2011; Lavoie and de la Noue, 1987). The irradiance and temperature have effects on microalgae EPS production (Wolfstein and Stal, 2002). Although any microalgae species is able to produce the EPS, blue-green filamentous species are the most suitable organisms (Lavoie and de la Noue, 1987). In addition, activated sludge is one of the best sources of organisms to produce EPS and it also has a good settleability (Lee et al., 2009). Using activated sludge as bacterial inoculum in algal-bacterial culture for municipal wastewater treatment could enhance the biomass sedimentation (Paper II).

Some bacteria could excrete mucous material as a microbial flocculant which enhances the algal flocculation activities. By comparison of several bacteria isolated from different types of soil samples, it was shown that microbial flocculants from *Paenibacillus* sp. AM49 were the best for harvesting *Chlorella vulgaris* (Oh et al., 2001). Furthermore, using microalgae species with self-aggregation properties is also a potential and low cost harvesting strategy. An algae-bacteria aggregate was formed with a good settlability which could settle down from initial 0.55 g VS/l to 18 mg VS/l after 10 min sedimentation (Gutzeit et al., 2005). In papers I, III and IV, a special cultivation strategy named alternate mixing and non-mixing cultivation was used to train and select microalgae species (both wastewater-borne algae and unicellular microalgae species) with good settling property. After 1 month of cultivation, the

systems showed good biomass settleability, which indicated that the proper cultivation strategies are also very important for the breeding of settleable algae species.

## 4.4 Immobilization systems

Immobilization systems as effective harvesting methods have been widely described and tested for biological water treatment. Living cells are immobilized by natural or artificial means to prevent from moving independently of their original location to all parts of an aqueous phase of a system (de-Bashan and Bashan, 2010; Tampion and Tampion, 1987). There are several types of immobilization methods: covalent coupling, affinity immobilization, adsorption, confinement in liquid-liquid emulsion, capture behind semi-permeable membranes and entrapment (or encapsulation) in polymers (de-Bashan and Bashan, 2010; Mallick, 2002).

Among them, entrapment-based immobilization technology is one of the most frequently used immobilization methods for algae-based wastewater treatment (Pittman et al., 2011). In this system, the substrates from the water and excreted products can diffuse to and from the cells through pores in the polymers materials (Mallick, 2002). The chosen polymers should be hydrophilic, nontoxic and could keep the cells to live as long as possible and allow the substrates to diffuse into the matrix with low cell leakage (de-Bashan and Bashan, 2010; Pittman et al., 2011). Several synthetic (acrylamide, polyurethane, polyvinyl, resins, etc), natural polymers (agar, alginate, carrageenan, collagen cellulose, agarose, etc), chitosan have been used as the immobilized matrix (Mallick, 2002; Olguin, 2012). Carrageenan, agar and alginate are three preferred natural polysaccharide matrices as they are renewable resource extracted from red algae or brown algae (Olguin, 2012). Little changes on the morphology of algal cell after immobilization are observed with microscopes (Musgrave et al., 1983). The previous study also showed that immobilized microalgae can grow well within gel beads and their growth curves were similar to those observed with free cells (Chevalier and de la Noue, 1985b). The freshwater microalga *Chlorella vulgaris* and plant-growth-promoting bacterium *Azospirillum brasilense* were co-immobilized in small alginate beads resulting in a significantly increased growth of the microalgae (Gonzalez and Bashan, 2000). Although the algal growth could be guaranteed after immobilization, the nutrient removal efficiencies decreased after several cycles. It was reported that the ammonium and phosphate removal efficiencies within 48 h were 100% and 83%, respectively, in semi-continuous culture during the first cycle, but decreased after several cycles (de-Bashan et al., 2002).

Besides, $Ca^{2+}$ is used in the process to form gel entrapments in these natural

matrices. This kind of immobilization beads may become unstable if phosphate is present or even sequester phosphate salts (Moreno-Garrido, 2008; Olguin, 2012). Compared with natural polymers, synthetic polymers are more stable during long-term operation and not easy to be degraded by microbes. However, natural polymers are less hazardous during the production process and higher in diffusivity (de-Bashan and Bashan, 2010). The immobilized microalgae systems are used for nutrient removal and heavy metal removal (Table 4.1).

**Table 4.1** Wastewater treatment with immobilized microalgae

| Microalgae or with bacteria | Immobilization material | Pollutant | Treatment performance | Reference |
|---|---|---|---|---|
| Eight microalgae species isolated from pig manure samples | Alginate | Nitrogen and phosphorus | The maximum N and P removal rates: 0.016 mg N/h and 0.012 mg P/h | (Perez-Martinez et al., 2010) |
| *Chlorella vulgaris* and plant-growth-promoting bacterium *Azospirillum brasilense* | Alginate | Nitrogen and phosphorus from municipal wastewater | $NH_4^+$:100%; $NO_2^-$: 15%; P: 36% within 6 days | (de-Bashan et al., 2004) |
| *Phormidium* | Chitosan | Tertiary treatment | Removal rates: 61.1±7.0 μg P/l/h; 370 ±50 μg N/l/h | (de la noue and Proulx, 1988) |
| Hyperconcentrated *Scenedesmus quadricauda* | Carrageenan | Tertiary treatment | Maximum removal rates: 3.4 μmol N/min; 0.186 μmol P/min | (Chevalier and de la Noue, 1985a) |
| *Anacytis nidulans* | Agar | Chromium | Cr removal efficiency: 86% within 6 h at flow rate of 0.05 ml/min | (Khattar et al., 1999) |

Attached algal culture systems as passive immobilization methods are intended to promote the growth of microalgae or formation of biofilms on the surface of carriers such as polystyrene foam thus to simplify the cell harvest (Zhang and Hu, 2012). Guzzon et al. (2008) developed a phototrophic biofilm reactor with polycarbonate slides for phosphorus removal and up to 112 mg/$m^2$/d maximal P removal rate was achieved. A system where microalgae attached onto fiber-bundle carrier was used to treat secondary wastewater with the HRT of 2 days (He and Xue, 2010). The algal turf scrubbing (ATS) system is designed to let filamentous algae grow on a turf scrubber (screen) to reduce the harvesting cost. The yearly mean of algal production was 35

$g/m^2/d$ and the yearly mean removal rates of N and P were 1.1 ± 0.5 and 0.7 ± 0.2 $g/m^2/d$, respectively (Adey et al., 2011). A twin-layer system was used to immobilize *Chlorella vulgaris* and *Scenedesmus rubescens* by self-adhesion on a wet, microporous and ultrathin substrate layer to remove nitrogen and phosphorus. This novel system could keep 100% immobilization efficiency and the removal efficiencies of ammonium, nitrate and phosphate were more than 90% within 9 days (Shi et al., 2007).

## 4.5 Other methods

Flotation is a harvest method to make microalgae cells to float on the surface of the medium with dispersed micro-air bubbles and removed as scum (Brennan and Owende, 2009). A commonly used flotation method in wastewater treatment for algae removal is dissolved air flotation (DAF) (Christenson and Sims, 2011). In this system, an air compressor is used to supply fine bubbles to supersaturate flotation water (Wiley et al., 2009). The microalgae cells are adhered by bubbles resulting in floating to the surface (Wiley et al., 2009). Some flocculants are added to improve the harvest efficiency (Christenson and Sims, 2011). It is energy-intensive and causes downstream processing of algae (Greenwell et al., 2010; Henderson et al., 2008; Wiley et al., 2009). An alternative method is to use surfactants to replace a compressor and saturator to create small bubbles for the algal cells to adhere (Henderson et al., 2008; Wiley et al., 2009). And the removal efficiency is dependent on the efficiency of the surfactants adsorbed at the bubble interface (Henderson et al., 2008).

Besides, the algae cell can be concentrated and harvested by the motion of dispersed microalgae in an electric field (Christenson and Sims, 2011). This harvest method is based on the electrophoresis phenomenon and the negative charge of algal cells. The energy need is at least 0.3 $KWh/m^3$ (Poelman et al., 1997). The high power investment together with the expensive electrode is still the obstacle of this technology (Uduman et al., 2010).

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# Chapter 5

# Process factors in algal-bacterial systems

Some abiotic (temperature, pH, dissolved oxygen, light, mixing, nitrogen and phosphorus concentration and ratios) and biotic (microalgal species, relationship between microalgae and bacteria, inoculum concentration) factors affect the growth rates and chemical composition of microalgae leading to differences in the wastewater treatment performances. Therefore, a deep understanding of these important factors is necessary for overall optimization.

## 5.1 Abiotic factors

### 5.1.1 Temperature

The optimum temperature for microalgae growth usually varies with the species and also depends on the acclimated environment (Cho et al., 2007). Below the optimum temperature, microalgal growth rates would increase with an increase of temperature. Above the optimum temperature, microalgal growth rates would decrease with increasing temperature. Generally, temperatures higher than 35 $^{\circ}$C are lethal for some algal species while temperatures below 16 $^{\circ}$C would slow down algal growth unless isolated from extreme environments. The previous study revealed that the green microalgae of *Chlorella* species show good adaptation capacities for low-temperature conditions and it could even sustain photosynthetic oxygen production at the temperature nearly as low as freezing (Oswald and Gotaas, 1957). In the meantime, *Chlorella* also showed better tolerance to high temperature (Hanagata et al., 1992). For the extreme microalgae such as sea-ice microalgae or microalgae in polar regions, a special adaptive mechanism to protect against freezing is developed in order to survive at freezing temperature and seasonal changes of light intensity (Gomez et al., 2009; Rochet et al., 1985).

Besides, for the consortium formed by various microalgae and bacteria species, the influence of the temperature is the results of all microorganisms. It was reported that the removal efficiency doubled when increasing the temperature from 25 $^{\circ}$C to 30 $^{\circ}$C with a symbiotic culture constituted of *Chlorella. sorokiniana* and *Ralstonia. basilensis* (Munoz et al., 2004).

### 5.1.2 pH

Freshwater microalgae species have a tolerance to both acidic and alkaline pH to adapt to widely fluctuating pH levels where they live and many marine microalgae species are unlikely to grow in extreme pH environments (such as pH ≥10) due to the well regulated pH of sea water (Goldman et al., 1982). Different microalgae species could grow at similar pH ranges but with a different optimum pH (Hoham et al., 2007). The optimum pH of *Dunaliella* sp. and *D. salina* DCCBC2 is 8.0, 7.0 of *Chlorella* sp. and 7.2 of *Synechocystis* sp.(Kim et al., 2012; Martinez et al., 2011).

Besides, pH does not only affect the growth of microalgae but also modify the $CO_2/HCO_3^-/CO_3^{2-}$, $NH_3/NH_4^+$ equilibria and phosphorus and heavy metal availability (Laliberte et al., 1994; Munoz and Guieysse, 2006). Furthermore, pH is important for processes of abotic nutrient removal as at a pH of 9 to 11 some of the inlet nitrogen and phosphorus are removed via ammonia stripping and orthophosphate precipitation (Munoz, 2005; Nurdogan and Oswald, 1995; Zhang et al., 2012). Changes in pH levels influence the bacterial activity in algae-bacteria culture. In addition, as discussed in chapter 4.2, the increase in pH would promote flocculation thus offering benefits for the biomass separation.

During wastewater treatment with algae-bacteria-based systems, several factors may influence the pH. Microalgal $CO_2$ uptake and nitrate uptake could cause an alkalinity concomitant (Gutzeit et al., 2005; Perez-Garcia et al., 2011). Nitrification process and ammonium consumption would result in pH decrease due to $H^+$ releasing (Gonzalez et al., 2008a; Li et al., 2010a). And the finial pH is the result of a combination of the above factors in the systems.

The inorganic/organic carbon ratio of the treated wastewater is also related to pH changes in the culture. A high inorganic/organic carbon ratio would lead to high pH, and vice versa (Hende et al., 2011). Similar results were observed in paper III and IV as well. A high pH (above 9) was observed after the first few days for tertiary treatment and the tertiary influent is high in inorganic/organic carbon ratio. But in papers I and II, the maximum pH remained around 8 in the reactors fed with pretreated municipal wastewater with low inorganic/organic carbon ratio.

### 5.1.3 Dissolved oxygen (DO)

Dissolved oxygen concentration increases during algal photosynthesis, as the growth of 1 g of microalgal cells is accompanied by the release of 1.587 g of dissolved molecular oxygen (Oswald and Gotaas, 1957). The generated oxygen would

be consumed immediately by heterotrophic bacteria or heterotrophic microalgae for organic carbon degradation. And the finial DO levels are the combined results of oxygen generation and consumption. For instance, in the experiments of Papers I and II, the DO concentration was as low as approximately 0 mg/l after starting the batch test indicating that the generated oxygen was completely used for organic carbon mineralization. DO increased immediately to around 5 mg/l as soon as the organic carbon source was eliminated. In Papers III and IV, above 15 mg/l of DO was observed after beginning the test and this high DO value remains stable till the end of the test for tertiary wastewater treatment. The possible reasons for this phenomenon might be that the low organic carbon concentration in the tertiary influent could not support the intensive growth of heterotrophic microorganisms thus resulted in the low oxygen consumption.

It is also common to find $O_2$ supersaturation up to 400% in enclosed algal cultivation photobioreactors (Munoz and Guieysse, 2006). Similarly, dissolved oxygen supersaturation on the top water layers of algal ponds can reach between 300% and 400% on warm sunny afternoons in the tropical regions (Tadesse et al., 2004). High dissolved oxygen levels can inhibit photosynthesis and this byproduct of microalgal photosynthesis must be removed in order to prevent photo-oxidative damage thus decrease the treatment efficiency (Christenson and Sims, 2011; Munoz, 2005). Gas-liquid contactor reactors such as rotation biological contactors are used to realize both carbon dioxide transfer and oxygen release if properly designed (Christenson and Sims, 2011).

### 5.1.4 Light

Among the abiotic factors affecting the autotrophic growth of microalgae, light is described as one of the most important factors because the photosynthesis is totally light powered. A typical light intensity curve for algal growth rate can be divided into three periods as shown in Figure 5.1 (Munoz, 2005; Sorokin and Krauss, 1958). I. Period of increase: The growth rates rise with the increase in light intensity. II. Light independent or plateau period: When the light intensity reaches a certain value (saturation light intensity), the growth rates remain stable with the increase in light intensity. III. Period of decrease: The additional light is not utilized and the growth rates declines with the increase in light intensity due to photoinhibition.

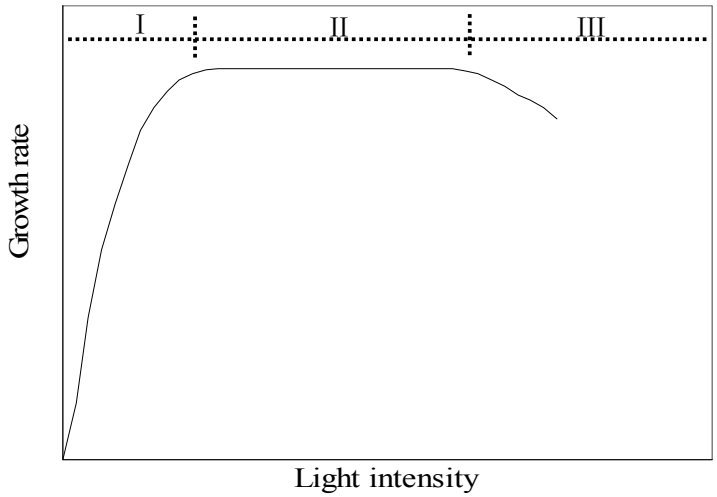


**Fig.5.1** Typical light intensity curve for algal growth rate

The characteristics of this curve (Fig. 5.1) are strongly dependent on the microalgal species, the concentration of microalgae and other environmental factors such as temperature, nutrient supply and $CO_2$ concentration (Munoz and Guieysse, 2006; Sorokin and Krauss, 1958). Temperature is of primary importance among the environmental factors and the total different trends of light intensity curves for algal growth rates were shown for the same species under 25 $^{\circ}$C and 39 $^{\circ}$C (Sorokin and Krauss, 1958). Each microalgae has a unique light saturation point but normally within 200-400 $\mu E/m^2/s$ (Cheirsilp and Torpee, 2012; Munoz, 2005). The saturation light intensity would decrease at high microalgal concentration as high algal cell densities could lead to the mutual shading within the cells thus inhibit the growth (Guieysse et al., 2002).

Sunlight intensity is fluctuant with the seasonal change and during daytime. The hydraulic retention time (HRT) needs to be adjusted during the year in order to stabilize the system performances (Garcia et al., 2000; Tadesse et al., 2004). Besides, natural sunlight is periodically absent and this diurnal cycle also varies during the year. The photosynthesis and pollutant removal would stop once light is not available and no leak of phosphorus occurs during darkness (Azad and Borchard, 1970; Munoz and Guieysse, 2006). As shown in Paper III, there was no difference of nutrient removal efficiencies with whole day illumination and 12 h light/12 h darkness (L/D) per day. The algal biomass generation rate with 24 h illumination (9.38±0.1 $g/m^2/d$) was only 1.87 $g/m^2/d$ higher than that of 12/12 (L/D) illumination due to the natural selection and long-term adaptation.

### 5.1.5 Mixing

Mixing may affect the microalgal growth in several important ways. First, it provides the microalgal cells intermittent contact with the light, thereby increasing the available light utilization efficiency (Oswald and Gotaas, 1957). Second, it may enhance the contact between the algal cells and nutrients and limits nutritional gradients in the cultivation medium (Azad and Borchard, 1970; Munoz, 2005). Third, it also helps the $CO_2$ transfer and $O_2$ release into atmosphere. Fourth, it keeps all the microalgal cells suspension and avoids the formation of anaerobic zones and further anaerobic decomposition (Munoz and Guieysse, 2006). Too much mixing would cause cell damage from shear stress and increase the operation stress due to the intensive energy requirement. On the other hand, low mixing velocity may have adverse effects on algal growth.

Several types of mixing equipments are used in algae-based wastewater treatment technologies. Paddle wheels as low cost mixing devices are commonly used in open ponds (Oswald and Gotaas, 1957). But the energy required in a paddle wheel-driven system could be reduced by as much as 80% with an airlift-driven raceway reactor (Ketheesan and Nirmalakhandan, 2011). Previous studies also indicate that microalgal productivity would increase up to 75% when replacing pump by airlift systems (Gudin and Chaumont, 1991; Munoz and Guieysse, 2006). Rotary-shaker or magnetic stirrers are always used in lab-scale batch test (Guieysse et al., 2002). In Paper IV, it was shown that the mixing velocity of 300 rpm (revolutions per minute) could realize a better nutrient removal efficiency and higher algal growth rates compared with 100 rpm.

### 5.1.6 The concentration and ratio of nitrogen and phosphorus

Nitrogen is the most important nutrient for algal biomass production (Richmond, 2004). The nitrogen content of the algal biomass is around 9.1% of the dry weight of microalgae and it varies between different algal species and also depends on the nutrients supply and availability (Oswald and Gotaas, 1957; Richmond, 2004). Phosphorus dose not normally exceed 1.5% of the dry weight of microalgae which is an essential element for microalgae for biosynthesis of nucleic acids, and supports energy transfer (Richmond, 2004). It rarely becomes a limiting factor for algal growth in municipal wastewater (Oswald and Gotaas, 1957). The N and P contents in municipal wastewater are around 20-85 mg N/l and 4-16 mg P/l respectively, which is adequate to support the growth of most of the microalgae (Christenson and Sims,

2011; Olguin, 2012).

N/P ratios ranging from 6.8-10 are considered optimal for microalgal growth (Olguin, 2012). Chevalier and de la Noue (1985a) pointed that the optimal N/P ratio for maximum nutrient uptake was 30. Wang and Lan (2011) indicated that for the green alga *Neochloris oleoabundans*, N removal was sensitive to N/P ratios and P removal was independent of N/P ratios when N:P was below 30. Besides, free ammonia would inhibit the photosynthesis of most microalgal strains due to the uncoupling effect of ammonia on photosynthetic processes in isolated chloroplasts (Crofts et al., 2004; Yuan et al., 2011). High ammonium concentration combined with high pH (above 9) could promote the free ammonia stripping thus inhibit the algal growth.

## 5.2 Biotic factors

### 5.2.1 Microalgae species selection

Many different microalgae species have been selected and tested for their potentials in wastewater treatment performance, microalgal biomass production or lipid content under various experimental conditions (Table 5.1). However, these results are difficult to compare as they are obtained under different operation conditions or with various bioreactors configurations. Among the different algae, *Chlorella*, *Chlamydomonas* and *Nitzschia* seem to be the preferred species of microalgae due to their good tolerance of different environmental conditions (Godos et al., 2009). Oswald (2003) showed that *Chlorella*, *Scenedesmus* and *Micractinium* were dominant algal species in algae wastewater treatment ponds, and species of *Euglena*, *Chlamydomonas* and *Oscillatoria* may occur in ponds with excessive loadings or long residence times, indicating that these 6 species may play important roles during wastewater treatment. Especially, *Chlorella* is viewed as indigenous species usually found in wastewater treatment plants (Olguin, 2012).

de Godos et al. (2010) compared two green microalgae (*Scenedesmus obliquus* and *Chlorella sorokiniana*), one cyanobacterium (*Spirulina platensis*), one euglenophyta (*Euglena viridis*) and two microalgae consortia isolated from a swine manure stabilization pond according to the degradation efficiency of piggery wastewater (de Godos et al., 2010). Based on the comparison of the above-mentioned 6 algae, it was indicated that *C. sorokiniana* and *E. viridis* had better removal performance for piggery wastewater treatment and *C. sorokiniana* showed the highest ammonia tolerance (de Godos et al., 2010). A mixed algal culture composed of

*Chlorella vulgaris* and *Scenedesums obliquus* could reach a nitrogen elimination capacity of 26 mg N/l/d (Gonzalez-Fernandez et al., 2011). Zhou et al. (2011) selected 17 top-performing algal strains for lipid accumulation from 60 algae-like microorganisms collected from different sampling sites in Minnesota. And these 17 strains were identified as *Chlorella* sp., *Heynigia* sp., *Hindakia* sp., *Micractinium* sp. and *Scenedesmus* sp. A mixed algal culture dominated by *Chlorella*, *Micractinium* and *Actinastrum* had a maximum lipid productivity of 24 mg/l/d with peak lipid contents ranging from 14-29% during primary clarifier effluent treatment and over 99% removal of N and P were achieved in 3 HRT (Woertz et al., 2009). In Paper I, a mixed algal culture obtained from the wall of the secondary clarifier of wastewater treatment plant was used. The maximum removal rates of TKN and P were 3.8 mg N/l/d and 0.35 mg P/l/d and average biomass productivity was 10.9 $g/m^2/d$. As presented in Paper III, three green microalgae (*Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Scenedesmus rubescens*) showed better N and P removal rates and settleabilities over the cyanobacteria (*Phormidium* sp.).

Normally, the ideal algal strains for wastewater treatment and biofuel production should: (1) have high lipid productivity and content; (2) have high tolerance to a wide range in environmental changes such as temperature, illumination, etc; (3) have a good settleability to harvest; (4) have a rapid growth capacity; (5) be easy to cultivate; (6) have a high ammonia tolerance; (7) have high $O_2$ generation rates and high $CO_2$ sinking capacity; (8) be able to dominate in open system; (9) have a good tolerance to shear stresses (10) have good tolerance to toxic pollutants (Brennan and Owende, 2009). However, no known algal strains could satisfy all these requirements and the selection of appropriate algae strains is heavily dependent on the aim of the project.

Future use of genome technology would develop genetically modified algae to enhance the algal function and thus to realize the overall optimization. The changes of the size of microalgal antenna by molecular tools could increase the microalgal photosynthetic rates and biomass productivity (Munoz, 2005).

**Table 5.1** Wastewater treatment efficiency, biomass production or lipid content with different microalgae species

| Algal species | Reactors type | Removal performance (removal rates or efficiency) | Biomass production | Lipid content (W/W) | Reference |
|---|---|---|---|---|---|
| *Botryococcus braunii* [a] | 1 L capacity Erlenmeyer flasks with 500 ml | | 0.034 g/l/d | 13.2% | (Chinnasamy et al., 2010) |
| *Chlamydomonas reinhardtii* | Biocoil photobioreactor | 55.8 mg N/l/d<br>17.4 mg P/l/d | 2.0 g/l/d | 25.25% | (Kong et al., 2010) |
| *Chlorella vulgaris* | Pilot-scale reactor | 33.2 mg N/l/d<br>2.9 mg P/l/d | | | (Gutzeit et al., 2005) |
| *Chlorella* sp. | Coiled reactor | 7.4 mg N/l/d [b]<br>12.3 mg P/l/d [c] | 0.92 g/l/d | 11.04% (FAME [d] content) | (Li et al., 2011) |
| *Chlorella* sp. | culture plate or conical flasks | | 0.241 g/l/d | 30.91% | (Zhou et al., 2011) |
| *Dunaliella tertiolecta* [a] | 1 L capacity Erlenmeyer flasks with 500 ml | | 0.028 g/l/d | 15.2% | (Chinnasamy et al., 2010) |
| Scenedesmus | Tubular photobioreactor | 36.06 mg N/l/d<br>2.53 mg P/l/d | 0.25 g/l/d | | (Di Termini et al., 2011) |
| *Scenedesmus* sp. LX1 | 250 ml flask | 1.04 mg N/l/d [e]<br>0.10 mg P/l/d [f] | 0.54 $d^{-1}$ | | (Li et al., 2010a) |
| *Scenedesmus* sp. LX1 | 250 ml flask | 0.76 mg N/l/d [g]<br>0.10 mg P/l/d [h] | | 20% | (Li et al., 2010a) |
| *Spirulina platensis* | Airlift photobioreactor [i] | 23.1 mg N/l/d<br>3.06 mg P/l/d [j] | 5.1 g/$m^2$/d | | (Yuan et al., 2011) |
| *Scenedesums rubescens* | Indoor photobioreactor [k] | | 0.539 g/l/d (AFDB [l]) | 26.7% (FAME content) | (Lin and Lin, 2011) |
| *Scenedesmus* sp. | culture plate or conical flasks | | 0.247 g/l/d | 30.09% | (Zhou et al., 2011) |

[a]: Cited with carpet industry untreated wastewater

[b]: Estimated from initial N concentration of 116 mg/l with removal efficiency of 89.1% after 14 days

[c]: Estimated from initial P concentration of 212 mg/l with removal efficiency of 80.9% after 14 days

[d]: FAME: fatty acid methyl ester

[e]: Estimated from initial $NO_3^-$-N concentration of 15 mg/l with removal efficiency of 90.4% after 13 days

[f]: Estimated from initial P concentration of 1.3 mg/l with removal efficiency of 99% after 13 days

[g]: Estimated from initial TN of 10 mg/l with removal efficiency of 99% after 13 days

[h]: Estimated from initial TP of 1.3 mg/l with removal efficiency of 99% after 13 days

[i]: Cited from experimental phase 4 with $NH_4^+$/TN=50%

[j]: Estimated from initial P concentration of 90 mg/l with removal efficiency of 51% and 15 HRT

[k]: Cited from nitrogen source with $(NH_4)_2CO_3$

[1]: AFDB: ash free dry biomass

### 5.2.2 Microalgal-bacterial symbiotic system

In nature, aerobic or anaerobic bacteria are always found in algal habitats, indicating the symbiotic relationship between microalgae and bacteria (Fukami et al., 1997; Subashchandrabose et al., 2011). During wastewater treatment, the symbiotic relationship between microalga and bacteria is also the base to complete the treatment. Their mutual interactions include both positive roles such as $O_2$ and $CO_2$ supply, and also negative mutual influence (Table 5.2). The special environment with high oxygen concentration and high pH created during algal photosynthesis growth could impede the bacterial growth (Schumacher and Sekoulov, 2003). Algae would also excrete some anti-bacterial substances which are especially toxic to gram-positive bacteria (Davies-Colley et al., 1999; Schumacher and Sekoulov, 2003). Certain bacteria such as *Azospirillum brasilense* strain Cd would excrete vitamins or algal growth-promoting substances which are used as "helper" for microalgal growth (de-Bashan et al., 2004; Fukami et al., 1997). However, two aerobic bacteria (*Pseudomonas diminuta* and *P. vesicularis*) can promote algal growth by reducing the photosynthetic oxygen tension within the microenvironment of the algal cells rather than releasing any growth promoting substance (Mouget et al., 1995). It can be concluded from the above discussions that the selection of suitable algae and bacteria species to form a powerful algal-bacterial culture is of great importance for achieving good wastewater treatment performance.

**Table 5.2** The positive and negative interactions between microalgae and bacteria

| | Positive (+) | Negative (-) |
|---|---|---|
| Algae to bacteria | $O_2$ supply | pH increase |
| | $CO_2$ consumption | Temperature increase |
| | | Antibacterial effects |
| Bacteria to algae | Dissolved oxygen decrease | Algicidal effects |
| | $CO_2$ generation | |
| | Stimulative effects | |

Many special unicellular microalgae and pollutant-specific degrading bacteria are used to treat municipal or toxic wastewater. But in open or outdoor systems, the mixed culture or natural-selected culture is always expected to suffer from changing

environmental conditions. It was reported that the dominant algal species changed during wastewater treatment process (Godos et al., 2009). In Paper I, it is indicated that the main bacteria community of the wastewater-borne and settleable algal-bacterial culture were *Flavobacteria*, *Gammaproteobacteria*, *Bacteroidia* and *Betaproteobacteria*. In Paper II, it is shown that the bacterial compositions were diverse in reactors inoculated with different algae/sludge ratios, which showed that different dominant bacteria were enriched and played important roles during wastewater treatment in algal-bacterial cultures with different inoculum ratios. To the best of our knowledge, these parts of works are the first investigation in the identifications of the dominating bacteria in wastewater-borne algal-bacterial systems.

### 5.2.3 Inoculum concentration

The proper inoculum concentration optimizes the removal process and ensures good removal efficiencies. Within certain concentrations, the removal performance improved with the increase of the initial algal concentration. After reaching certain concentrations, further increase of the algal inoculum concentration had no influence on the nutrient uptake rates. The possible explanation may be that about the same number of algae is actually working in these systems (Chevalier and de la Noue, 1985a). And once a critical microalgal density is reached, the processes are limited by the available light supply and the self-shading phenomenon, thus resulting in the collapse of the systems (Guieysse et al., 2002). Paper IV shown that different algae/bacteria inoculum ratios led to different treatment efficiencies which indicated that proper inoculum strategy was vital for the treatment performance.

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# Chapter 6

# Conclusions

This thesis has mainly focused on the cultivation of settleable algal-bacterial culture, improvement of municipal wastewater treatment efficiency and better understanding of these algal-bacterial systems including N, P mass balance and bacterial community compositions. The major contributions of this thesis work are summarized as below:

- In Paper I, an algal-bacterial culture, cultivated from algae from the second clarifier of Suderburg wastewater treatment plant (Germany) and wastewater bacteria with alternate mixing and non-mixing strategy, were used for municipal wastewater treatment and to accumulate biomass simultaneously. The algal-bacterial culture showed good settleablility, resulting in a reduction of total suspended solids (TSS) from initially 1.84 to 0.016 g/l within 20 min. The average removal efficiencies of COD, TKN and phosphate were 98.2 ± 1.3%, 88.3 ± 1.6% and 64.8 ± 1.0% within 8 days, respectively. The average TN and P removal rates were 2.4 mg N/l/d and 0.35 mg P/l/d. The average biomass productivity was 10.9 ± 1.1 $g/m^2 \cdot d$. Accumulation into biomass was identified as the main nitrogen and phosphorus removal mechanism, which accounted for around 44.9 ± 0.4% and 61.6 ± 0.5% of total inlet nitrogen and phosphorus, respectively. Microscopic analysis showed the main algae species in the bioreactor were filamentous blue-green algae. Furthermore, microbial community analysis revealed that the main bacteria present in the photobioreactor were consortia with sequences similar to those of *Flavobacteria, Gammaproteobacteria, Bacteroidia and Betaproteobacteria*.

- In Paper II, aerobic activated sludge was used as bacterial inoculum in algal-bacterial culture to enhance the settleability. The effects of different algae/sludge inoculation ratios were investigated in terms of carbon, nutrients removal and biomass settleability. The COD removal efficiency was above 91.2% for the four tested algae/sludge inoculation ratios (10:1, 5:1, 1:1 and 1:5) within 8 days. To the contrary, nutrient removal was enhanced with proper inoculation ratios. The highest nitrogen and phosphorus removal efficiencies

were observed with a 5:1 (algae/sludge) culture (91.0 ± 7.0% and 93.5 ± 2.5%, respectively) within 10 days, which was 5% - 40% higher and 2 - 4 days faster than those of other cultures with 10:1, 1:1 or 1:5 inoculation ratio. Biomass incorporation was identified as the main nitrogen removal mechanism for 5:1 and 1:1 cultures, which accounted for 60.0 ± 0.3% and 41.6 ± 0.5% of inlet nitrogen. $NH_4^+$ was mainly oxidized to $NO_2^-$ other than biomass uptake at ratios of 1:5 and 10:1, which accounted for 41.4 ± 1.6% and 26.8 ± 0.9% of inlet nitrogen. Biomass uptake was the main P removal mechanism. The settleability of algae biomass was enhanced after inoculating with sludge. The culture with 1:5 algae/sludge showed the best settleability, resulting in reduction of total suspended solids from 1.64 ± 0.02 to 0.05 ± 0.02 g/l in 30 min followed by cultures with 1:1 and 5:1, which took 36 and 42 min to settle down. Furthermore, microbial community analysis revealed that the bacterial communities were varying with different algae and activated sludge inoculation ratios.

- In Paper III, four common used microalgae species were compared concerning their settleability, nutrient removal capacity and biomass productivity. After one month of training, except cyanobacteria *Phormidium* sp., three green microalgae species, *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Scenedesmus rubescens* showed good settleability, from initially 508.3 ± 2.5, 510.8 ± 1.5 and 522.4 ± 2.5 FTU (Formazin Turbidity Unit) to 2.76 ± 2.5, 1.84 ± 1.5 and 4.79 ± 2.5 FTU after one hour of sedimentation. The N and P removal efficiency was all above 99% within 7, 4, 6 and 6 days for N and 4, 2, 3 and 4 days for P, resulting in N removal rates of 3.66 ± 0.17, 6.39 ± 0.20, 4.39 ± 0.06 and 4.31 ± 0.18 mg N/l/d and P removal rates of 0.56 ± 0.07, 0.89 ± 0.05, 0.76 ± 0.09 and 0.60 ± 0.05 mg P/l/d for *Phormidium* sp., *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Scenedesmus rubescens*, respectively. *Phormidium* sp. had the lowest algal biomass productivity (2.71 ± 0.7 $g/m^2/d$) and the other three green microalgae showed higher and similar algal biomass productivity (6 $g/m^2/d$). More than 92.9%, 89.4%, 90.1% and 88.8% of removed nitrogen was assimilated into algal biomass and 96.7%, 96.1%, 96.9% and 97.9% of removed $PO_4^{3-}$-P was mainly uptaken by algae and partly precipitated when the pH was above 9 for *Phormidium* sp., *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Scenedesmus rubescens*, respectively.

- In Paper IV, a mixed algal culture, composed of three unicellular green microalgae (*Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Scenedesmus rubescens*) selected according to the results of Paper III, was cultivated for nutrient removal and algal biomass accumulation simultaneously. The mixed algal culture showed higher nutrient removal rates than those of the individual microalgae species. The influences of biotic (algal inoculum concentration) and abiotic factors (illumination cycle, mixing velocity and nutrient strength) on the treatment efficiency, biomass generation and settleability were investigated. Dark condition led to poor nutrient removal efficiency. No significant difference in the N, P removal and biomass settleability between continuous and alternate illumination was observed, but a higher biomass generation capability for the continuous illumination was obtained (9.38 ± 0.1 $g/m^2/d$). Different mixing velocity led to similar phosphorus removal efficiencies (above 98%) with different retention time (6, 10 and 11 days for 300, 100 and 0 rpm, respectively). The reactor with 300 rpm had the best N removal capability, and nitrification plays the important role in nitrogen removal for the other two reactors with 0 and 100 rpm. For the low strength wastewater (around $NH_4^+$ -N: 45.2 mg/l; $PO_4^{3-}$ -P: 3.7 mg/l), the N and P removal efficiencies were above 98% for all the tested algal inoculum concentrations but with different retention time, resulting in the daily N removal rate of 5.4 ± 0.2, 9.1 ± 0.3 and 10.8 ± 0.3 mg/l/d and P removal rate of 0.57 ± 0.03, 0.56 ± 0.03 and 0.72 ± 0.05 mg/l/d for reactors with the algal inoculum concentration of 0.2, 0.5 and 0.8 g/l, respectively. Low nutrient removal efficiency and poor biomass settleability were obtained for high strength wastewater (around $NH_4^+$ -N: 90.2 mg/l; $PO_4^{3-}$ -P: 11.5 mg/l).

Overall, the thesis has explored a specific cultivation strategy (alternate mixing and non-mixing strategy) to train and enhance the settleability of microalgae-based systems. Besides, activated sludge can be used as bacterial inoculum to improve the settleability of biomass for this algal-bacterial system. It was found that the mixing velocity of 300 rpm could offer good removal performance for tertiary treatment and light-cycle (full-day light or 12 h light/12 h darkness) had slight influence on the removal efficiencies. Mixed algal culture can enhance the treatment performance over individual algae species. This study explores a better understanding of an algal-bacterial system through analyzing the bacterial community compositions and offers new options to solve the harvesting problems. Although the good settleability and removal performance could be realized in the lab-scale test, further large-scale

and long-term investigation with these settleable algae-based systems is still needed. Future research efforts should go towards investigating the biogas and biofuel generation potentials of the settleable algal biomass accumulated during treatment to evaluate and optimize the whole process.

# Acknowledgements

I would hereby like to express my gratitude to the following organizations and people for their generous help and great support during my three-year PhD life. Without them, my PhD journey would not be such smoothly, meaningful and unforgettable like this.

First of all, the financial support for a PhD scholarship from German-Academic Exchange Service (DAAD) is gratefully acknowledged.

Europäischer Fonds für regionale Entwicklung (EFRE) is also acknowledged.

I would like to express my million thanks to my two PhD supervisors, Professor Brigitte Urban and Professor Artur Mennerich, for supervising, supporting and encouraging me through the whole PhD project. Both of you have always been able to increase my enthusiasm and give me unlimited inspirations and valuable guidance on scientific work. Thanks for your involvement and contribution in both my research work and personal development.

Now, I would like to express my gratitude to:

- Prof. Dr. Wolfgang Ruck for his patient guidance and great help.
- The examiners of this thesis for their precious time.
- The technical staffs Sabine Henschel, Kerstin Lammers, Margot Seeber and Christiane Hilmer (Ostfalia University of Applied Science) for their assistant and help on chemical water analysis.
- Our project leaders, Dr. Manfred Schuetze, for introducing this interesting research topic when I was in China in 2009 and giving me this opportunity to work for LiWa (Lima wasser) project.
- Mrs. Irmgard Kasperek, the secretary of German-Academic Exchange Service (DAAD), for her generous help during my stay in Germany.

- Mrs. Karin Dziarski (Leuphana University Lüneburg) for being all the time available to help me out, inside and outside the department.

- All my former and present colleagues of our group in Ostfalia for their encouragement, support, nice discussion and creating a pleasant and inspiring working environment: Ingeborg Joost, Pamela Holweg, Sascha Ogasa, Patrick Stohri, Woitas Vanessa.

- All the PhD members of Prof. Urban's group, Leuphana University Lüneburg, Institute of Ecology for so many interesting discussions, warm-hearted helps and supports.

- My dear friend Patricia Bedon-Minaya (PhD student) and Anjana Koju (MSc student) for all the help in the lab, all the funny and spontaneous chats at our free time and also all the fun in the dinners and trips we shared.

- Last but not the least, I must express my special gratitude to my husband Dr. Yifeng Zhang for his support and advice on the molecular biology work which is very important for my PhD research work. Also thanks for his continuous inspiration and support on the scientific research work, sharing of my stresses and burdens and suffering from my bad temper. Finally, my heartfelt appreciation should go to all my family members, especially my mother Yonghong Yu (于永红) and my father Yong Su (苏勇) for all the endless support and encouragement.

Just let this thesis in memory of my past three precious years…

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3. **Su, Y.**, Mennerich, A., Urban, B. (2011). Municipal wastewater treatment and biomass accumulation with a wastewater-born and settleable algal-bacterial culture. *Water Research*. 45, 3351-3358. **(SCI, IF 4.865)**
4. **Su, Y.**, Mennerich, A., Urban, B. (2012). Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: Influence of algae and sludge inoculation ratios. *Bioresource Technology*. 105, 67-73. **(SCI, IF 4.980)**
5. **Su, Y.**, Mennerich, A., Urban, B. (2012). Coupled nutrient removal and biomass production with mixed algal culture: Impact of biotic and abiotic factors. *Bioresource Technology*. 118, 469-476. **(SCI, IF 4.980)**
6. **Su, Y.**, Mennerich, A., Urban, B. (2012) Comparison of nutrient removal capacity and biomass settleability of four high-potential microalgal species. *Bioresource Technology*. In press. DOI: 10.1016/j.biortech.2012.08.037. **(SCI, IF 4.980)**

## Patents:

1. Wang, J., **Su**, **Y**., Zhou, J., Lu, H., Jin, R.
   Enhanced biodegradation of organics by immobilization of redox mediator and bacteria. China patent (2009).

## Other scientific contributions:

1. **Su**, **Y**., Wang, J., Zhou, J. (2008) Enhanced biodecolorization of azo dyes by the catalysis of anthraquinone dyes intermediators. *Environment science*. 29(7):240-245. (Chinese Journal, EI index, Abstract in English).

## Conference contributions:

1. **Su, Y**., Mennerich, A., Urban, B. (2010) Low-cost municipal wastewater treatment and biomass accumulation with settleable and wastewater-born mixed algae culture. ["Future Megacities in Balance" Young Researchers' Symposium in Essen, 9-10. October. 2010]- Poster.

# Selbständigkeitserklärung

Ich versichere, dass ich die eingereichte Dissertation “Settleable algal-bacterial culture for municipal wastewater treatment.” selbständig und ohne unerlaubte Hilfsmittel verfasst habe. Anderer als der von mir angegebenen Hilfsmittel und Schriften habe ich mich nicht bedient. Alle wörtlich oder sinngemäß den Schriften anderer Autorinnen oder Autoren entnommenen Stellen habe ich kenntlich gemacht.

Yanyan Su

Suderburg, den 11.07.2012

# Declaration 1

This PhD thesis titled "Settleable algal-bacterial culture for municipal wastewater treatment" consists of the following publications:

**I** Su, Y., Mennerich, A., Urban, B. 2011. Municipal wastewater treatment and biomass accumulation with a wastewater-born and settleable algal-bacterial culture. *Water Research* 45, 3351-3358.

**II** Su, Y., Mennerich, A., Urban, B. 2012. Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: Influence of algae and sludge inoculation ratios. *Bioresource Technology* 105, 67-73.

**III** Su, Y., Mennerich, A., Urban, B. Comparison of nutrient removal capacity and biomass settleability of four high-potential microalgal species. *Bioresource Technology*. In press. DOI: 10.1016/j.biortech.2012.08.037.

**IV** Su, Y., Mennerich, A., Urban, B. 2012. Coupled nutrient removal and biomass production with mixed algal culture: Impact of biotic and abiotic factors. *Bioresource Technology* 118, 469-476.

These published papers were reviewed by two or three international peers and accepted after the revision according to the reviewers' suggestions and comments. All four publications were written by the first author Yanyan Su. The first author also carried out the field work, sampling, preparation, measurement and analysis of the samples and the interpretation and discussion of the data. All the figures and tables used in the published papers were created by the first author.

Yanyan Su

Suderburg, 11.07.2012

# Declaration 2

This PhD thesis titled “Settleable algal-bacterial culture for municipal wastewater treatment” has neither as a whole nor in part been submitted to assessment in a doctoral procedure at another university.

Yanyan Su

Suderburg, 11.07.2012

# Appendices

**I** Su, Y., Mennerich, A., Urban, B. 2011. Municipal wastewater treatment and biomass accumulation with a wastewater-born and settleable algal-bacterial culture. *Water Research* 45, 3351-3358.

**II** Su, Y., Mennerich, A., Urban, B. 2012. Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: Influence of algae and sludge inoculation ratios. *Bioresource Technology* 105, 67-73.

**III** Su, Y., Mennerich, A., Urban, B. Comparison of nutrient removal capacity and biomass settleability of four high-potential microalgal species. *Bioresource Technology*. In press. DOI: 10.1016/j.biortech.2012.08.037.

**IV** Su, Y., Mennerich, A., Urban, B. 2012. Coupled nutrient removal and biomass production with mixed algal culture: Impact of biotic and abiotic factors. *Bioresource Technology* 118, 469-476.

I

# Municipal wastewater treatment and biomass accumulation with a wastewater-born and settleable algal-bacterial culture

Su, Y., Mennerich, A., Urban, B.

Water Research 45(11), 3351-3358 (2011)

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/watres

# Municipal wastewater treatment and biomass accumulation with a wastewater-born and settleable algal-bacterial culture

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ARTICLE INFO

*Article history:*
Received 17 October 2010
Received in revised form
20 March 2011
Accepted 23 March 2011
Available online 31 March 2011

*Keywords:*
Algal-bacterial culture
Nutrient removal
Biomass accumulation
N and P accumulation
16S rDNA gene

ABSTRACT

A wastewater-born and settleable algal-bacterial culture, cultivated in a stirred tank photobioreactor under lab conditions, was used to remove the carbon and nutrients in municipal wastewater and accumulate biomass simultaneously. The algal-bacterial culture showed good settleable property and could totally settle down over 20 min, resulting in a reduction of total suspended solids from an initial 1.84 to 0.016 g/l. The average removal efficiencies of chemical oxygen demand, total kjeldahl nitrogen and phosphate were 98.2 ± 1.3%, 88.3 ± 1.6% and 64.8 ± 1.0% within 8 days, respectively, while the average biomass productivity was 10.9 ± 1.1 g/m$^2$·d. Accumulation into biomass, identified as the main nitrogen and phosphorus removal mechanism, accounted for 44.9 ± 0.4% and 61.6 ± 0.5% of total inlet nitrogen and phosphorus, respectively. Microscopic analysis showed the main algae species in the bioreactor were filamentous blue-green algae. Furthermore, denaturing gradient gel electrophoresis and 16S rDNA gene sequencing revealed that the main bacteria present in the photobioreactor were consortia with sequences similar to those of *Flavobacteria, Gammaproteobacteria, Bacteroidia and Betaproteobacteria*. This study explores a better understanding of an algae-bacteria system and offers new information on further usage of biomass accumulated during treatment.



## 1. Introduction

The concept of algal-bacterial culture as an engineered system in domestic and industrial wastewater treatment has experienced increased momentum over the past few years (Bordel et al., 2009; de-Bashan et al., 2002; Garcia et al., 2000; Gutzeit et al., 2005; Medina and Neis, 2007; Munoz et al., 2005). It is especially favorable in regions with year-round high solar radiation and temperature as the removal is an entirely natural process. When illuminated, algae produce oxygen that can be used by aerobic bacteria to biodegrade pollutants whilst, in return, they consume the carbon dioxide released from bacterial respiration (Oswald, 1988), which provides a cheaper and safer alternative to mechanical aeration and contributes to $CO_2$ mitigation (Guieysse et al., 2002; Munoz and Guieysse, 2006).

Another advantage of this technology is that more nitrogen and phosphorus could accumulate into the algal and bacterial biomass during the removal process. When using a bacterial system to treat acetonitrile (1 g/l), only 26% N–$NH_4^+$ was assimilated into biomass. Under the same conditions using algal-bacterial culture, 53% N–$NH_4^+$ was assimilated into algae

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0043-1354/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.watres.2011.03.046

and bacteria biomass (Munoz, 2005). Oswald also inferred that, under optimal operating conditions (e.g., sufficient light intensity and a proper bioreactor configuration), almost all the available ammonia nitrogen appeared in the form of algal cell material (Oswald and Gotass, 1957). All the above gives algal-bacterial biomass, accumulated during wastewater treatment, the potential to be used as a fertilizer in agriculture (Benemann et al., 1977; Mulbry et al., 2005).

The major limitation of the exploitation of this technology is the requirement for cost-effective biomass harvesting techniques. A technical separation unit consisting of filtration or centrifugation has to be applied (Mohn, 1988), but this will raise the operation cost. Adding chemicals such as $Ca^{2+}$ or slaked lime will result in secondary pollutants (Imase et al., 2008; Nurdogan and Oswald, 1995). Using an immobilization system is another possible solution (Mallick, 2002; Moreno-Garrido, 2008), but all media are costly and inefficient over a long operation time. Therefore, a more effective biomass harvesting strategy such as a settleable algae-bacteria system is required.

The identification and biometry of the dominant algal species in the algal-bacterial culture were well-studied in previous works (Godos et al., 2009; Garcia et al., 2000; Oswald, 2003), but little information was available about the bacterial community involved in this symbiotic system. Investigation of the microbial composition and their functionalities could provide some insights into the biological catalytic and symbiotic mechanisms. Moreover, the purification capacity could potentially be improved by addressing microbial constraints.

In this study, for the first time, a settleable algal-bacterial culture was cultivated from domestic wastewater, and its treatment efficiencies, biomass generation, N and P accumulation processes and microbial diversity were investigated.

## 2. Material and methods

### 2.1. Settleable algal-bacterial culture enrichment

The algae inoculum was obtained from the second clarifier wall of the Suderburg municipal wastewater treatment plant (County of Uelzen, Lower Saxony, Germany). The collected algae solution (exposure to bacteria was unavoidable) was firstly settled down for 1 h, and then 30 g (wet weight) of settled solid was used as algae inoculum for algal-bacterial culture enrichment. The wastewater collected from the second clarifier at the same site was used as medium, and 600 ml pretreated wastewater collected from the same plant (after preliminary screening, grit removal and primary sedimentation process) was used as bacterial inoculum and nutrient supply. The settleable algal-bacterial culture was cultivated under laboratory conditions at around 19 °C. The stirred tank photobioreactor (for culture enrichment) was made of transparent PVC 40 cm in depth and 29 cm in diameter. The total volume of the medium in the reactor was 14 l (approx. 25 cm in depth). Constant mixing was maintained using a magnetic stirring bar (100 rpm) to avoid algae sedimentation. Two compact fluorescent lamps (Sylvania, F20W/860/E27) were used to irradiate the tank with about 360 $\mu E\, s^{-1}\, m^{-2}$ (measured at the top of the liquid surface) for a period of 12 h per day. In order to cultivate the settleable algal-bacterial culture, the mixing procedure was stopped every 23 h for 1 h and the floating biomass was discarded with a screen (0.5 mm). 600 ml pretreated wastewater was exchanged after sedimentation every 3 days to maintain a nutrient supply. After one month of cultivation, dark green and pea green microalgae were visible and distributed evenly in the reactor.

### 2.2. Experimental operation

The same laboratory-scale reactor system as in the cultivation process was used for the batch mode. The pretreated wastewater was used as feed for the reactor in the following experiments, unless otherwise stated. The characterization of the pretreated wastewater used in the different batches is shown in Table 1. Before starting the batch experiment, the algal-bacterial biomass was allowed to settle by stopping the stirrer for half an hour. At the end of each 8-days cycle, 12.5 l of suspension was removed and replaced by fresh wastewater as above. The irradiation device was the same as that for the cultivation process. The photoperiod was a 12 h light-12 h dark cycle. 150 ml samples for further analysis (see below) were collected near the midway of the reactor with a pipe every day, 4 h after starting the irradiation period.

### 2.3. Analytical procedures

The temperature and dissolved oxygen (DO) were measured near the midway of the reactor by using a microprocessor oximeter (Oxi 320/SET. WTW, Germany) coupled with an $O_2$ sensor (CellOx 325, WTW, Germany). pH was determined using a Crison pH electrode (pH 197-S). Chemical Oxygen Demand (COD), Total Kjeldahl Nitrogen (TKN) and Total Suspended Solid (TSS) were analyzed according to DIN 38409-H 41(44), DIN EN 25663-H11 and DIN ISO 11465 (DEV., 2002). $NH_4^+$, total phosphorus and dissolved phosphorus ($PO_4^{3-}$) were determined according to DIN 38406-E5-1 and DIN EN ISO 6878-D11 (DEV., 2002) using a UV/Vis Spectrometer (Perkin Elmer, Lambda 40, USA). $NO_3^-$ and $NO_2^-$

**Table 1 – Characterization of wastewater.**

| Parameter | Unit | First batch | Second batch | Third batch | Fourth batch |
|---|---|---|---|---|---|
| Chemical oxygen demand (COD) | mg $O_2$/l | 132.7 ± ±3.0 | 103.0 ± ±5.0 | 190.9 ± ±3.0 | 140.8 ± ±4.0 |
| Total organic carbon (TOC) | mg C/l | 49.8 ± 1.2 | 36.9 ± 1.5 | 63.6 ± 1.0 | 52.1 ± 0.8 |
| TKN | mg N/l | 25.7 ± 0.3 | 25.3 ± 0.2 | 23.1 ± 0.6 | 35.4 ± 0.3 |
| Ammonium | mg N/l | 14.6 ± 1.0 | 18.4 ± 0.8 | 17.0 ± 1.2 | 18.9 ± 1.0 |
| Total phosphorus | mg P/l | 4.9 ± 0.1 | 3.9 ± 0.1 | 4.7 ± 0.1 | 3.8 ± 0.1 |

were determined using an Ion Chromatograph (Dionex DX-100, USA) according to DIN EN ISO 10304-1 (DEV., 2002). Total Organic Carbon (TOC) and Inorganic Carbon (IC) were determined using a TOC analyzer (Elementar liqui TOC II, Germany) according to DIN EN 1484-H3 (DEV., 2002). Before analysis of the above parameters in liquid, samples were membrane filtered (0.45 μm). To measure nitrogen or phosphorus in biomass, the samples were first divided into two identical parts. One part was homogenized, while another part was filtered to remove the solids. Nitrogen or phosphorus in biomass was calculated as the total nitrogen or phosphorus difference between the homogenized samples and the filtered samples. All the experiments were performed in duplicate. An optical microscope (OLYMPUS CHT, Japan) was used for morphological characterisation of microalgae.

### 2.4. *Community analysis*

Bacteria of the algae-bacteria system were collected at the end of each batch test by centrifugation at 10,000 rev $min^{-1}$ for 10 min at 4 °C. Each sample was washed twice with phosphate buffer (pH 7.0). Genomic DNA was isolated using the QIAamp™ DNA Stool Mini Kit (QIAGEN, 51504) according to the manufacturer's instructions. The polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE) and 16S rDNA analysis were done as described previously (Su et al., 2009; Schauer et al., 2000). Dominant bands were sequenced (Macrogen, the Netherlands). Sequences were subjected to Basic Local Alignment Search Tool (BLAST) and Ribosomal Database Project (RDP) analysis (Zhang et al., 2009). Phylogeny was determined with the RDP classifier and Sequmatch. The sequences used here have been deposited in the GenBank under the accession numbers HQ327478–HQ327485.

## 3. Results and discussion

### 3.1. *Settleability of the algal-bacterial culture*

The settleability of the cultivated algal-bacterial culture was investigated and is shown in Fig. 1. It showed good settleability, as nearly all the algal-bacterial biomass settled to the bottom of glass cylinder within 20 min, resulting in a reduction of TSS from an initial 1.84 to 0.016 g/l. The corresponding sludge settling ratio (SV %) was 12%, which also implied its good settleability (Sekine et al., 1984). Compared with an uncultivated algal-bacterial culture, the good settleability of the system might be due to the special cultivation strategy used in this study. The alternate mixing and non-mixing operation in the cultivation period promoted the selection of settleable algae and bacteria, which provided an effective way to harvest algal-bacterial biomass. The harvest technology of this settleable system has three advantages over other algal-bacterial harvesting technologies (Mohn, 1988; Imase et al., 2008; Nurdogan and Oswald, 1995). First, the operating cost was greatly reduced, as no extra energy (or equipment) was required. Second, secondary pollutants were eliminated during operation and biomass harvest, as no extra chemicals were added. Third, the settleability could be guaranteed during long-term operation. The sedimentation was the characteristic of the cultivated algal-bacterial culture and not dependent on any immobilization medium which was inefficient over long time operation (Mallick, 2002; Moreno-Garrido, 2008). A microscopic photograph of the developed algal-bacterial flocs is shown in Fig. 2. From Fig. 2A, it can be seen that the main species in the bioreactor were filamentous blue-green algae. Fig. 2B shows the wastewater bacteria attached to the algae filaments, which forms the cooperative system. Obviously, binding mechanisms supporting the bio-flocculation process led to the formation of settleable biomass. There may be some factors responsible for the settling process, such as the algae cell surface properties, extracellular polymeric substances (EPS) and the content of cations, which will influence the formation and the stability of the settleable algal-bacterial biomass (Gutzeit et al., 2005).

### 3.2. *Carbon and nutrients removal in municipal wastewater with algal-bacterial culture*

#### 3.2.1. *Temperature, dissolved oxygen and pH*

Changes in temperature, pH and dissolved oxygen during operation were monitored to determine their effects on the treatment process. As shown in Fig. 3A, the culture temperature

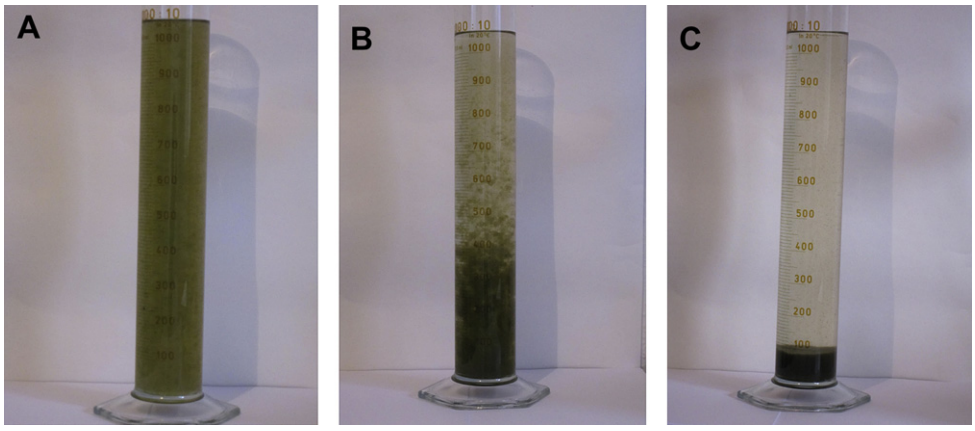


**Fig. 1 – Settleability of algal-bacterial biomass. (A) Initial completely mixed sample. (B) After 5 min of sedimentation. (C) After 20 min of sedimentation.**

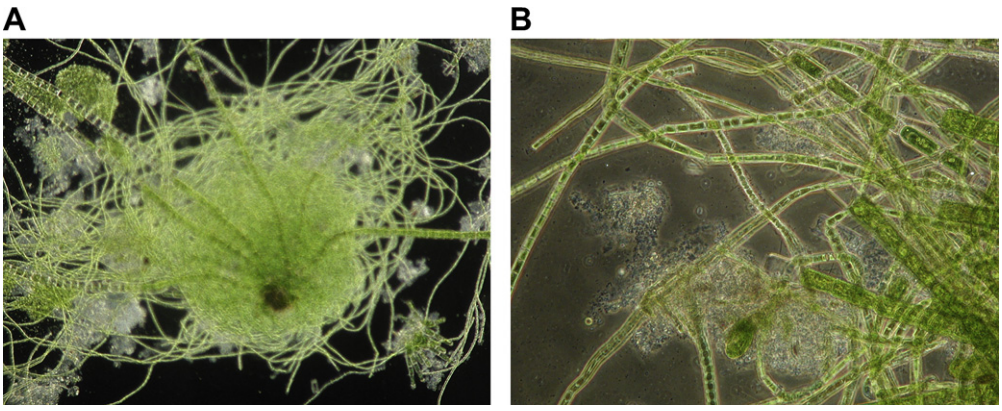


**Fig. 2 – Microscopic photographs of algal-bacterial flocs. (A) Light microscope × 100. (B) Light microscope × 400.**

was around 12 °C (the same as outdoor temperature) at the beginning of each batch test. It might be due to the fact that the wastewater was added into the bioreactor immediately after collection from the municipal wastewater plant. After that, the temperature increased to room temperature and remained stable until the end of each batch test.

Similarly, at the beginning of each batch test, the dissolved oxygen concentration (DO) was the same as that of the pre-treated wastewater. When starting the batch run, the DO values dropped significantly to around zero, which indicated that after initial consumption of the DO in the wastewater, the $O_2$ released from the algal photosynthesis was almost consumed by processes such as heterotrophic carbon oxidation and nitrification. After three days, the oxygen concentration increased gradually to around 2 mg/l and continued to increase until it was around 5.5 mg/l (70% saturation) at the end of each batch run (Fig. 3A).

No significant variation in culture pH during the four batches was detected in the system. Only a slight pH decrease occurred due to the intensive nitrification over the first five days. After that, pH increased gradually to 8.4 (see Fig. 3B). There are several factors which may influence the culture pH, such as micro-algal growth (pH increase as a result of $CO_2$ uptake), $NH_4^+$ nitrification (pH decrease due to the release of $H^+$) and the excretion of acidic or basic metabolites from organic matter biodegradation (Gonzalez et al., 2008b).

### 3.2.2. *Elimination of organic carbon*

As shown in Fig. 4, the COD decreased with time and was lower than 3 mg/l at the end of each batch test. The COD and TOC removal efficiencies were around 98% and 75.2% for the four batches, respectively (Fig. 4). Both algae and bacteria were able to use organic carbon through mixotrophic or heterotrophic metabolism (Abeliovich and Weisman, 1978). It was obvious that carbon sources were eliminated significantly

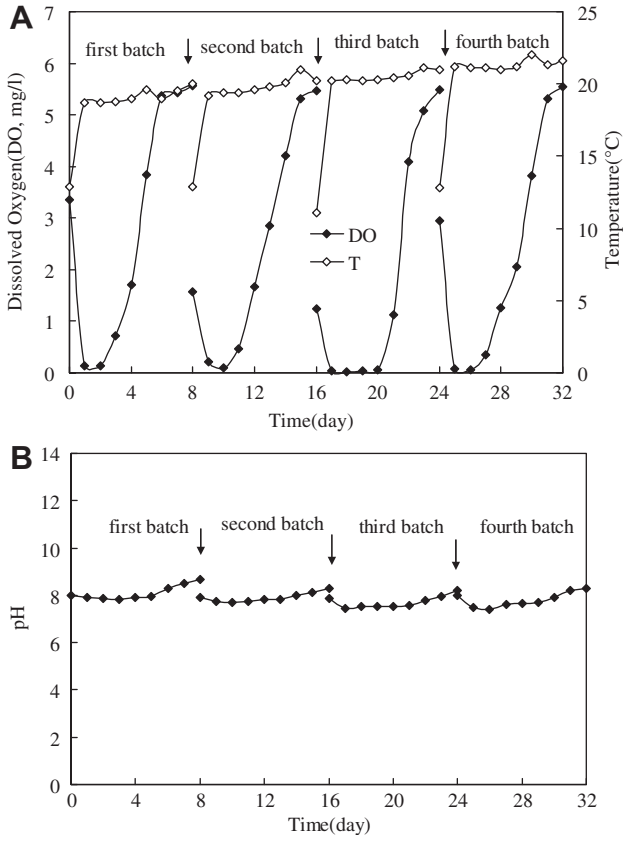


**Fig. 3 – Changes in temperature, dissolved oxygen and pH in the algal-bacterial system. (A) Temperature and dissolved oxygen. (B) pH. Arrows indicate the start of a new batch test.**

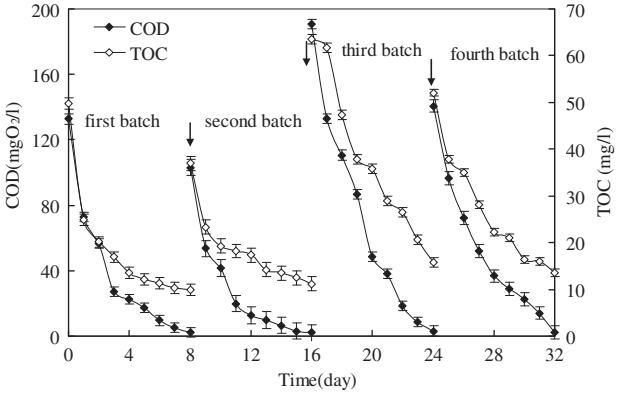


**Fig. 4 – The concentration of COD and TOC in the algal-bacterial system over four batch runs. Arrows indicate the start of a new batch test.**

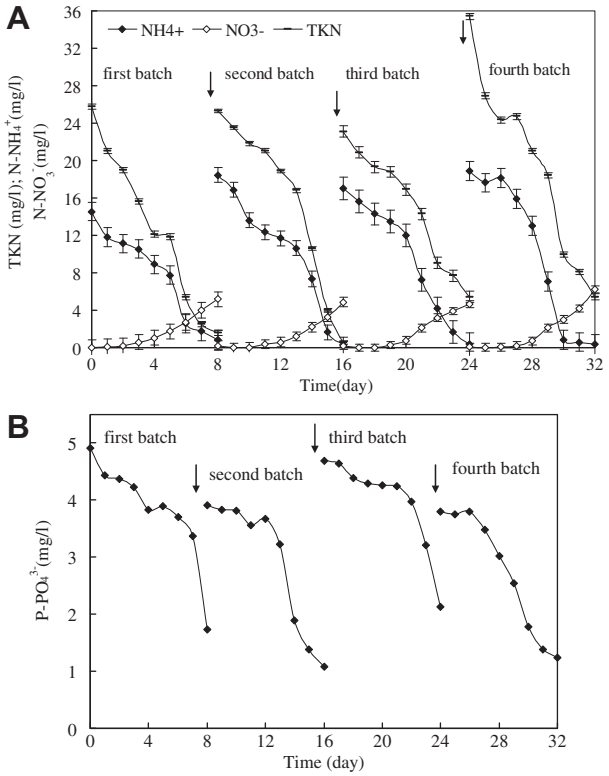


**Fig. 5 – The concentration of TKN, $NH_4^+$, $NO_3^-$ and $PO_4^{3-}$ over four batch runs. (A) TKN, $NH_4^+$ and $NO_3^-$. (B) $PO_4^{3-}$. Arrows indicate the start of a new batch test.**

within the first four days (Fig. 4). This result was in agreement with that of previous studies (Gutzeit et al., 2005). The COD and TOC removal were slow after the fourth day. There were two possible reasons for this. First, the carbon source left in the system was small after the fourth day. Second, the remaining carbon may be some colloidal, slowly biodegradable material. Usually, carbon was the limiting factor when algae were cultured in sewage. However, algae may also obtain $CO_2$ from the air in an open system, when the amount of carbon (inorganic and organic) in wastewater is inadequate for algal photosynthesis (Gonzalez et al., 2008a; Oswald, 1988).

### 3.2.3. *Elimination of nitrogen*

The influent nitrogen was mainly in the form of $N–NH_4^+$ (70–90%), followed by total organic nitrogen (10–30%). Fig. 5A shows the TKN, $N–NH_4^+$ removal and $N–O_3^-$ generation process. $N–NH_4^+$ removal efficiencies for all the four batches were nearly 100%, while the TKN removal efficiency were slightly different, ranging from 76.6% to 97.8% (Fig. 5A). The TKN removal efficiency in the last two batches was slightly lower compared with that in the first two batches. Obviously, there was still some organic nitrogen (ON, the difference of TKN and $N–NH_4^+$) in the effluent. The ON might be made of a small amount of inseparable organic matter produced during algae growth and wastewater treatment processes Oswald and Gotass, 1957. Additionally, $N–NO_3^-$ was always detected in the effluent due to incipient nitrification (around 5.2 mg/l). Nitrite was never detected at the end of each batch run.

The nitrogen balance was also investigated for a better understanding of nitrogen removal mechanisms. Based on total nitrogen removal and biomass concentration (discussed later), nitrogen assimilation into biomass accounted for approx. 52.9 ± 0.3%, 43.1 ± 0.4%, 43.0 ± 0.5% and 40.7 ± 0.4% of the total inlet nitrogen in the four batches, respectively (Table 2). The contribution of ammonia volatilization to total nitrogen removal in this system could be negligible due to the low $NH_4^+$ concentrations and relatively low pH (<8.5). Conversion into nitrate only accounted for 20.0 ± 0.2%, 18.3 ± 0.3%, 19.0 ± 0.1% and 17.4 ± 0.1% of total inlet nitrogen in the four batches, respectively (Table 2). The remaining missing nitrogen might be due to denitrification processes, which could occur at DO below 2 mg/l (Godos et al., 2009).

### 3.2.4. *Elimination of phosphate*

The time course of $P–PO_4^{3-}$ is shown in Fig. 5B. The removal of phosphate was a much slower process compared to that observed for nitrogen, but the same general pattern was apparent (Fig. 5B). The removal efficiencies of phosphate ranging from 54.5% to 72.6% were observed. The relatively slower and lower $P–PO_4^{3-}$ removal efficiencies, compared with $N–NH_4^+$, were probably due to the fact that nitrogen was the limiting nutrient, not phosphate, in this system. Previous studies have shown that the optimal ratio for maximum nitrogen and phosphorus uptake by algal-bacterial culture is N:P = 30:1 (Chevalier and de la Noue, 1985). However, the ratio of nitrogen to phosphorus was lower than 3 in this study.

Similarly, the balance of phosphorus was also investigated. Phosphorus accumulation into the biomass was still the main mechanism in this system, which accounted for 62.6 ± 0.5%, 66.6 ± 0.4%, 50.1 ± 0.6% and 67.1 ± 0.5% of the total inlet phosphorus for the four batches, respectively (Table 2). Phosphorus can be eliminated through both biotic phosphorous assimilation into the biomass and abiotic phosphorous precipitation (Godos et al., 2009). Nurdogan and Oswald (1995) reported on abiotic P removal which took place mainly in the

**Table 2 – Nitrogen and phosphorus balance over the four batch tests.**

| Batch | Inlet TN (mg N/l) | Outlet TN (mg N/l) | Inlet nitrogen oxidized in $NO_3^-$ (%) | Inlet nitrogen accumulated in biomass (%) | Inlet Phosphorus (mg P/l) | Outlet Phosphorus (mg P/l) | Inlet phosphorus accumulated in biomass (%) |
|---|---|---|---|---|---|---|---|
| 1 | 25.8 ± 1.3 | 6.8 ± 1.5 | 20.0 ± 0.2 | 52.9 ± 0.3 | 4.9 ± 0.1 | 1.7 ± 0.1 | 62.6 ± 0.5 |
| 2 | 25.5 ± 1.0 | 5.4 ± 0.9 | 18.3 ± 0.3 | 43.1 ± 0.4 | 3.9 ± 0.1 | 1.1 ± 0.1 | 66.6 ± 0.4 |
| 3 | 23.3 ± 1.8 | 10.1 ± 1.0 | 19.0 ± 0.1 | 43.0 ± 0.5 | 4.7 ± 0.1 | 2.1 ± 0.1 | 50.1 ± 0.6 |
| 4 | 35.5 ± 1.3 | 11.7 ± 0.7 | 17.4 ± 0.1 | 40.7 ± 0.4 | 3.8 ± 0.1 | 1.2 ± 0.1 | 67.1 ± 0.5 |

form of orthophosphate precipitation at high pH (9–11). In our system, the pH below 9 was not sufficient to promote this removal mechanism.

### 3.3. Biomass generation and nutrients accumulation processes

#### 3.3.1. Biomass generation during the operation

As shown in Fig. 6A, the TSS increased during operation, from 0.6 g/l at the beginning to 1.84 g/l at the end. The mean biomass generation rate was 10.9 ± 1.1 g/m$^2$ d. These values were lower than those reported by previous studies, in which maximum biomass productivities of 27.7 g/m$^2$ d were observed with 10 times diluted swine manure (2417 ± 481 mg COD/l; 214 ± 53 mg $NH_4^+$/l) during June and August in high rate algae ponds (HRAPs) in Valladolid, Spain (average irradiations 7062 ± 81 Wh/m$^2$ d) (de Godos et al., 2009). The lower availability of carbon and nitrogen in municipal wastewater compared with diluted swine manure, together with the lower irradiation, may be possible reasons for this.

Although there was continuous algal and bacterial growth in the system during the treatment process, the removal efficiencies of both carbon and nutrients and their general patterns were quite similar for the four batches. One possible reason for this might be that the further increased algae concentration has less effect on the uptake rate, since the nutrient uptake by algae was also determined by other factors (e.g., illumination conditions and the irradiated water surface area of the reactor) (Chevalier and de la Noue, 1985).

#### 3.3.2. Nutrient accumulation processes

The accumulation of nitrogen and phosphorus in biomass during the treatment process is shown in Fig. 6B. The nitrogen concentration in biomass increased from 27.8 to 32.6 mg/g, at a rate of nearly 0.15 mg/g d. Meanwhile, the phosphorus concentration in the biomass increased from 0.72 to 4.74 mg/g, at a rate of 0.12 mg/g d. The most rapid accumulation period of nitrogen and phosphorus occurred in the first batch. After that, the accumulation rates were much lower and the amount of nitrogen and phosphorus in biomass was stable. It has been reported that the biomass generated from a swine manure removal process, ranging from 22.9 to 76.6 mg N/g biomass and from 4.3 to 25.2 mg P/g biomass, had fertilizer value to promote plant growth (Mulbry et al., 2005; Mulbry et al., 2006). The concentrations of nitrogen and phosphorus in biomass in this study were comparable to those of the swine manure treatment system. However, it is worth noting that the nutrient concentration in municipal wastewater is much lower than that of swine manure, so it leaves room to improve the accumulation of nitrogen and phosphorus in the settleable algal-bacterial culture.

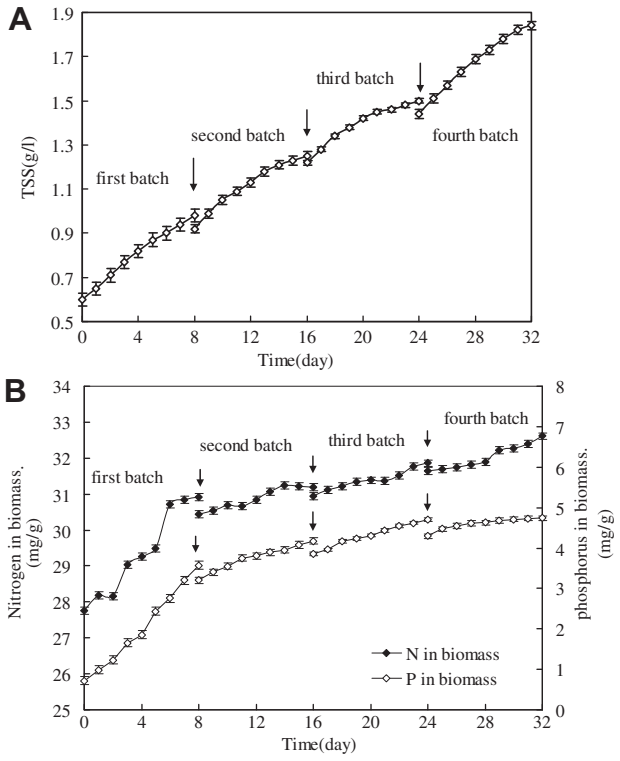


**Fig. 6 – Changes in total suspended solids, nitrogen and phosphorus accumulation in biomass over four batch runs. (A) Total suspended solids. (B) Nitrogen and phosphorus concentration in biomass. Arrows indicate the start of a new batch test.**

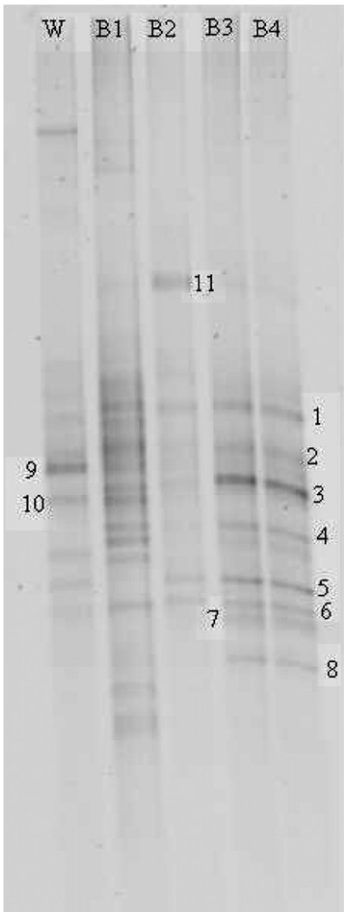


**Fig. 7 – DGGE bands of bacteria communities. W: wastewater; B1 to B4: batch 1 to 4; 1 to 11: the name of each band.**

**Table 3 – DGGE 16S rDNA band identifications.**

| Band | Run | | | | | Genbank accession no. | Closest relatives (%Sequence similarity[d]) | Class[c] |
|---|---|---|---|---|---|---|---|---|
| | W[a] | B1 | B2 | B3 | B4 | | | |
| 1 | | •[b] | • | • | • | HQ327478 | Uncultured *Flavobacteria* bacterium clone ATB-LH-7295 (96%) | *Flavobacteria* |
| 2 | | • | | • | • | HQ327479 | Uncultured bacterium isolates DGGE gel band a3 (81%) | *Gammaproteobacteria* |
| 3 | | | | • | • | HQ327480 | Uncultured bacterium clone sls1360 (97%) | *Flavobacteria* |
| 4 | | • | | • | • | HQ327481 | Uncultured bacterium clone LL141-8H16 (92%) | *Bacteroidia* |
| 5 | • | | • | • | • | HQ327482 | *Dysgonomonas* sp. enrichment culture clone YFZ1 (100%) | *Bacteroidia* |
| 6 | • | • | • | • | • | HQ327483 | Uncultured bacterium 6week9 (94%) | *Bacteroidia* |
| 7 | | | | • | • | HQ327484 | Uncultured *Bacteroidetes* bacterium clone 298 (83%) | *Bacteroidia* |
| 8 | | | | • | • | HQ327485 | Uncultured bacterium clone RW6944 (86%) | *Betaproteobacteria* |

a Inoculum.
b Existence under the condition.
c The phylotypes were assigned to phyla based on Ribosomal Database Project II (RDP II) taxonomy classifications.
d Percent values represent similarities between the associated DGGE band sequence and the closest match sequence from GenBank.

### 3.4. *Community analysis*

The DGGE profiles of the bacteria community sampled from the reactor at the end of each batch run as shown in Fig. 4 are summarized in Fig. 7. It was clear that the bacterial populations changed with time and became stable after operation through three batches (Fig. 7). At the same time, bacteria related to nutrient removal might have been enriched and stable, as indicated by the high removal efficiency observed along the successive tests, as shown in Fig. 4 and Fig. 5. Some bacteria in the acclimatized bacteria consortium were not present in the inoculum, suggesting that some new communities were enriched after operation. The above results also indicate that the bacteria community in algal-bacterial culture required time to acclimate to this commensalism system.

In order to provide greater insight into microbial ecology and diversity, eight predominant species extracted from DGGE bands were sequenced. Based on the 16S rDNA gene library results (Table 3), the acclimatized bacteria consortium was predominated by *Bacteroidia* (50% of clones), followed by *Flavobacteria* (25% of clones), *Betaproteobacteria* (12.5% of clones) and *Gammaproteobacteria* (12.5% of clones). It has been observed that *Flavobacteria* and *Bacteroidetes* phylotypes were present in high numbers in ammonia-oxidizing processes (Ducey et al., 2010; Nakano et al., 2008; Zang et al., 2008). Bafana et al. (2007) also observed that a *Gammaproteobacteria* (54%) phylotype was dominant in the acclimatized sludge of wastewater treatment plants. It was noticed that the band 1, 3 and 5 were stronger than other bands during operation (Fig. 7), thus their respective microorganisms might play a very important role in wastewater nutrient removal.

## 4. Conclusion

A settleable algal-bacterial culture, cultivated from wastewater, was successfully used to treat municipal wastewater in a stirred tank photobioreactor. The algal-bacterial culture showed good settleability, while the total suspended solid could be reduced to 0.016 g/l within 20 min sedimentation. The average removal efficiencies of COD, TKN and phosphate were 98.2 ± 1.3%, 88.3 ± 1.6% and 64.8 ± 1.0% within 8 days, respectively, while the average biomass productivity was 10.9 ± 1.1 g/m$^2$·d. Biomass uptake was the main mechanism for nutrient removal. The main algae species in the bioreactor were filamentous blue-green algae, while the main bacteria present in the photobioreactor were a consortium with sequences similar to *Flavobacteria, Gammaproteobacteria, Bacteroidia and Betaproteobacteria*. This study provides new insights into rapidly settleable algal-bacterial culture enrichment strategies and supplements the information on microbial ecology and diversity in algal-bacterial culture.

## Acknowledgments

The authors thank Yifeng Zhang (Technical University of Denmark, Demark) for help and advice on the molecular biotechnology work, Sabine Henschel and Kerstin Lammers-for advice on chemical water analysis work and Ingeborg Joost and Pamela Holweg (Ostfalia University of Applied Sciences, Germany) for their kind assistance. This work was supported by Deutscher Akademischer Austausch Dienst (DAAD) and the LiWa project.

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# II

# Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: Influence of algae and sludge inoculation ratios

Su, Y., Mennerich, A., Urban, B.

Bioresource Technology 105, 67-73 (2012)

Bioresource Technology 105 (2012) 67–73

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: Influence of algae and sludge inoculation ratios

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ARTICLE INFO

*Article history:*
Received 12 October 2011
Received in revised form 26 November 2011
Accepted 27 November 2011
Available online 3 December 2011

*Keywords:*
Algal–bacterial culture
Algae/activated sludge inoculum ratio
Wastewater treatment
Settleability
16S rDNA gene sequencing

ABSTRACT

An algal–bacterial culture, composed of wastewater-born algae and activated sludge, was cultivated to treat domestic wastewater and accumulate biomass simultaneously. The influence of algae and sludge inoculation ratios on the treatment efficiency and the settleability of the accumulated biomass were investigated. There was no significant effect of the inoculation ratios on the chemical oxygen demand removal. Comparatively, the nutrients removal and related mechanism were varied with different inoculation ratios. The highest nitrogen and phosphorus removal efficiencies were observed with 5:1 (algae/sludge) culture (91.0 ± 7.0% and 93.5 ± 2.5%, respectively) within 10 days, which was 5–40% higher and 2–4 days faster than those with other inoculation ratios. The biomass settleability was improved with the assistance of sludge, and the 1:5 (algae/sludge) culture showed the best settleability. Furthermore, 16S rDNA gene analysis showed that the bacterial communities were varying with different algae and sludge inoculation ratios and some specific bacteria were enriched during operation.



## 1. Introduction

Algal–bacterial culture biotechnology has received more attention in recent years, especially in the tropical and subtropical regions, as an alternative biosystem for municipal and industrial wastewater treatment (Garcia et al., 2000; Godos et al., 2009; Oswald, 2003). Under illuminated conditions, algae produce $O_2$ by photosynthesis, which is required by the bacteria to mineralize organic matter, realizing simultaneously cost-efficient aeration (Guieysse et al., 2002). The $CO_2$ released by the bacteria is then consumed by algal photosynthesis, thereby mitigating greenhouse gas emission (Munoz and Guieysse, 2006; Munoz et al., 2003; Oswald, 1988). In addition, nutrients such as ammonium and phosphate known as the main cause of water body eutrophication are concomitantly eliminated by algal uptake (Chevalier et al., 2000; de Godos et al., 2009; Zhang et al., 2011a,b). Moreover, the algal photosynthesis favors the elimination of many pathogens and viruses by increasing the temperature, pH and dissolved oxygen concentration of the treated effluent (Munoz et al., 2003; Oswald, 1988). The most important thing is that the biomass harvested at the end of treatment process is a new sustainable source of liquid transport fuel or valuable products (e.g., biodiesel, drugs, fertilizer) (Rittmann, 2008).

The treatment process occurring in the algal–bacterial system may therefore be said to result from a symbiosis of algae and bacteria. The initial algae and bacteria inoculum ratios may influence the cooperation relationship and then result in different treatment efficiencies. Guieysse et al. (2002) described that the initial *Chlorella sorokiniana* and *Ralstonia basilensis* inoculation ratios have great influences on the salicylate degradation efficiency in a closed system. However, there are no relevant reports about the influence of the initial composition and algae/bacteria inoculum ratios on the municipal wastewater treatment, especially in an open system.

Besides, one of the hurdles of this technology is the harvest or separation of the algal–bacterial biomass from the treated wastewater discharge (Mallick, 2002; Nudogan and Oswald, 1995). The conventional harvesting technologies, such as filtration, centrifugation, adding chemicals or using immobilization medium have their own disadvantages such as the high operation costs, secondary contamination and low efficiency for long-term operation (Imase et al., 2008; Mallick, 2002; Mohn, 1988). Considering this, microalgae bioflocculation followed by gravity sedimentation is an easy and low-cost harvesting technique during wastewater treatment (Nudogan and Oswald, 1995; Oh et al., 2001). But this approach is not always efficient, especially in the case of the small size and rapidly growing unialgal cell (Munoz, 2005; Munoz and Guieysse, 2006). Therefore, selection of algal–bacterial culture with a natural tendency to settle down is recommended (Olguin, 2003; Su et al., 2011). Activated sludge has better gravity settleability compared with microalgae, and higher organic matter removal capacity than the normal bacteria contained in domestic wastewater (Gutzeit et al., 2005), which

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doi:10.1016/j.biortech.2011.11.113

could be used as bacterial inoculum in algal–bacterial culture to enhance the biomass settleability and treatment efficiency. However, no report of this approach is available. Furthermore, how different algae/activated sludge inoculation ratios influence the biomass settleability and which ratio is better for the settleability are still unknown.

In this study, for the first time, the influence of initial inoculum ratios of wastewater-born algae and aerobic activated sludge on the municipal wastewater treatment and the biomass settleability were investigated. Besides, the microbial community compositions in the cultures with different inoculum ratios were investigated.

## 2. Methods

### 2.1. Inoculum ratio tests

Wastewater treatment tests were performed in stirred tank photobioreactors which were made of transparent PVC (40 cm in depth and 29 cm in diameter) at room temperature (around 23 °C). The total volume of the liquid in the reactor was 14 l (approx. 25 cm in depth). The algae inoculum was obtained from the second clarifier wall of the Suderburg municipal wastewater treatment plant (County of Uelzen, Lower Saxony, Germany). The collected algae inoculum (mainly filamentous blue–green algae observed under a microscopy) was firstly settled down for 1 h and then the settled solids were used as algae inoculum. The aerobic activated sludge obtained from the same plant (first settled down for 1 h and discarded the supernatant) was used as bacteria inoculum. The total suspended solid (TSS) of initial algae inoculum was 8 g/l. The TSS of initial activated sludge inoculum was 8 g/l. The six reactors were filled with 1200, 1090.9, 1000, 600, 200 and 0 ml algae inoculum and 0, 109.1, 200, 600, 1000, 1200 ml activated sludge inoculum to obtain the following algae/sludge ratios of only algae, 10:1, 5:1, 1:1, 1:5 (w/w) and only activated sludge, respectively. The total inoculum volume was 1.2 l. 12.8 l pretreated wastewater (after preliminary screening, grit removal and primary sedimentation process) from the same plant was added into the bioreactors to investigate the carbon and nutrient removal efficiencies. The characterization of the pretreated wastewater was COD: 380.0 ± 15.3 (mg $O_2$/l), total nitrogen (TN): 50.1 ± 3.0 (mg N/l), $NH_4^+$-N: 39.4 ± 5.5 (mg/l), $PO_4^{3-}$-P: 8.8 ± 0.9 (mg/l), $NO_3^-$-N: 0.02 ± 0.02 (mg/l). $NO_2^-$-N: 0 (mg/l). Constant mixing was maintained using a magnetic stirring bar (100 rpm) to avoid algae sedimentation. Two compact fluorescent lamps (Sylvania, F20W/860/E27) were used to irradiate the tank with about 7000 lux (measured at the top of liquid surface) for a 12 h light–12 h dark cycle (TES-1335 Digital light Meter). The above batch tests were performed in duplicate.

### 2.2. Analytical procedures

The dissolved oxygen (DO) and pH were measured near the midway of the reactor using a microprocessor oximeter (Oxi 320/SET, WTW, Germany) coupled with an $O_2$ sensor (CellOx 325, WTW, Germany) and a Crison pH electrode (pH 197-S). Eighty milliliters of samples for further analysis (see below) were collected near the midway of the bioreactors with a pipe every day, 4 h after starting the illumination period. Soluble chemical oxygen demand (COD), Total Kjeldahl Nitrogen (TKN) and TSS were analyzed according to DIN 38409-H 41(44), DIN EN 25663-H11 and DIN ISO 11465 (DEV, 2002). $NH_4^+$, total phosphorus and $PO_4^{3-}$ were determined according to DIN 38406-E5-1 and DIN EN ISO 6878-D11 (DEV, 2002) using an UV/Vis Spectrometer (Perkin Elmer, Lambda 40, USA). $NO_3^-$ and $NO_2^-$ were determined using an Ion Chromatograph (Dionex DX-100, USA) according to DIN EN ISO 10304-1 (DEV, 2002). Before analysis of the above parameters, the liquid samples were membrane filtered (0.45 μm). To measure nitrogen or phosphorus in biomass, the samples were first divided into two equal parts. One part was homogenized (IKA T25 digital, ULTRA-TURRAX), while another part was filtered to remove the solids. Nitrogen or phosphorus in biomass was calculated as the total nitrogen or phosphorus difference between the homogenized samples and the filtered samples.

### 2.3. The biomass settleability

At the end of the test, 1 l mixed sample was transfer to 1 l measurement cylinder to investigate the settleability of the algal–bacterial biomass with different algae/sludge inoculum ratios (Su et al., 2011). The TSS samples were taken every 6 min from 10 cm below the liquid surface of the cylinder with a pipe. This procedure lasted for 1 h.

### 2.4. Community analysis

The algal–bacterial culture with different initial algae/bacteria inoculum ratios were collected at the end of each batch by centrifugation at 10,000 rev $min^{-1}$ for 10 min at 4 °C. Each sample was washed twice with phosphate buffer (pH 7.0). Genomic DNA was isolated using the QIAamp™ DNA Stool Mini Kit (QIAGEN, 51504) according to the manufacturer's instructions. The polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) were accomplished as described previously (Su et al., 2009; Zhang et al., 2009). The similarities between lanes from DGGE and band intensity were analyzed with Quantity One Software (Bio-RadLaboratories) (Zhang et al., 2011a,b). Dominant bands were sequenced (Macrogen, the Netherlands). Sequences were subjected to Basic Local Alignment Search Tool (BLAST) and Ribosomal Database Project (RDP) analysis (Zhang et al., 2009). Phylogeny was determined with the RDP classifier and SEQMATCH. Parsimony phylogenetic tree was constructed by the neighbor-joining method using the MegAlign program (DNASTAR, Madison, WI, USA) (Fig. S1, Supplementary data). The sequences used here have been deposited in the GenBank under the accession numbers JN157636–JN157643.

## 3. Results and discussion

### 3.1. The variation of dissolved oxygen and pH

Change in DO and pH of the reactors inoculated with different algae/activated sludge ratios are shown in Fig. 1. At the beginning of the test, the DO was the same as that of the pretreated wastewater (around 2 mg/l). After that, it decreased to around 0.1 mg/l in all the reactors. This means that after consumption of the remaining $O_2$ in the wastewater, the $O_2$ released from the algal photosynthesis was immediately and almost completely consumed by the aerobic bacteria. The low DO lasted till the end of the test in the reactor with only sludge. However, for the reactor with only algae, the low DO was only observed during the first day and increased gradually to 8.5 mg/l because the main oxygen consumer (aerobic sludge) was not added. For the other four reactors with both algae and sludge, the low DO below 0.33 mg/l lasted for different durations (11 days for algae:activated sludge = 10:1, 7 days for 5:1, 8 days for 1:1 and 12 days for 1:5) and increased gradually to around 5 mg/l till the end of the test (Fig. 1a). The possible reason for the longer duration time of low DO in the reactor with 10:1 ratio may be due to the aerobic process such as nitrification. For the reactor with algae/sludge inoculation ratio of 1:5, the fact that the $O_2$ generated by the low concentration of algae could not fulfill the respiration demand of the high concentration of bacteria

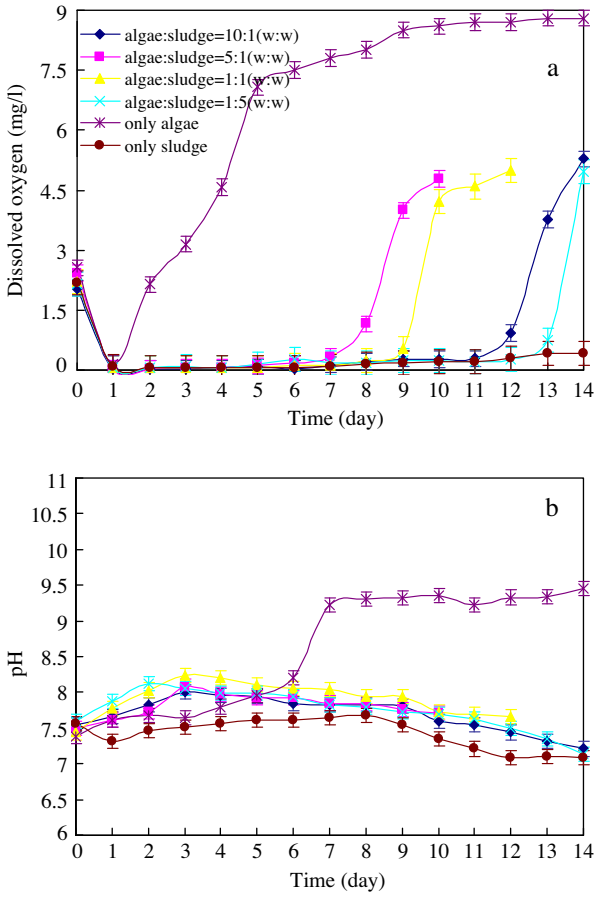


**Fig. 1.** The dissolved oxygen concentration (a) and pH (b) in the reactors with different algae/sludge inoculation ratios.

might be responsible for the relatively longer duration of low oxygen concentration.

pH in the reactor with only sludge was stable during the first 10 days and dropped softly to around 7 due to the nitrification. pH in the reactor with only algae showed different trend comparing with others. It increased to around 9.2 after the first 7 days and became stable till the end of the test. Dissolved $CO_2$ is correlated with solution pH. The initial increase of pH may due to the intensive $CO_2$ utilization from the medium by algal autotrophic growth. After that, as there were no enough $CO_2$ supply from bacterial respiration, the balance between $CO_2$ capture from air and algal uptake lead to the pH stability. pH in the other four reactors with different algae/sludge inoculum ratios showed the similar trend and level. It increased slightly during the first 4 days from 7.5 to around 8.3 (Fig. 1b), which might be caused by $CO_2$ uptake for algal photosynthesis (Munoz and Guieysse, 2006). After that, pH dropped back to 7.5 again, which could be due to the nitrification process (Gutzeit et al., 2005).

### *3.2. The influence of algae/activated sludge inoculum ratios on carbon source removal*

The abilities of the reactors inoculated with different algae/sludge ratios (10:1, 5:1, 1:1 and 1:5) to mineralize the organic carbon were investigated. The pattern of COD removal was similar in these four reactors, indicating that there was no strong relationship between COD removal and initial algae/activated sludge inoculum ratios (Fig. 2). A maximal COD removal was attained within 8 days, during which the COD content decreased from 364.9 ± 5.4,

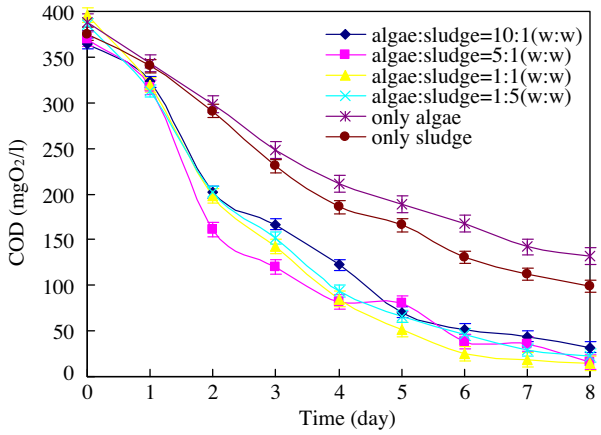


**Fig. 2.** The COD concentration in the reactors with different algae/activated sludge inoculation ratios.

369.7 ± 7.1, 395.8 ± 4.2 and 385.4 ± 5.9 mg $O_2$/l to 32.2 ± 5.4, 15.4 ± 7.1, 14.9 ± 4.2 and 23.1 ± 5.9 mg $O_2$/l with algae/activated sludge ratios of 10:1, 5:1, 1:1 and 1:5, respectively. The corresponding COD removal efficiencies were 91.2 ± 1.7%, 95.8 ± 2.0%, 96.2 ± 1.1% and 94.0 ± 1.6%, respectively. In addition, the COD removal in the reactors with only algae and only sludge was also investigated. It was obviously that the COD removal efficiencies with only algae (66.0 ± 6.0%) and only activated sludge (73.6 ± 5.1%) were much lower than those of the cultures with both of them, indicating the importance of cooperation between algae and sludge. For the reactor with only algae culture, the low removal efficiency could be due to the lack of sludge to enhance the organic carbon degradation. For the reactor with only sludge culture, there was no enough oxygen supply from algae for their aerobic respiration, and thereby resulting in low COD removal. Some algae-based bio-systems were used to treat highly concentrated municipal wastewater and the corresponding COD was removed from initial 2500 mg/l to around 250 mg/l (Zhou et al., 2011; Li et al., 2011). The performance of the algae–sludge system proposed in this study for high concentration wastewater treatment could be our further research goal.

### *3.3. The influence of algae/activated sludge inoculation ratios on nitrogen removal*

The nitrogen removal was also investigated in the above reactors. The removal of TKN and ammonia were much slower than that observed for carbon source, which took 2–6 days more. The reactor with algae/activated sludge ratios of 5:1 exhibited the highest and fastest TKN removal (95.8% within 10 days). Above 93.7% of TKN was degraded after 14, 12 and 14 days in the reactors with algae/sludge inoculum ratios of 10:1, 1:1 and 1:5, respectively. The TKN removal efficiency in the reactor with only algae was also reached to 89% after 14 days. While, for the reactor with only sludge, the TKN removal efficiency was only 31.4 ± 3.0% due to the long duration of low DO concentration which could not support the oxygen demand of the nitrification (Fig. 3a). These results matched with the $NH_4^+$-N removal efficiency shown in Fig. 3b. The better performance of the reactor with algae/sludge ratio of 5:1 was probably due to its proper inoculation ratio which promoted the cooperation between the algae and sludge.

On the other hand, $NO_3^-$-N was always detected in the effluent of all the reactors, up to 7.0 ± 0.2, 5.2 ± 0.3, 2.3 ± 0.3, 1.9 ± 0.3, 1.6 ± 0.2 and 6.6 ± 0.3 mg/l for the reactors with only algae, 10:1, 5:1, 1:1, 1:5 (algae/sludge) and only sludge, respectively (Fig. 3c). $NO_2^-$ was never detected in the reactors with algae/sludge ratio of 5:1 and only sludge. 17.0 ± 0.4, 14.3 ± 0.2, 1.8 ± 0.3 and 20.6 ± 0.3 mg/l of $NO_2^-$-N

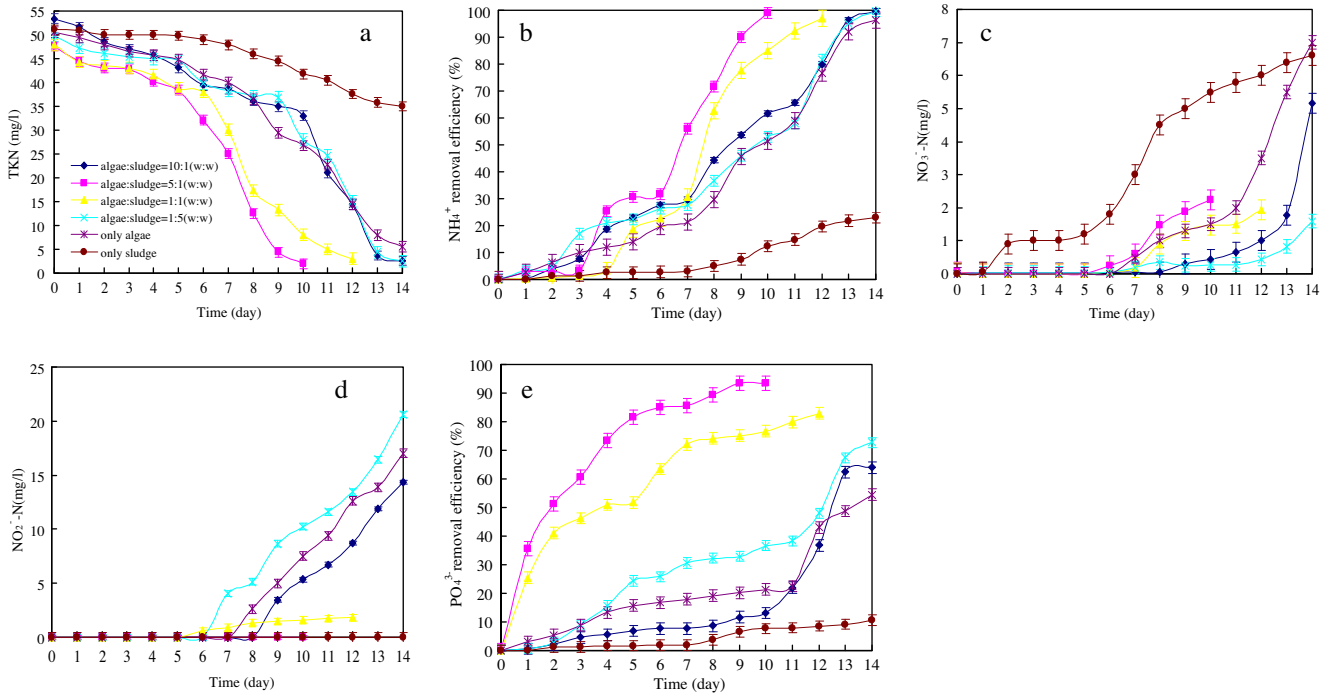


**Fig. 3.** Nutrients removal in the reactors with different algae/activated sludge inoculation ratios. (a) TKN; (b) $NH_4^+$ removal efficiency; (c) $NO_3^-$-N; (d) $NO_2^-$-N; (e) $PO_4^{3-}$ removal efficiency.

were detected in the final effluent of the other four reactors (only algae, 10:1, 1:1 and 1:5, respectively) (Fig. 3d).

In order to investigate the nitrogen removal mechanism with different algae/activated sludge ratios, the mass balance of nitrogen were calculated (Table 1). The total nitrogen removal efficiency was 41.7 ± 6.4%, 58.6 ± 5.8%, 91.0 ± 7.0%, 86.0 ± 8.5%, 50.2 ± 7.0% and 18.6 ± 6.0% for only algae, 10:1, 5:1, 1:1, 1:5 (algae/activated sludge) and only sludge cultures. With algae/sludge inoculum ratios of 5:1 and 1:1, assimilation into algal–bacterial biomass represented the main nitrogen removal mechanism, accounting for 60.0 ± 0.3% and 41.6 ± 0.5% of the total inlet nitrogen, while nitrification only represented 4.6 ± 0.7% and 7.8 ± 1.3% in the above-referred system, respectively. In contrast, in the other systems with only algae, 10:1, 1:5, (algae/sludge) and only sludge, about 47.4 ± 2.2%, 36.4 ± 1.6%, 44.6 ± 2.1% and 12.9 ± 2.9% of inlet nitrogen were oxidized into $NO_3^-$ and $NO_2^-$ by nitrification and not further removed. Accumulation of nitrogen into algal–bacterial biomass only accounted for 20.0 ± 0.4%, 24.9 ± 0.7%, 14.9 ± 0.9% and 11.7 ± 0.3% of the inlet nitrogen in these four reactors, respectively. The above results indicated that different algae and bacteria compositions lead to different algal and bacterial activity which resulted in different nutrient removal mechanism.

### *3.4. The influence of algae/activated sludge inoculation ratios on phosphorus removal*

The $PO_4^{3-}$-P removal in these reactors was also investigated. As shown in Fig. 3e, the fastest and highest phosphorus removal was found in the reactor inoculated with algae/activated sludge ratio of 5:1 (93.5 ± 2.5% reduction within 10 days), followed by the reactor with algae/activated sludge ratio of 1:1 (82.9 ± 2.0% in 12 days). While, only 72.7 ± 1.8% and 64.0 ± 2.0% $PO_4^{3-}$ was removed within 14 days in the reactors inoculated with algae/activated sludge ratio of 1:5 and 10:1, respectively (Fig. 3e). In the control test with only algae, the P removal efficiency was 54.4 ± 2.0% within 14 days. There were two possible reasons for this low P removal efficiency. Firstly, light inhibition among algae cells led to low algae autotrophic growth. Secondly, without the main $CO_2$ supply partner (sludge), the photosynthesis of algae was limited as the only $CO_2$ source was from the air. Similarly, only 10.6 ± 1.8% phosphate removal was observed in the reactor with sole activated sludge, which may be due to the lack of algal involvement and poor phosphorus removal capability of activated sludge.

The mass balance of phosphorus indicated that accumulation into the biomass was the main removal mechanism of phosphorus, which accounted for 50.5 ± 2.3%, 60.8 ± 1.2%, 91.4 ± 2.4%, 80.9 ± 1.0%, 71.8 ± 1.4% and 5.2 ± 1.2% of the total inlet phosphorus for the only algae, 10:1, 5:1, 1:1, 1:5 (algae/sludge) and only sludge cultures (Table 1). It has been reported that both biomass uptake and phosphate precipitation can account for the decrease in phosphate levels (Godos et al., 2009). The abiotic P removal process (normally occurred at pH 9–11) could have a minor influence in this study due to the relative low pH (Nudogan and Oswald, 1995).

The above results indicated that different inoculation ratios have great influences on nitrogen and phosphorus removal. It is obvious that the inoculation ratio of 5:1 (algae/activated sludge) gave the best results concerning nutrient removal efficiency and retention time. Either too high or too low algae/sludge ratio in the inoculum was not good for nutrients removal.

The algae concentration determines light utilization efficiency and also controls the oxygenation supply and removal efficiencies achieved in the algal–bacterial system (Janssen et al., 2003; Munoz and Guieysse, 2006; Munoz et al., 2004). However, it is not as expected that the higher the algae concentration in the symbiotic system, the higher nutrient removal efficiency. There were several explanations for this behavior. The nutrient removal was always limited by the $O_2$ generation rate in the initial phase of the experiments, which was initially proportional to the algal density within certain range, but rapidly became limited by the availability of light once the algae concentration reached a certain value (Guieysse et al., 2002). After the algae concentration reached a certain level, it had no effect on the nutrient uptake rate, since about the same numbers of algae were actually working in this system (Burrell et al., 1985). In addition, it should be noticed that the high

**Table 1**
Nitrogen and phosphorus balance for the cultures with the different algae/activated sludge ratios.

| Algae:activated sludge | Inlet total nitrogen (mg N/l) | Outlet total nitrogen (mg N/l) | Total nitrogen removal efficiency (%) | Inlet nitrogen oxidized in $NO_3^-$ (%) | Inlet nitrogen oxidized in $NO_2^-$ (%) | Inlet nitrogen accumulated in biomass (%) | Inlet total phosphorus (mg P/l) | Outlet total phosphorus (mg P/l) | Inlet phosphorus accumulated in biomass (%) |
|---|---|---|---|---|---|---|---|---|---|
| Only algae | 50.6 ± 1.1 | 29.5 ± 1.7 | 41.7 ± 6.4 | 13.8 ± 0.7 | 33.6 ± 1.5 | 20.0 ± 0.4 | 8.8 ± 0.2 | 4.0 ± 0.2 | 50.5 ± 2.3 |
| 10:1 | 53.4 ± 1.0 | 22.1 ± 1.5 | 58.6 ± 5.8 | 9.6 ± 0.7 | 26.8 ± 0.9 | 24.9 ± 0.7 | 7.8 ± 0.1 | 3.0 ± 0.1 | 60.8 ± 1.2 |
| 5:1 | 47.6 ± 1.0 | 4.3 ± 1.5 | 91.0 ± 7.0 | 4.6 ± 0.7 | 0 | 60.0 ± 0.3 | 8.6 ± 0.2 | 0.6 ± 0.2 | 91.4 ± 2.4 |

(*continued on next page*)

algal concentration would lead to mutual shading within the algal population, a reduction in photosynthetic efficiency and the increase of $O_2$ consumption due to the algal dark respiration (Guieysse et al., 2002; Lau et al., 1995).

### *3.5. The influence of initial algae/activated sludge inoculation ratios on the biomass settleability*

At the end of the test, the settleability of the algal–bacterial biomass with different initial algae/activated sludge inoculum ratios was investigated. Obviously, the culture with only activated sludge showed the best settleability (from 1.60 ± 0.02 to 0.05 ± 0.02 g/l within only 24 min) (Fig. 4). The biomass from the reactor with inoculum ratio of 1:5 (algae:sludge) also showed a good settleability, which could settle down from 1.64 ± 0.02 to 0.05 ± 0.02 g/l (TSS) within 30 min, followed by 1:1, 5:1 and 10:1 (algae/sludge) cultures (from 1.85 ± 0.02 g/l to 0.05 ± 0.02 g/l within 36 min, from 1.91 ± 0.03 g/l to 0.06 ± 0.03 g/l within 42 min and from 1.72 ± 0.02 g/l to 0.21 ± 0.02 g/l within 60 min, respectively). The culture with only algae showed the lowest settleability (from initial 1.68 ± 0.03 to 0.41 ± 0.03 g/l).

The above results proved the hypothesis that activated sludge could enhance the settleability of algae biomass. Besides, the binding mechanisms influenced by algae cell surface properties, extracellular polymeric substances and the cations amount supported the bio-flocculation process leading to the formation of settleable biomass (Gutzeit et al., 2005) and the high pH could decrease the biomass settleability and lead to reactor washout (Hende et al., 2010).

### *3.6. The microbial community*

The DGGE profiles of bacteria community sampled from the four reactors at the end of the test are summarized in Fig. 5. The similarities between lane A (algae/sludge ratio of 1:1) and lane B (5:1) were more than 80%, indicating these two reactors had similar dominant bacterial community. While, the similarities between lane A (B), lane C (1:5) and lane D (10:1) were lower than 30%, only band 1, 2 and 10 universally occurred in lane A, B and C. The intensities of band 8 and 9 in lane A and lane B were strong, while the intensities of band 4 and 5 were strong in lane C. Bacteria represented by these bands might take part in wastewater treatment. The above results indicate that initial algae/sludge inoculation ratios can affect the bacterial community compositions of the algal–bacterial culture, which might contribute to the different removal efficiencies as shown in Figs. 2 and 3. In addition, some of the bacteria in the cultivated cultures were not the dominant species or undetected in the original inoculum, which indicated that specific bacteria were enrich during operation.

In order to provide more insight into microbial consortium, eight predominant species extracted from DGGE bands were sequenced and matched based on the 16S rDNA gen library results (Table 2). Band 1 and 10 are classified as *Alphaproteobacteria*. The gene sequence from band 1 showed 97% similarity to uncultured bacterium

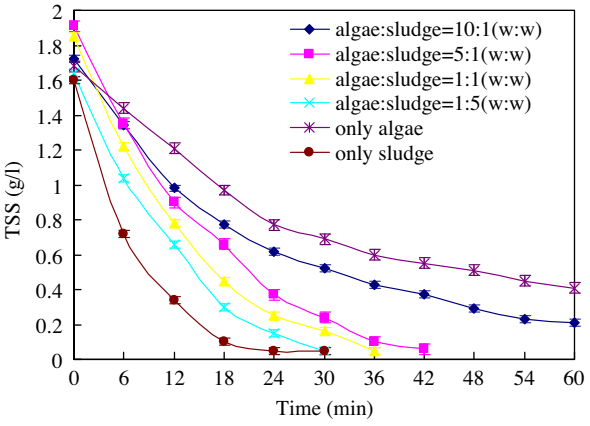


**Fig. 4.** The settleability of biomass with different algae and activated sludge inoculation ratios within 1 h.

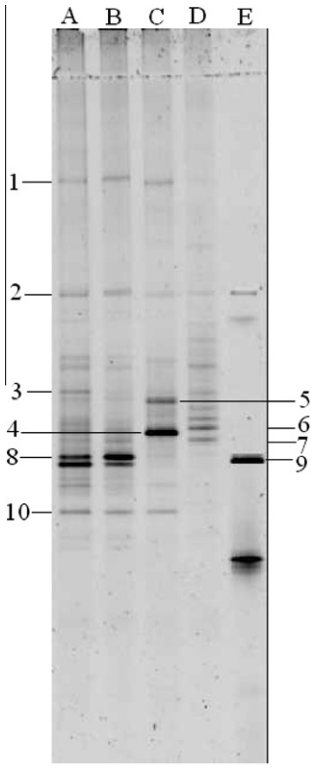


**Fig. 5.** DGGE bands of bacteria communities. Lane A: algae and sludge inoculation ratio of 1:1; Lane B: algae and sludge inoculation ratio of 5:1; Lane C: algae and sludge inoculation ratio of 1:5; Lane D: algae and sludge inoculation ratio of 10:1; Lane E: sludge inoculum. Numbers represented bands that were excised and sequenced for further analysis.

**Table 2**
DGGE 16S rDNA band identifications.

| Band | Run | | | | | Genbank accession No. | Closest relatives (%Sequence similarity[d]) | Class[c] |
|---|---|---|---|---|---|---|---|---|
| | A[a] | B | C | D | E | | | |
| 1 | •[b] | • | • | • | | JN157636 | Uncultured bacterium isolate DGGE gel band DM18 (97%) | *Alphaproteobacteria* |
| 2 | • | • | • | • | • | JN157637 | Uncultured bacterium A7 (98%) | *Gammaproteobacteria* |
| 3 | • | | | | | JN157638 | Uncultured bacterium clone TBM.13SEP-3 (96%) | Unclassified Bacteria |
| 4 | | | • | | | JN157639 | Uncultured bacterium clone F1Q32TO05GFJ9Y (96%) | *Betaproteobacteria* |
| 5 | | | • | | | JN157640 | Uncultured bacterium clone F1Q32TO05FZEFF (96%) | *Flavobacteria* |
| 8 | • | • | | | | JN157641 | *Kaistella koreensis* strain Chj707 (98%) | *Flavobacteria* |
| 9 | • | • | | | • | JN157642 | Uncultured bacterium clone BACd-6E3 (100%) | *Sphingobacteria* |
| 10 | • | • | • | | | JN157643 | Uncultured bacterium clone THPA.0912.142 (95%) | *Alphaproteobacteria* |

[a] Corresponding to A, B, C, D, E in the DGGE profile.
[b] Existence under the condition.
[c] The phylotypes were assigned to phyla based on Ribosomal Database Project II (RDP II) taxonomy classifications.
[d] Percent values represent similarities between the associated DGGE band sequence and the closest match sequence from GenBank.

DM18, which was an uncultured bacterium isolated from sludge from a submerged membrane bioreactor treating synthetic inorganic wastewater. The gene sequence from band 10 showed 95% similarity to uncultured bacterium THPA.0912.142, which was a particle-attached bacterial sample from lake. Band 5 and 8 are classified as *Flavobacteria*. The gene sequence from band 5 showed 96% similarity to uncultured bacterium F1Q32TO05FZEFF, which was isolated from suspended biomass for Guri wastewater treatment (Soondong et al., 2010). The gene sequence from band 8 showed 98% similarity to *Kaistella koreensis* Chj707, which was isolated from industrial wastewater as phenolic compounds degrading bacteria. Band 2 is classified as *Gammaproteobacteria* isolated from an aerobic activated sludge system. Band 4 is classified as *Betaproteobacteria* isolated from biomass of Guri wastewater treatment plant (Soondong et al., 2010). Band 9 is classified as *Sphingobacteria* isolated from Kobresia Meadow soil as ammonia degrading bacteria. The above results further indicated that the bacterial communities were diverse in reactors inoculated with different algae/sludge ratios, which could be an explanation of different treatment performance in these reactors. Further identification of the cultivated algal species and study the efficiency of each identified dominant microorganism may be helpful for active species selection and better understanding and optimization of the treatment system.

## 4. Conclusion

The wastewater treatment and biomass settleability were enhanced through the synergistic cooperation between wastewater-born algae and activated sludge. More than 91.2% of COD was removed regardless of algae and sludge inoculation ratios. The reactor with 5:1 (algae/sludge) culture reached the highest total nitrogen and phosphorus removal efficiency (91.0 ± 7.0% and 93.5 ± 2.5%, respectively) within the shortest time (10 days). Biomass uptake was identified as the main nitrogen and phosphorus removal mechanism. Besides, the biomass settleability was improved with proper inoculation ratio. Furthermore, different algae/bacteria inoculation ratios lead to different bacterial community compositions, which might contribute to the different treatment performance.

## Acknowledgements

The authors thank Yifeng Zhang (Technical University of Denmark, Demark) for help and advice on the molecular biotechnology work, Sabine Henschel and Kerstin Lammers for their advice on chemical water analysis (Ostfalia University of Applied Sciences, Germany). This work was supported by German-Academic Exchange Service (DAAD) and the LiWa (Future Megacities in Balance-Lima Water) project.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.11.113.

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**Table 1** Nitrogen and phosphorus balance for the cultures with the different algae/activated sludge ratios (continued from previous page)

| Algae: Activated sludge | Inlet total nitrogen (mg N/l) | Outlet total nitrogen (mg N/l) | Total nitrogen removal efficiency (%) | Inlet nitrogen oxidized in $NO_3^-$ (%) | Inlet nitrogen oxidized in $NO_2^-$ (%) | Inlet nitrogen accumulated in biomass (%) | Inlet total phosphorus (mg P/l) | Outlet total phosphorus (mg P/l) | Inlet phosphorus accumulated in biomass (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1:1 | 48.0±1.2 | 6.7±1.8 | 86.0±8.5 | 4.0±0.6 | 3.8±0.7 | 41.6±0.5 | 9.5±0.1 | 1.6±0.1 | 80.9±1.0 |
| 1:5 | 49.8±1.2 | 24.8±1.7 | 50.2±7.0 | 3.2±0.5 | 41.4±1.6 | 14.9±0.9 | 9.7±0.1 | 2.6±0.1 | 71.8±1.4 |
| Only sludge | 51.1±1.0 | 41.6±1.3 | 18.6±6.0 | 12.9±2.9 | 0 | 11.7±0.3 | 8.2±0.1 | 7.3±0.1 | 5.2±1.2 |

**Supplementary data**

# Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: influence of algae and sludge inoculation ratios

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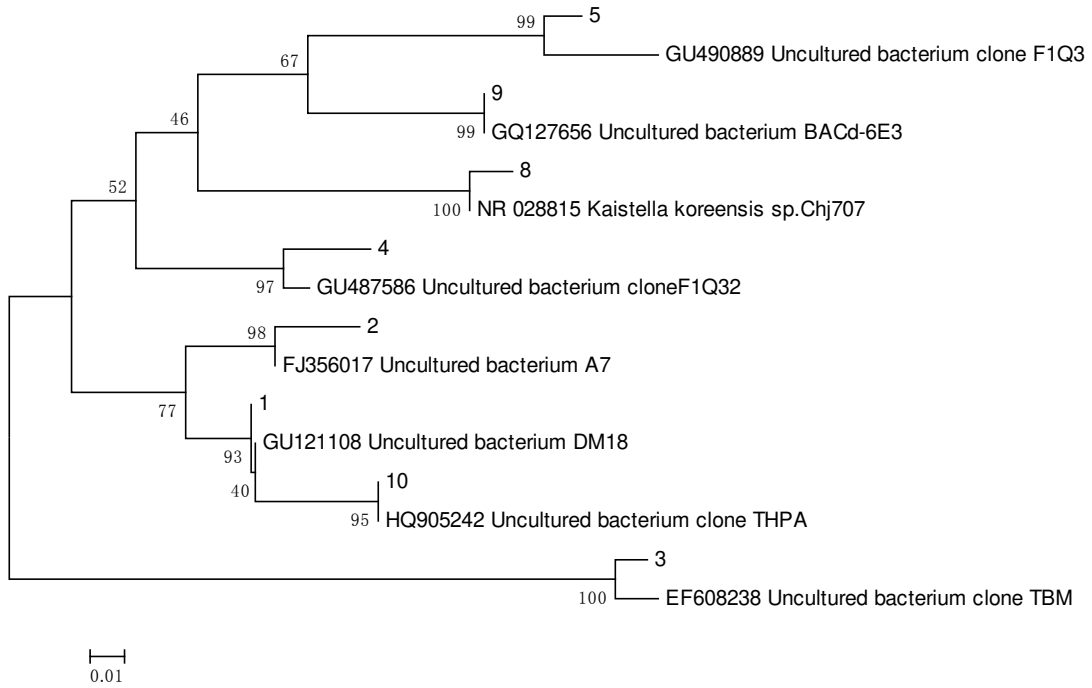


Fig.S1 The phylogenetic tree of the predominant bacterial community in algal-bacterial culture. The bacteria labeled with full scientific names were the most common affiliated species in the NCBI. The bacterial labeled with 1-10 indicate the 16S rDNA sequences obtained in this study.

# III

# Comparison of nutrient removal capacity and biomass settleability of four high-potential microalgal species

Su, Y., Mennerich, A., Urban, B.

Bioresource Technology 124, 157-162 (2012)

Bioresource Technology 124 (2012) 157–162

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Comparison of nutrient removal capacity and biomass settleability of four high-potential microalgal species

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## HIGHLIGHTS

- Comparison of four algae for nutrient removal, biomass settleability and generation.
- Algal uptake was the main N and P removal mechanism for all the four algae.
- Three green algae species were suitable for water treatment and biomass production.
- Algal settleability should be concerned in the species selection of coupling system.

## ARTICLE INFO

*Article history:*
Received 5 June 2012
Received in revised form 9 August 2012
Accepted 10 August 2012
Available online 19 August 2012

*Keywords:*
Unicellular microalgae species
Algal biomass settleability
Nutrient removal
Biomass productivity
High-potential

## ABSTRACT

Four common used microalgae species were compared in terms of settleability, nutrient removal capacity and biomass productivity. After 1 month training, except cyanobacteria *Phormidium* sp., three green microalgae species, *Chlamydomonas reinhardtii*, *Chlorella vulgaris* and *Scenedesmus rubescens*, showed good settleability. The N and P removal efficiency was all above 99% within 7, 4, 6 and 6 days for N and 4, 2, 3 and 4 days for P, resulting in the N removal rates of 3.66 ± 0.17, 6.39 ± 0.20, 4.39 ± 0.06 and 4.31 ± 0.18 mg N/l/d and P removal rates of 0.56 ± 0.07, 0.89 ± 0.05, 0.76 ± 0.09 and 0.60 ± 0.05 mg P/l/d for *Phormidium* sp., *C. reinhardtii*, *C. vulgaris* and *S. rubescens*, respectively. *Phormidium* sp. had the lowest algal biomass productivity (2.71 ± 0.7 g/m$^2$/d) and the other three green microalgae showed higher algal biomass productivity (around 6 g/m$^2$/d). Assimilation into biomass was the main removal mechanism for N and P.



## 1. Introduction

Algal-based biotechnology has been investigated for municipal wastewater treatment over the past few years (Oswald, 1988; Su et al., 2011b; Nurdogan and Oswald, 1995). Some researchers also tested the feasibility of algal–bacterial symbiotic system for the agricultural and industrial wastewater treatment (Munoz, 2005; Munoz and Guieysse, 2006). As algae could increase the dissolved oxygen concentration in the culture, assimilate nutrient and $CO_2$ through photosynthetic metabolism thus integration reducing energy requirement for mechanical aeration, wastewater purification and green gas mitigation. The by-product of this treatment process is a high yield of protein-rich algal biomass which could be further used for agricultural fertilizer, biofuels and biogass production (Logan and Ronald, 2011; Rawat et al., 2011). All of these make algal-based biotechnology attractive compared with the conventional treatment technologies.

However, two bottlenecks are still the major limitations of the exploitation of this technology. The first one is the selection of highly-effective microalgae species. Some algae species are carefully chosen in terms of nutrient removal rate and biofuel generation potential. Among them, cyanobacteria *Phormidium* sp. with a high tolerance to extreme temperatures was an efficient strain for tertiary wastewater treatment (Olguin, 2003). The previous study also showed that *Chlamydomonas* and *Chlorella* were two dominant algae strains and played important roles during long-term piggery wastewater treatment (de Godos et al., 2009; Kong et al., 2010). And *Scenedesmus* was a high-potential algae species for industrial wastewater treatment and high in lipid concentration which could be further used for biodiesel production (Termini et al., 2011; Martinez et al., 2000; Zhang et al., 2008). But most of these algae were tested separately with different bioreactor configurations and environmental conditions, and few reports focused on

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http://dx.doi.org/10.1016/j.biortech.2012.08.037

the comparison of the nutrient removal capability and biomass generation rate of these high-potential microalgae species.

The second challenge is how to harvest the algal biomass in a low-cost way (Mohn, 1988; Nurdogan and Oswald, 1995). A physical separation unit consisting of filtration or centrifugation has been applied (Mohn, 1988), but they are not economical and unsuitable for large-scale microalgae collection. The chemical flocculation followed by either sedimentation or flotation by addition chemicals such as alum, lime or calcium chloride would lead to the increase of effluent salinity and causes the secondary pollutants (Oh et al., 2001; Imase et al., 2008; Lee et al., 1998). Using immobilization system by alginate, carrageenan or chitosan could solve the biomass harvest problem (Gonzalez and Bashan, 2000; Chevalier and de la Noue, 1985), but these matrices are costly and inefficient during long-term operation (Su et al., 2009). Therefore, naturally fast settleable algae species are required. At present, the common standard for selection of algae species candidates for wastewater treatment generally focuses on the nutrient removal capacity, growth rate and lipid content in cells (de Godos et al., 2010; Zhou et al., 2011). However, good algal biomass settleability is also a crucial factor associated with the success of algal-based system for both wastewater treatment and further utilization, which need to be investigated in detailed. So far, the information about the settling capacity of these high-potential microalgae is still missing.

In this study, for the first time, four common used and high-potential microalgae species were compared in terms of settling capacity, biomass productivity and nutrient removal. This study is helpful for better understanding and controlling the wastewater treatment and value-added biomass accumulation with algal-based technology, thus resulting in overall optimization.

## 2. Methods

### 2.1. Settleable microalgae strains cultivation

One cyanobacteria strain (*Phormidium* sp.), two green microalgae strains (*Chlamydomonas reinhardtii* and *Scenedesmus rubescens*) were obtained from the Institute for Cereal Processing Ltd. (IGV, Germany) and grown on BG11, TAP and 1/2 Tamiya media respectively at room temperature. Another green microalga (*Chlorella vulgaris*) was obtained from Scandinavian Culture Collection of Algae & Protozoa (Denmark) and grown on a modified MWC media at room temperature (Zhang et al., 2011). The initial volatile suspended solids (VSS) of the four microalgae cultures were around 0.8 g/l, respectively. Five litres glass beakers were used as photobioreactor with a consistent mixing (300 rpm, VWR 984VW0CSTEUS, USA). A fluorescent lamp (Philips TL-D36w/840, Poland) was used to irradiate from the side of the reactors in a light:dark cycle of 12:12 h, to mimic natural solar day–night cycle, with 7000 lux (measured at the side of bioreactor with TES-1335 Digital light meter). In order to cultivate the settleable algal culture, the mixing procedure was stopped every 23 h for 1 h and the floating algae biomass was discarded with a screen (0.5 mm). Two litres wastewater, collected from the effluent of the second clarifier in wastewater treatment plant (WWTP) of Holthusen (Germany), was exchanged after sedimentation every 3 days to maintain a nutrient supply. The characterization of the wastewater used here was COD: 30.2 ± 2.5 (mg $O_2$/l), Total Kjeldahl Nitrogen (TKN): 26.4 ± 0.7 (mg N/l), $NH_4^+$—N: 25.2 ± 0.3 (mg/l), $PO_4^{3-}$—P: 1.74 ± 0.12 (mg/l), $NO_3^-$—N: 0.75 ± 0.06 (mg/l) and $NO_2^-$—N: 0.10± 0.06 (mg/l). After 1 month of cultivation, the turbidity of the culture during 1 h sedimentation was measured (Turbidity photometer, Dr. Lange, Type-Nr. LPG239, Germany) according to DIN EN27027 (DEV, 2002).

### 2.2. Experimental operation

After 1 month cultivation, four microalgae suspension were centrifuged (10,000 × g), washed three times with deionized water to remove the residual nutrient. The initial algae concentration of the four microalgae was 0.4 g/l (VSS). The same laboratory-scale reactor system as the cultivation process was used, and all the following tests were carried out in batch mode. Another reactor filled with 5 L wastewater without algae addition was set up for control experiments. All the experiments were carried out in duplicate.

### 2.3. Analytical methods

The temperature, dissolved oxygen (DO) and pH were measured near the midway of the reactor by using a digital multi parameter meter (Multi 3430 WTW, Germany) coupled with an $O_2$ sensor (CellOx 325, WTW, Germany) and a pH electrode (pH SenTix 940, Germany). Total Kjeldahl Nitrogen (TKN) and VSS were analyzed according to DIN EN 25663-H11 and DIN ISO 11465 (DEV, 2002). $NH_4^+$, total phosphorus and dissolved phosphorus ($PO_4^{3-}$) were determined according to DIN 38406-E5-1 and DIN EN ISO 6878-D11 (DEV, 2002) using an UV/Vis Spectrometer (Perkin Elmer, Lambda 40, USA). $NO_3^-$ and $NO_2^-$ were determined using an Ion Chromatograph (Dionex DX-100, USA) according to DIN EN ISO 10304-1 (DEV, 2002). Before analysis of the above parameters in liquid, the samples were membrane filtered (0.45 μm). To measure nitrogen or phosphorus in biomass, the samples were first divided into two identical parts. One part was homogenized, while another part was filtered to remove the solids. Nitrogen or phosphorus in biomass was calculated as the total nitrogen or phosphorus difference between the homogenized samples and the filtered samples.

## 3. Results and discussion

### 3.1. The settleability of the four microalgae strains

After 1 month cultivation and training, the microalgal biomass settleability within 1 h was investigated. As shown in Fig. 1, three green microalgae species *C. vulgaris*, *C. reinhardtii* and *S. rubescens* showed good settleability, as most of the algal biomass could settle to the bottom of the bioreactor in the first 15 min. The corresponding turbidity decreased from initial 510.8 ± 1.5, 508.3 ± 2.5 and 522.4 ± 2.5 FTU to 6.22 ± 1.5, 8.27 ± 2.5 and 6.34 ± 2.5 FTU during the first 15 min and then decreased slightly to 1.84 ± 1.5, 2.76 ± 2.5 and 4.79 ± 2.5 FTU after 1 h sedimentation for *C. vulgaris*,

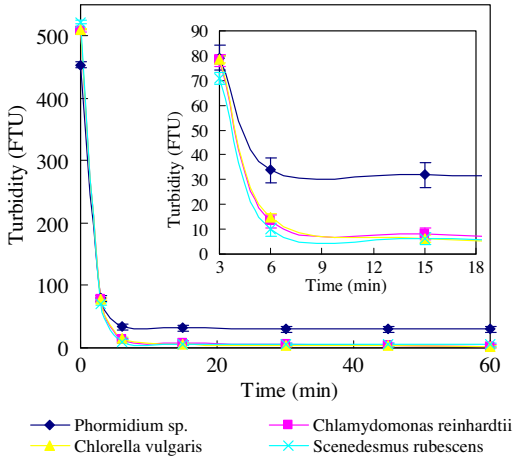


**Fig. 1.** The turbidity of the reactors with four different unicellular microalgae during 1 h sedimentation.

*C. reinhardtii* and *S. rubescens*, respectively. The cyanobacteria (*Phormidium* sp.) showed a poor settleability which dropped from initial 453.8 ± 5.0 FTU to around 31.9 ± 5.0 FTU after first 15 min sedimentation and no further reduction. This result was consistent with the previous study which reported that the settleability of the algal-based system became poor with the growth of filamentous cyanobacteria. The other three green unicellular microalgae showed good settleabilities which had good prospects for the utilization.

Only around 5% of reduction in FTU was observed before cultivation and training (data not shown). Comparatively, the settleabilities of the four algal species were greatly improved after the cultivation and training, which indicated that the special cultivation strategy (alternate mixing and non-mixing operation) used contributes to the improvement of the settleability of the four algal species. Besides, the different cell surface properties and extracellular polymeric substances (EPS) of *C. vulgaris*, *C. reinhardtii* and *S. rubescens* might contribute to the better settleability than that of *Phormidium* sp. It has been reported that the change of cell surface properties and the quantity of EPS are related with the algal settleability capacity (Lavoie and de la Noue, 1987; Gutzeit et al., 2005).

### 3.2. The variation of dissolved oxygen and pH

Changes in DO and pH of the reactors inoculated with four different unicellular microalgae are recorded in Fig. 2. The DO for the reactors inoculated with *C. vulgaris*, *C. reinhardtii* and *S. rubescens* increased to the peak value (around 16 mg/l) as soon as starting the batch test. Comparing with the above green algae species, the DO value for *Phormidium* sp. increased slower to the peak value. The possible reasons for this phenomenon may be the oxygen consumption by intensive nitrification process of the reactor inoculated with *Phormidium* sp. (Fig. 3C and D) and the relatively low oxygen production capacity of *Phormidium* sp. The DO level was an indication of algal photosynthesis activity but excessive DO concentration (>35 mg/l) could severely inhibit microalgal growth (Kumar et al., 2010). For the control test, the DO decreased from the initial 6 mg/l to around 1.5 mg/l within the first 3 days due to nitrification and then recovered to 8 mg/l which are close to the saturation. The constant mixing and possible growth of phototrophic bacteria might contribute to the recovery of DO after Day 4.

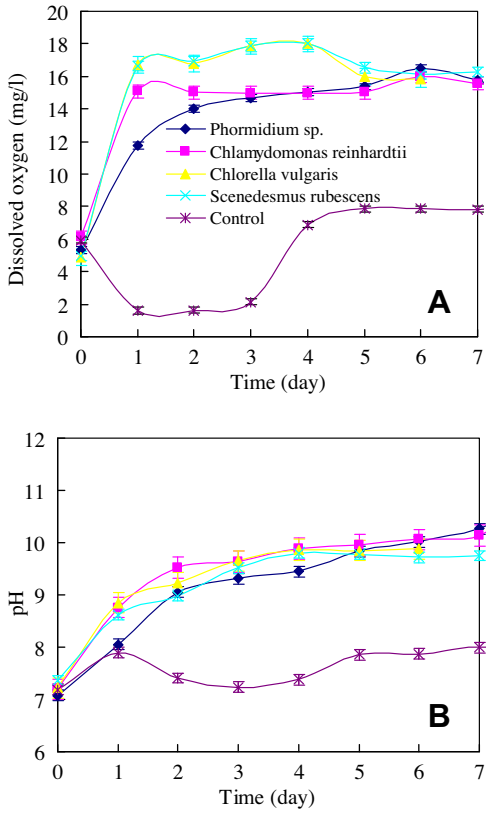


**Fig. 2.** Changes in dissolved oxygen (A) and pH (B) in four species of algae.

The pH trend of the four unicellular microalgae was quite similar, which jumped to around 9 during the first 2 days and increased gradually till 10 at the end of the test (Fig. 2B). The increase in pH may be due to the algal photosynthesis activity (Oswald, 1988). While, it is worth noting that the increase of pH for the cyanobacteria *Phormidium* sp. in the first 5 days was lower than that of the other three green algae, the intensive nitrification in the reactor with *Phormidium* sp. was the possible reason for this phenomenon (Fig. 3C and D). The culture pH was much lower and stable (below 9) when treating pretreated municipal wastewater (Su et al., 2011a) as there were more organic carbon in the pretreated municipal wastewater (Hende et al., 2011). Comparing with reactors with algae, a rather low pH level was recorded in the control test due to the intensive nitrification process (as shown in Fig. 3). The possible growth of phototrophic bacteria which led to the $CO_2$ consumption might be the possible reason for the slight raise of pH after Day 4.

Many unicellular freshwater microalgae are capable of growing at both acidic and alkaline as an adaptative response to widely fluctuating environments conditions, even though their pH optima are closer to 8 (Goldman et al., 1982). It was reported that certain algae species such as *C. vulgaris* were extremely sensitive to alkaline pH so that the biomass levels at the upper pH were lower than attained at lower pH. But *Scenedesmus obliquus* was relatively unaffected by a varying pH. While, acidic condition (<6) would have an inhibitory effect on the growth of *Scenedesmus* sp. LX1 (Li et al., 2010). The detail information on the effects of pH on the growth and nutrient removal rate of different algae species should be further investigated as it is relatively easy to control the pH in biological system.

### 3.3. The nutrient removal and biomass productivity

The nutrient removal processes of four unicellular microalgae are shown in Fig. 3. Nearly all of the $NH_4^+$—N was removed within 4, 4, 6 and 6 days and the $PO_4^{3-}$—P removal efficiency was above 98% within 4, 2, 3 and 4 days for *Phormidium* sp., *C. reinhardtii*, *C. vulgaris* and *S. rubescens*, respectively. The $NO_3^-$—N for the three green microalgae showed the similar trend which kept the initial value (around 0.8 mg/l) during the first 3, 5 and 5 days for *C. reinhardtii*, *C. vulgaris* and *S. rubescens*, respectively, but decreased immediately to below 0.05 mg/l as soon as the deletion of ammonium. For *C. reinhardtii*, the $NO_2^-$—N concentration was always below 0.25 mg/l during the first 3 days and decreased to 0.07 mg/l at the end of the batch (4 days). For the other two green microalgae, the $NO_2^-$—N increased from initial 0.06 mg/l to a peak value of around 0.61 and 1.42 mg/l within 5 days and decreased to below 0.1 mg/l at the end of the test (6 days) for *C. vulgaris* and *S. rubescens*, respectively. This phenomenon showed that microalgae prefer to utilize ammonium first when ammonium, nitrate and nitrite were all available, which was in accordance with the previous studies (Li et al., 2010; Perez-Garcia et al., 2011). However, Li et al. (2010) reported that the algal biomass concentration was significantly lower when feeding with ammonium than that with nitrate. The cyanobacteria *Phormidium* sp. showed different trend for the removal of $NO_3^-$—N and $NO_2^-$—N compared with those of the three green microalgae species (Fig. 3C and D), as nitrification pro-

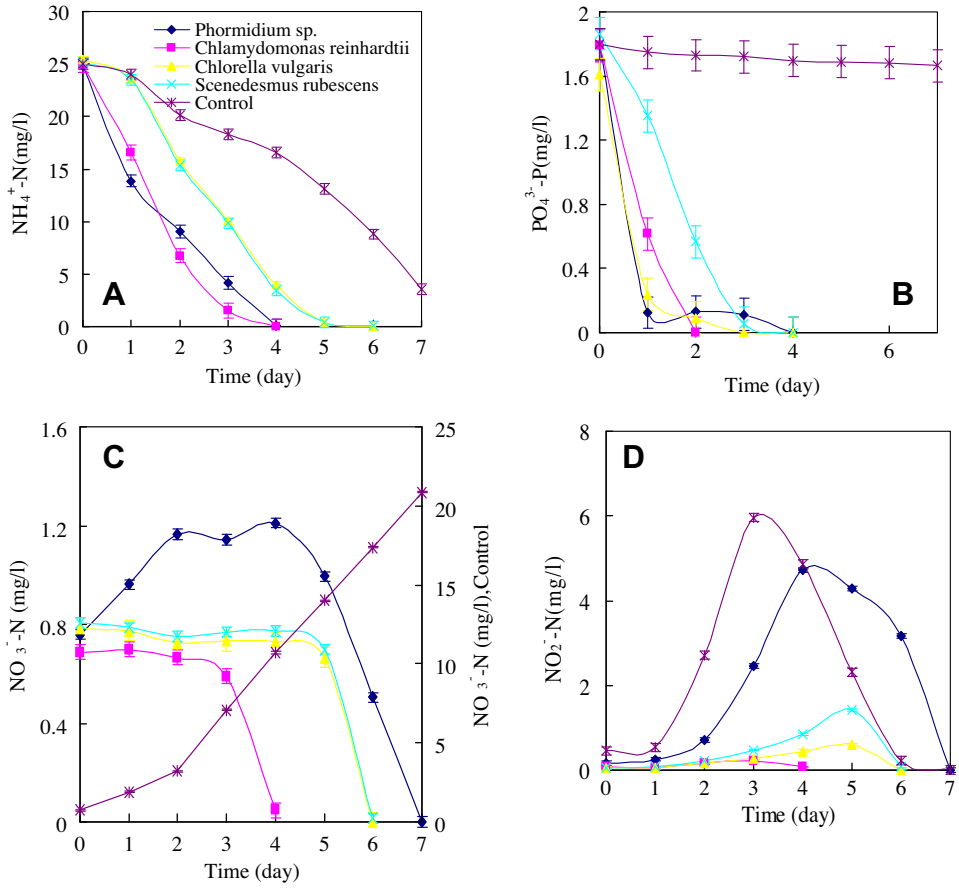


**Fig. 3.** Nutrient removal in the reactors with four different unicellular microalgae.

cess played an important role and a peak value of $NO_3^-$—N (1.21 mg/l) and $NO_2^-$—N (4.72 mg/l) was appeared at the fourth day. But both of them were assimilated by *Phormidium* sp. within the following 3 days.

Much lower phosphorus removal (7.44%) was observed in the control reactor without microalgae addition within 7 days, which indicated the high phosphorus removal potential of the four selected microalgae species. Comparing with the phosphate, ammonium was much easier to remove in the control reactor, as 85.7% of $NH_4^+$—N was removed but most of the removed $NH_4^+$—N (around 93.7%) was transferred into $NO_3^-$—N through nitrification process. Only 6.3% of the lost nitrogen in the control test might be removed through denitrification process rather than ammonia volatilization as the rather low pH value (<8) was recorded in the control test. There are two possible reasons for the weak denitrification in the control test. The low organic carbon source and the relatively high DO level for denitrification could not support intensive denitrification process. The nitrification process through nitrifiers always competed with the ammonium uptake by microalgae as $NO_3^-$—N and $NO_2^-$—N were two important byproducts during wastewater treatment by algal-based technology (de Godos et al., 2009; Su et al., 2011b; Zhang et al., 2011). From the above results, the cyanobacteria showed relatively lower capability compared with the other three green microalgae species in competition with nitrifier for ammonium utilization, as the high $NO_3^-$—N and $NO_2^-$—N concentrations were recorded during the operation in the reactor inoculated with cyanobacteria.

The algal biomass productivity, N and P removal rates of the four different unicellular microalgae species were investigated (Table 1). *S. rubescens* had the highest biomass productivity (6.56 ± 0.8 g/m$^2$/d), followed by *C. vulgaris* (6.28 ± 0.8 g/m$^2$/d) and *C. reinhardtii* (6.06 ± 1.2 g/m$^2$/d). The cyanobacteria *Phormidium* sp. had the lowest biomass productivity capacity which was only 2.71 ± 0.7 g/m$^2$/d. *C. reinhardtii* showed the best N and P removal capacity which were 6.39 ± 0.20 mg N/l/d and 0.89 ± 0.05 mg P/l/d. The N and P removal rates for *C. vulgaris* were 4.39 ± 0.06 mg N/l/d and 0.76 ± 0.09 mg P/l/d, followed by 4.31 ± 0.18 mg N/l/d and 0.60 ± 0.05 mg P/l/d for *S. rubescens*. The cyanobacteria *Phormidium* sp. still showed the lowest N and P removal rate which was only 3.66 ± 0.17 mg N/l/d and 0.56 ± 0.07 mg P/l/d.

From the above results, it was clear that the three green microalgae *C. reinhardtii*, *C. vulgaris* and *S. rubescens* were suitable for integration of wastewater treatment and algae cultivation in terms of biomass settleability, nutrient removal rate and biomass productivity. The N and P removal rates of all the four unicellular microalgae species were much higher than those of previously reported wastewater-born algae obtained from the wall of the secondary clarifier of the wastewater treatment plant (around 3.7 mg N/l/d and 0.4 mg P/l/d), but the latter had a higher algal biomass generation rates (10.9 ± 1.1 g/m$^2$/d) (Su et al., 2011a).

### 3.4. Nitrogen and phosphorus balance

Both nitrogen and phosphorus balance were investigated (Table 2). Based on the nitrogen balance, more than 92.9%, 89.4%, 90.1% and 88.8% of the removed nitrogen (25.0 ± 0.1, 24.3 ± 0.2, 24.8 ± 0.1 and 24.2 ± 0.1 mg N/l) was assimilated into algae biomass for *Phormidium* sp., *C. reinhardtii*, *C. vulgaris* and *S. rubescens*, respectively. And 6.8%, 10.2%, 10.0% and 10.5% of removed nitrogen (1.82 ± 0.87, 2.76 ± 1.0, 2.74 ± 0.47 and 2.85 ± 0.78 mg N/l) was in the form of $N_2$ or $N_2O$ through nitrification–denitrification process or lost through ammonia volatilization for above four algae species,

**Table 1**
Algal biomass productivities and nutrient removal rates of the four microalgae species.

| Algal species | Algal biomass productivity ($g/m^2/d$) | Daily removal per reactor volume (mg/l/d) | |
|---|---|---|---|
| | | N | P |
| *Phormidium* sp. | 2.71 ± 0.7 | 3.66 ± 0.17 | 0.56 ± 0.07 |
| *Chlamydomonas reinhardtii* | 6.06 ± 1.2 | 6.39 ± 0.20 | 0.89 ± 0.05 |
| *Chlorella vulgaris* | 6.28 ± 0.8 | 4.39 ± 0.06 | 0.76 ± 0.09 |
| *Scenedesmus rubescens* | 6.56 ± 0.8 | 4.31 ± 0.18 | 0.60 ± 0.05 |

**Table 2**
Nitrogen and phosphorus balance for the four unicellular microalgae.

| | | *Phormidium* sp. | *Chlamydomonas reinhardtii* | *Chlorella vulgaris* | *Scenedesmus rubescens* |
|---|---|---|---|---|---|
| Inlet N | TKN (mg/l) | 26.0 ± 0.6 | 26.4 ± 0.7 | 26.7 ± 0.3 | 26.4 ± 0.5 |
| | $NO_3^-$—N (mg/l) | 0.76 ± 0.02 | 0.69 ± 0.03 | 0.79 ± 0.04 | 0.81 ± 0.02 |
| | $NO_2^-$—N(mg/l)N | 0.16 ± 0.05 | 0.09 ± 0.02 | 0.05 ± 0.03 | 0.04 ± 0.02 |
| Outlet N | TKN (mg/l) | 0.1 ± 0.1 | –[b] | – | 0.1 ± 0.1 |
| | $NO_3^-$—N (mg/l) | – | 0.05 ± 0.03 | – | 0.02 ± 0.02 |
| | $NO_2^-$—N mg/l | – | 0.07 ± 0.02 | – | 0.08 ± 0.02 |
| | Biomass uptake (mg/l) | 25.0 ± 0.1 | 24.3 ± 0.2 | 24.8 ± 0.1 | 24.2 ± 0.1 |
| | Other[a] | 1.82 ± 0.87 | 2.76 ± 1.0 | 2.74 ± 0.47 | 2.85 ± 0.78 |
| Inlet P | $PO_4^{3-}$—P (mg/l) | 1.80 ± 0.1 | 1.79 ± 0.1 | 1.61 ± 0.1 | 1.86 ± 0.1 |
| Outlet P | $PO_4^{3-}$—P (mg/l) | – | – | – | – |
| | Biomass uptake and phosphorus precipitation (mg/l) | 1.74 ± 0.03 | 1.72 ± 0.05 | 1.56 ± 0.04 | 1.82 ± 0.03 |
| | Other | 0.06 ± 0.13 | 0.07 ± 0.15 | 0.05 ± 0.14 | 0.4 ± 0.13 |

[a] Ammonia volatilization, $N_2$ or $N_2O$.
[b] Below detection.

respectively. And nearly no nitrogen was left in the forms of $NO_3^-$—N and $NO_2^-$—N at the end of test in the above four reactors. As shown in Fig. 3, $NO_3^-$—N and $NO_2^-$—N were the byproducts during the wastewater treatment through nitrifiers but removed afterward through algal uptake and denitrification. The gas of $N_2$ or $N_2O$ was not measured in this open system, but based on the nitrogen balance, around 10% of the inlet nitrogen was lost through ammonia volatilization and transfer into $N_2$ or $N_2O$ through denitrification (Liao et al., 1995; Clauwaert et al., 2007). But the low organic carbon and rather high DO concentration could not support the denitrification process in this system. Besides, the high pH value after the first 2 days (Fig. 2) was also the evidence which indicated that the left removed nitrogen was more likely lost through ammonia volatilization (Liao et al., 1995). $N_2O$ and $N_2$ are common products in denitrification process. $N_2O$ is a strong greenhouse gas and even has been observed as main product over $N_2$ in conventional denitrification process (Zeng et al., 2003). The direct uptake of most of the nitrogen in the culture by microalgae resulted in the reduction of $N_2O$ emission in this study, thereby the conventional nitrogen reduction process ($NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$) becoming less dominant. The above results indicated that algal biomass uptake was the main nitrogen removal mechanism in these systems. This result is consistent with the previous studies in which algal uptake was the main N removal mechanism for municipal or artificial wastewater treatment (Su et al., 2011a; Zhang et al., 2011). While, for piggery wastewater treatment, nitrification-denitrification was identified as the main N removal mechanism as more inlet nitrogen was transfer into $NO_3^-$ or released into atmosphere (de Godos et al., 2009). The different nutrient composition of wastewater and different algal culture composition may be the possible reasons for the different N removal mechanisms within the systems.

The mechanism of phosphorus removal has significant relationship with the culture pH change as abiotic phosphorus precipitation would take place when pH is above 9 (Nurdogan and Oswald, 1995). From Fig. 2B, the culture pH was all below 9 in the first day for all the four algal species and this low pH trend (below 9) even lasted till the second day for *S. rubescens*. After that, culture pH of 9.05, 9.52, 9.24 were observed in the second day for *Phormidium* sp., *C. reinhardtii* and *C. vulgaris*, and 9.52 were recorded on the third day for *S. rubescens*, which could promote the abiotic phosphorus precipitation. However, it is worth mentioning that nearly 93%, 65.5%, 85.2% and 69.5% of the inlet phosphorus was already removed through algal uptake before the pH increased to 9 for *Phormidium* sp., *C. reinhardtii*, *C. vulgaris* and *S. rubescens*, respectively. At the end of the test, more than 96.7%, 96.1%, 96.9% and 97.9% of the removed $PO_4^{3-}$—P (1.74 ± 0.03, 1.72 ± 0.05, 1.56 ± 0.04 and 1.82 ± 0.03 mg P/l) were tested in the biomass and sediment which showed that they were removed through algal biomass assimilation and precipitation (Table 2) for *Phormidium* sp., *C. reinhardtii*, *C. vulgaris* and *S. rubescens*, respectively. Although it is difficult to quantify the proportion of the abiotic precipitation as the sediment is mixed with the algal biomass, the above results already indicated that the algal uptake is still the main mechanism of phosphorus removal in this study. Similar results were found in the previous studies that assimilation was the main P removal mechanism for algal–bacterial culture (Su et al., 2011a; Zhang et al., 2011).

## 4. Conclusion

Three unicellular green microalgal species, *C. reinhardtii*, *C. vulgaris* and *S. rubescens* were, one cyanobacteria, *Phormidium* sp., were trained for tertiary wastewater treatment and compared in terms of settleability capacity, nutrient removal capacity and biomass productivity. Compared with the cyanobacteria, the three green microalgae showed the better settleability, nutrient removal rate and biomass productivity. Based on the N and P balance, assimilation into algal biomass was the main removal mechanism for both N and P.

## Acknowledgements

This work was funded by German-Academic Exchange Service (DAAD), LiWa project and Europäischer Fonds für regionale

Entwicklung (EFRE). The authors wish to thank Margot Seeber, Sabine Henschel and Kerstin Lammers for their advice on chemical analysis.

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# IV

# Coupled nutrient removal and biomass production with mixed algal culture: Impact of biotic and abiotic factors

Su, Y., Mennerich, A., Urban, B.

Bioresource Technology 118, 469-476 (2012)

Bioresource Technology 118 (2012) 469–476

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

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# Coupled nutrient removal and biomass production with mixed algal culture: Impact of biotic and abiotic factors

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## HIGHLIGHTS

- Compare the performance of individual algae species and mixed algal culture.
- Cultivate a mixed algal culture consisted of three unicellular microalgae.
- Investigate the effect of illumination cycle, mixing velocity on nutrient removal.
- Monitor the effect of nutrient, algal inoculum concentration on removal performance.

## ARTICLE INFO

*Article history:*
Received 18 March 2012
Received in revised form 16 May 2012
Accepted 19 May 2012
Available online 26 May 2012

*Keywords:*
Mixed algal culture
Biotic and abiotic factors
Nutrient treatment
Algal biomass production
Biomass settleability

## ABSTRACT

The influence of biotic (algal inoculum concentration) and abiotic factors (illumination cycle, mixing velocity and nutrient strength) on the treatment efficiency, biomass generation and settleability were investigated with selected mixed algal culture. Dark condition led to poor nutrient removal efficiency. No significant difference in the N, P removal and biomass settleability between continuous and alternating illumination was observed, but a higher biomass generation capability for the continuous illumination was obtained. Different mixing velocity led to similar phosphorus removal efficiencies (above 98%) with different retention times. The reactor with 300 rpm mixing velocity had the best N removal capability. For the low strength wastewater, the N rates were 5.4 ± 0.2, 9.1 ± 0.3 and 10.8 ± 0.3 mg/l/d and P removal rates were 0.57 ± 0.03, 0.56 ± 0.03 and 0.72 ± 0.05 mg/l/d for reactors with the algal inoculum concentration of 0.2, 0.5 and 0.8 g/l, respectively. Low nutrient removal efficiency and poor biomass settleability were obtained for high strength wastewater.



## 1. Introduction

Municipal wastewater containing both organic carbon and nutrient could cause eutrophication and deterioration of aquatic ecosystems. These substances need to be removed or captured from wastewater before reuse or return to the environment. A stable and high carbon removal could be easily achieved with many conventional biotechnologies, while nutrient removal is a complex and costly process which involves several steps and technologies (Ahn, 2006; Oehmen et al., 2007; Yeoman et al., 1988). Therefore, optimizing cost effective and efficient technologies for one-step tertiary treatment of wastewater is now given high priority.

Microalgae are an important bioresource that could be further used for biofuels production (Rawat et al., 2011). They are regarded as a potential bioenergy source to face the global threats of fuel shortage. However, cost-effective algae cultivation and harvesting are two obstacles to the exploitation of this technology (Mohn, 1988; Nurdogan and Oswald, 1995). Municipal wastewater is rich in nitrogen, phosphorus and trace metal elements and thus could offer a readily available and cost-effective growth medium for microalgae. The microalgae could assimilate nutrient ($NH_4^+$, $PO_4^{3-}$, $NO_3^-$ and $NO_2^-$) in the wastewater thus integrating of wastewater purification and algal biomass production (Su et al., 2011).

Certain unicellular microalgae such as *Chlorella*, *Scenedesmus*, *Phormidium* and *Chlamydomonas* have been reported as high-potential candidates for domestic wastewater treatment and biofuel production (Gutzeit et al., 2005; Lin and Lin, 2011; Olguin, 2003; Rawat et al., 2010). However, it is difficult to maintain pure culture during the operation because of constant airborne contamination in the open system and impacts from the wastewater (Perez-Garcia et al., 2011). Furthermore, to the best of our knowledge, there are few studies on the mixture of unicellular microalgae for municipal wastewater treatment and whether mixed algal culture could

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http://dx.doi.org/10.1016/j.biortech.2012.05.093

enhance the treatment performance over an algal monoculture is uncertain. Besides algae species, algal inoculum concentration is another important biotic factor resulting in different nutrient removal rates, biomass growth rate and settleability (Guieysse et al., 2002), which should be investigated in detail for the mixed culture.

On the other hand, certain abiotic factors such as the light-dark cycle, mixing velocity and initial nutrient concentration have been described as the main physical parameters affecting algal activity that could result in differences in wastewater treatment efficiency (Gonzalez-Fernandez et al., 2011). Although some researchers have explored the effect of light intensity (Cheirsilp and Torpee, 2012), few studies focus on the illumination cycle. As sunlight in the natural environment is periodically absent, an additional artificial light might be supplied during darkness in order to get better nutrient removal efficiency within shorter retention time if the whole-day illumination is favorable for nutrient removal and algal growth. Moreover, mixing to keep the algae suspended and periodically illuminated is indispensable for both algae cultivation and wastewater treatment in algae-based systems (Azad and Borchard, 1970; Olguin, 2003). However, which mixing velocity could optimize both energy conservation and high nutrient removal efficiencies is still largely unknown. In addition, the nutrient concentration of wastewater after the secondary treatment is always below the range of medial strength domestic wastewater (Christenson and Sims, 2011). Whether this nutrient concentration is sufficient and suitable for algal growth and whether the different nutrient concentration might have an effect on nutrient removal efficiency, biomass growth and settleability are largely uncertain.

The object of the present study was to investigate the effects of biotic (algal inoculum concentration) and abiotic factors (illumination cycle, mixing velocity and nutrient strength) on nutrient removal, biomass accumulation and biomass settleability during tertiary wastewater treatment with a mixed algal culture consisting of three high-potential algal candidates.

## 2. Methods

### 2.1. Settleable microalgae cultivation

Three unicellular microalgae (*Chlamydomonas reinhardtii*, *Scenedesmus rubescens* and *Chlorella vulgaris*) which have high-potential for wastewater treatment and are high in lipid content have been selected. *C. reinhardtii* and *S. rubescens* were purchased from the Institut für Getreideverarbeitung GmbH (Germany) and grown on TAP and 1/2 Tamiya media at room temperature, respectively. *C. vulgaris* was obtained from Scandinavian Culture Collection of Algae and Protozoa (Denmark) and grown on a modified MWC media at room temperature (Zhang et al., 2011). The initial volatile suspended solids (VSS) of the three algae cultures were around 0.8 g/l, respectively. The three algae species were cultivated individually before carrying out all the tests. 5 L glass beakers were used as photo-bioreactors with a consistent mixing (300 rpm, VWR 984VW0CSTEUS, USA). A fluorescent lamp (Philips TL-D36w/840, Poland) was used to irradiate from the side of the reactors in a light: dark cycle of 12:12 h, to mimic natural solar day-night cycle, with 7000 lux (measured at the side of bioreactor with TES-1335 Digital light meter). In order to cultivate the settleable algal culture, the mixing procedure was stopped every 23 h for 1 h and the floating algae biomass was discarded with a screen (0.5 mm). Two liters of wastewater, collected from the effluent of the second clarifier in the wastewater treatment plant (WWTP) of Holthusen (Germany), was exchanged after sedimentation every 3 days to maintain a nutrient supply. The characterization of the wastewater used here was COD: 32.9 ± 3.0 (mg $O_2$/l), total kjeldahl nitrogen (TKN): 48.4 ± 4.0 (mg N/l), $NH_4^+$—N: 45.2 ± 3.8 (mg/l), $PO_4^{3-}$—P: 3.7 ± 0.6 (mg/l), $NO_3^-$ —N: 5.8 ± 1.6 (mg/l), $NO_2^-$—N: 1.8 ± 0.8 (mg/l), T: 22.3 ± 0.2 °C. After 1 month of cultivation, all the three algae species showed good settleability (within 1 h).

### 2.2. Experimental operation

After individual cultivation, three algae suspensions (*C. reinhardtii*, *S. rubescens*, *C. vulgaris*) were centrifuged (10,000×*g*), washed 3 times with deionized water to remove the residual nutrient. The mixed algal culture was obtained by mixing the three species of microalgae in ratios of 1:1:1 (dry weight). In the test to compare the nutrient removal rates with *C. reinhardtii*, *S. rubescens*, *C. vulgaris* and mixed algal culture, the algal inoculum concentrations were all 0.2 g/l (VSS). The same laboratory-scale reactor system used for the cultivation process was used for the experiments. The wastewater from the same location was used as feed for the reactors in the following experiments unless otherwise stated. The same lamp was used to irradiate the bioreactors with 7000 lux for a period of 12 h (7:00 to 19:00) per day unless otherwise stated. In the test to investigate the influence of the illumination cycle, the same lamps ran for a period of 0 h (with reactor covered with a black box to avoid natural light), 12 h (7:00 to 19:00) or 24 h per day. Constant mixing (300 rpm) was supplied unless otherwise stated. In the test to investigate the influence of mixing velocity, the same mixing devices were set at 0, 100 or 300 rpm. The mixed algae inoculum concentration was 0.2 g/l (VSS), unless otherwise stated. In the test to investigate the influence of the algal inoculum concentration, the mixed algae inoculum concentrations were 0.2, 0.5 or 0.8 g/l (VSS). In order to test the influence of nutrient strength, the above-mentioned wastewater and the same wastewater supplemented with 50 mg/l $NH_4^+$ – N and 8 mg/l $PO_4^{3-}$—P were compared. To test algal biomass settleability under different light-dark cycles, mixing velocities, nutrient strengths and algal inoculum concentrations, the turbidity of the culture after 1 h of sedimentation was measured (Turbidity photometer, Dr. Lange, Type-Nr. LPG239, Germany) according to DIN EN27027 (DEV, 2002).

### 2.3. Analytical methods

The temperature, dissolved oxygen (DO) and pH were measured at around 12 o'clock near the middle of each reactor by using a digital multi parameter meter (Multi 3430 WTW, Germany) coupled with an $O_2$ sensor (CellOx 325, WTW, Germany) and a pH electrode (pH SenTix 940, Germany). Total Kjeldahl Nitrogen (TKN) and VSS were analyzed according to DIN EN 25663-H11 and DIN ISO 11465 (DEV, 2002). $NH_4^+$, total phosphorus and dissolved phosphorus ($PO_4^{3-}$) were determined according to DIN 38406-E5-1 and DIN EN ISO 6878-D11 (DEV, 2002) using an UV/Vis Spectrometer (Perkin Elmer, Lambda 40, USA). $NO_3^-$ and $NO_2^-$ were determined using an Ion Chromatograph (Dionex DX-100, USA) according to DIN EN ISO 10304-1 (DEV, 2002). Before analysis of the above parameters in liquid, the samples were membrane filtered (0.45 μm). To measure nitrogen or phosphorus in biomass, the samples were first divided into two identical parts. One part was homogenized, while another part was filtered to remove the solids. Nitrogen or phosphorus in biomass was calculated as the total nitrogen or phosphorus difference between the homogenized samples and the filtered samples. All the experiments were performed in duplicate. Analysis of variance (ANOVA) test was employed to examine any significant difference (at 0.05 level) among the treatments.

## 3. Results and discussion

### 3.1. Comparison of individual microalgae species and mixed algal culture on nutrient removal

The nitrogen and phosphorus removal rates with *C. reinhardtii*, *S. rubescens*, *C. vulgaris* and mixed algal culture are shown in Table 1. Table 1 indicates that the nutrient removal rates by using mixed algal culture are higher than those of the three individual microalgae species, which show that the mixed algal culture could enhance the nutrient removal capacity even the improvement is not very significant according to ANOVA analysis. Some researchers presumed that mixed cultures of certain favorable microalgae candidates may improve the tolerance for environmental impacts, exploit their own advantage and optimize the system performance (Olguin, 2003). On the other hand, compared with pure cultivation, mixed cultivation is cheap and easy to operate and maintain making the wastewater treatment and algae cultivation more cost-effective and efficient. Therefore, the mixed algal culture was used in the following tests.

### 3.2. Changes in pH and dissolved oxygen under different illumination cycles

The effect of illumination cycles on the system performance was investigated. The pH and DO of cultures under different illumination conditions are shown in Fig. 1. In both reactors with continuous and alternating illumination, the DO increased significantly to the peak value as soon as starting the batch test and became stable till the end of the test. In the reactor without illumination, DO concentration remained stable during the first two days and dropped to around 0.5 mg/l afterward. This low DO level lasted for the following 5 days and then recovered to around 6 mg/l which was the same as that of the beginning (Fig. 1a). The possible reason for this low DO concentration in the reactor without illumination may be the low algal autotrophic activity under dark condition and the intensive nitrification process between day 2 and day 8 as shown in Fig. 2c and d.

A shift in pH in the above processes was also observed. Reactor with alternating illumination (12 h light per day) shifted up from around 7.9 to 9.6 (Fig. 1b). The increase in pH was found to be greater in the reactor under continuous illumination compared to that in the reactor operated with alternating illumination. The pH increase in the above two reactors (with illumination) could be due to photosynthetic inorganic carbon uptake. This was also consistent with the previous study that inorganic carbon uptake led to a high pH of 9.5 (Hende et al., 2011). Moreover, the continuous illumination caused high photosynthesis activity thus leading to a higher pH (above 10.6). In the reactor with no illumination, the pH remained stable within the range of 8–9.

### 3.3. Nutrient removal, algal biomass production and settleability under different illumination cycles

The daily concentrations of $NH_4^+$—N,$PO_4^{3-}$—P,$NO_3^-$—N *and* $NO_2^-$—N are shown in Fig. 2. In the two reactors with illumination (12 or 24 h per day), a high removal of nitrogen and phosphate was obtained after a similar detention time (Fig. 2). Ammonium removal efficiency was more than 98% for a detention time of 6 and 7 days in the reactors with continuous (24 h per day) and alternating (12 h per day) illumination, respectively. The removal of phosphate was almost 99% within 6 days for both the reactors. The above results suggest that N and P removal was not significantly enhanced by continuous illumination. The possible reason for this might be due to the outcome of long-term adaptation and natural selection. Under the continuous and alternating illumination, the removal of nitrate and nitrite was a much slower

**Table 1**
Nitrogen and phosphorus removal rates by using mixed algal culture and three individual species of unicellular microalgae.

| Algal species | Algal inoculum concentration (g/l) | Inlet $NH_4^+$—N (mg/l) | Inlet $PO_4^{3-}$—P (mg/l) | Retention time for ammonium removal (d) | Retention time for phosphorus removal (d) | Daily removal per reactor volume (mg/l/d) | |
|---|---|---|---|---|---|---|---|
| | | | | | | $NH_4^+$—N | $PO_4^{3-}$—P |
| Mixed algae culture | 0.2 | 48.9 ± 1.0 | 4.0 ± 0.2 | 9 | 7 | 5.4 ± 0.2 | 0.57 ± 0.03 |
| *Chlamydomonas reinhardtii* | 0.2 | 46.7 ± 1.0 | 4.0 ± 0.2 | 9 | 7 | 5.2 ± 0.1 | 0.55 ± 0.03 |
| *Chlorella vulgaris* | 0.2 | 44.7 ± 1.0 | 3.8 ± 0.2 | 10 | 8 | 4.5 ± 0.1 | 0.48 ± 0.02 |
| *Scenedesmus rubescens* | 0.2 | 43.1 ± 1.0 | 4.3 ± 0.2 | 10 | 9 | 4.3 ± 0.1 | 0.48 ± 0.02 |

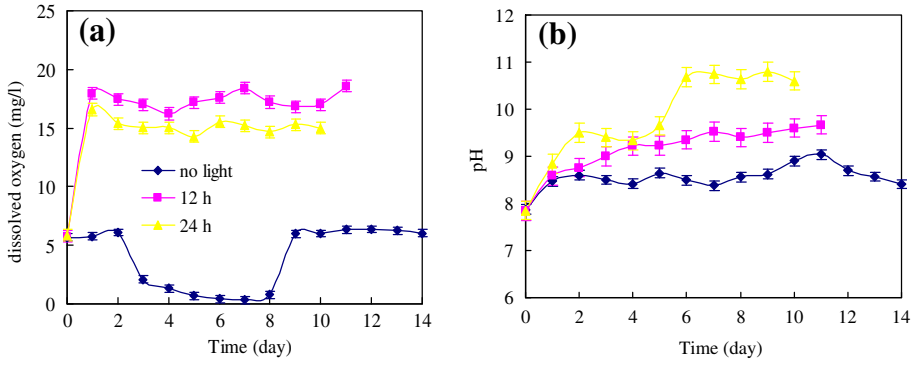


**Fig. 1.** Changes in dissolved oxygen (a) and pH (b) of the mixed algae culture under different illumination cycles.

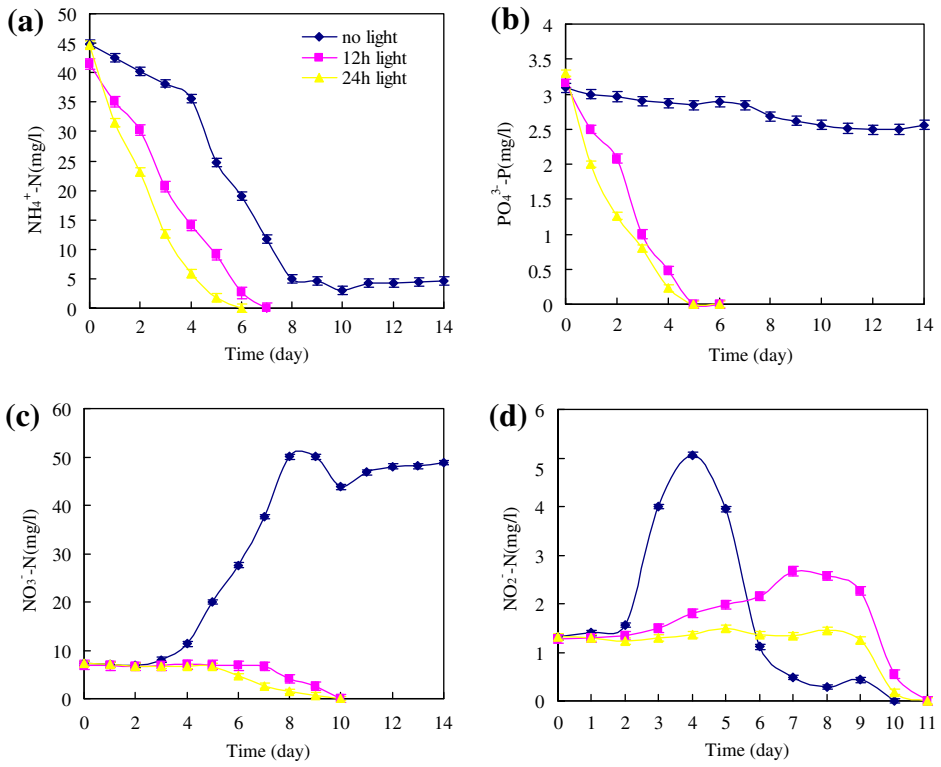


**Fig. 2.** The nutrient removal under different illumination cycles. (a) $NH_4^+$—N. (b) $PO_4^{3-}$—P. (c) $NO_3^-$—N. (d) $NO_2^-$–N.

**Table 2**
Algal biomass generation rates and turbidity.

| Influencing factor | Reactors with different conditions | Algal biomass generation rate ($g/m^2/d$) | Turbidity (FTU) after 1 h sedimentation |
|---|---|---|---|
| Light-dark cycle | no light | 0.93 ± 0.1 | 9.2 ± 2.0 |
| | 12 h illumination per day | 7.51 ± 0.2 | 4.8 ± 2.0 |
| | 24 h illumination per day | 9.38 ± 0.1 | 5.2 ± 1.8 |
| Mixing velocity | no mixing | 1.53 ± 0.2 | 6.8 ± 1.7 |
| | 100 rpm | 2.49 ± 0.1 | 6.0 ± 1.7 |
| | 300 rpm | 7.51 ± 0.2 | 4.8 ± 2.0 |
| Different algal inoculum concentrations in low nutrient concentration | 0.2 g/l | 7.51 ± 0.2 | 4.8 ± 2.0 |
| | 0.5 g/l | 2.54 ± 0.2 | 2.8 ± 1.7 |
| | 0.8 g/l | 1.46 ± 0.2 | 1.8 ± 1.6 |
| Different algal inoculumconcentrations in highnutrient concentration | 0.2 g/l | 7.27 ± 0.1 | 4.3 ± 2.0 |
| | 0.5 g/l | 2.40 ± 0.2 | 9.0 ± 1.8 |
| | 0.8 g/l | 1.44 ± 0.2 | 10.8 ± 1.6 |

process compared to that observed for ammonium or phosphate, which needed 4 to 5 days more.

In the dark condition only 17.2% of phosphate was removed by the end of the experiment (after 14 days). The ammonium removal efficiency was around 90% after 8 days and became stable till the end of the test. From the results of nitrogen removal, it was obvious that the removed ammonium was completely transferred to nitrate through nitrification process under dark condition (Fig. 2a, c and d). Some microalgal species (such as *Chlorella* and *Scenedesmus*) have the ability to switch between phototrophic and heterotrophic metabolism, depending on environmental conditions (Perez-Garcia et al., 2011). But the shortage of organic carbon source in the effluent of the secondary clarifier used here might inhibit the heterotrophic metabolism.

The mean biomass generation rates for the reactors with 0, 12 and 24 h illumination per day were 0.93 ± 0.1, 7.51 ± 0.2 and 9.38 ± 0.1 $g/m^2/d$, respectively (Table 2). The algal biomass settleability after 1 h sedimentation was also investigated. The corresponding turbidities were 9.2 ± 2.0, 4.8 ± 2.0 and 5.2 ± 1.8 FTU for the reactors with 0, 12 and 24 h illumination per day, respectively (Table 2).

The above results show that one of the key issues for algal autotrophic activity which may affect the nutrient removal is the illumination. No significant difference in the nutrient removal between alternating and continuous illumination was observed, which was consistent with the previous study (Zhang et al., 2011).

### *3.4. Changes in pH and dissolved oxygen under different mixing velocities*

The changes of DO and pH with different mixing velocities (0, 100 and 300 rpm) were determined (Fig. 3). As shown in Fig. 3, different mixing velocities had an effect on DO trends and peak value. For the reactor with 300 rpm, the DO increased from 5.8 to

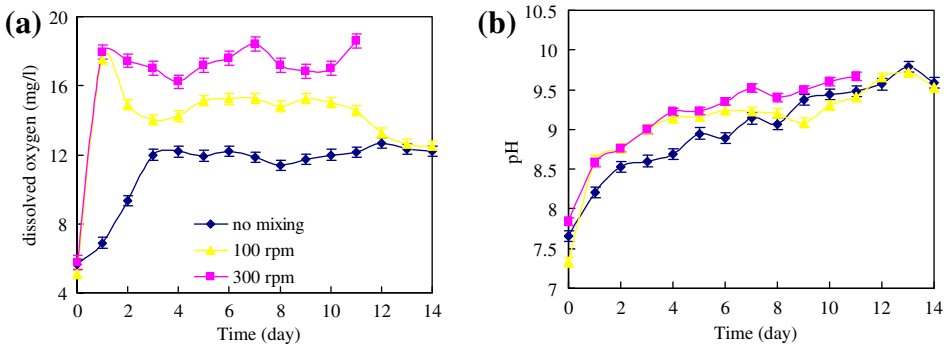


**Fig. 3.** Changes in dissolved oxygen (a) and pH (b) under different mixing velocities.

18.6 mg/l during the first day and became stable after that. For the reactor with 100 rpm, the same rise trend was observed (from 5.1 to 17.6 mg/l) for the first day. But this high DO level was only observed for one day and then it dropped to around 15 mg/l and became stable during the following 10 days. Finally, it decreased to 12 mg/l in the 11th day and kept this value till the end of the test. The possible reason for the difference in DO between 100 and 300 rpm mixing velocity after the first day may be that low mixing velocity could not supply enough periodic illumination to the algal cell thus resulted in low algal autotrophic activity and low DO. Besides, nitrifiers (autotrophic) might show high activity under 100 rpm mixing velocity thus resulted in low DO. For the reactor without mixing, the DO increased from 5.63 to 12 mg/l within 3 days and remained stable till the end of the test.

A similar pH trend which increased from 7.7 ± 0.3 to 9.6 ± 0.1 was appeared in the reactors with 0, 100 and 300 rpm mixing velocities (Fig. 3b). There are several factors influencing the culture pH, and the final pH in the above reactors is a result of a combination of factors. Algal photosynthesis could cause culture pH to rise through removing carbon dioxide from the water (Oswald, 1988). Nitrification process and ammonium consumption, the pH is acid due to $H^+$ releasing (Gonzalez et al., 2008; Li et al., 2010a). In addition, nitrate consumption by denitrification could cause an increase in pH (Perez-Garcia et al., 2011). Overall, different mixing velocities had no significant influence on pH.

### 3.5. Nutrient removal, algal biomass production and settleability under different mixing velocities

Nitrogen and phosphorus removal under different mixing velocities was also investigated. Nearly no ammonium was detected after 7, 9 and 14 days for the reactors with mixing velocity of 300, 100 and 0 rpm, respectively (Fig. 4a). The ammonium removal was probably due to algal uptake and ammonia stripping at high pH value (Munoz and Guieysse, 2006). The mean initial concentrations of $NO_3^-$—N *and* $NO_2^-$—N were 7.1 ± 0.2 and 1.2 ± 0.1 mg/l respectively for the three reactors. For the reactor with 300 rpm, the $NO_3^-$—N level was stable in the first 7 days and

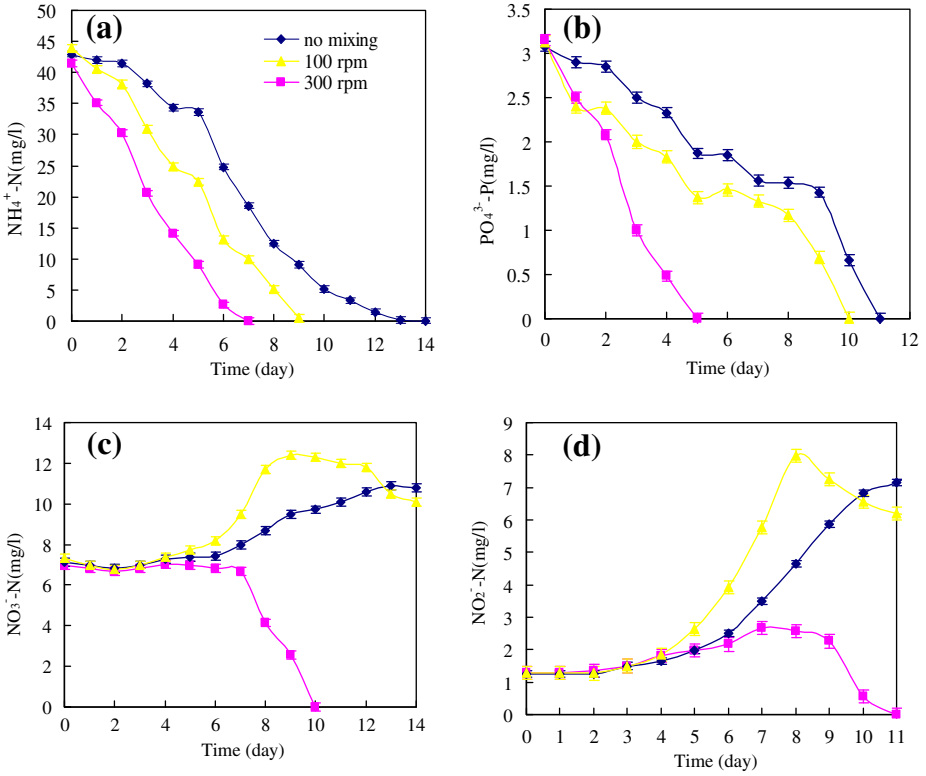


**Fig. 4.** The nutrient removal under different mixing velocities. (a) $NH_4^+$—N. (b) $PO_4^{3-}$—P. (c) $NO_3^-$—N. (d) $NO_2^-$—N.

a peak value of $NO_2^-$—N (2.7 ± 0.2 mg/l) was detected in the 7 days. But they were both removed afterwards through algal uptake or partly denitrification, resulting in total nitrogen removal efficiency above 99% at the end of the batch run (Fig. 4c and d). Obviously, microalgae prefer to use ammonium as nitrogen source when ammonium, nitrate and nitrite are all available in the medium. Similar results were reported in the literature showing that the order of using a nitrogen source by most microalgal species is, in declining order: ammonium > nitrate > nitrite > urea (Perez-Garcia et al., 2011). Around 10 ± 0.1 mg/l of $NO_3^-$—N and 5.5 ± 0.2 mg/l of $NO_2^-$—N were detected at the end of the test in the reactor with 100 rpm. Similarly, the final values of $NO_3^-$—N and $NO_2^-$—N were 10.8 ± 0.2 and 7.7 ± 0.1 mg/l respectively when no mixing was provided which shows that around 8.4% and 15.2% of inlet ammonium was converted to nitrate or nitrite through nitrification processes.

Similarly, $PO_4^{3-}$ – P decreased gradually from 3.1 ± 0.1 to 0.1 ± 0.1 mg/l with different retention times (5 days for 300 rpm, 10 days for 100 rpm and 11 days for 0 rpm). The removal efficiency of phosphate was above 98% in each case. The removal of phosphate was a faster process compared to that observed for ammonium, but the same general pattern was apparent (Fig. 2a and b, Fig. 4a and b). The opposite result has been reported in previous studies that phosphate removal process was a few days slower than that of ammonium removal (Su et al., 2012; Zhang et al., 2011). This contradiction may be due to the fact that the N/P ratio of the wastewater used in the present study was around 15:1 and much higher than that of the previous studies (around 5:1). Unlike illumination, mixing velocity had no significant influence on phosphate removal ($p > 0.05$, ANOVA analysis), but did affect retention time needed to reach the same percentage. The biomass generation rate was 1.53 ± 0.2, 2.49 ± 0.1 and 7.51 ± 0.2 g/m$^2$/d for the reactors with 0, 100 and 300 rpm of mixing velocity, respectively (Table 2). The turbidities after 1 h sedimentation were 6.8 ± 1.7, 6.0 ± 1.7 and 4.8 ± 2.0 FTU for the reactors with 0, 100 and 300 rpm of mixing velocity, respectively (Table 2).

### 3.6. Changes in pH and dissolved oxygen with different algal inoculum concentrations for low and high nutrient strength wastewater treatment

The changes of culture pH and dissolved oxygen under different algal inoculum concentrations for low and high nutrient strength wastewater treatment are shown in Fig. 5. During the first 4 days, the pH increased from around 7.8–8.8 in each of the six reactors. After that, the trends of pH between low and high nutrient strength were quite different. For the three reactors with high nutrient strength, a drop in pH (below 9) was observed in the reactors with different algal inoculum concentrations. The reactors with low nutrient strength showed a similar pH level which increased gradually to around 9.6. As mentioned in Section 3.4, the culture pH was the result of several factors. It was clear that the different nutrient strength had significant influence on the culture pH. For the low nutrient concentration, the culture pH was independent of the algal inoculum concentration. However, for the high nutrient concentration, different pH levels were observed for different algal inoculum concentrations.

At the beginning of the test, the DO level was the same as that of the pretreated wastewater. When starting the batch run, for the algal inoculum concentration of 0.2 g/l in low nutrient strength, the DO increased significantly to around 18 mg/l and became stable till the end of the experiment. For the other two reactors with 0.5 and 0.8 g/l in low nutrient strength wastewater, after the rise in DO at the beginning of the run, a gradual increase was recorded till the end of the experiment. For the reactors with 0.2 g/l algal inoculum concentration in high nutrient strength wastewater, the DO increased slightly till the 6th day, dropped in the following 3 days and became steady until the end of the experiment. For the reactors with 0.5 and 0.8 g/l in high nutrient strength wastewater, the DO increased during the first 3 days, then decreased till the 5th to 6th day and became stable. The drop of DO in the reactors with high nutrient concentration may be due to the intensive nitrification.

### 3.7. Nutrient removal, algal biomass production and settleability with different algal inoculum concentrations for low and high nutrient strength wastewater treatment

The nutrient removal performance was further tested with different algal inoculum concentrations under both low and high strength wastewater. The results are presented in Table 3. For the wastewater of low nutrient strength (around 53 mg N/l and 4 mg P/l), an above 98% nitrogen removal efficiency was achieved within 9, 5 and 4 days for the reactors with algal inoculum concentrations of 0.2, 0.5 and 0.8 g/l, respectively, leading to a daily nitrogen removal rate of 5.4 ± 0.2, 9.1 ± 0.3 and 10.8 ± 0.3 mg/l/d, respectively. It was clear that the better nitrogen removal efficiencies were obtained with all the tested algal inoculum concentrations for low nutrient wastewater treatment but the higher algal inoculum concentration led to higher nitrogen removal rates. Similarly, nearly 100% of phosphate removal efficiencies were obtained within 7, 7 and 6 days for the reactors with algal inoculum concentrations of 0.2, 0.5 and 0.8 g/l, respectively. The corresponding phosphorus removal rates were 0.57 ± 0.03, 0.56 ± 0.03 and 0.72 ± 0.05 mg/l/d, respectively. The above results indicated that the removal of phosphorus was independent of the algal inoculum concentration when treating the wastewater in the low strength wastewater. Similar P removal efficiencies and rates were achieved for the three tested algal inoculum concentrations (0.2, 0.5 and 0.8 g/l). However, the mean biomass generation rates were different (7.51 ± 0.2, 2.54 ± 0.2 and 1.46 ± 0.2 g/m$^2$/d) for the algal

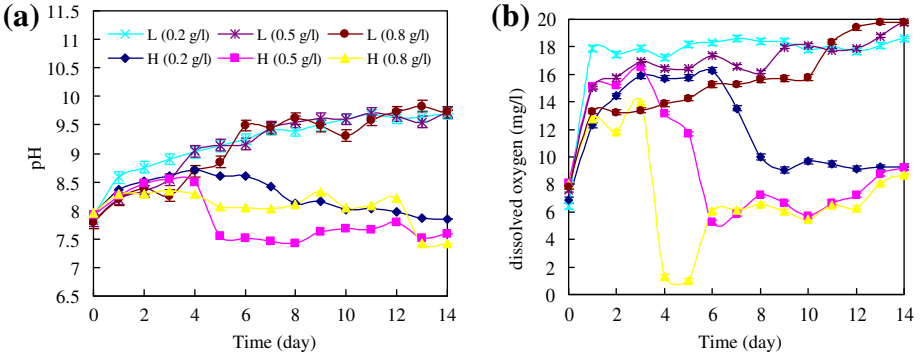


**Fig. 5.** Changes in pH (a) and dissolved oxygen (b) with algal inoculum concentrations of 0.2, 0.5 and 0.8 g/l for low (L) and high (H) nutrient strength wastewater treatment.

**Table 3**
Nitrogen and phosphorus removal performance under different algal inoculum concentrations and nutrient strength.

| Reactor | Algal inoculum concentration (g/l) | Inlet $NH_4^+$—N (mg/l) | Inlet $PO_4^{3-}$—P (mg/l) | Inlet $NO_3^+$—N and $NO_2^+$—N (mg/l) | Daily removal per reactor volume (mg/l/d) | | Retention time for ammonium removal (d) | Total nitrogen removal efficiency (%) | Retention time for phosphorus removal (d) | Total phosphorus removal efficiency (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $NH_4^+$—N | $PO_4^{3-}$—P | | | | |
| 1 | 0.2 | 48.9 ± 1.0 | 4.0 ± 0.2 | 5.2 ± 1.0 | 5.4 ± 0.2 | 0.57 ± 0.03 | 9 | 99.2 ± 0.8 | 7 | 100 |
| 2 | 0.5 | 45.7 ± 1.0 | 3.9 ± 0.2 | 7.3 ± 0.9 | 9.1 ± 0.3 | 0.56 ± 0.03 | 5 | 99.4 ± 0.6 | 7 | 100 |
| 3 | 0.8 | 43.1 ± 1.0 | 4.3 ± 0.3 | 9.7 ± 1.5 | 10.8 ± 0.3 | 0.72 ± 0.05 | 4 | 100 | 6 | 100 |
| 4 | 0.2 | 92.6 ± 1.0 | 11.5 ± 0.2 | 5.3 ± 1.5 | 4.8 ± 0.2 | 0.36 ± 0.03 | 14 | 52.8 ± 3.8 | 14 | 43.6 ± 2.8 |
| 5 | 0.5 | 91.1 ± 1.0 | 11.5 ± 0.3 | 7.6 ± 1.5 | 4.8 ± 0.1 | 0.12 ± 0.04 | 14 | 53.6 ± 3.8 | 14 | 14.3 ± 4.0 |
| 6 | 0.8 | 86.9 ± 1.0 | 11.5 ± 0.2 | 9.3 ± 1.5 | 4.4 ± 0.2 | 0.10 ± 0.03 | 14 | 41.2 ± 4.0 | 14 | 12.2 ± 3.3 |

inoculum concentrations of 0.2, 0.5 and 0.8 g/l respectively and the lowest algal inoculum concentration (0.2 g/l) led to a higher biomass generation rate which had greater potential for algal cultivation. The wastewater used here is within the normal nutrient concentration range of wastewater from the secondary treatment worldwide. The results show that the mixed algal culture could potentially be employed for combined wastewater tertiary treatment and biofuel production.

In order to investigate the effect of nutrient concentration on the removal performance, biomass accumulation and settleability, the same three algal inoculum concentrations were also tested with wastewater of high nutrient strength (around 97 mg N/l and 11.5 mg P/l). The nitrogen removal efficiency reached up to 52.8 ± 3.8%, 53.6 ± 3.8% and 41.2 ± 4.0% during the first 8 days and then became stable until the end of the test (14 days) for the reactors with algal inoculum concentrations of 0.2, 0.5 and 0.8 g/l, respectively. Similarly, only 43.6 ± 2.8%, 14.3 ± 4.0% and 12.2 ± 3.3% of phosphorus were removed for the reactors with algal inoculum concentration of 0.2, 0.5 and 0.8 g/l, respectively. The observed removal efficiencies were much lower than those for the wastewater in low nutrient wastewater. The $NH_4^+ - N$ removal rates were 4.8 ± 0.2, 4.8 ± 0.1 and 4.4 ± 0.2 mg/l/d and the phosphorus removal rates were 0.36 ± 0.03, 0.12 ± 0.04 and 0.10 ± 0.03 mg/l/d for the reactors with the algal inoculum concentration of 0.2, 0.5 and 0.8 g/l, respectively. It was noticed that the high nutrient strength wastewater led to poor nutrient removal performance. Previous studies showed that microalgae *Chlorella* sp. could well purify highly concentrated municipal wastewater (around COD: 2300 mg/l; TN: 120 mg/l and TP 212 mg/l) within 15 days (Li et al., 2011; Zhou et al., 2011). The possible reason for this may be due to the different nutrient composition of the wastewater (high COD concentration in the previous study) and algal domestication. In addition, the biomass generation rates were 7.27 ± 0.1, 2.40 ± 0.2 and 1.44 ± 0.2 g/m$^2$/d for the reactors with algal inoculum concentration of 0.2, 0.5 and 0.8 g/l, which was similar with that of low nutrient concentration.

Obviously, for both low and high strength wastewater, the reactor with the same algal inoculum concentration showed the similar biomass generation capacities (Table 2), indicating that there was no strong relationship between nutrient strength in wastewater and algal growth rate. In addition, lower algal inoculum concentration led to higher biomass accumulation rate. There were two possible reasons for this phenomenon. First, the available nutrient per algal cell was high at low algal inoculum concentration which would promote the algal growth. Second, higher algal inoculum concentration may lead to mutual shading within the algal population and lower the algal growth (Guieysse et al., 2002). The similar results were also found in previous studies (Yuan et al., 2011), which showed that initial algal inoculation concentration was the key factor for the further biomass accumulation.

At the end of the test, the settleabilities of the algal biomass with different algal inoculum concentrations for treating low and high strength of secondary wastewater were investigated (Table 2). Obviously, the culture with all the algal inoculum concentrations for low strength wastewater treatment showed the similar and good settleability after 1 h sedimentation (0.2 g/l: 4.8 ± 2.0 FTU; 0.5 g/l: 2.8 ± 1.7 FTU and 0.8 g/l: 1.8 ± 1.6 FTU). The algal biomass from the reactor with 0.2 g/l algal inoculum concentration for high strength wastewater treatment also showed a good settleability, which was around 4.3 FTU after 1 h sedimentation. The culture with 0.5 and 0.8 g/l initial algal concentrations for high strength wastewater treatment had the poor settleability (9.0 ± 1.8 and 10.8 ± 1.6 FTU, respectively). The above results indicate that the biomass settleability would decrease with the increase of the algal inoculum concentration under high strength wastewater, and there was no strong relationship between the initial biomass concentration and biomass settleability when treating low strength wastewater. The relatively higher availability of nitrogen and phosphorus in high nutrient strength wastewater, together with the higher algal inoculum concentration could lead to different algal cell composition and lipid content (Li et al., 2010b), which might result in the lower settleability.

### 3.8. Nitrogen and phosphorus balance under different conditions

Certain conditions with high nutrient removal efficiencies which were worthy to be used were further investigated for the nitrogen and phosphorus balance. From Table 4, biomass uptake

**Table 4**
Nitrogen and phosphorus balance under different conditions.

| Reactor | Mixing velocity (rpm) | Algal inoculum concentration (mg/l) | Illumination (h/day) | Inlet TN (mg N/l) | Outlet TN (mg N/l) | Inlet N oxidized in $NO_3^-$ and $NO_2^-$ (%) | Inlet N accumulated in biomass (%) | Others[a] (%) | Inlet P (mg P/l) | Outlet P (mg P/l) | Inlet P accumulated in biomass and precipitant (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 100 | 0.2 | 12 | 55.6 ± 1.0 | 17.1 ± 1.0 | 28.1 ± 1.2 | 61.8 ± 1.5 | 10.1 ± 2.7 | 3.1 ± 0.1 | 0 | 96.1 ± 2.0 |
| 2 | 300 | 0.2 | 24 | 56.2 ± 1.3 | 0 | 0 | 92.4 ± 1.2 | 7.6 ± 1.2 | 3.3 ± 0.1 | 0 | 97.2 ± 2.0 |
| 3 | 300 | 0.2 | 12 | 57.1 ± 2.0 | 0 | 0 | 91.8 ± 1.3 | 8.2 ± 1.3 | 4.0 ± 0.2 | 0 | 97.9 ± 2.0 |
| 4 | 300 | 0.5 | 12 | 56.0 ± 1.9 | 0 | 0 | 90.9 ± 1.4 | 9.1 ± 1.4 | 3.9 ± 0.2 | 0 | 96.8 ± 2.0 |
| 5 | 300 | 0.8 | 12 | 55.8 ± 2.5 | 0 | 0 | 90.3 ± 1.5 | 9.7 ± 1.5 | 4.3 ± 0.3 | 0 | 98.7 ± 2.0 |

[a] Ammonia stripping and denitrification.

was the main N removal mechanism which accounted for more than 61.8% of inlet N for reactors 1 and 90% of the inlet N for the other reactors. Ammonia stripping usually occurring at high pH is also depended on temperature of air and wastewater (Liao et al., 1995). And it was slight in this system as most of the ammonium was already removed before that pH was above 9.5. Besides, denitrification process did not play an important role as the high DO and low organic carbon concentration would not promote the intensive denitrification. For the reactor 1, 28.1 ± 1.2% of inlet nitrogen was converted to $NO_3^-$ and $NO_2^-$ through nitrification. Based on the P mass balance, uptake through algae and phosphorus precipitant were the main P removal mechanisms in all the tested reactors which accounted for more than 96%, and P precipitant would only take place at high pH (9–11). Before the increase of pH (above 9), more than 61% of inlet P was already removed through algal uptake in the reactor of 2 and 3. For reactor of 1, 4 and 5, around 36%, 53.2% and 50.0% of inlet P was removed before that pH was above 9.

## 4. Conclusion

A mixed algal culture, constituted of three high-potential unicellular microalgae, was investigated in terms of nutrient removal, biomass production and settleability. This study showed that the mixed culture of certain favorable microalgae candidates may enhance the nutrient removal from wastewater. Illumination cycle and mixing velocity can influence the nutrient removal and biomass generation. 98% and 99% of total nitrogen and phosphate removal and a good settleability were obtained for the algal inoculum concentration of 0.2, 0.5 and 0.8 g/l for low strength wastewater.

## Acknowledgements

The authors thank Margot Seeber, Sabine Henschel and Kerstin Lammers for advice on chemical water analysis work, Ingeborg Joost and Pamela Holweg (Ostfalia University of Applied Sciences, Germany) for their kind assistance. This work was supported by German Academic Exchange Service (DAAD), LiWa project and Europäischer Fonds für regionale Entwicklung (EFRE).

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