Microalgae cultivation for nutrient recovery from human urine

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For my beloved family

Abstract

Nitrogen (N) and phosphorus (P) are key nutrients for all living organisms. At the same time, discharge of wastewaters containing these elements on surface waters has a strong negative impact on the environment. Urine is the major nutrient source in domestic wastewater and these nutrients cover 24% of the N, 20% the P and 32% of the potassium (K) in artificial fertilizers (year 2010). Separation of urine from other household wastewater streams and using it for nutrient recovery therefore is considered much more efficient and less energy consuming than treatment of large volumes of highly diluted domestic wastewater. This thesis aimed to apply microalgae cultivation as a tool to recover the nutrients from human urine. Because of the high N:P ratio of microalgae biomass, it was expected that all of the P and a significant amount of the N could be simultaneously recovered from source-separated urine.

At first, growth of a pre-selected microalga, *Chlorella sorokiniana*, on urine was tested in batch at different degrees of urine hydrolysis in order to assess possible limitations for microalgae growth. Hydrolysis of urea in urine resulted in a changing in urine composition and a higher pH, and stimulated precipitation of P, magnesium (Mg) and some trace elements, reducing their availability for the microalgae. Supplementation of these compounds therefore was required to enhance microalgae growth. Next, *C.sorokiniana* was cultivated in a short light-path photobioreactor under continuous illumination at a high light intensity and at various hydraulic retention times (HRTs) to optimize biomass productivity, biomass yield on light and nutrient uptake from both synthetic and human urine. The highest biomass productivity and nutrient recoveries respectively were 1.1 g-dw L^{-1} h^{-1}, 123 mg-N L^{-1} h^{-1} and 13 mg-P L^{-1} h^{-1} in synthetic urine, and 0.8 g-dw L^{-1} h^{-1}, 82 mg-N L^{-1} h^{-1} and 8 mg-P L^{-1} h^{-1} in human urine. The highest biomass yield on light in synthetic and human urine respectively were 0.98 and 0.74 g-dw mol-photons^{-1} showing high photosynthetic efficiency even at a high irradiance level of 1500 µmol-photons m^{-2} s^{-1} at the photobioreactor surface. With human urine free ammonia toxicity negatively affected system performances.

Experiments under outdoor conditions with simulated day/night cycles, showed that microalgae specific growth rate, biomass productivity and nutrient uptake rates were exerted during the day period in a short HRT reactor. At night, growth and nutrient removal could not be demonstrated. Hence, the microalgae reactor should be operated at short HRTs during the

day period and at long HRTs or in batch during the night. Model calculation showed that for Dutch conditions system optimization may be possible by increasing the ratio between illuminated surface and photobioreactor volume and by applying urine dilution. In Dutch summer conditions with system optimization, <1 m^2 of ground surface is needed for a photobioreactor treating the urine of a single person. However, the combination of microalgae production and treatment of concentrated urine probably is restricted to regions where light availability and temperature are higher than in The Netherlands.

Keywords: microalgae cultivation, source-separated urine, nutrient recovery, short light-path photobioreactors, biomass productivity, dilution rate, day/night cycle

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Introduction and thesis outline

1.1 Introduction

The growing world population increases the global food consumption and hence, fertilizer need for crop production. Nitrogen and phosphorus are, among the most abundant elements on earth, essential for living organisms and are major elements needed for crop production. The following paragraphs briefly describe the importance of these two essential elements for living organisms, the supply of these elements and the impacts on the environment.

1.1.1 Nitrogen

Nitrogen is abundantly present as dinitrogen gas in the earth's atmosphere. It plays an essential role in living organisms as a building block of amino and nucleic acids. However, nitrogen is only available to primary organisms when atmospheric nitrogen is converted into ammonia nitrogen (Bernhard, 2012) i.e. by biological nitrogen fixation or industrial processes for production of nitrogen fertilizers.

Nitrogen for organisms was formerly supplied by a nitrogen pool that was fixed biologically. Since the first nitrogen manufacturing plant was completed in 1914, nitrogen fertilizer produced via the Haber-Bosh process is lowered in its price and increasingly used in agriculture (Frink et al., 1999). Industrial nitrogen fixation and other human activities have influenced the nitrogen cycle, and herewith the environment, in many ways (Vitousek et al., 1997; Galloway et al., 2008). Nitrogen from the human metabolism only accounts for a trivial amount in the global nitrogen cycle but is essential in its contribution to water pollution (Larsen et al., 2007). Reducing artificial fertilizer use by recovery of nitrogen from human waste and recycling to agricultural fields can reduce nitrogen losses to the environment and as a consequence, reduce impacts to the environment.

1.1.2 Phosphorus

Phosphorus is essential for all forms of life on earth. It plays a major role in biological molecules, i.e. DNA and RNA, cell membranes, and in transporting cellular energy as adenosine triphosphate (ATP) in living cells (Childers et al., 2011). It is also found in bones and teeth enamel, nervous tissue, and cell protoplasm. In the past, bone meal, animal manure

and human excreta have been mainly used to supply phosphate in agriculture. Increasing world population has increased the need of phosphorus supply and artificial fertilizers nowadays are the major source for production of phosphorus-containing compounds (Neset and Cordell, 2012). Growing use of fertilizers affects surface water bodies by eutrophication due to water run-off from agriculture fields and discharge of nutrients from wastewater treatment plants (Oliveira and Machado, 2013).

The major global phosphorus supply comes from mined phosphate rock. However, in contrast to nitrogen, phosphorus reserves are not infinite resources. On-going studies are aiming at finding measures to sustain these reserves in order to ensure food security. In the meantime, surface water bodies need to be protected from accumulation of excess nutrients. Recovery and reuse of phosphorus from organic municipal and industrial waste, animal manure, and source-separated human excreta are, among several options, sustainable measures to guarantee a sufficient phosphorus supply in the future (Neset and Cordell, 2012; Cordell and White, 2013). In addition, in this way the discharge of nutrients onto surface waters, or other sinks e.g. landfills (Schröder et al., 2010) is reduced.

1.2 New sanitation concepts

1.2.1 Application of new sanitation concepts

Wastewater is considered more and more as a resource for energy, nutrients and water, and for the production of several kinds of bio-products. Recovery of energy and nutrients from diluted streams like collected within conventional sanitation concepts, is more energy intensive as compared to concentrated streams (Zeeman and Lettinga, 1999). Since the end of 20th century, source-separation based, 'new sanitation' concepts have been a subject of several studies (Larsen and Gujer, 1997; Otterpohl et al., 1997; Zeeman et al., 2008). In general, domestic wastewater is a mixture of different waste streams with different compositions and potential for resource recovery. They originate from diverse sources including the toilet, laundry, washbasin, bathroom, kitchen and often rainwater. Kujawa-Roeleveld and Zeeman (2006) reported the amount of the different compounds daily excreted by one individual in separate streams of feces, urine, grey water, and kitchen waste**.** It is clear that toilet wastewater, often referred to as black water, contains large amounts of organics and nutrients, but also pathogens, pharmaceutical residues and hormones. The major fraction

of nutrients (nitrogen, phosphorus, potassium) however is present in the urine fraction of the black water.

New sanitation concepts allow for the recovery of energy and nutrients from black water and/or urine. Separate treatment of concentrated black water using UASB (Upflow Anaerobic Sludge Blanket) technology produces energy as methane gas. Approximately 40% of the phosphorus is present in the sludge of this reactor and the liquid effluent contains the major fraction of the nitrogen and the remaining 60% of the phosphorus (de Graaff et al., 2010). In addition, grey water, the less polluted stream, can be treated to produce a relatively clean water stream, which after treatment can be reused for irrigation within the house hold (Abu Ghunmi et al., 2010; Hernández Leal et al., 2011).

The practical application and feasibility of these new sanitation concepts have been demonstrated in several projects. In the Netherlands, these projects are for example, 32 apartments at Lemmerweg-Oost in Sneek, a housing complex of 250 houses at Noorderhoek in Sneek, an office building of 160 employees in Wageningen and the office building Villa Flora in Venlo. Also in other countries projects have been conducted, for example a vacuum-biogas system for black water and a constructed wetland for grey water for 350 inhabitants of 'Flintenbreite' in Lübeck (Germany), a two-chamber composting system for brown water (toilet water without urine) in the rural water-mill museum 'Lambertsmúhle' in Cologne (Germany) and the Erdos Eco-town project in Dongsheng (China) (Otterpohl, 2000; Langergraber and Muellegger, 2005; Lixia et al., 2008).

1.2.2 Black water

Black water is wastewater generated from toilets which is a combined stream of feces, urine, and flush water. Its composition generally consists of organic compounds, organic and inorganic nutrients (nitrogen, phosphorus, potassium), and trace amounts of heavy metals and pharmaceuticals. Pollutant concentrations within black water depend upon toilet type, which influences the amount of flush water and, as such, the dilution of the black water. Table 1.1 shows typical concentrations of pollutants in black water collected via different toilet types.

Table 1.1 Comparison of black water concentration from different studies and different toilet types

Parameter	van Voorthuizen et al. (2008)[/1]	de Graaff et al. (2010)	Murat Hocaoglu et al. (2010)
Toilet type	Low flush toilet	Vacuum toilet	Conventional toilet
Flush volume (L flush^{-1})	5	1	~9
Sample preparation	n.r.	Filtered 4-5 mm	Filtered 3&6 mm
COD_{total} (g-O_2 L^{-1})	1.139	9.8±2.6	1.225±0.11
$COD_{suspended}$ (g-O_2 L^{-1})	0.391	5.1±2.7	n.r.
$COD_{soluble}$ (g-O_2 L^{-1})	0.530	3.4±0.47	0.407±0.12
$COD_{colloidal}$ (g-O_2 L^{-1})	0.215	1.3±0.42	n.r.
N_{total} (g-N L^{-1})	0.169	1.9±0.19	0.180±0.098[/2]
NH_4-N (g-N L^{-1})	0.138	1.4±0.15	0.147±0.089
P_{total} (g-P L^{-1})	0.121	0.22±0.067	0.025±0.076
PO_4-P (g-P L^{-1})	0.027	0.079±0.0085	n.r.

[/1] standard deviation not reported

[/2] reported number as TKN

n.r. = not reported

1.2.3 Source-separated urine

Urine is a sterile liquid with a transparent-amber color. Generated by the kidneys, urine contains a variety of water-soluble compounds which are eliminated from the human bloodstream. Urine contains high concentrations of urea, inorganic salts, creatinine, organic acids as well as toxins and pigments that give its color (Bouatra et al., 2013). Organic compounds (expressed as Chemical Oxygen Demand; COD) in urine can be as high as 10 g-O_2 L^{-1} (Udert et al., 2006) and are mainly aliphatic compounds (alcohols, X-CH_3, alkenes, alkines) and creatinine/creatine (Kuntke, 2013). A detailed overview of identified compounds in urine is reported in Bouatra et al. (2013). With a high concentration of aliphatic compounds, most of the COD in urine is easily biodegradable (Kuntke, 2013). Within this thesis the major focus is towards the nutrients, nitrogen and phosphorus in urine, and not as much towards the organic compounds and salts. Table 1.2 shows concentrations of several

compounds of interest in human urine as used in this thesis in comparison to values reported in literatures.

Table 1.2 Nutrient and COD concentrations (in g L^{-1}) in urine as used in this thesis and published values

Parameter	This thesis [/1]	Kuntke (2013)[/2]	Maurer et al. (2006)[/3]
Collection system	Directly & urine diverting toilet	Directly	Several toilet types
Dilution factor (times)	Not diluted	Not diluted	0.26-1
COD	2.5-8.3	9	1.6-10
TN	2.3-7.1	8.6	1.8-9.2
NH_4^+-N/NH_3-N [/4]	0.7-4.6	0.4	0.5-8.1
TP	n.r.	0.7	0.1-2
PO_4^{3-}-P	0.2-0.5	0.6	n.r.
K^+	1.1-1.7	1.9	0.8-3.3
Mg^{2+}	1.4×10^{-4}-0.025	0.08	1.5×10^{-3}-0.12

[/1] more detailed data reported in Chapter 2 and 3, concentration ranges were reported for different batches of urine.

[/2] data obtained from 106 urine samples.

[/3] reviewed ranges of compounds in urine from different collection systems from several sources.

[/4] low NH_4^+-N/NH_3-N values represent fresh urine data.

n.r. = not reported

The major nitrogen form in fresh urine is urea ($CO(NH_2)_2$), a waste product from the amino acid metabolism. With the bacterial enzyme 'urease', urea is readily hydrolyzed or broken down to ammonia and bicarbonate (Udert et al., 2003) according to:

$$CO(NH_2)_2 + 2H_2O \rightarrow NH_3 + NH_4^+ + HCO_3^- \quad (1).$$

This process is called 'ureolysis' and increases the pH. Phosphate is the major phosphorus form and accounts for 93-100% of the total amount of phosphorus in urine (Udert et al., 2006; Kuntke, 2013). At an increased pH due to 'ureolysis' precipitation of inorganic salts

like struvite ($MgNH_4PO_4 \cdot 6H_2O$), hydroxyapatite; HAP ($Ca_5(PO_4)_3(OH)$) and calcite ($CaCO_3$) (Udert et al., 2003) occurs. This lowers the availability of some inorganic nutrients such as phosphate and magnesium.

Urine volume accounts for only 1% of the total household wastewater volume while it contains the major source of nutrients in domestic wastewater: 69% nitrogen (N), 40% phosphorus (P), and 60% potassium (K) (Kujawa-Roeleveld and Zeeman, 2006). Hence, urine is an extremely interesting stream for nutrient recovery and it should not be diluted with grey water, toilet flushing water or rain water. In practice, urine source separation and/or recovery has been established in several locations, for example an office building of 60 employees (Sneek, the Netherlands), the Saniphos installation (Zutphen, the Netherlands) recovering ammonium sulphate and struvite from collected 'festival urine', the head offices of the German technical co-operation GTZ (Eschborn, Germany), Kullön residential area (Vaxholm, Sweden), Universeum-science and Discovery Museum (Gothenburg, Sweden), and the EAWAG research institute (Zurich, Switzerland). In Sweden more than 135,000 urine diverting toilet units have already been installed since the mid-1990s (Kvarnström et al., 2006; Lienert and Larsen, 2009).

1.2.4 Nutrient recovery from human excreta

Table 1.3 shows calculated global nutrient loads annually excreted via urine and feces and potential coverage (%) of the global artificial nutrient fertilizer consumption. Calculated from the average amount of nutrients excreted from one individual (Kujawa-Roeleveld and Zeeman, 2006) and the world population in 2010, urine based nutrients would account for 20-30% of world's N, P, and K artificial fertilizer consumption. Nutrients from feces would account for 5-8% of world N, P, and K fertilizer consumption. Obviously, if these nutrients are recycled to agriculture, the artificial fertilizer production could be reduced considerably.

Table 1.3 World fertilizer consumption in 2010 and nutrient production from human excreta from world population in 2010 (unit: Mt),

	Nitrogen	Phosphorus	Potassium
World fertilizer consumption*	104.7	17.7	23.0
World nutrient excretion via human urine	25.0 (23.8)	3.5 (19.8)	7.4 (32.0)
World nutrient excretion via human feces	5.6 (5.4)	1.4 (7.8)	1.9 (8.1)

* (International Fertilizer Industry Association, 2014)

Number in brackets represents % of world fertilizer consumption

Anaerobic treatment is proposed as the proper technology for treatment of black water because this allows for energy recovery in the form of methane (Zeeman et al., 2008). Anaerobic sludge produced during anaerobic treatment of black water can be used as a fertilizer. In addition, this sludge contains less heavy metals than sewage sludge that is produced during treatment of municipal wastewater (Tervahauta et al., 2014). Nutrients in the digester effluent can be precipitated as struvite which can be used as fertilizer. As such, globally 10% of the world artificial phosphorus fertilizer production could be substituted by struvite produced from anaerobically treated black water (de Graaff et al., 2011).

Likewise, source-separated urine has a potential use as nitrogen- and phosphorus-rich liquid fertilizer for crops/plants (Table 2) and already is applied in some areas such as in China and Sweden (Kvarnström et al., 2006). Application of urine as a fertilizer is feasible given that appropriate measures are taken with respect to pathogens (in case urine is contaminated with feces during collection), ammonia volatilization, storage, transportation and distribution costs (Höglund, 2001). In addition, in some countries regulatory restrictions exist for applying urine on land (Lienert and Larsen, 2009).

Maurer et al. (2006) reviewed and discussed techniques for removing and recovery of nitrogen and phosphorous from source-separated urine. Of these, only struvite precipitation and ammonia stripping have been applied at full scale. Struvite precipitation is usually applied for simultaneous recovery of phosphorus and part of the nitrogen. However, the fraction of nitrogen that can be recovered is very small (Zeeman and Kujawa-Roeleveld, 2011) because the N:P molar ratio of struvite is 1:1, whereas in urine this ratio can be higher than 25:1. Ammonia stripping is applied for nitrogen recovery but requires a high ammonia concentration for an energy efficient performance (Maurer et al., 2003) and the phosphorus is

not recovered. For all the techniques reviewed by Maurer et al. (2006), the high energy consumption is the major constraint for full-scale application. Recently, Kuntke et al. (2012) proposed a new bio-electrochemical method based on a microbial fuel cell for energy production combined with nitrogen recovery from urine. With this technique, organic compounds in urine are oxidized at an anode compartment while ammonium passes a membrane to a cathode compartment where it can be stripped at the prevailing high pH and is recovered as an ammonium salt. A surplus energy of 3.46 kJ $g\text{-}N^{-1}$ and an ammonium recovery rate of 3.29 g-N d^{-1} m^{-2}-membrane surface area were obtained (Kuntke et al., 2012).

1.3 Microalgae and wastewater treatment

1.3.1 Coupling microalgae biomass production with wastewater treatment

Microalgae are photoautotrophic microorganisms that take up and accumulate nutrients using light as an energy source and carbon dioxide as an inorganic carbon source. Microalgae biomass is a potential source for biofuels, biochemicals, and bio-fertilizer. One particular application, i.e. the cultivation of microalgae to produce biofuels is considered promising, but at the moment not economically feasible and sustainable (Clarens et al., 2010; Wijffels and Barbosa, 2010). Among the major aspects determining the feasibility of large-scale microalgae cultivation, the availability of nutrients, especially nitrogen and phosphorus is of importance. The use of nutrient-rich wastewaters as cultivation media has been considered an option for environmentally and economically viable large-scale biofuel production from microalgae (Yang et al., 2011). Microalgae, *Chlorella* species for example, consist of 6-8% nitrogen and 1-2% phosphorus on a dry-weight basis (Oh-Hama and Miyachi, 1988). The nitrogen to phosphorus atomic ratio of microalgae grown in a balanced medium is reported to be 16:1 (Hillebrand and Sommer, 1999). Utilizing microalgae for removing/recovering nutrients from urine would allow full recovery of phosphorus from urine and recovery of a large fraction of the nitrogen. Compared to struvite precipitation the fraction of nitrogen that can be recovered by microalgae is 16 times higher.

Microalgae in pond systems have been used to treat wastewaters since the early 20th century (Oswald, 1988). Recently, cultivation of microalgae on domestic and diluted (digested) animal waste has been executed to couple wastewater treatment with biofuel production (Levine et al., 2011; Wang and Lan, 2011; Chang et al., 2013; Dickinson et al.,

2013; Ruiz et al., 2013), with the aim to reduce microalgae production costs and make microalgae biofuel more sustainable. Using different algal species and growth conditions, several studies have shown effective nutrient removal and a potential to economically produce algal biomass for biofuel production. Studies towards microalgae and/or cyanobacteria cultivation on nutrient-rich human urine and anaerobically treated black water, however, are scarce. So far only diluted synthetic urine or 50-180 times diluted human urine were used as a growth medium (Dao-lun and Zu-cheng, 2006; Feng and Wu, 2006; Yang et al., 2008; Chang et al., 2013). Furthermore, only a few species were used, for example *Scenedesmus acuminatus* (Adamsson, 2000) and *Arthrospira* (*Spirulina*) *platensis* (Feng and Wu, 2006; Yang et al., 2008).

1.3.2 Microalgae cultivation systems

Microalgae can be cultivated in several ways. The traditional way to cultivate microalgae is in open systems such as raceway ponds (Borowitzka and Moheimani, 2013). Although investment and operational costs are lower than in closed systems, in open systems algae cultivation is subject to lower productivity, less control of operating conditions due to climatic and seasonal changes, and to contamination. Since closed systems, such as tubular, flat panel, or bubble column photobioreactors (PBR), are costly, their application is so far restricted to the production of high-value products (Acién Fernández et al., 2013; Dillschneider and Posten, 2013; Zittelli et al., 2013) including poly-unsaturated fatty acids (PFUAs), carotenoids or other chemicals for pharmaceutical and cosmetics industries. Microalgae can be cultivated in suspension, immobilized in a matrix, or within a biofilm. Suspended microalgae systems have higher accessibility to light and nutrients than immobilized systems but harvesting of the small algae cells is energy consuming. Immobilized microalgae systems are designed to lower harvesting costs while maintaining a high affinity for light and nutrients. However, the cost of the immobilization matrix and the economics of the system as a whole need further investigation (Christenson and Sims, 2011). Biofilm systems, i.e. algal turf scrubber, trickling filters, and rotating biological contactors, are also designed to reduce harvesting cost but control of the target species as well as land requirement still need to be improved (Roeselers et al., 2008).

With concentrated urine as a growth medium, the system has to support a dense culture with special emphasis on light utilization and toxicity control. In dense cultures light

is available only at the surface of the culture and a considerable part of the culture can become a 'dark zone'. Minimizing this 'dark zone' to enhance biomass productivity is possible in a short light-path photobioreactor (PBR), i.e. a flat panel PBR. Because urine is rich in nutrients and has a high N:P ratio, remaining nitrogen present as ammonium/ammonia can inhibit microalgae growth. Free ammonia at 1.2 mM (~17 mg-N L^{-1}) has shown to negatively affect microalgae growth by inhibition of the photo-assimilation (Azov and Goldman, 1982). In this study, a short light-path photobioreactor was chosen as a proper system to improve microalgal biomass productivity and nutrient removal efficiency.

1.4 Thesis scope and outline

The main focus of this thesis is on source-separated human urine. Figure 1.1 presents a scheme of microalgae cultivation using source separated urine, while other domestic wastewater streams are conventionally transported and treated. Such concept can be used in a transition phase towards full source separation and recovery of resources. The objectives of the research were to (1) utilize microalgae as a tool to recover/remove nutrients from human urine and (2) optimize the production of microalgae biomass while maximizing nutrient recovery/removal efficiency. Figure 1.2 provides an overview of the research that was carried out to meet these objectives.

Chapter 2 shows the feasibility to cultivate microalgae on concentrated urine. For this, small scale lab experiments were carried out using micro-plates with a selected strain; *Chlorella sorokiniana*. The focus of this chapter is on requirements for microalgae growth on urine at different degrees of hydrolysis. Since the composition of urine can change during transport and storage, this might affect microalgae growth by potential toxicity or an improper nutrient balance. Having confirmed rapid growth of microalgae on concentrated urine, a short light-path photobioreactor was selected as a suitable algae cultivation system.

In **Chapter 3**, *C.sorokiniana* was cultivated in a short light-path photobioreactor (PBR) with continuous supply of light. Different urine dilutions were applied at a fixed hydraulic retention time (HRT) of 1 day to determine the minimum dilution factor that would support a stable microalgae production and urine treatment. In these experiments high biomass productivities and high nutrient removal efficiencies were obtained when shortening the photobioreactor light-path and optimizing the urine N:P ratio with additional magnesium.

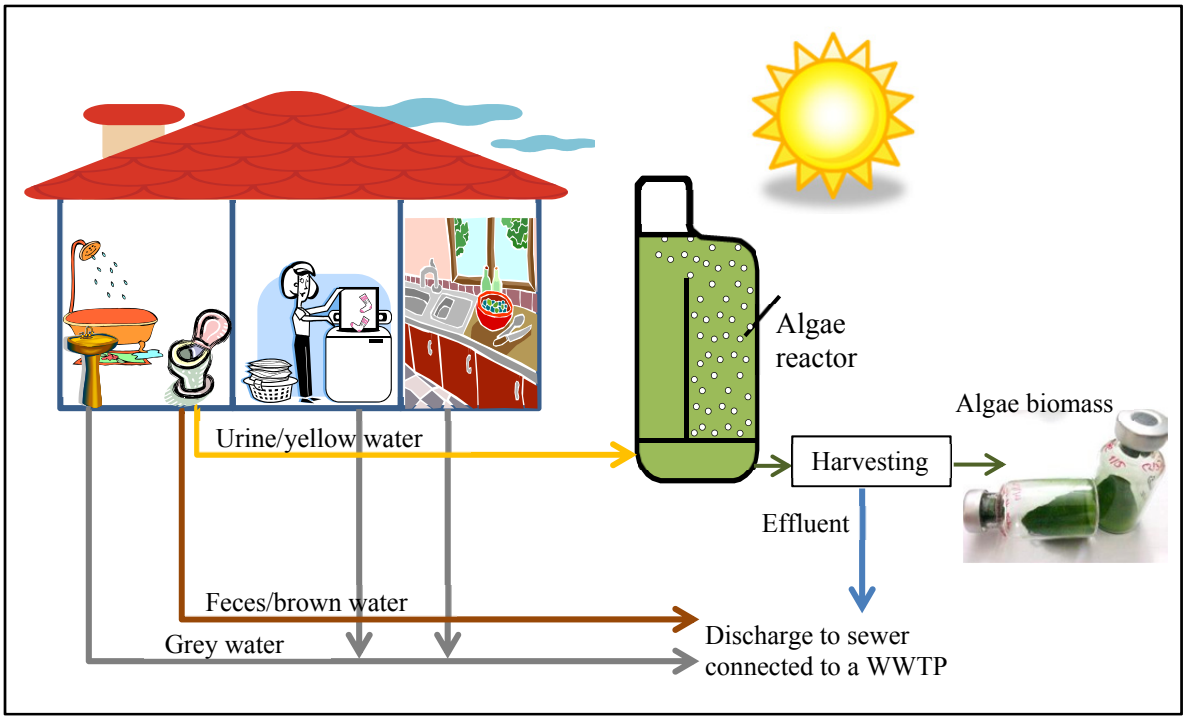

Figure 1.1 A concept of microalgae cultivation for nutrient recovery/removal from source separated urine in combination with conventional transport and treatment of remaining wastewater streams

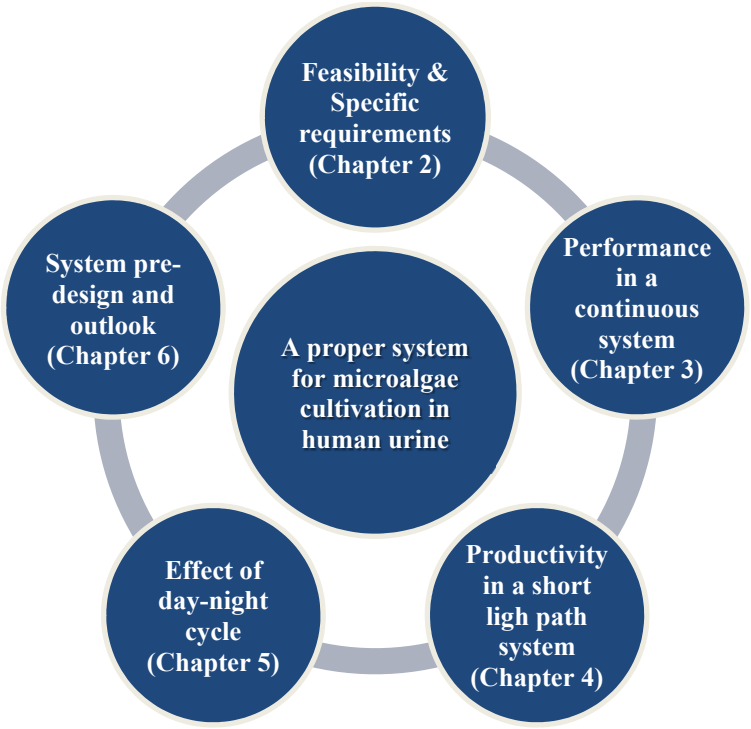

Figure 1.2 Overview of the research that was carried out to utilize microalgae to recover nutrients from human urine and to optimize the production of microalgae growing in urine.

To further optimize the performance parameters, the effect of the HRT (or dilution rate), on biomass productivity, biomass yield on supplied light, and nutrient removal efficiency was further investigated (**Chapter 4)**. At optimal HRT or in other words, optimal biomass density, the efficiency of light use is maximized resulting in the maximal biomass productivity as well as nutrient removal rates. In this chapter, synthetic urine was tested. At optimal HRT obtained on synthetic urine, performances on real urine was investigated. In **Chapter 5**, a day-night cycle was applied to simulate an outdoor cultivation system. This was done for slowly- and rapidly-growing microalgae cultures. Diurnal change of irradiance certainly affects the microalgae growth rate, productivity, and nutrient removal rate especially, when applying a short HRT. Implications of the findings for outdoor microalgae cultivation were also discussed in this chapter.

The first preliminary design for a microalgae cultivation system treating human urine was made. Based on calculations using a previously published model in combination with data described in Chapter 4, the biomass productivity and area requirement for this reactor were estimated for Dutch conditions (**Chapter 6**). The potential of this process is discussed in relation to presently available treatment technologies for source-separated urine. Finally, a discussion is presented on microalgae cultivation within new sanitation concepts ending in an outlook to future applications.

2

Microalgae growth on concentrated human urine

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Abstract

In this study, for the first time, a microalga was grown on non-diluted human urine. The essential growth requirements for the species *Chlorella sorokiniana* were determined for different types of human urine (fresh, hydrolyzed, male and female). Batch experimental results using microplates showed that both fresh and synthetic urine supported rapid growth of this species, provided additional trace elements (Cu, Fe, Mn, and Zn) were added. When using hydrolyzed urine instead of fresh urine, additional magnesium had to be added as it precipitates during hydrolysis of urea. *C.sorokiniana* was able to grow on non-diluted urine with a specific growth rate as high as 0.104 h^{-1} under light-limited conditions (105 µmol-photons m^{-2} s^{-1}), and the growth was not inhibited by ammonium up to a concentration of 1400 mg-NH_4^+-N L^{-1}. The highest growth rate on human urine was as high as 0.158 h^{-1}. Because it was demonstrated that concentrated urine is a rich and good nutrient source for the production of microalgae, its application for a large-scale economical and sustainable microalgal production for biochemicals, biofuels and biofertilizers becomes feasible.

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2.1 Introduction

Conventional municipal wastewater treatment is costly and inefficient in nutrient and energy recovery because the wastewater is highly diluted. New sanitation concepts, in which separate wastewaters are collected and treated at their source, have been proposed and are considered more efficient for this purpose (Zeeman et al., 2008). In domestic wastewater, more than half of the nutrient load originates from urine. Within a volume of only 1% of the total wastewater volume, urine contains 40% of the phosphorus load, 69% of the nitrogen load, and 60% of the potassium load (Zeeman et al., 2008; Zeeman and Kujawa-Roeleveld, 2011). Clearly this indicates that a large fraction of the nutrients may be directly recovered from source-separated urine.

Methods for nutrient recovery from source-separated urine were discussed by Maurer et al.(2006). Recently, a new bio-electrochemical method based on a microbial fuel cell for energy production combined with nitrogen recovery from urine was proposed by Kuntke et al. (2012). So far, only struvite precipitation and ammonia stripping have been applied at full scale. Struvite precipitation is usually applied for simultaneous recovery of phosphorus and nitrogen. The end product can be used as a fertilizer. However, the fraction of nitrogen that can be recovered is very small. Ammonia stripping requires a large energy input (Maurer et al., 2003) and only recovers nitrogen.

Another option for nutrient recovery from human urine is microalgae cultivation. Microalgae are photoautotrophic microorganisms that take up and accumulate nutrients. *Chlorella* species, for example, consist of 6-8% nitrogen and 1-2% phosphorus on a dry-weight basis (Oh-Hama and Miyachi, 1988). In line with these numbers, cultivation of microalgae on urine could maximize phosphorus recovery along with nitrogen recovery compared to other methods such as struvite precipitation. Large-scale cultivation of microalgae would then create a sink for these nutrients.

Microalgae biomass can be used as a biofertilizer and as a potential source of biochemicals and biofuels. Large-scale microalgae production for biofuels is not yet economically feasible (Kovacevic and Wesseler, 2010). The algal culture medium usually is made up of fertilizers and this accounts for 7-11% of the total microalgae production costs (Norsker et al., 2011). This can become even higher due to depletion of natural resources, in particular phosphate rock. Coupling microalgae cultivation for biofuels to urine treatment therefore is considered a realistic option to reduce the costs for microalgae production

(Pittman et al., 2011) although it is appreciated that several logistic problems need to be solved to achieve this. Eventually, to close nutrient cycles, microalgae biomass or the remaining nutrients from processed biomass can be used as fertilizer.

Using human urine for cultivation of microalgae and cyanobacteria (blue-green algae) was first studied for life-support systems for missions in outer space. Several studies demonstrated that cyanobacteria and microalgae can grow on highly diluted human urine (Adamsson, 2000; Feng and Wu, 2006; Yang et al., 2008). The urine sources used in these studies were either diluted synthetic urine, or urine which was diluted more than 100 times. No information is available at all about microalgal growth on concentrated urine and about the specific needs of microalgae growing in concentrated human urine. Moreover, the composition of urine changes due to hydrolysis and precipitation while it is transported and stored (Udert et al., 2003). This could have an effect on microalgae growth because of the presence of adverse concentrations of free ammonia or the precipitation of essential nutrients.

In this study, for the first time, microalgae were grown on pure urine. In addition, the essential requirements for microalgae growth on different types of urine (fresh, hydrolyzed, male, female) were determined.

2.2 Materials and Methods

2.2.1 Microalgae

The selected species, *Chlorella sorokiniana*, demonstrated better growth than *Chrolella vulgaris*, *Scenedesmus obliquus*, and mixed algae culture in preliminary tests on human urine (unpublished results) and therefore was selected for further experiments. *C.sorokiniana* CCAP211/8K was obtained from the Culture Collection of Algae and Protozoa, Oban, UK. Pre-cultivation was done with M8a medium (Kliphuis et al., 2010) in 250-ml enclosed shake flasks with 100 mL of liquid culture at 2% CO_2 (v/v), 25ºC, a light intensity of 20-40 µmol-photons m^{-2} s^{-1}, and a 16/8 h day/night cycle. A culture in its linear growth phase was used as an inoculum for an experiment on the requirement of trace elements (Experiment I). The inoculum in a linear growth phase used for growth experiments on diluted and concentrated urine (Experiment II and III) was first concentrated by centrifuging for 3 min at 13,800 relative centrifugal force (RCF) and re-suspending the pellet in demineralized water.

2.2.2 Collection of urine

Two batches of male and female urine were collected without dilution from healthy employees of the Sub-department of Environmental Technology, Wageningen University, the Netherlands. Male and female urine were tested separately to see whether or not differences in gender-related compositions e.g. sex hormones would affect microalgae growth. The urine was kept at 4ºC prior to the experiments. Urine hydrolysis, a decomposition of urea into ammonia and bicarbonate, was done by letting urine bottles stand with continuous mixing on a shaker at 30ºC. The ammonia concentration was regularly analyzed and urine taken at different incubation times represented different degrees of hydrolysis.

2.2.3 Urine-based growth media

Experiment I: The requirement of trace elements

The first batch of male and female urine was used in this experiment. The composition of male urine was as follows; chemical oxygen demand (COD) of 7,480 mg-O_2 L^{-1}, total N (TN) of 6,340 mg L^{-1}, ammonium nitrogen (NH_4^+-N) of 442 mg L^{-1}, and total phosphorus (TP) of 401 mg L^{-1}. The composition of female urine was as follows; COD of 6,305 mg-O_2 L^{-1}, TN of 6,500 mg L^{-1}, NH_4^+-N of 361 mg L^{-1}, and TP of 510 mg L^{-1}. These male and female urine were diluted 20 times with demineralized water (1 urine volume to the total volume of 20, named MU 1/20 and FU 1/20). With addition of iron and micronutrients (B, Cu, Mn, and Zn) that are referred to as trace elements in this study, final concentrations of added compounds in 20 times diluted urine were equal to those on M8a medium: 320 µM EDTA ferric sodium salt, 100 µM $Na_2EDTA.2H_2O$, 1 µM H_3BO_3, 66 µM $MnCl_2.4H_2O$, 11 µM $ZnSO_4.7H_2O$, and 7.3 µM $CuSO_4.5H_2O$. $NaHCO_3$ was added to all diluted media with a final concentration of 10 mM and the pH was adjusted to 7 with 3 M HCl.

Experiment II: Growth experiment on diluted urine

The second batch of male and female urine was used in this experiment. Fresh and hydrolyzed urine with different degrees of hydrolysis were diluted 5 and 10 times with demineralized water (1 urine volume to the total volume of 5 and 10, named 1/5 and 1/10). For the experiments with additional magnesium, the urine pH was adjusted to 7 prior to addition of $MgCl_2.H_2O$ and diluting with demineralized water. Magnesium concentration in undiluted urine was set to 0.02 mM. Trace elements were added while diluting urine with

demineralized water resulting in the following concentrations in 5 and 10 times diluted urine: 32 µM EDTA ferric sodium salt, 10 µM $Na_2EDTA.2H_2O$, 0.1 µM H_3BO_3, 6.6 µM $MnCl_2.4H_2O$, 1.1 µM $ZnSO_4.7H_2O$, and 0.73 µM $CuSO_4.5H_2O$.

Synthetic urine modified from Feng and Wu (2006) consisted of (in 1 liter) 10.72 g urea, 4.12 g K_2HPO_4, 4.83 g NaCl, 2.37 g Na_2SO_4, 1 g creatine, 0.65 g tri-sodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$), 0.5 g $CaCl_2.2H_2O$, 0.47 g $MgCl_2.H_2O$, and 0.29 g KCl. To mimic hydrolyzed urine, 28.8 g NH_4HCO_3 was added to serve as a nitrogen source instead of urea. Trace elements were added to synthetic urine resulting in the following concentrations: 320 µM EDTA ferric sodium salt, 100 µM $Na_2EDTA.2H_2O$, 1 µM H_3BO_3, 66 µM $MnCl_2.4H_2O$, 11 µM $ZnSO_4.7H_2O$, and 7.3 µM $CuSO_4.5H_2O$. Synthetic urine with either urea or ammonium as a nitrogen source was then diluted 5 and 10 times with demineralized water and the pH was adjusted to 7.

Table 2.1 shows the composition of different stocks of non-diluted urine used in this experiment, as well as the composition of synthetic urine and M8a medium.

Experiment III: Growth experiment on concentrated urine

The same batch of male and female urine as for Experiment II was used. For non-diluted urine and 2 times diluted urine (1 urine volume with 1 volume of demineralized water). The concentration of trace elements was doubled from that used in 5 and 10 times diluted urine. $NaHCO_3$ was added to all diluted media with a final concentration of 10 mM and the pH was adjusted to 7.

2.2.4 Experimental set-up

The experiments were done in 24-well microplates under non-axenic conditions and all growth media were not sterilized. For Experiment I and II, each well was filled with 1 mL of growth media. After filling growth media, each well was inoculated with 20-50 µl of microalgae culture to obtain an optical density at 750 nm (OD_{750}) of 0.05-0.10. For Experiment III, each well was filled with only 0.35 mL of growth media and inoculated with 10 µl of microalgae culture. The smaller volume was used to simulate a short optical path and to increase biomass concentration and volumetric productivity while still working within the linear measuring range of the well-plate reader.

Table 2.1 Compositions of media used in this study (all values are in mg L^{-1})

Media	NH_4^+-N	PO_4^{3-}-P	TN	COD_{sol}	K^+	Na^+	Ca^{2+}	Mg^{2+}	B
Male urine (MU)	733	387	3,550	4,885	1,747	1,588	36.11	4.18	13.90
Female urine (FU)	312	215	2,260	2,520	1,077	1,080	48.19	24.71	1.95
Hydrolyzed male urine (HMU)	3,260	341	3,500	3,555	1,879	1,707	2.18	0.15	5.30
Hydrolyzed female urine (HFU)	2,150	295	2,220	2,795	1,073	1,220	0.78	0.17	1.09
40% Hydrolyzed male urine (40%HMU)	1,470	386	3,680	4,920	1,670	1,560	11.89	0.72	12.22
60% Hydrolyzed male urine (60%HMU)	2,150	272	3,420	3,460	1,706	1,582	2.44	0.14	9.04
20% Hydrolyzed female urine (20%HFU)	549	251	2,310	2,520	1,083	1,085	2.81	0.66	1.78
70% Hydrolyzed female urine (70%HFU)	1,595	242	2,220	2,325	1,047	1,190	1.79	0.09	1.06
Hydrolyzed male urine + Mg^{2+} (HMU+Mg)	3,360	350	3,500	3,235	1,906	1,721	2.44	57.37	4.14
Hydrolyzed female urine + Mg^{2+} (HFU+Mg)	2,150	298	2,220	2,085	1,076	1,208	0.75	43.07	1.04
Ammonium-based synthetic urine (SYNam)*	5,000	733	5,370	1,428	2,002	2,830	136	56	0.01
Urea-based synthetic urine (SYNur)*	-	733	5,370	1,428	2,002	2,830	136	56	0.01
M8a**	-	214	415**	-	1,373	79	3.5	39.4	0.01

* calculated values

** nitrate served as a nitrogen source

Each medium was tested in triplicate. The plates were incubated in an orbital shaking incubator (Infors HT Multitron, Infors, Switzerland) operated at 100 rpm, 35ºC, and 80% humidity. The cultures were continuously illuminated with fluorescence lamps at an average photon flux density of 105 µmol-photons PAR m^{-2} s^{-1}. 4% CO_2 (v/v) was supplied to prevent CO_2 limitation.

A repeated batch mode was applied. After incubating for 24 hours, the culture was diluted by first re-suspending the cultures in each well, transferring them to clean 1.5-ml eppendorf tubes, re-filling each well with 1 mL of fresh growth media (with the same composition as the previous day), and re-inoculating each well with 40-80 µl of the cultures from the eppendorf tubes. The amount of inoculum depended on the OD_{750} of the culture in order to maintain the OD_{750} at the start of each consecutive day between 0.05 and 0.1. This repetitive procedure allowed to test whether or not the microalgae would be able to maintain their growth in a continuous cultivation system. For the experiment on the trace element requirement, after 48 hours of growth the trace elements were added to diluted urine media containing no initial trace elements.

The OD_{750} nm was measured 5 times a day with a BIOTEK (EL800) spectrophotometer (i.e. a well-plate reader). Prior to optical density measurement, cultures were re-suspended to detach precipitated algae from plate surface. The pH was measured daily before and after daily diluting of algal cultures by a Sentron pH meter. Because the urine has color, an OD_{750} of the urine media without microalgae was subtracted from the OD_{750} of samples containing microalgae. Reported values were averaged over triplicated samples and error bars in all figures indicated standard deviation of three replicates.

2.2.5 Analytical methods

NH_4^+-N and PO_4^{3-}-P concentrations were determined according to Standard Methods (APHA, 1998) using the continuous flow analyzer (SKALAR). Chemical oxygen demand (COD_{sol}) and total nitrogen (TN) were measured photometrically according to Standard Methods (APHA, 1998) using Dr Lange® test kits. Prior to analyses, urine samples were filtered (0.45 µm) for solid removal. The ICP-OES (VISTA-MPX) was used to determine B, Ca, Fe, K, Mg, Na, and Zn concentrations. Prior to an ICP analysis, samples were acidified with nitric acid (HNO_3) to the final concentration of 1% acid.

2.2.6 Calculations

To be able to compare microalgae growth under different conditions, the specific growth rate of the microalgae was calculated although there was no clear exponential growth under the light-limiting conditions applied. The reported specific growth rate was calculated by a linear regression of the natural logarithm of OD_{750} versus culturing time and specific growth rate at a certain time was calculated according to the following equation:

$$\mu = \frac{\ln\left(\frac{OD_{750-t2}}{OD_{750-t1}}\right)}{t_2 - t_1} \qquad (h^{-1})$$

where μ is a specific growth rate, $OD_{750\text{-}t1}$ and $OD_{750\text{-}t2}$ are the optical density at 750 nm at time t_1 and t_2, respectively. Calculated growth rates were analyzed using multivariate analysis in IBM SPSS Statistics 19 computer software. Significant differences in growth rate were tested using Sidak (multiple comparisons) with 95% confidence interval.

2.3 Results

2.3.1 Experiment I: The need for trace elements for microalgae growth on urine

Figure 2.1 shows growth as OD_{750} of *C.sorokiniana* on 20 times diluted fresh male and female urine with and without additional trace elements (B, Cu, Fe, Mn, and Zn); MU1/20, MU1/20+trace, FU1/20, and FU1/20+trace. Growth of *C.sorokiniana* on both male and female urine, with and without addition of trace elements, were similar during the first 24 hours (p=1.0 for all paired samples). When cultures were diluted, growth on urine without additional trace elements decreased drastically to about half of that on the medium with trace elements. This suggests that the small amount of trace elements contained in the inoculum could support microalgae growth during the first 24 hours but after dilution insufficient trace elements were available to support substantial growth on the second day.

During the second daily dilution, trace elements were added to the urine media lacking these elements and this resulted in similar growth on both urine media on day 3. Re-growth after adding trace elements confirmed that decreasing growth was a result of low trace element concentrations. The specific growth rate of this algae ranged from 0.06-0.07 h^{-1} at

low concentrations of trace elements on day 2 to 0.10-0.16 h^{-1} with addition of trace elements on day 3 (Table 2.2). Although similar growth was observed, the highest specific growth rate took place on 20 times diluted male urine with trace elements (MU1/20+trace) on day 3. There is no significant difference between the specific growth rates on male and female urine (p=1.0), except for the growth rate on MU1/20+trace on day 3 which is higher than growth rates on any other media.

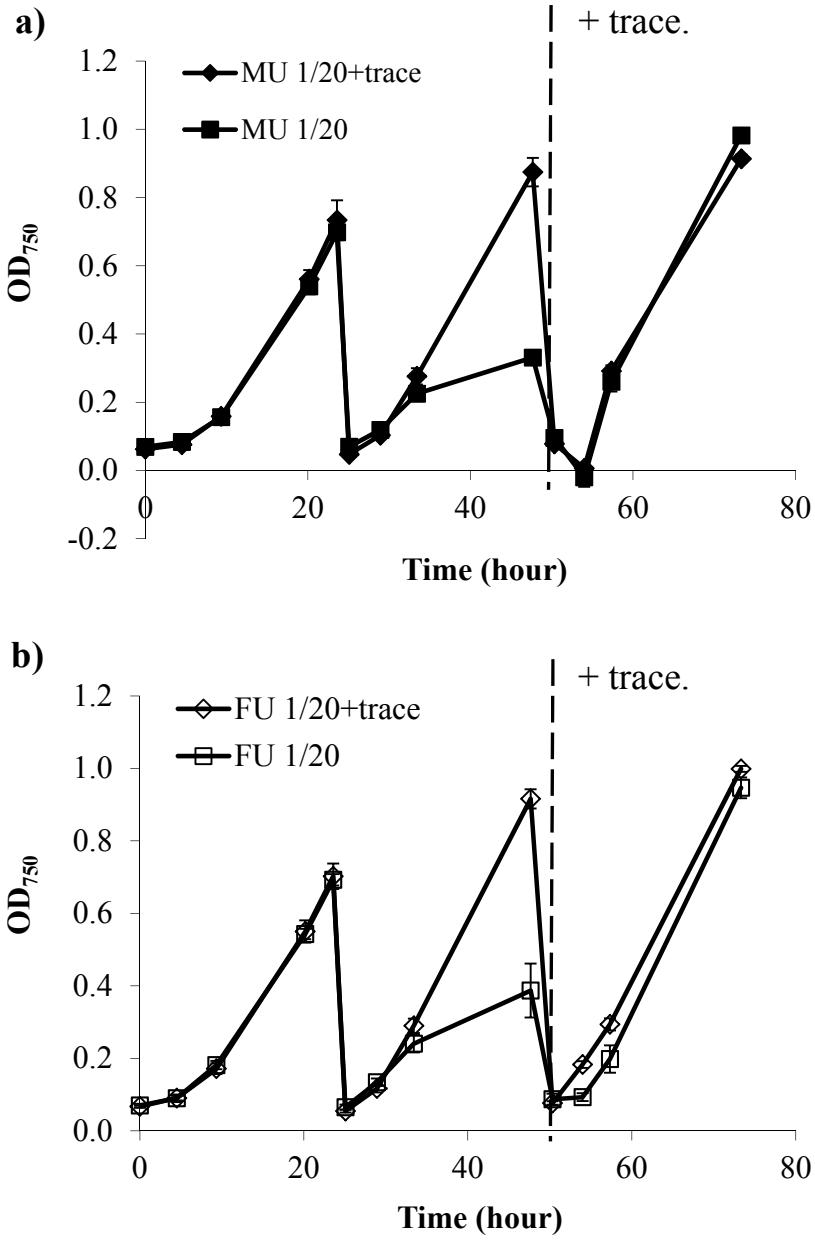

Figure 2.1 OD_{750} in time of *Chlorella sorokiniana* grown on 20 times dilution with and without addition of trace elements (Fe, B, Cu, Mn, and Zn); a) fresh male urine, and b) fresh female urine. Error bars show standard deviation (n=3).

Table 2.2 Calculated specific growth rate (h^{-1}) of *Chlorella sorokiniana* on different diluted urine media and the pH ranges of urine media during microalgae cultivation. The numbers in brackets represent standard deviation of triplicate samples.

Media	Day 1	Day 2	Day 3	pH
MU1/20+trace[/1]	0.111(0.003)	0.124(0.003)	0.158(0.022)	7.6-8.4
MU1/20[/1]	0.105(0.004)	0.064(0.008)	0.108(0.015)[/2]	7.6-8.3
FU1/20+trace[/1]	0.104(0.001)	0.119(0.004)	0.102(0.002)	7.7-8.3
FU1/20[/1]	0.102(0.004)	0.071(0.008)	0.109(0.004)[/2]	7.7-8.4
MU1/5	0.113(0.004)	0.100(0.004)	0.069(0.001)	5.7[/3]-8.0
MU1/10	0.116(0.005)	0.080(0.004)	0.088(0.003)	5.9[/3]-7.9
FU1/5	0.101(0.004)	0.093(0.006)	0.104(0.007)	7.1-7.7
FU1/10	0.114(0.006)	0.095(0.003)	0.104(0.003)	7.1-7.9
HMU+Mg1/5	0.108(0.006)	0.095(0.004)	0.101(0.001)	5.6-7.8
HMU+Mg1/10	0.103(0.003)	0.103(0.002)	0.104(0.002)	4.8[/3]-7.7
HFU+Mg1/5	0.107(0.002)	0.098(0.004)	0.096(0.006)	5.3[/3]-7.8
HFU+Mg1/10	0.101(0.002)	0.111(0.002)	0.101(0.005)	3.4[/3]-7.7
SYNam1/5	0.105(0.005)	0.113(0.001)	0.091(0.005)	7.5-8.2
SYNam1/10	0.123(0.012)	0.092(0.001)	0.094(0.003)	7.7-8.0
SYNur1/5	0.100(0.006)	0.083(0.002)	0.092(0.006)	6.8-7.3
SYNur1/10	0.101(0.002)	0.084(0.002)	0.096(0.002)	6.9-7.3
M8a	0.119(0.005)	0.096(0.004)	0.094(0.003)	6.5-7.6

[/1] different batch of urine media was used for these diluted media

[/2] on day 3 trace elements were added to MU1/20 and FU1/20

[/3] low pH was observed at the end of day 1. Averaged pH at the end of day 2 and 3 was within a range of 7.0-7.7.

2.3.2 Experiment II: Microalgae growth on diluted urine

Growth of Chlorella sorokiniana *on synthetic urine*

Most of the nitrogen in fresh urine is present as urea. When urine is stored, urea is hydrolyzed into free ammonia (NH_3), ammonium (NH_4^+) and bicarbonate (HCO_3^-). Consequently, urea and ammonium are the major forms of nitrogen in urine and both are known to support growth of microalgae (Birdsey and Lynch, 1962). However, high free

ammonia concentrations can inhibit microalgae growth. Therefore, synthetic urine with either urea or ammonium was tested.

Figure 2.2 shows growth as an increase of OD_{750} of *C.sorokiniana* on 5 and 10 times diluted synthetic urine, with urea and ammonium as a nitrogen source, and on M8a medium as a reference: SYNur1/5, SYNur1/10, SYNam1/5, SYNam1/10, and M8a. The experimental results showed similar growth on synthetic urine and M8a medium, indicating that urea and ammonium support microalgae growth as well as nitrate, the nitrogen source in M8a medium. On day 1, growth on all synthetic urine media was similar ($p>0.05$) and ranged from 0.100 to 0.123 h^{-1}. After daily dilution, re-growth could be observed on all synthetic urine media with a specific growth rate between 0.083 and 0.113 h^{-1}. Calculated specific growth rates of *C.sorokiniana* on different dilutions of synthetic urine are given in Table 2.2. The highest growth rate of 0.123 h^{-1} was observed on SYNam1/10 on day 1.

During cultivation, the pH of diluted synthetic urine with urea was between 6.8 and 7.3 and the pH of diluted synthetic urine with ammonium was between 7.5 and 8.2 (Table 2.2). At a pH higher than 8, the fraction of free ammonia in synthetic urine with ammonium may have affected growth of the microalgae. For SYNam1/5, the ammonium concentration was around 940 mg-N L^{-1}. At a pH of 8.2 and temperature of 35ºC, the free ammonia (NH_3) concentration could reach 140 mg-N L^{-1}, which is much higher than the reported free ammonia level causing inhibition of photo-assimilation (1.2 mM) (Azov and Goldman, 1982). *C.sorokiniana* apparently could tolerate this ammonia level as demonstrated by a specific growth rate as high as 0.113 h^{-1} on day 2. In addition, it was calculated that the specific growth rate on this medium is not statistically different from that on M8a medium.

Growth of Chlorella sorokiniana *on fresh urine*

On fresh or slightly hydrolyzed urine, the microalgae were able to grow on all media. Figure 2.3 shows the increase of OD_{750} representing microalgae growth on 5 and 10 times diluted male and female urine; MU1/5, MU1/10, FU1/5, and FU1/10. On day 1, specific growth rates on all urine media ranged between 0.101 to 0.114 h^{-1} and no significant difference was observed among these media and on M8a ($p>0.05$) . After the cultures were daily diluted on day 2, re-growth could be maintained and specific growth rates on all urine media were similar ($p>0.05$). However, the specific growth rate on day 2 was slightly lower

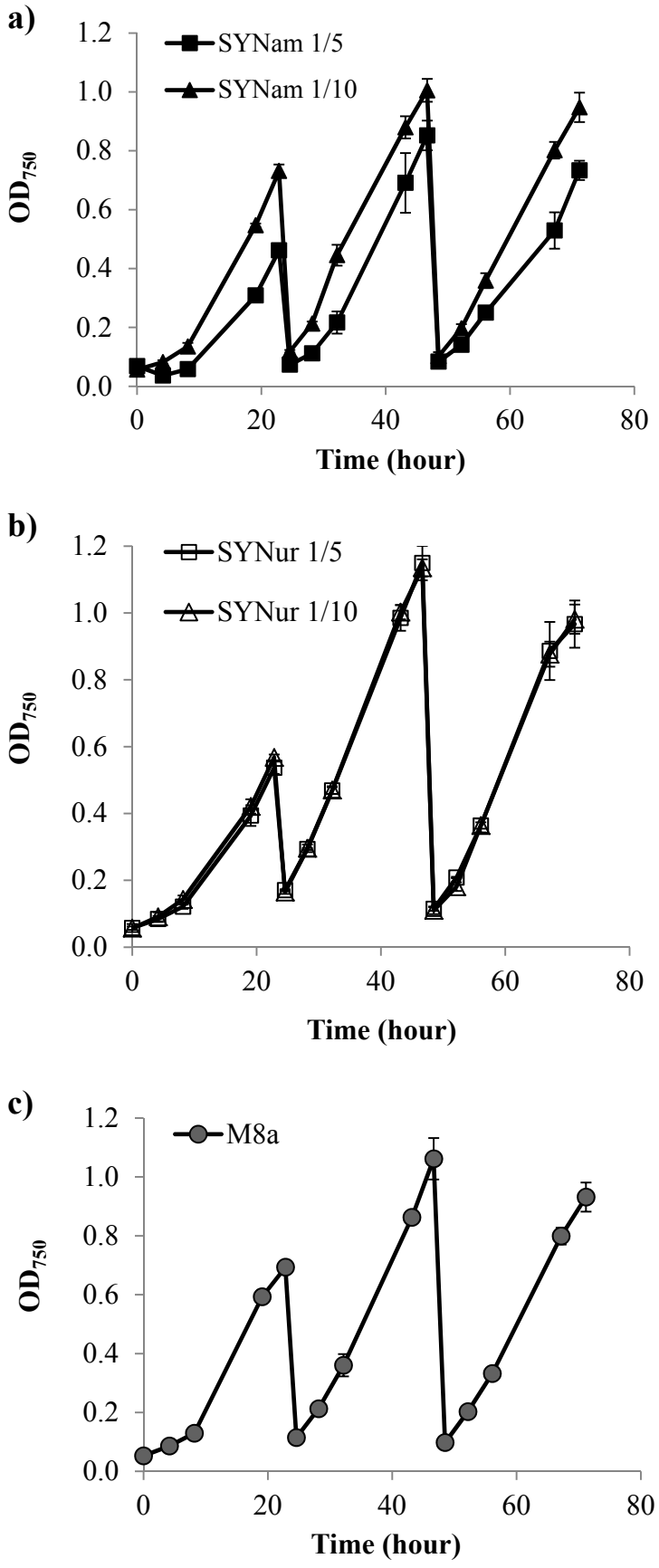

Figure 2.2 OD_{750} in time of *Chlorella sorokiniana* grown on 5, and 10 times dilution of a) synthetic urine with ammonium as a nitrogen source with additional trace elements, b) synthetic urine with urea as a nitrogen source, and c) M8a (standard medium). Error bars show standard deviation (n=3).

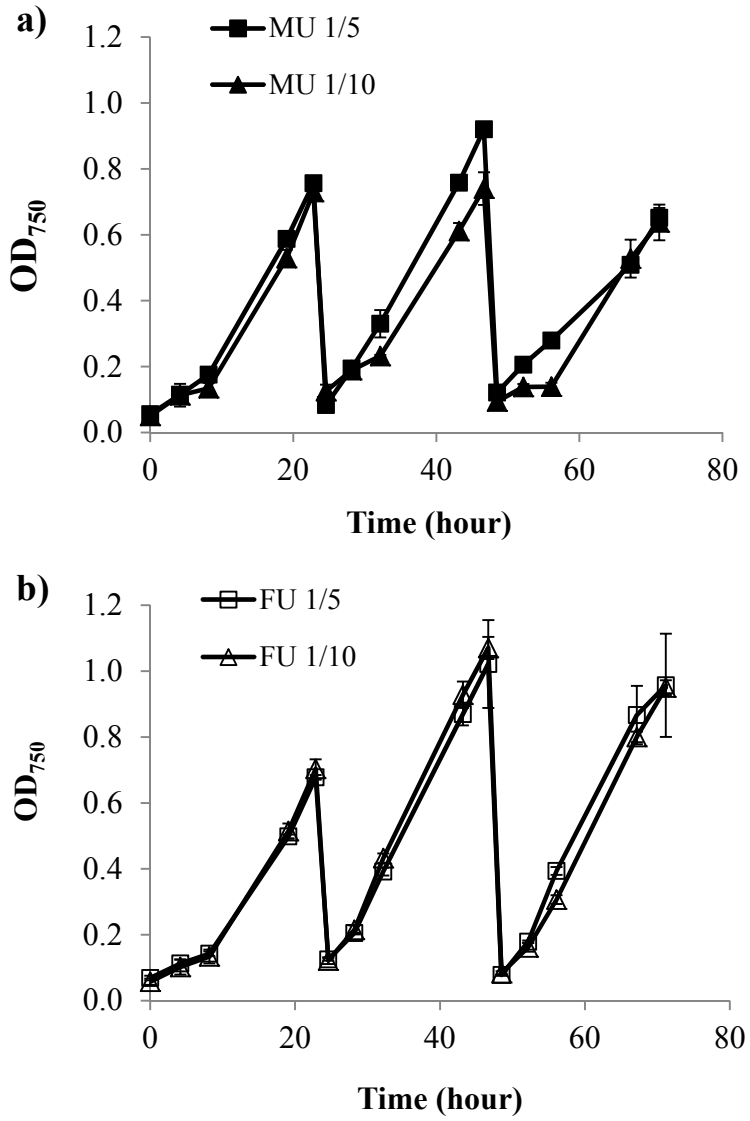

Figure 2.3 OD_{750} in time of *Chlorella sorokiniana* grown on 5 and 10 times dilution of a) fresh male urine, and b) fresh female urine with additional trace elements. Error bars show standard deviation (n=3).

than that of day 1 (0.08-0.10 h^{-1}). Again after daily dilution on day 3, rapid growth was observed in all media with a slight increase in specific growth rate in comparison to day 2 except for MU1/5. Calculated specific growth rates of *C.sorokiniana* on different dilutions of male and female urine are shown in Table 2.2. The highest growth rate on real urine media was observed on MU1/10 on day 1 (0.116 h^{-1}).

When comparing growth rates on male and female urine with synthetic urine, specific growth rates on both male and female urine were similar to that on synthetic urine with either

urea or ammonia. Similar growth on real and fresh urine in comparison to synthetic urine indicated no inhibitory effect that could possibly result from organic compounds or other compounds present in real urine. Moreover, similar growth on male and female urine from both Experiment I and II showed that differences in gender related urine composition (e.g. presence of different hormones) did not significantly affect microalgae growth in this study.

During the experiments, the pH of male urine media ranged between 5.7 and 8.0, whereas the pH of female urine media ranged between 7.1 and 7.9 (Table 2.2). The lowest pH was observed on male urine on day 1. However, this low pH did not seem to affect microalgal growth because there was no significant difference among growth rates on male urine, female urine, and M8a medium ($p>0.05$). These results show that it is feasible to cultivate *Chlorella sorokiniana* on fresh urine with minimal dilution.

Growth of Chlorella sorokiniana *on hydrolyzed urine*

When urine is collected and stored, hydrolysis can readily occur resulting in increasing ammonia concentrations and this could be toxic to microalgae. Figure 2.4a and b show growth of *C.sorokiniana* as the increase of OD_{750} on 5 and 10 times dilution of fully hydrolyzed male and female urine: HMU1/5, HMU1/10, HFU1/5, and HFU1/10. In addition, Figure 2.4c and 2.4d show growth on 5 and 10 times dilution of partially hydrolyzed male and female urine: 40%HMU, 60%HMU, 20%HFU, and 70%HFU. On fully hydrolyzed male and female urine, the specific growth rate was significantly lower than that on synthetic and fresh urine.

Specific growth rate on urine media with a degree of hydrolysis of 60% and 70% was similar to that on fully hydrolyzed urine media. Slightly higher growth rate was observed on urine with lower degrees of hydrolysis; 40% and 20%. The specific growth rates calculated over 24 hours for microalgae grown on fully hydrolyzed male and female urine and 60% and 70% hydrolysis of male and female urine were within a range of 0.064-0.075 h^{-1}. Specific growth rates on 40%HMU and 20%HFU respectively were 0.088 and 0.092 h^{-1}. The culture pH ranged between 6.8 and 7.9. After 24 hours, microalgae stopped growing. This poor growth could have been caused by either high ammonia concentrations, which are known to be toxic to microalgae, or by specific nutrient limitations.

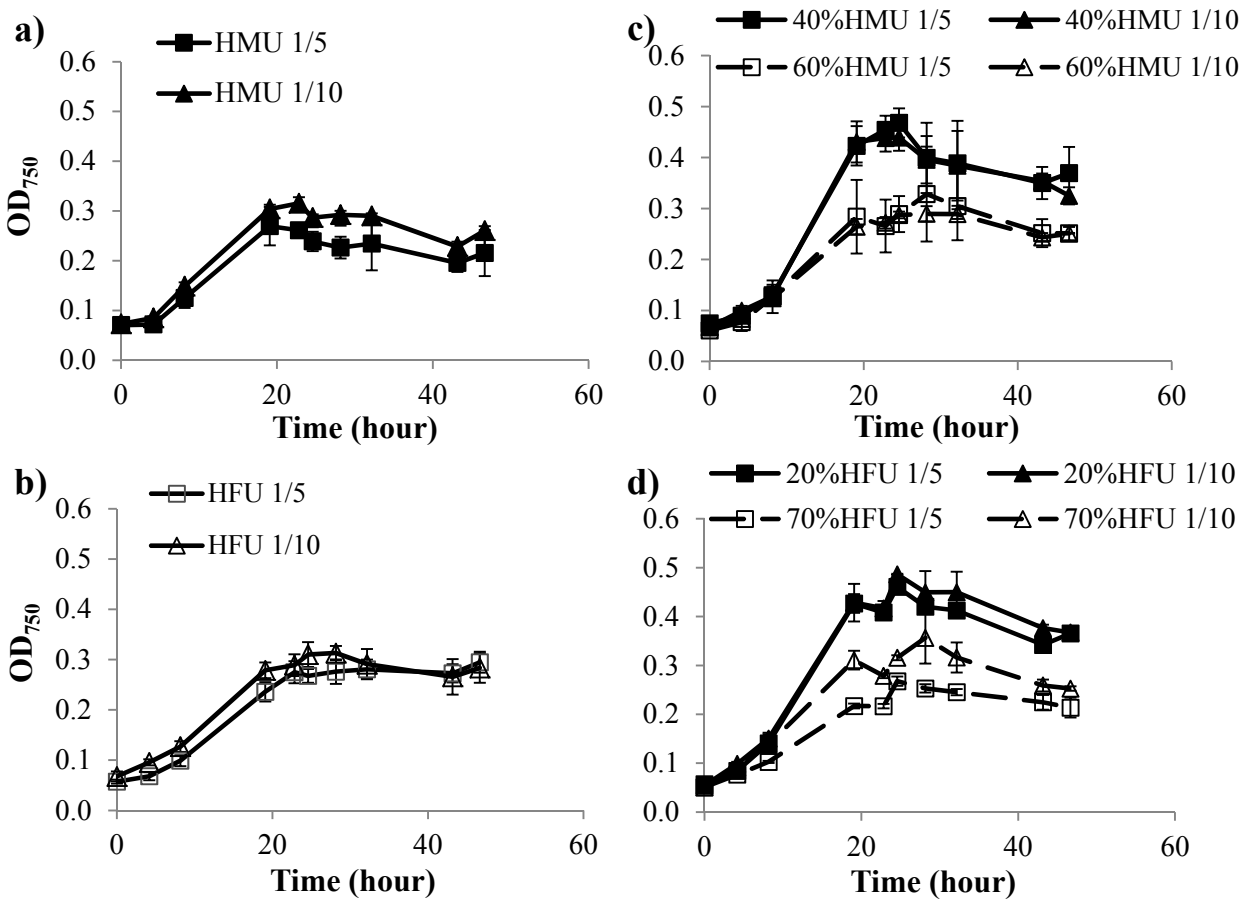

Figure 2.4 OD_{750} in time of *Chlorella sorokiniana* grown on 5 and 10 times dilution of a) fully hydrolyzed male urine, b) fully hydrolyzed female urine, c) 40% and 60% hydrolyzed male urine, and d) 20% and 70% hydrolyzed female urine with additional trace elements. Error bars show standard deviation (n=3).

When comparing growth on hydrolyzed urine with synthetic urine with ammonium, higher growth was observed on synthetic urine even though synthetic urine contains a higher ammonium concentration: 4,700 mg-NH_4^+-N L^{-1} compared to 3,260 and 2,150 mg-NH_4^+-N L^{-1} respectively for undiluted hydrolyzed male and female urine. The ammonium concentrations in 40% and 20% hydrolysis male and female urine were even lower but still growth on these media was poor. Moreover, during the experiments the pH of the hydrolyzed urine was within a range of 6.6 to 7.9 (Table 2.2). This pH range was slightly lower than the pH of synthetic urine. Consequently, the fraction of free ammonia in hydrolyzed urine must be lower than that of synthetic urine. Apparently free ammonia was not the major factor limiting microalgae growth on hydrolyzed urine.

From the composition of urine shown in Table 2.1, hydrolyzed urine contains less magnesium (Mg^{2+}) than fresh urine. The reported magnesium content of *Chlorella* sp. ranges between 0.36-0.8% on dry weight basis (Oh-Hama and Miyachi, 1988). The Mg^{2+} concentration of diluted hydrolyzed urine never was higher than 0.15 mg L^{-1} which could support only 40 mg-dry biomass L^{-1} at 0.36% magnesium content. Hence, this could explain the poor growth observed in this study.

The effect of magnesium addition on growth of Chlorella sorokiniana *on hydrolyzed urine*

To check the hypothesis of magnesium (Mg^{2+}) limited growth, we tested the effect of Mg^{2+} addition to hydrolyzed urine. Figure 2.5 shows growth of *C.sorokiniana* on 5 and 10 times diluted fully hydrolyzed male and female urine with additional Mg^{2+}. When Mg^{2+} was added to hydrolyzed urine, the microalgae were able to grow and maintain growth after repeated daily dilution. Specific growth rates on hydrolyzed male and female urine with additional Mg^{2+} ranged between 0.095 and 0.111 h^{-1} (Table 2.2). There was no significant difference of the specific growth rate on hydrolyzed urine with additional Mg^{2+} as compared to that on synthetic and fresh urine confirming that Mg^{2+} limited growth of microalgae on hydrolyzed urine.

2.3.3 Experiment III: Microalgae growth on concentrated urine

The previous results showed the possibility to cultivate microalgae on minimally diluted urine. On concentrated urine, microalgae might not be able to maintain growth. In experiment III it was therefore attempted to grow *C.sorokiniana* on concentrated urine. At high nutrient concentrations, as those in concentrated urine, high biomass densities are needed to remove all nutrients. To maintain a high productivity in such dense cultures, however, it is needed to increase the light supply to the cultures. This can be done by increasing the ratio between the light-exposed surface area and the culture volume (S/V ratio). To test this strategy the wells of the microplates were filled with a smaller volume of culture medium. Consequently, the S/V ratio was increased resulting in a higher light input to the cultures.

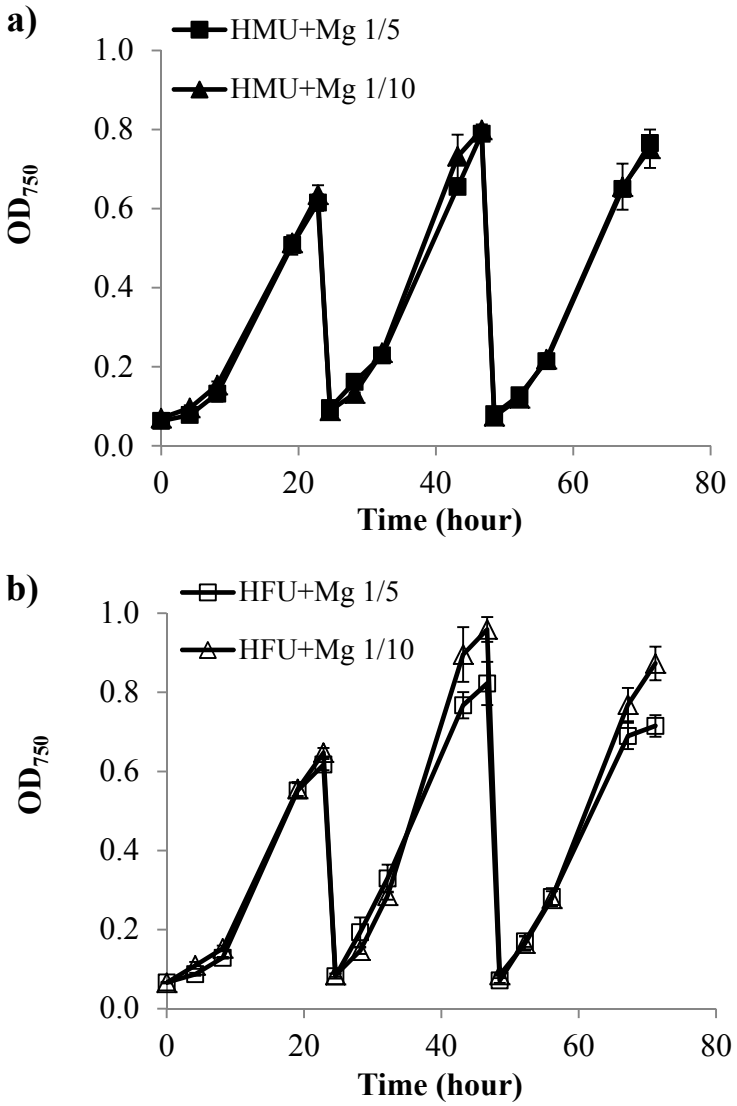

Figure 2.5 OD_{750} in time of *Chlorella sorokiniana* grown on 5 and 10 times dilution of a) fully hydrolyzed male urine with addition of magnesium and trace elements, and b) fully hydrolyzed female urine with addition of magnesium and trace elements. Error bars show standard deviation (n=3).

In Figure 2.6a and b, growth of *C.sorokiniana* is shown on non-diluted and 2 times diluted fresh male and female urine: MU, MU1/2, FU, and FU1/2. In addition, growth on 2 times diluted hydrolyzed female urine with additional Mg^{2+}, synthetic urine with urea, and M8a are shown in Figure 2.6c. The results demonstrate that microalgae can grow on concentrated urine. The corresponding specific growth rates are shown in Table 2.3. Although there was almost no growth on non-diluted male urine (MU), growth could still be

observed on 2 times diluted male urine (MU1/2). On the contrary, growth rate on non-diluted female urine (FU) was better than that of male urine. On day 2 and 3, the specific growth rate on FU had increased to the same level as observed on FU1/2, MU1/2, and M8a. Apparently the microalgae adapt to this concentrated medium. The pH of the urine media in this experiment was within a range of 7.4-8.4, indicating the occurrence of hydrolysis.

On non-diluted synthetic urine with urea (SYNur), good growth was observed with the specific growth rate ranging between 0.072 and 0.116 h^{-1}. The specific growth rate was relatively low on day 1 but on day 2 and 3 it was equal to that of diluted fresh and synthetic urine. The pH of SYNur was maintained at 7.2-7.7 (Table 2.2). As expected based on the high ammonium concentration, no growth was observed on non-diluted synthetic urine containing ammonium instead of urea.

C.sorokiniana was also successfully grown on hydrolyzed urine with only a dilution factor of two with additional Mg^{2+}. This urine medium had an ammonium concentration of about 1,400 mg-NH_4^+-N L^{-1} and the pH ranged between 7.6 and 8.0. During the cultivation, the highest specific growth rate on this medium was up to 0.149 h^{-1}. This shows that *C.sorokiniana* can tolerate ammonium levels as high as 1,400 mg-NH_4^+-N L^{-1}.

As compared to results from Experiment II, an OD_{750} obtained in this experiment was somewhat lower but relatively high specific growth rates calculated according to Section 2.6 were still obtained. Due to the fact that only 1/3 of culture media was used for simulating a short light-path system, 1 OD_{750} unit in this experiment would correspond to a 3 times higher biomass density if 1 mL of growth medium was used. With a factor of 3, biomass density on concentrated urine in this experiment would be comparable to that from Experiment I and II. Results of this experiment clearly show the feasibility to cultivate microalgae on concentrated human urine provided that trace elements and (if needed) magnesium were supplemented.

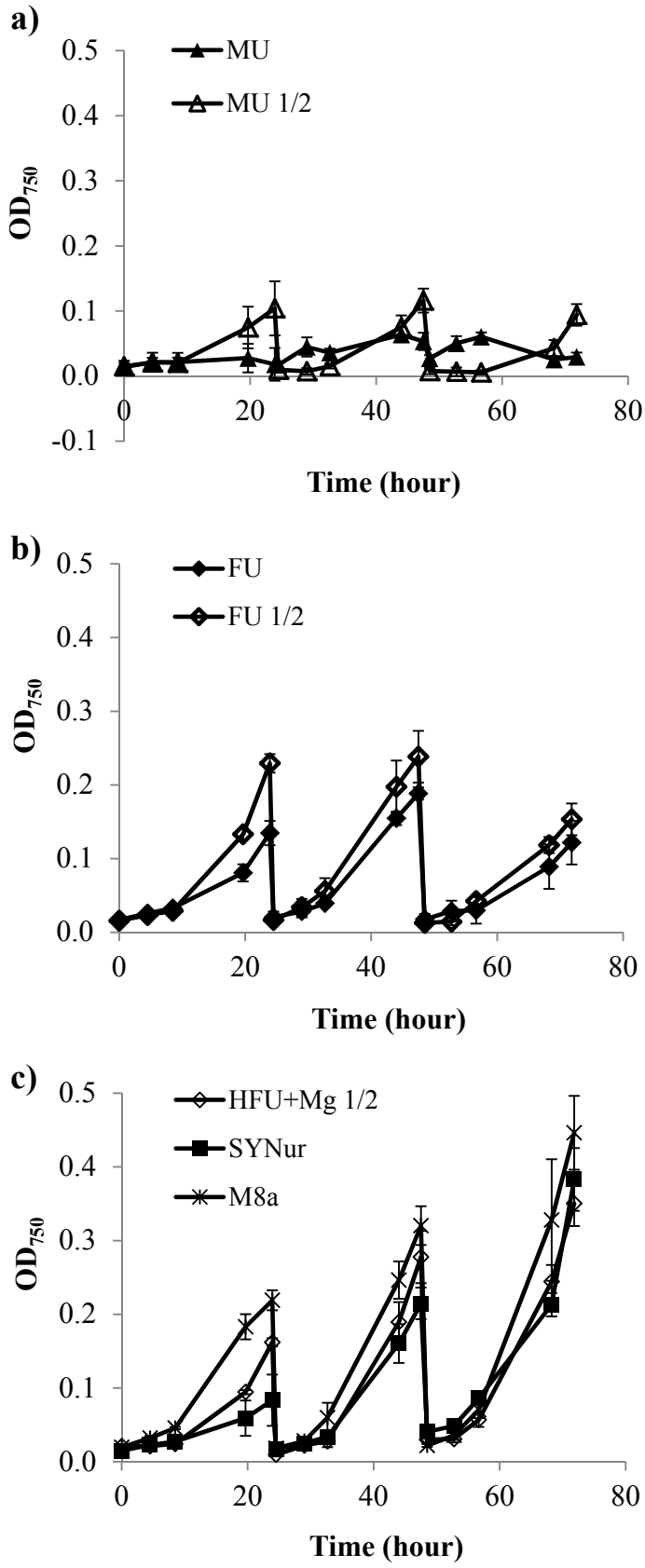

Figure 2.6 OD_{750} in time of *Chlorella sorokiniana* grown on a) concentrated male urine with additional trace elements, b) female urine with additional trace elements, and c) hydrolyzed female urine with magnesium and trace elements, synthetic urine with urea, and M8a. Error bars show standard deviation (n=3). In this experiment, 0.35 mL of growth media was used to mimic a short light-path system.

Table 2.3 Calculated specific growth rate (h^{-1}) of *Chlorella sorokiniana* on minimally diluted urine media and the pH ranges of urine media during microalgae cultivation. The numbers in brackets represent standard deviation of triplicate wells.

Media	Day 1	Day 2	Day 3	pH
MU1/2	0.083(0.004)	0.126(0.012)	0.129(0.054)	7.4-8.2
FU	0.085(0.006)	0.104(0.017)	0.084(0.012)	7.4-8.2
FU1/2	0.115(0.005)	0.116(0.008)	0.114(0.008)	7.4-8.4
HFU+Mg1/2	0.093(0.004)	0.149(0.007)	0.115(0.003)	6.9-7.5
SYNur	0.072(0.012)	0.116(0.001)	0.095(0.004)	7.2-7.7
M8a*	0.106(0.002)	0.130(0.005)	0.132(0.004)	6.7-7.9

* This media was tested twice.

2.4 Discussion

Chlorella sorokiniana is a fast-growing microalgae with reported maximum specific growth rates up to 0.25 h^{-1} (Sorokin, 1967; Cuaresma et al., 2009) under autotrophic and light saturating conditions. In this study, the highest specific growth rate of *C.sorokiniana* achieved on real urine was 0.158 h^{-1} (MU1/20+trace elements, day 3). Nevertheless, this is still lower than the reported maximum specific growth rate. This is most likely related to the relatively low light intensity of 105 μmol-photons m^{-2} s^{-1} considering that the growth of *C.sorokiniana* only saturates above 300 μmol-photons m^{-2} s^{-1} (Janssen et al., 1999). Moreover, mutual shading would further decrease the average light supply per microalgae cell during batch cultivation.

In our study, we minimized urine dilution and achieved relatively high growth rate which was maintained for several days when daily diluting the cultures. Although the tested period lasted for only 3 days, rapid growth can be observed already on the first day for most experiments. As can be seen in almost all figures even in the first day a clear lag phase cannot be distinguished. In most cases microalgae growth increased further during day 2 and day 3 showing adaptation of the microalgae to the growth media used.

In this study, microalgae biomass density was measured and expressed as the optical density measured at 750 nm. In addition the OD at 680 nm was measured as well. At 680 nm the absorbance of light by chlorophylls adds to the OD measured (OD_{680}). At 750 nm it is only scattering of light that determines the OD (OD_{750}). If the bacterial population increases

this would be reflected in a change in the ratio OD_{680}/OD_{750}. This ratio would become lower and approach 1 in case the bacteria would dominate. The ratio OD_{680}/OD_{750} of microalgae grown on fresh urine and on hydrolyzed urine with additional magnesium ranged between 1.8±0.2 and 2.2±0.1. This was comparable to the OD_{680}/OD_{750} ratio on M8a medium (2±0.2) which did not contain any organic compounds. Consequently, microalgae dominated all the cultures and the optical density is a reliable measure for microalgae biomass density.

In this study under light limited conditions, the highest growth rate on 5 and 10 times diluted fresh urine and synthetic urine respectively were 0.116 h^{-1} and 0.123 h^{-1}. This growth is comparable to growth on a standard medium, M8a, showing that urine with additional trace elements is a good growth medium for this microalga. Fresh urine well supports growth of microalgae with supplemental iron and micronutrients. Additional magnesium is needed for growing microalgae on hydrolyzed urine. The required amount of these compounds depends upon the initial concentration in the urine, which can vary from person to person according to dietary differences.

Re-growth after trace element addition to fresh urine clearly showed the need for these elements. The trace elements Fe, Cu, Mn, and Zn that were added have been shown to play an important role in microalgal metabolism, whereas B is only required for some algal species (O'Kelley, 1968; Oh-Hama and Miyachi, 1988). The minimum concentrations of trace elements required for growth of *C.sorokiniana* reported by Eyster (1978) were 1 µM of Fe, 10^{-6} µM of Cu, 0.3 µM of Mn, and 1 µM of Zn. The requirement of B was not indicated as neither positive nor negative effect was observed at a concentration of 0.001- 10 mg L^{-1} (McBride et al., 1971).

In urine, these trace elements are present mostly at low concentrations. The measured Fe concentration was lower than the detection limit (0.9 µM or 0.05 mg L^{-1}), whereas the measured concentration of Cu and Zn of the urine used in this study were within a range of 3-9 and 1-11 µM, respectively. Reported concentrations of trace elements in urine in other studies are respectively 1.2-7.2 µM of Fe (Ronteltap et al., 2007; Chang et al., 2013), 0.04-2.25 µM of Cu (Ronteltap et al., 2007; Ronteltap et al., 2007), 0.001-0.05 µM of Mn (Tsalev, 1984), and 1.1-8.2 µM of Zn (Ronteltap et al., 2007). Initial concentrations of these elements are relatively low except for Cu that is present at higher concentration than the minimum concentration needed for *C.sorokiniana* (Eyster, 1978). However, when the urine is hydrolyzed, Cu potentially precipitates (Ronteltap et al., 2007), and this could result in a

deficiency condition. Among these trace elements, manganese (Mn) seems to be the most critical element lacking in urine but also Fe, Cu, and Zn are present at vary concentrations and can potentially precipitate.

Magnesium is needed for production of chlorophyll and it is also believed to play a role in algae metabolism (Sydney et al., 2010). The results of this study confirms that Mg^{2+} is important for microalgae growth and this finding agrees with other studies that showed enhancement of microalgae growth with additional Mg^{2+} (Mandalam and Palsson, 1998; Adamsson, 2000). The Mg^{2+} requirement can be estimated from the growth results on fresh urine. Fresh male urine diluted 10 times (MU1/10) contained 0.42 mg-Mg^{2+} L^{-1} (Table 1) and resulted in a biomass increase to an OD_{750} of 0.7, which is comparable to an algae density of 200 mg-dw L^{-1}. Assuming that Mg^{2+} was fully utilized, the calculated Mg^{2+} content of microalgae would then be 2 mg g-biomass^{-1}. This mass fraction can be used to assess the maximum amount of Mg^{2+} required to support microalgae growth up to a given biomass density.

A major loss of Mg^{2+} could be due to precipitation of struvite ($MgNH_4PO_4.6H_2O$) during hydrolysis of urea. Udert et al. (2003) simulated precipitation of struvite and octacalcium phosphate (OCP) in urine and reported 87% precipitation potential at only 11% hydrolysis. At 24% hydrolysis, the precipitation potential increased to 97%. This phenomenon could explain the significant decrease in Mg^{2+} concentration in the urine with 20% and 40% hydrolysis. In addition to Mg^{2+}, *Chlorella* biomass contains 0.005-0.08%-Calcium (Ca). Precipitation of hydroxyapatite (HAP; $Ca_{10}(PO_4)_6(OH)_2$) causes a reduction of Ca^{2+} when the Mg^{2+} concentration decreases (Udert et al., 2003). However, the Ca^{2+} content of microalgae is relatively low and urine contains higher Ca^{2+} than Mg^{2+} concentration. This explains why we did not observe Ca^{2+} deficiency affecting microalgae growth.

In most cases, the urine N:P atomic ratio is relatively higher (up to 28:1) than a balance value (16:1) for microalgae known as the Redfield ratio (Redfield, 1958). This ratio increases when urine is hydrolyzed because a significant amount of phosphate precipitates as struvite, hydroxyapatite, or other forms (Udert et al., 2003). However, in this study the effect of the high N:P ratio is minimal because of light being the real growth limiting factor in the short cultivation period applied. In practice, phosphorus, light, or both can limit growth and that has to be taken into account when designing microalgae based treatment systems.

As mentioned earlier, urea hydrolysis immediately occurs leading to struvite precipitation and depletion of Mg^{2+} and a decrease in its availability. Struvite has low solubility (Ronteltap et al., 2007). Nevertheless, dissolution of struvite in hydrolyzed urine is possible with addition of chemicals. To prevent urine hydrolysis, there are several methods that can be applied including acidification, microfiltration, ultrafiltration, and the use of urease inhibitors which are aiming at removing, or inactivating bacterial growth (Maurer et al., 2006). But some of these techniques are costly and energy consuming. Acidification needs acid to stabilize urine but later the pH needs to be neutralized again in order to grow microalgae. Using urease inhibitors is not reliable and has potential negative effects on human and environment (Benini et al., 1999). Unless the urine is freshly collected and used for microalgae growth, addition of Mg^{2+} directly to the microalgae culture would then be a less complicated and more cost-effective option. The estimated Mg^{2+} requirement for *C.sorokiniana* observed in this study is about 2 mg- Mg^{2+} g-dw^{-1}.

2.5 Conclusions

Chlorella sorokiniana is able to grow on concentrated human urine up to 1.4 g-NH_4^+-N L^{-1} at a pH lower than 8.0. Maximum growth was obtained on 20 times diluted urine with additional trace elements. Microalgae growth on pure urine is possible at slightly reduced growth rates. Human urine supports rapid microalgae growth with additional trace elements and/or magnesium, depending on the urine composition. Urine can serve as a rich source of major nutrients for the large-scale sustainable production of microalgae biomass.

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3

Nutrient removal and microalgal biomass production on urine in a short light-path photobioreactor

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Abstract

Due to the high nitrogen and phosphorus content, source-separated urine can serve as a major nutrient source for microalgae production. The aim of this study was to evaluate the nutrient removal rate and the biomass production rate of *Chlorella sorokiniana* being grown continuously in urine employing a short light-path photobioreactor. The results demonstrated, for the first time, the possibility of continuous microalgae cultivation in human urine. The lowest dilution factor successfully employed was a factor of 2 (50% v/v urine). Microalgae dominated a smaller bacterial population and were responsible for more than 90% of total nitrogen and phosphorus removal. With a light-path of 10 mm, a maximum volumetric biomass productivity as high as 9.3 g-dw L^{-1} d^{-1} was achieved. The co-existing bacterial population removed up to 70% of organic pollutants from the urine at a rate of 1300 mg COD L^{-1} d^{-1}. Enriching the urine with magnesium, adjusting the N:P molar ratio, and shortening the reactor light-path further increased the volumetric biomass productivity to 14.8 g-dw L^{-1} d^{-1}. The corresponding nitrogen and phosphorus removal rates were 1300 mg-N L^{-1} d^{-1} and 150 mg-P L^{-1} d^{-1}, respectively. The subsequently produced biomass contained 43-53% w/w proteins and 16-25 % w/w total fatty acids.

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3.1 Introduction

Innovative, decentralized sanitation concepts have been the subject of several studies (Larsen and Gujer, 1997; Otterpohl et al., 1997; Zeeman et al., 2008). Although source separation concepts continue to be immature, various pilot projects have demonstrated their potential to save water, recover energy, and recover nutrients from black water and/or urine (de Graaff et al., 2010; de Graaff et al., 2011). In the Netherlands, several projects for new sanitation concepts have been established i.e. in 32 apartments in Lemmerweg-Oost in Sneek, a housing complex of 250 houses of Noorderhoek in Sneek, and an office building of 160 employees in Wageningen. Urine separation projects were established in several countries such as an office building of 60 employees (Sneek, the Netherlands), the Saniphos installation (Zutphen, the Netherlands), the building 'Villa Flora' (Venlo, the Netherlands), the head offices of the German technical co-operation GTZ (Eschborn, Germany), Kullön residential area (Vaxholm, Sweden), Universeum-science and Discovery Museum (Gothenburg, Sweden), and the EAWAG research institute (Zurich, Switzerland), etc (Kvarnström et al., 2006; Lienert and Larsen, 2009).

Because urine is rich in the nutrients, nitrogen and phosphorus, it has potential as a liquid fertilizer or as a nutrient source for the production of microalgae biomass which are a potential source of feedstock for the bio-based production of chemicals and biofuels (Wijffels et al., 2010), although, large-scale cultivation is not yet economically feasible. Coupling microalgae production to wastewater treatment is considered a more economic and sustainable option (Yang et al., 2011). In only a few studies human urine has been employed as the growth medium for microalgae cultivation. Although these studies have demonstrated that microalgae and cyanobacteria can grow and remove nutrients from urine, the studies were conducted in batch systems, and the urine or synthetic urine that was employed was extensively diluted from 50 to more than100 times (Adamsson, 2000; Dao-lun and Zu-cheng, 2006; Yang et al., 2008; Yang et al., 2008; Chang et al., 2013).

Studies have shown the feasibility to use microalgae to recover nutrients from different types of wastewaters (Cai et al., 2013). However, no knowledge has yet been made available regarding the potential of coupling microalgae cultivation with the treatment of concentrated urine. From a theoretical point of view, high cell density cultivation is required to afford high nutrient removal efficiencies from high strength wastewaters such as urine. This can only be achieved with short light-path reactor systems that effectively supply light to

all of the cells encapsulated inside the microalgal culture. In addition, the complex and partially unknown composition of concentrated urine may affect microalgae growth. Finally, with concentrated urine, hydrolysis of urea into ammonium will result in increased free ammonia concentrations which may inhibit microalgal growth (Azov and Goldman, 1982).

The aim of this study was to assess the minimum dilution factor that would allow for stable treatment of urine and concomitant microalgae production. In addition, it was investigated whether the process could be continuously operated at consistently high rates for nutrient removal and biomass production. For this purpose, *Chlorella sorokiniana* was selected as the inoculum as it had previously demonstrated significant potential in urine treatment. A flat panel photobioreactor with short light-paths of 1.0 and 0.5 cm was selected to support the high density microalgae cultivation. Finally, the possibility was explored to further stimulate biomass production by optimizing the urine N:P molar ratio and by supplementing magnesium.

3.2 Materials and Methods

3.2.1 Microorganism and urine media

Chlorella sorokiniana CCAP211/8K was obtained from the Culture Collection of Algae and Protozoa, Oban, UK. Pre-cultures were grown in M8a medium (Kliphuis et al., 2010) in 250-ml shake flasks with 100 mL of liquid culture at 25ºC. The microalgae were initially cultivated in a light intensity of 20-40 µmol-photons m^{-2} s^{-1} and a 16/8 h day/night cycle followed by continuous light of 165 µmol-photons m^{-2} s^{-1} and 2% CO_2 enriched air.

In Experiment 1, synthetic urine modified from Yang et al. (2008) was employed. It consisted of (per L): 0.11 g $(NH_4)_2SO_4$, 22.49 g NH_4HCO_3, 0.7 g K_2HPO_4, 0.75 g $Na_2HPO_4.2H_2O$, 0.53 g $CaCl_2.2H_2O$, 1.23 g $MgSO_4.7H_2O$, 1.4 g K_2SO_4, 9.6 g NaCl, 1 g creatine, 0.1 g phenol, 10 mL Fe-EDTA stock solution and 10 mL micronutrient stock solution. Fe-EDTA stock solution contained per L: 11.6 g EDTA ferric sodium salt and 3.72 g $Na_2EDTA.2H_2O$. Micronutrient stock solution consisted of (per 1 L): 1.3 g $MnCl_2.4H_2O$, 0.32 g $ZnSO_4.7H_2O$, 0.18 g $CuSO_4.5H_2O$, and 0.006 g H_3BO_3. The synthetic urine pH was adjusted to 7 prior to utilizing. In synthetic urine, creatine was mistakenly used instead of creatinine which is the second most-abundant organic metabolite in urine (Bouatra et al., 2013). However, this did not affect synthetic urine COD concentration since creatine and

creatinine both require equal amount of oxygen for their oxidation (the same COD input) (Kuntke, 2013).

In Experiments 2 and 3, various batches of urine were employed. Urine batch A was applied in Experiment 2; Urine batch B was administered in Experiment 3 from day 1 to 90; and batch C was employed from day 91 to the completion of Experiment 3. Urine batch A was directly collected using bottles from male employees from the Sub-department of Environmental Technology, Wageningen University, the Netherlands. Batch B was collected with urine diverting toilets from male and female employees of the Sub-department of Environmental Technology. Batch C was extracted from urine diverting toilets collecting urine from offices of a participating company (Landustrie BV, the Netherlands). The urine batches were maintained in the dark at 4ºC during all experiments. The composition of the various urine batches is depicted in Table 3.1. Non-diluted urine was supplemented with Fe-EDTA stock solution and micronutrient stock solution at a concentration level of each solution at 33.25 mL per liter. As demonstrated in Table 3.2, the urine with supplemental iron and micronutrients was diluted 50, 20, 10, 5, 3, and 2 times (1 urine volume in a total volume of 50, 20, 10, 5, 3, and 2, respectively). An addition of 0.02% (v/v) of antifoam® B (J.T.Baker) was combined with all diluted urine media.

Table 3.1 Composition of synthetic urine and different batches of human urine

Composition (g L^{-1})	Synthetic urine[/1]	Human urine		
		Batch A	Batch B[/2]	Batch C[/2]
Total nitrogen (TN)	4,326	7,167	4,358	5,310
Phosphate phosphorus (PO_4^{3-}-P)	255	466	200	260
Ammonium nitrogen (NH_4^+-N)[/3]	4,006	844	393	4,660
Chemical oxygen demand (COD)	-	8,349	2,886	5,160
N:P atomic ratio	38:1	34:1	48:1	45:1

[/1] calculated values based on synthetic urine composition

[/2] urine mixture of males and females from urine diverting toilet

[/3] concentration of ammonium nitrogen was measured at the beginning of the experiments

Table 3.2 Applied urine dilutions and light intensities for *C.sorokiniana* grown on synthetic urine (Experiment I) and human urine (Experiment II and III)

Experiment/time		Urine dilution (times) and treatment	Light intensity ($\mu mol\ m^{-2}\ s^{-1}$)	Average flow rate ($L\ d^{-1}$)
Experiment 1: Synthetic urine (HRT 0.92±0.04 d^{-1})				
Day	1-5	20	490	0.85
	6-13	10	490	0.95
	14-20	5	990	0.92
	21-29	2 (0.5P)[/1]	990	0.89
	30-55	2	1,800	0.97
	56-72	2 (2P)[/2]	1,800	0.92
Experiment 2: Male urine (batch A) (HRT 1.0±0.02 d^{-1})				
Day	1-12	50	1,180	0.91
	13-19	20	1,180	0.92
	20-35	10	1,180	0.90
	36-44	5	1,180	0.90
	45-66	5	1,500	0.91
Experiment 3: Male and female urine (batch B and C) (HRT 1.0±0.02 d^{-1})				
Day	1-8	10	1,050	0.97
	9-57	5	1,050	0.95
	58-64	5	1,540	0.94
	65-76	2	1,540	0.97
	77-142	3	1,540	0.94
	143-170	3 (N:P 36:1 +Mg^{2+}[/3])	1,540	0.97
	171-190	3 (N:P 36:1 +Mg^{2+}[/3])	2x1,550[/4]	0.91
	191-219	3 (N:P 25:1 +Mg^{2+}[/3])	2x1,550[/4]	0.91
	220-239	3 (N:P 15:1 +Mg^{2+}[/3])	2x1,550[/4]	0.93
	240-247	3 (N:P 15:1, +Mg^{2+}[/5])	2x1,550[/4]	0.91
	248-269	3 (N:P 23:1, +Mg2+/6)	2x1,550/4	0.94

[/1] phosphorus concentration was halved

[/2] phosphorus concentration was doubled

[/3] magnesium concentration with supplemental magnesium in the final diluted urine was 17.3 mg L^{-1}

[/4] illuminating both sides of the reactor

[/5] magnesium concentration with supplemental magnesium in the final diluted urine was 28.5 mg L^{-1}

[/6] magnesium concentration with supplemental magnesium in the final diluted urine was 21.8 mg L^{-1}

3.2.2 Photobioreactors and culturing system

In experiment 1, a flat panel photobioreactor, PBR 1, was employed. The influent and effluent lines, as well as the pH sensor, were situated on top of the reactor whereas the temperature sensor was inserted from the side. The reactor was aerated along the entire bottom surface, and the air was enriched with carbon dioxide (CO_2). In Experiments 2 and 3, an additional flat panel photobioreactor, PBR 2, was employed. This photobioreactor (PBR) was only aerated over two-thirds of its width which created an air-lift mixing, resulting in a thoroughly mixed liquid with no stagnant zones. To further strengthen this mixing pattern, the riser and down comer were separated by a baffle. The air was once again enriched with CO_2. PBR 1 and 2 are exhibited in Figure 3.1a. Both reactors possess a light path of 10 mm. The illuminated surface area of PBR 1 was 0.140 m^2, and the illuminated area of PBR 2 was 0.0904 m^2. The working volume of PBR 1 was 1 L. Due to sub-atmospheric pressure that developed within the water jacket, PBR 2 experienced a working volume of 0.925 L during Experiment 2, and this volume ranged between 0.930 and 0.950 L during Experiment 3.

Figure 3.1b demonstrates a schematic diagram of the complete microalgae culturing system consisting of 6 components; the photobioreactor, influent and effluent pumps, a temperature control system, a pH controller, an aeration system, and a light source. The photobioreactor consists of two chambers including a water jacket for temperature control and a cultivation chamber. Peristaltic pumps (Watson Marlow 120U, Watson-Marlow pumps, UK) were employed to feed and withdraw the influent and effluent. The influent and effluent bottles were placed in a refrigerator and maintained at 4ºC in the dark to prevent urine hydrolysis. The culture temperature was controlled via a temperature sensor located inside the photobioreactor which was connected to a waterbath (Julabo-F25-HE, Julabo Labortechnik GMBH, Germany) circulating water through the water jacket. The temperature was maintained at 35ºC for Experiment 1 and 38ºC for Experiments 2 and 3.

The culture pH was controlled at 7.0±0.2 with 2M HCl and 2M NaOH solutions. Air enriched with 5% v/v CO_2 was regulated by mass flow controllers and fed into the reactor to serve as a carbon source as well as to provide mixing. The employed aeration rates were 1 L L^{-1} min^{-1} (Experiment 1); 0.76 L L^{-1} min^{-1} (Experiment 2); and 0.67 L L^{-1} min^{-1} (Experiment 2 and Experiment 3).

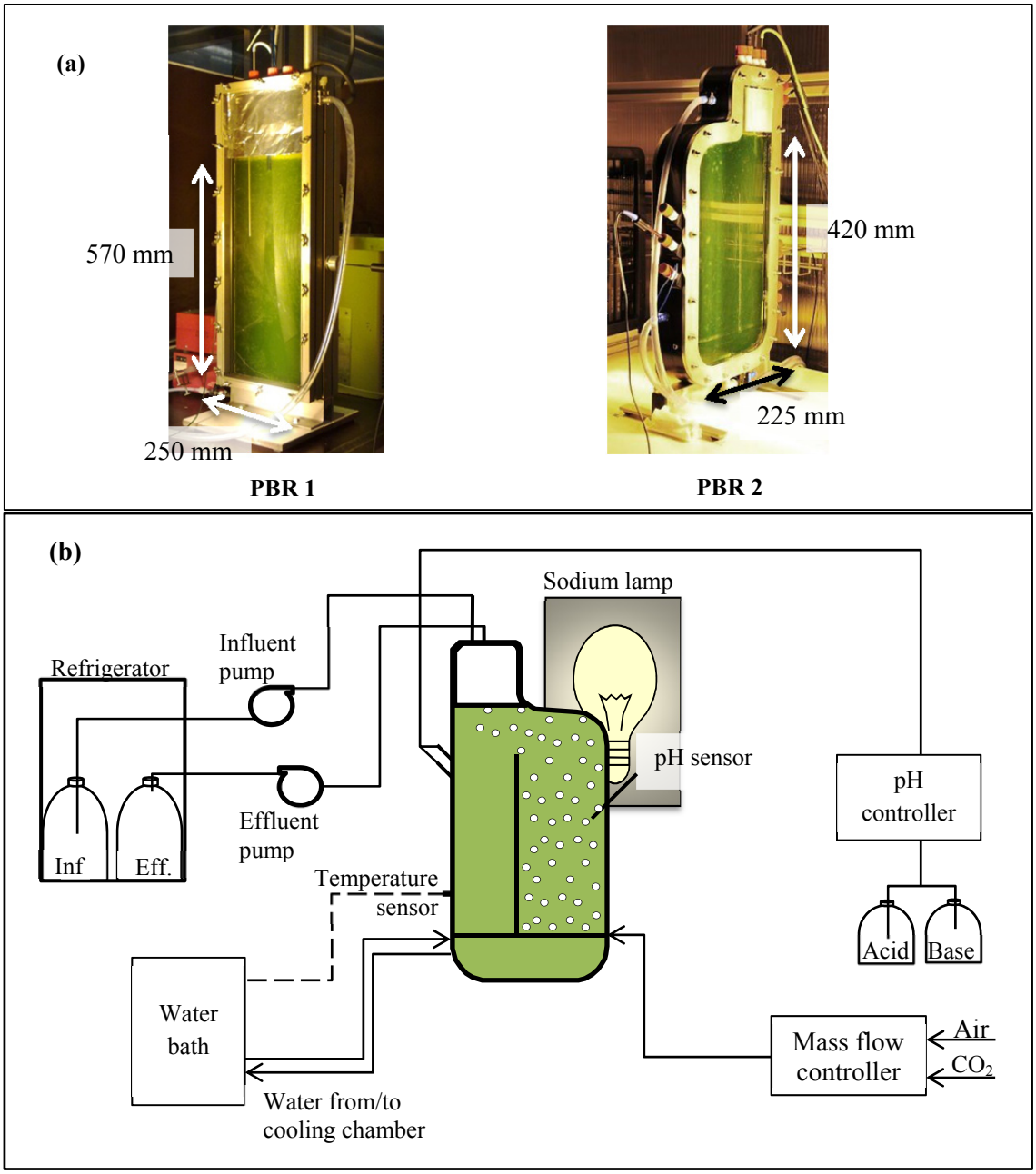

Figure 3.1 (a) Flat panel photobioreactors used for cultivation of *Chlorella sorokiniana* on synthetic urine (PBR 1) and on human urine (PBR 2) and (b) schematic diagram of the complete microalgae culturing system with auxiliary equipment

The photobioreactor was continuously illuminated with high-pressure sodium lamps (MIG 400W, LEG Illumination, Italy). The light intensity was measured at various sites which were distributed homogeneously inside an empty growth chamber of the reactor before and after the experiments utilizing a 2π PAR quantum sensor (LI-190SA, LiCOR, USA) connected to a Li-COR handheld meter (LI-250A, Li-COR, USA). Reported light intensity

was averaged over the reactor surface. To prevent evaporation from the microalgae culture, a condenser was installed at the gas outlet which was cooled with water at a temperature of 4°C. The average hydraulic retention time (HRT) was maintained at 1 day. Biofilms on the bioreactor surface were withdrawn daily employing a teflon-coated magnetic stirring bar situated within the reactor. Table 3.2 depicts the applied urine dilutions and light intensities (photon flux density; PFD) during each experiment.

3.2.3 Biomass density and dry weight determination

The biomass density was followed by measuring the optical density (OD) at 680 and 750 nm with an Ultraspec 2000 spectrophotometer (Pharmacia Biotech Ltd., UK) for Experiment 1 and a Xion 500 spectrophotometer (Hach Lange, Germany) for Experiment 2 and 3. Based on corresponding measurements of the dry weight concentration, a calibration factor between dry weight and OD_{750} was calculated and used to estimate the dry weight concentration from OD_{750}.

Dry weight was determined by collecting suspended biomass on Whatman GF/F glass microfiber filters (Ø 55 mm, pore size 0.7 µm). Pre-washed and pre-dried filters were first weighed. Aliquots of 5 mL of culture broth withdrawn from the reactor were diluted 15 times with pre-filtered, demineralized water. The diluted samples were subsequently filtered followed by washing with 50 mL of pre-filtered, demineralized water to remove adhering inorganic salts. Filters were dried overnight at 105ºC and cooled in a desiccator for a minimum of 2 hours. The dry weight was expressed as the difference in weight of the dry filters and the dry filters containing microalgae.

3.2.4 Microalgae settling efficiency

Microalgae cultures were diluted in a cuvette, blended, and left to settle at room temperature in the dark in a spectrophotometer (DU730 spectrophotometer, Beckman Coulter Inc., US). The optical density at 750 nm (OD_{750}) was monitored during the settling period. The following equation was employed to calculate the settling efficiency;

$$Settling\ efficiency\ (\%) = \frac{OD_{750}(t_0) - OD_{750}(t)}{OD_{750}(t_0)}$$

where $OD_{750}(t_0)$ is the OD_{750} at time zero and $OD_{750}(t)$ is the OD_{750} at time t. The size of microalgal cells and flocs in the suspension was measured utilizing a Mastersizer (Hydro 2000SM, Malvern, AU).

3.2.5 Elemental composition of biomass

Biomass accumulated overnight was harvested by centrifugation at 3,620 relative centrifugal force (rcf) for 10 min at 5°C. The pellet was washed with demineralized water and once again centrifuged. This washing step was repeated twice. The pellets were stored at -20°C prior to freeze drying overnight. Freeze dried biomass was manually ground into a fine powder and subsequently transferred to glass serum bottles, freeze dried overnight, and sealed with a metal cap under vacuum. The carbon (C), hydrogen (H), and nitrogen (N) content was analyzed in duplicate by combustion at 960°C using an elemental analyzer (ThermoQuest Interscience, Breda, the Netherlands). The ash content of the freeze-dried biomass was ascertained by burning it at 550°C in an oven to remove all organic materials. Phosphorus (P), sulphur (S), and magnesium (Mg) content was established utilizing the ICP-OES (VISTA-MPX, Varian Inc., Australia) after acid extraction of the freeze-dried biomass with a mixture of 7.5 mL hydrochloric acid (37%) and 2.5 mL nitric acid (65%). During extraction, the total volume of the sample, water, and acid mixture was 20 mL and, following extraction, the volume was conformed to 100 mL with demineralized water. The O content and dry biomass molar mass were calculated in accordance with Duboc et al. (1999).

3.2.6 Total fatty acid content of biomass

Extraction of total fatty acids was modified in accordance with Lamers et al. (2010). 5-10 mg of ground and freeze-dried biomass was further disrupted by bead beating with a Precellys 24 bead beater (Bertin Technologies, France). Biomass was placed into bead beating tubes (Lysing Matrix E, MP Biomedicals, Germany), and 1 mL of chloroform/methanol mixture (2:2.5) containing 45 μg mL^{-1} of the internal standard tripentadecanoin (Sigma Aldrich T4257) was added to each tube. Eight beating cycles of 60 s and 2,500 rpm were performed with 120 s pauses in between. The homogenates were transferred to 15 mL-glass tubes with an additional 3 mL of the chloroform/methanol mixture containing internal standard employed for rinsing the bead beating tubes. The 4 mL homogenates were then treated according to the steps provided in supplemental material from

Santos et al. (2012). The analysis and quantification of total fatty acids were conducted in accordance with Santos et al. (2012).

3.2.7 Protein content of biomass

Ground and freeze dried biomass was employed for protein analysis. Cell disruption was accomplished by bead beating. An amount of 10-20 mg of biomass was added to each bead beating tube together with 1 mL of cell lysisbuffer (pH 7-9). The cell lysis buffer consisted of 7.272 g L^{-1} tris(hydroxymethyl)-aminomethane and 20 g L^{-1} of sodium dodecyl sulphate. Three beating cycles of 60 s and 6,500 rpm were performed with 120 s pauses in between. The homogenates were subsequently transferred to 15 mL-glass tubes with an additional 1 mL of cell lysisbuffer employed for rinsing the bead beating tubes. The homogenates were vortexed, incubated at 100°C for 30 min, allowed to cool to room temperature, and finally centrifuged at 3,500 rpm for 10 min. The supernatant was then analyzed for protein content utilizing the BioRad *DC* protein assay (BioRad Laboratorie, Inc., USA). Bovine serum albumin (BSA) was assigned as a standard protein. The absorbance at 750 nm of protein standard and samples was measured with a BioTek EL800 plate reader.

3.2.8 Nutrient analyses

The samples withdrawn from the effluent tube were centrifuged at 10,000 rpm for 10 min, and the supernatant was maintained at 4ºC prior to analyses. Synthetic urine samples were analyzed for ammonium (NH_4^+-N) and phosphate (PO_4^{3-}-P) according to Standard Methods (APHA, 1998) using a continuous flow analyzer (SKALAR). Actual urine samples were analyzed photometrically for total nitrogen (TN), ammonium nitrogen (NH_4^+-N), total phosphorus (TP), and chemical oxygen demand (COD) according to Standard Methods (APHA, 1998) using Dr Lange® test kits (Hach Lange GMBH, Germany).

3.3 Results and Discussion

3.3.1 pH controlling system

pH is one of important parameters affecting microalgae growth. *C.sorokiniana* has shown a high specific growth rate within the pH range from 4 to 7 (Morita et al., 2000). When urine is collected and stored, urea is hydrolyzed and the urine pH increases up to

around pH 9 (Udert et al., 2003). Concomitantly with elevating pH, free ammonia concentration increases up to the level that can inhibit microalgae growth (1.2 mM) (Azov and Goldman, 1982; Tuantet et al., 2013). In principle all the ammonia (or urea) entering the photobioreactor should be consumed by the microalgae and built into microalgae biomass. In our experiments discussed later we optimized process conditions and there was still substantial nitrogen not taken up in the effluent. Based on the pH control the alkalinity was directly neutralized requiring acid. But, in an optimized process this ammonia would have been taken up directly by the microalgae and built in the biomass with minimal pH effect for the water phase and we will come back to this later.

3.3.2 Biomass productivity and nutrient removal

Experiment 1: Chlorella sorokiniana *cultivated in synthetic urine*

In this experiment, *Chlorella sorokiniana* was cultivated in synthetic urine in a short light-path (10 mm) photobioreactor in order to detect the minimum dilution factor that would continue to allow for an efficient removal of nutrients and high production of microalgae biomass with ammonium as the nitrogen source. Different dilutions were applied beginning with 20 times and ending with 2 times diluted synthetic urine. In addition to decreasing urine dilutions, increased light intensities were applied to compensate for microalgae self-shading at higher biomass densities.

Table 3.2 exhibits the experimental settings during the different experiments, including the applications of urine dilutions and light intensities. Figure 3.2 exhibits biomass density, influent and effluent NH_4^+-N and PO_4^{3-}-P concentrations, and the removal efficiencies for nitrogen and phosphorus. The biomass densities and nutrient removal efficiencies were averaged over the respective periods. The biomass density increased with decreasing synthetic urine dilutions and concomitantly increasing light intensity. The average biomass density at 20 times diluted synthetic urine and a light intensity of 490 μmol-photons m^{-2} s^{-1} was 2.9 g-dw L^{-1} but increased to 6.0 g-dw L^{-1} at 5 times dilution and 990 μmol-photons m^{-2} s^{-1}.

During the first time frame when 2 times diluted synthetic urine was applied, the phosphorus (as phosphate) concentration was mistakenly halved, resulting in a decrease in biomass density to 5.1 g-dw L^{-1}. With increased light intensity and a corrected phosphate

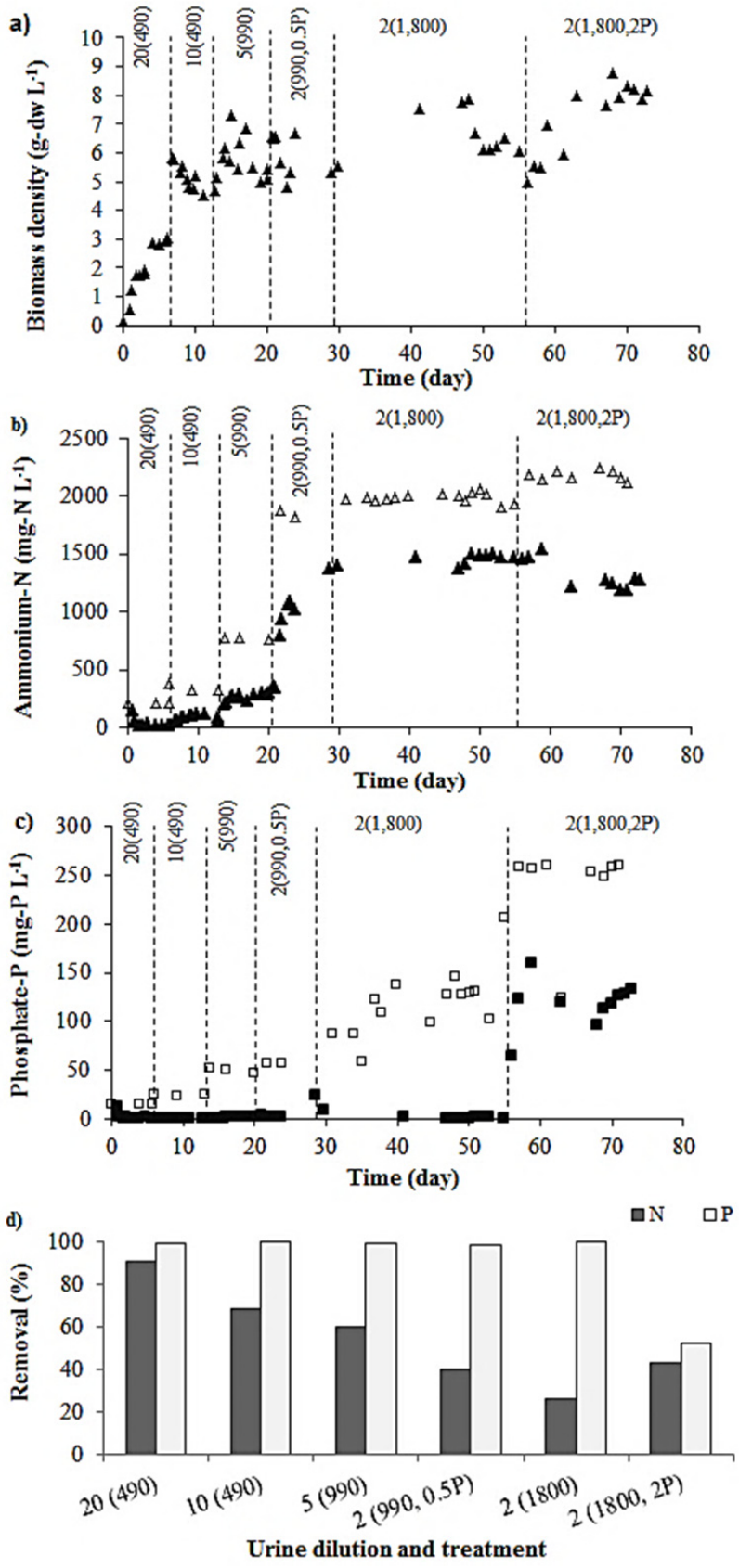

Figure 3.2 Experiment 1: cultivation of *C.sorokiniana* grown on synthetic urine. Time course of (a) biomass density; (b) concentration of influent (△) and effluent (▲) ammonium nitrogen; (c) concentration of influent (□) and effluent (■) phosphate; (d) removal efficiency of nitrogen and phosphorus (x-axis shows urine dilution and, in brackets, light intensity, and phosphorus supply)

concentration, the biomass density recovered to 6.2 g-dw L^{-1}. Because of the higher N:P molar ratio of synthetic urine (38:1) compared to the average algal N:P ratio known as Redfield ratio (16:1), phosphorus consistently restricted microalgae growth. When the phosphate concentration of the synthetic urine was doubled once again to 255 mg-P L^{-1}, the biomass increased to 8.1 g-dw L^{-1} with a light intensity of 1800 µmol-photons m^{-2} s^{-1}. The maximum volumetric biomass productivity with this urine dilution was 7.5 g-dw L^{-1} d^{-1}.

Because phosphorus limited microalgae growth, phosphate removal was almost 100% during the entire experiment except when the phosphate concentration was increased to 255 mg-P L^{-1} at the end of the experiment at 2 times diluted synthetic urine. The ammonium removal, on the other hand, decreased with an increasing urine load (Figure 3.2d). With 2 times diluted urine, nearly all of the phosphate was removed, but the removal of ammonium significantly decreased. The N:P molar ratio of nitrogen and phosphorus taken up by the culture reduced from >20:1 to 7:1, indicating that phosphorus was no longer the limiting nutrient. The observation that phoshorus removal was 100% but, at the same time, was not the limiting nutrient may be explained by possibly precipitation or luxury uptake of phosphorus, a phenomenon known to occur at increased light intensities and temperatures (Powell et al., 2009).

When the phosphate concentration was doubled toward the end of Experiment 1, the biomass density and ammonium removal increased. The ratio of N to P removal also increased again from 7:1 to 16:1. As phosphate was not depleted, another factor must have limited microalgae growth and concomitant nutrient uptake. Most likely, at this stage, the culture was growing under limited light conditions. The maximum volumetric removal rates of ammonium and phosphate in this experiment were 865 mg-N L^{-1} d^{-1} and 123 mg-P L^{-1} d^{-1}, respectively. This experiment regarding synthetic urine illustrates the potential to cultivate microalgae in concentrated urine at increased nutrient removal efficiencies and high volumetric biomass productivities.

Experiment 2: Chlorella sorokiniana *grown in human urine-batch A*

To investigate whether microalgae can be cultivated in human urine and if high biomass productivities and nutrient removal efficiencies can be maintained, *Chlorella sorokiniana* was cultivated in various dilutions of human urine. Urine concentration is shown in Table 3.1 and Table 3.2 exhibits the urine dilutions and light intensities that were applied

during this experiment. The results in Figure 3.3 demonstrate that *C.sorokiniana* was able to grow in human urine and that the biomass density increased with decreasing urine dilutions and increasing light intensities. The biomass density increased from 1 g-dw L^{-1} in 50 times diluted urine at a light intensity of 450 µmol-photons m^{-2} s^{-1} to 8.1 g-dw L^{-1} in 5 times diluted urine at 1,500 µmol-photons m^{-2} s^{-1}. The biomass productivity at 5 times diluted urine was 8.6 g-dw L^{-1} d^{-1}.

The removal efficiency of total phosphorus was approximately 100% during the entire experiment which is not surprising as the human urine contained an N:P molar ratio of 34:1, and phosphorus plausibly limited microalgae growth. When the urine dilution was decreased from 50 to 10 times, the biomass density increased with a stable removal rate of total nitrogen between 58% and 62%. However, when 5 times diluted urine was applied, total phosphorus removal only minimally decreased whereas total nitrogen removal substantially declined, indicating a factor other than phosphorus was limiting microalgae growth. Increasing the light intensity from 1,180 to 1,500 µmol-photons m^{-2} s^{-1} resulted in an increased biomass density and improved nitrogen and phosphorus removal efficiencies. Apparently, light continued to limit algal growth at intensity of 1180 µmol-photons m^{-2} s^{-1} and a biomass density of 6.5 g-dw L^{-1}. With 5 times diluted urine and a light intensity of 1,500 µmol-photons m^{-2} s^{-1}, the removal efficiencies for total nitrogen and total phosphorus were 70% and more than 99%, respectively. This corresponds to a total nitrogen removal rate of 1,125 mg-N L^{-1} d^{-1} and a total phosphorus removal rate of 99 mg-P L^{-1} d^{-1}.

Not only nitrogen and phosphorus were removed from the urine. Organic matter, expressed as COD, experienced a removal efficiency of 71% which occurred toward the end of the experiment with a corresponding removal rate of 1,354 mg-COD L^{-1} d^{-1}. Figures 3.3(d) and (e) exhibit a development of COD removal during the cultivation period. Although *Chlorella sorokiniana* was the dominant microorganism in the culture, it was evident that a heterotrophic bacterial population capable of degrading the organic pollutants in urine gradually developed in the reactor.

This experiment with human urine performed similarly to the synthetic urine in Experiment 1 whereby the microalgae were able to grow and maintain high nutrient removal efficiencies and a high volumetric productivity at low urine dilutions. The high concentrations of organic compounds in the urine of 8,350 mg-COD L^{-1} and the ammonium

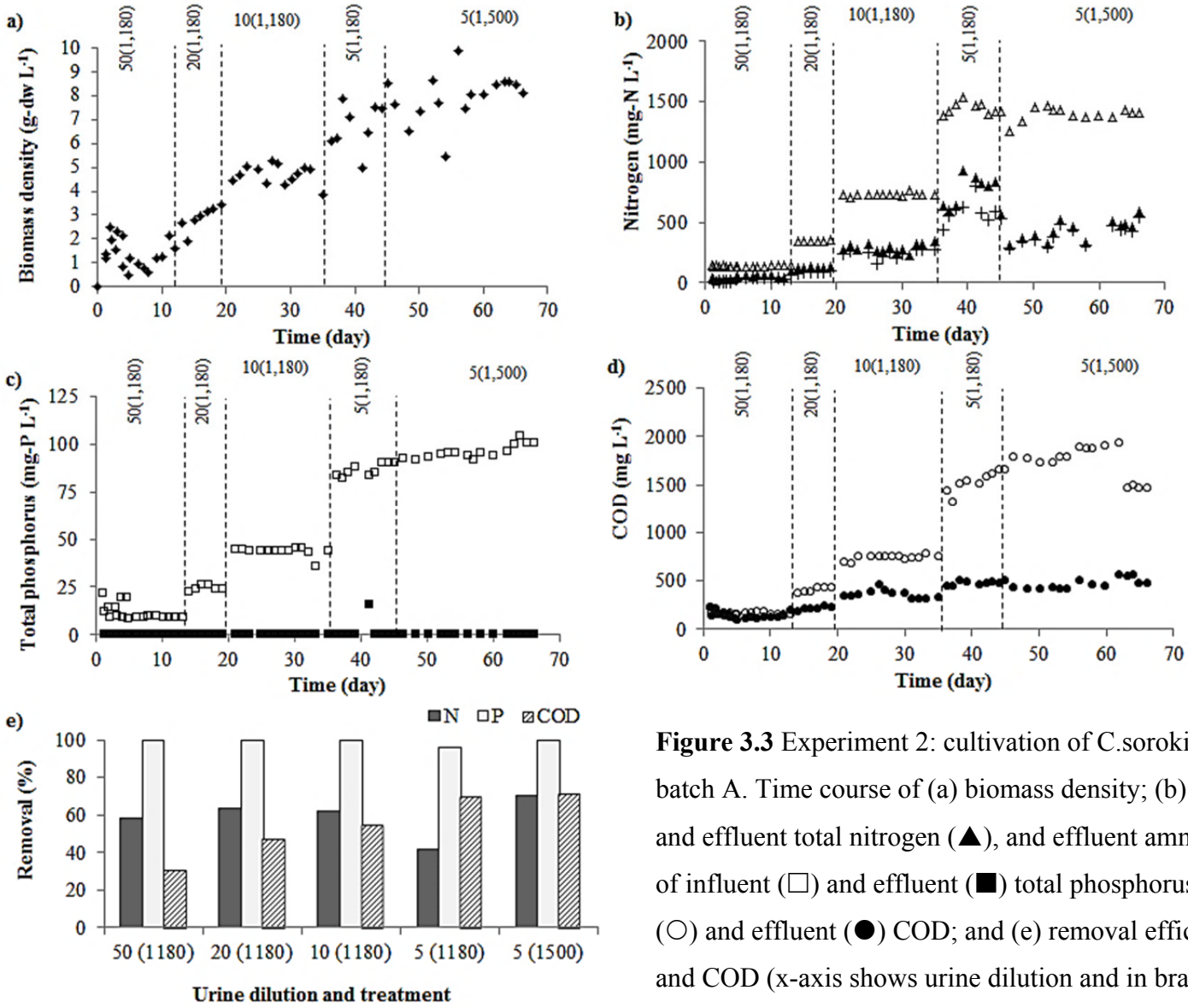

Figure 3.3 Experiment 2: cultivation of C.sorokiniana grown on human urine batch A. Time course of (a) biomass density; (b) concentration of influent (△) and effluent total nitrogen (▲), and effluent ammonium (+); (c) concentration of influent (□) and effluent (■) total phosphorus; (d) concentration of influent (○) and effluent (●) COD; and (e) removal efficiency of nitrogen, phosphorus, and COD (x-axis shows urine dilution and in brackets, light intensity)

concentration of 7,167 mg-N L^{-1} (600 mg-N L^{-1} within the reactor) did not negatively affect microalgae growth. The nitrogen removal rate in this study is comparable to that reported by Udert et al. (2003) with a series of nitritration and anaerobic ammonium oxidation processes. Udert and co-workers demonstrated a total nitrogen removal rate from urine of 75-85% or 1,000-1,300 mg-N L^{-1} d^{-1} concomitantly with 82% of COD removal. This study reveals a potential utilization of microalgae for considerable high nitrogen removal efficiency and complete phospohrus removal. In addition, opportunities remain to enhance the biomass productivity and nutrient removal efficiency.

Experiment 3: Chlorella sorokiniana *grown in human urine batches B and C*

This experiment was designed to determine the minimum dilution factor for removal of nutrients from human urine and to maximize the biomass productivity and nutrient removal efficiency. Table 3.1 shows concentrations of urine batch B and C, and Table 3.2 presents the urine dilutions and light intensities that were applied. With decreasing urine dilutions and increasing light intensities, biomass density increased from 3.8 g-dw L^{-1} in 10 times diluted urine and a light intensity of 1,050 µmol-photons m^{-2} s^{-1} to 6.6 g-dw L^{-1} in 2 times diluted urine and 1,540 µmol-photons m^{-2} s^{-1}. Similar to Experiment 2, a removal of phosphorus (as total phosphorus) of almost 100% was observed during the entire experiment. Total nitrogen removal efficiency increased from 47% with 10 times diluted urine to 51% with 5 times diluted urine. When 2 times diluted urine was applied, the biomass density slightly increased, but this was accompanied by a significant decrease in total nitrogen removal efficiency from 51% to 36 %, and the ammonium concentration increased to 1.2 g-NH_4^+-N L^{-1}. To prevent ammonia toxicity, 3 times diluted urine was applied which resulted in a significant increase of biomass density to 9.7 g-dw L^{-1}. The change in dilution was of the urine known as batch B to batch C.

In the new batch of urine (batch C), urea was already substantially hydrolyzed to ammonium (4.7 g-NH_4^+-N L^{-1} of 5.3 g-TN L^{-1}) and magnesium was almost nonexistent. Because supplemental magnesium is known to support dense cultures for microalgae cultivation in urine (Tuantet et al., 2013), extra magnesium was continuously fed to the reactor, leading to a magnesium concentration in the diluted urine medium of 17 mg-Mg^{2+} L^{-1}. With batch C and 3 times dilution, several factors were tested to maximize biomass productivity and nutrient removal efficiencies: (1) lowering N:P molar ratio by adding

phosphate beginning at 45:1 to 14:1; (2) shortening the light path from 10 to 5 mm; and (3) further increasing the magnesium supply from 1.4 mg-Mg^{2+} g-dw^{-1} to 1.9 and 1.5 mg-Mg^{2+} g-dw^{-1}.

First, the N:P ratio was reduced from 45:1 to 36:1. As a result, removal of total nitrogen slightly increased from 58% to 62%, but biomass density decreased from 9.7 to 9.0 g-dw L^{-1}. This indicates a factor other than phosphorus, probably light, was limiting algae growth. Therefore, the light path was shortened from 10 to 5 mm by illuminating the photobioreactor from both sides. This significantly increased the biomass density (from 9.0 to 12.6 g-dw L^{-1}) and total nitrogen removal (from 62 to 76%), proving that light, indeed, had been the limiting factor with a light path of 10 mm. Subsequently lowering the N:P ratio to 25:1 and 14:1 did not result in obtaining an increased biomass density nor removal of total nitrogen. In contrast, an increase of the magnesium supply from 1.4 to 1.9 mg-Mg^{2+} g-dw^{-1} raised the biomass density from 12.7 to 15.4 g-dw L^{-1} and the nutrient removal efficiency from 73% to 87% for total nitrogen and 57% to 76% for total phosphorus. This strongly suggested the urine itself contained insufficient amounts of magnesium to sustain satisfactory phototrophic growth.

At this point, the biomass density achieved a maximum of 15.4 g-dw L^{-1}. The removal of total nitrogen and total phosphorus were 87% and 76%, respectively, corresponding to the removal rates of 1,479 mg-N L^{-1} d^{-1} and 163 mg-P L^{-1} d^{-1}, respectively. However, the effluent still contained certain nutrients. Finally, by adjusting the N:P ratio to 23:1 with a magnesium supply of 1.5 mg-Mg^{2+} g-dw^{-1}, an increased total phosphorus removal occurred with a relatively high biomass density and total nitrogen removal efficiency (Figure 3.4a and e). Although an average N:P ratio of microalgae biomass of 16:1 was mentioned by Ho and co-workers (2003), the optimum N:P ratio to maximize biomass productivity and nutrient removal in this study was approximately 23:1 with a magnesium supply of 1.5 - 1.8 mg-Mg^{2+} g-dw^{-1} which lies within the range of magnesium required for microalgae in general (0.5-75 mg-Mg^{2+} g-dw^{-1}) (Grobbelaar, 2004). This is, however, relatively low compared to the magnesium content reported for *Chlorella sp*. which ranges between 0.36 and 0.8% on a dry weight basis (Oh-Hama and Miyachi, 1988).

A substantial COD removal efficiency of 59-66 % was also observed. Similar to Experiment 2, the COD removal efficiency gradually increased during the experiment and

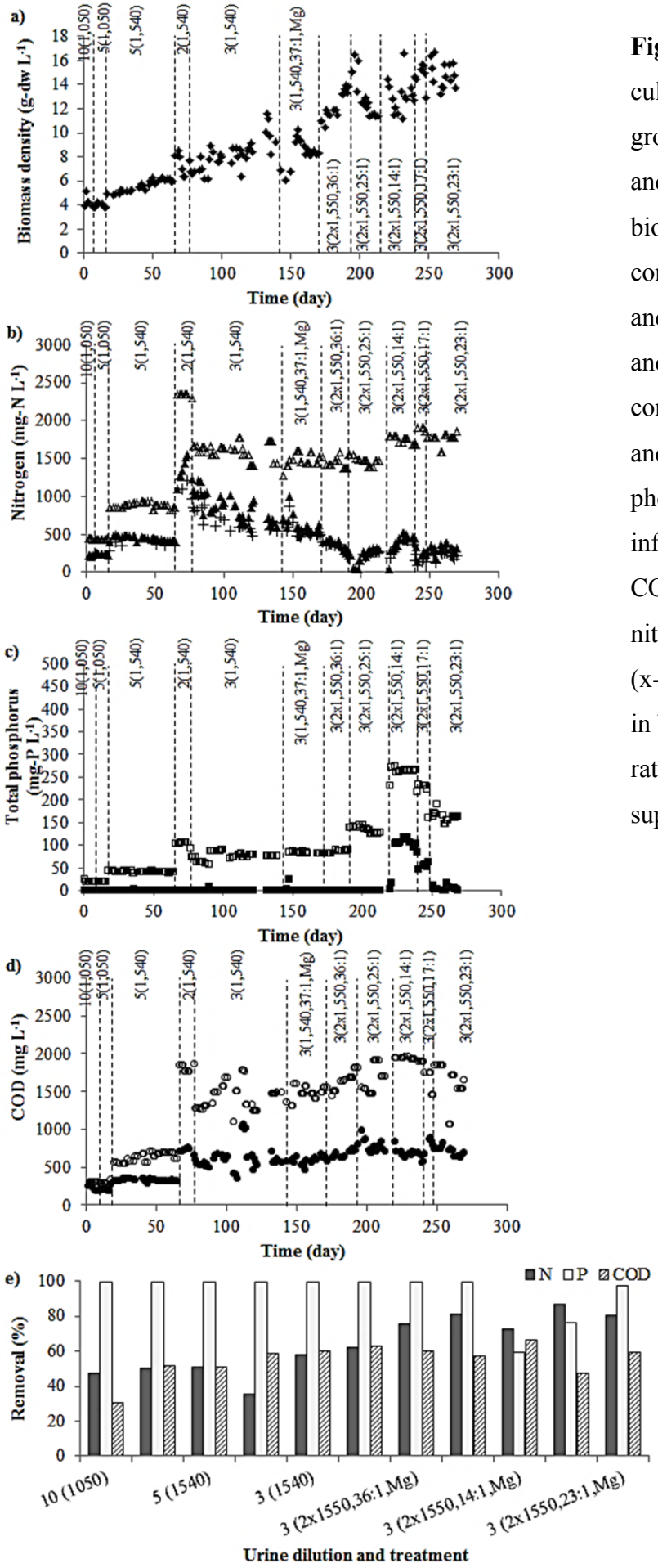

Figure 3.4 Experiment 3: cultivation of *C.sorokiniana* grown on human urine batch B and C. Time course of (a) biomass density; (b) concentration of influent (△) and effluent total nitrogen (▲), and effluent ammonium (+); (c) concentration of influent (□) and effluent (■) total phosphorus; (d) concentration of influent (○) and effluent (●) COD;(e) removal efficiency of nitrogen, phosphorus, and COD (x-axis shows urine dilution and, in brackets, light intensity, N:P ratio, and magnesium supplement)

became stable at 3 times diluted urine. Bacteria was not inoculated into the system, a gradual increase in COD removal could be due to the development of bacterial community over time. However, a slightly lower COD removal was observed in this experiment (66% compared to 71% in Experiment 2) possibly due to a variation in biodegradable COD fractions from different urine batches. Due to the short hydraulic retention time (1 day) of the system, only the easily biodegradable COD fraction (or BOD; Biological Oxygen Demand) could be removed. Therefore, the COD removal efficiency was influenced by the BOD of urine. The substantial biodegradability of human urine is due to the presence of aliphatic compounds, and reported BOD of urine lies within a range of 67-85% of the urine's COD (Udert et al., 2003; Kuntke, 2013). The highest COD removal in Experiment 3 was at a N:P ratio of 14:1, i.e., 66% removal or 1,137 mg-COD L^{-1} d^{-1}. At high biomass densities of 14.5 - 15.4 g-dw L^{-1}, COD removal somewhat diminished. This could possibly be due to low sludge retention time being applied or because the microalgae began competing with heterotrophic bacteria for space or certain micronutrients since phosphorus and nitrogen were not completely removed. Another possible cause is bacteria inhibitory substances released by microalgae (Cole, 1982).

When comparing results of 5 times diluted urine batch A of Experiment 2 with 3 times diluted urine batch B and C from Experiment 3, nutrient concentrations were comparable and higher biomass density was obtained from Experiment 3 with slightly lower nitrogen removal. Hydrolyzed urine did not significantly affect biomass productivity and nutrient removal when ammonium concentration within the reactor was maintained below 1 g-N L^{-1}. However, with hydrolyzed urine there is the need to neutralize the culture pH. With system optimization i.e. at a N:P ratio of 23:1, the acid used was minimal. Table 3.3 shows nutrient and ionic species balance of microalgae cultivation on hydrolyzed urine when the process was optimized. The average microalgae composition ($CH_{1.75}O_{0.42}N_{0.15}P_{0.008}$) was used. On day 266, only 1.4 mM of acid was needed to neutralize the pH in combination with 25.5 mM of additional carbonic acid (H_2CO_3) originating from the dissolution of excess CO_2 gas. This amount is equivalent to 4% of the CO_2 required for microalgae growth. Hydrolysis of urea increases urine alkalinity (reaction 3) whereas microalgae growth on ammonium (NH_4^+) leads to proton release (reaction 5) neutralizing alkalinity. In this way, the N:P ratio of urine and microalgae biomass both influence the amount of alkalinity and proton produced, and hence, the acid need to neutralize the system alkalinity. With optimization of

the urine N:P ratio and enhancing microalgal biomass density, the acid need can be significantly minimized. An additional factor which will help stabilize the pH in case there is ammonia left is the dissolution of excess CO_2 in the water creating carbonic acid. This excess CO_2 is also mandatory to support rapid growth of microalgae.

Table 3.3 Balance of nutrient and ionic species for microalgae cultivation on hydrolyzed urine when the process was optimized

Influent: hydrolysed urine	
pH	9.00
TN	127.07
NH_3	60.44
NH_4^+	44.13
HCO_3^-	52.29
TP	5.21
HPO_4^{2-}	5.21

Unit: mmol L^{-1}

Other additions	
H^+ (pH control)	1.40
CO_2 (from gas, for growth)	587.10
H^+ (from CO_2 gas)	31.54
HCO_3^- (from CO_2 gas)	31.54

Reactor	Consumed	Produced
TN	104.71	-
NH_3	60.24	-
NH_4^+	29.26	-
biomass-N	-	95.91
TP	5.07	-
HPO_4^{2-}	5.07	-
$H_2PO_4^-$	-	0.07
biomass-P	-	5.12
HCO_3^-	52.29	-
CO_2	587.10	-
biomass-C	-	639.39
H^+ (reaction 3)	31.54	-
H^+ (pH control)	1.40	
H^+ (reaction 5)	-	27.70
OH^- (reaction 2 and 4)	-	55.50
OH^- (reaction 1)	-	5.14

Effluent	
pH	7.00
TN	22.36
NH_3	0.20
NH_4^+	14.87
TP	0.14
HPO_4^{2-}	0.07
$H_2PO_4^-$	0.07
HCO_3^-	31.54
biomass-C	639.39
biomass-N	95.91
biomass-P	5.12

Note: because averaged N content was used 7% of TN is missing from N balance and 2% of ionic charge is missing from the charge balance

Reactions in Reactor

1) $\mathbf{H}^+ + HPO_4^{2-} \rightarrow H_2PO_4^-$

2) $HCO_3^- \rightarrow CO_2 + \mathbf{OH}^-$

3) $CO_2(g) \rightarrow CO_2(aq) + H_2O \rightarrow HCO_3^- + \mathbf{H}^+$

4) Biomass grown on NH_3: $CO_2 + 0.15NH_3 + 0.008H_2PO_4^- + 0.646H_2O \rightarrow CH_{1.75}O_{0.42}N_{0.15}P_{0.008} + 1.125O_2 + \mathbf{0.008OH}^-$

5) Biomass grown on NH_4^+: $CO_2 + 0.15NH_4^+ + 0.008H_2PO_4^- + 0.638H_2O \rightarrow CH_{1.75}O_{0.42}N_{0.15}P_{0.008} + 1.125O_2 + \mathbf{0.142H}^+$

3.3.3 Volumetric and areal productivity

Figure 3.5 compares the volumetric biomass productivities obtained during the three experiments in synthetic and actual human urine. The productivity of microalgae grown in synthetic urine with ammonium as the nitrogen source increased with decreased urine dilutions and increasing light intensities. Additional phosphorus enhanced productivity when more concentrated synthetic urine was employed. In actual urine, a similar trend was observed with increasing volumetric productivities at lower urine dilutions and increasing

light intensities. When 2 times diluted urine was applied, the biomass productivity increased only marginally, possibly due to increased ammonia concentration within the reactor. Decreasing the urine loading to 3 times diluted urine resulted in higher productivity. More light and magnesium indicated an improvement in biomass productivity and nutrient removal. In this study, the highest productivity of *C.sorokiniana* grown in synthetic and actual urine obtained in the 10 mm light-path photobioreactor were 7.5 and 9.3 g-dw L^{-1} d^{-1}, respectively, which corresponds to 74.6 and 93.3 g-dw m^{-2} d^{-1} where the surface area represents the illuminated photobioreactor surface. With a light path of 5 mm, an adjusted N:P molar ratio and additional magnesium, the productivity with actual urine increased to 14.8 g-dw L^{-1} d^{-1}. The areal productivity with the light path of 5 mm, however, decreased to 74 g-dw m^{-2} d^{-1} due to the doubling of the illuminated area.

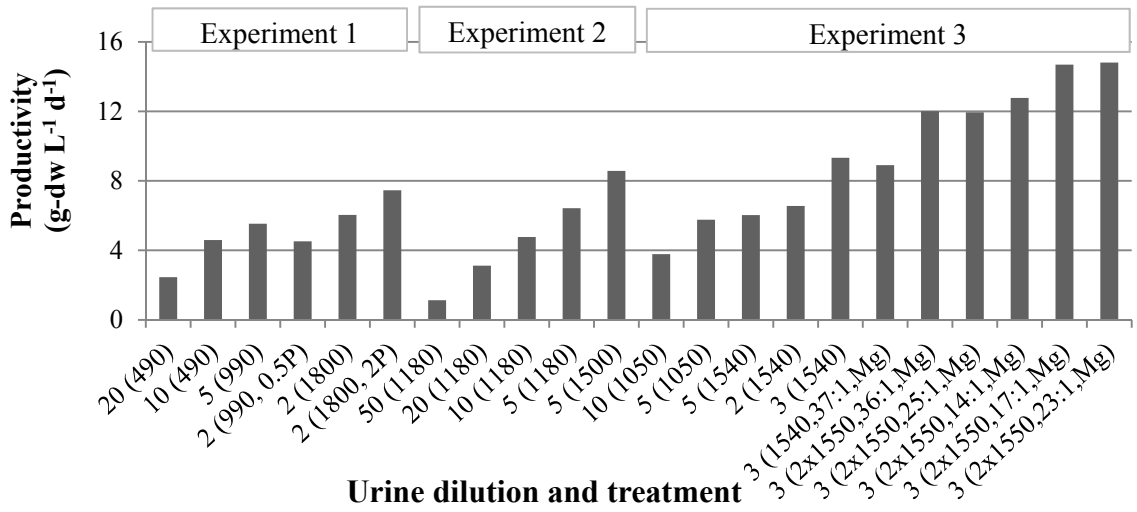

Figure 3.5 Comparison of volumetric biomass productivity of the three experiments (x-axis shows urine dilution and in brackets treatments with different light intensities, phosphorus supply, N:P molar ratio, and additional magnesium)

Volumetric biomass productivities obtained in other studies employing different types of wastewater were much lower: 0.92 g-dw L^{-1} d^{-1} for *Chlorella* sp. grown in municipal wastewater in a continuous system with light/dark cycles (Li et al., 2011); 0.33 g-dw L^{-1} d^{-1} for a mixed culture grown on digested swine slurry in a continuous system (Molinuevo-Salces et al., 2010); 0.088 g-dw L^{-1} d^{-1}, for *Neochloris oleoabundans* grown on digested dairy manure in a batch system with light/dark cycle (Levine et al., 2011); and 0.072 g-dw L^{-1} d^{-1} for *Spirulina platensis* grown on synthetic urine in a continuous system (Yang et al., 2008). It

must be mentioned, however, that these studies were conducted with lower nutrient concentrations as well as different algal species and light intensities. The results of our study with urine, therefore, were also compared to those regarding dense cultures employing standard media at high light intensities and in short light-path systems. Cuaresma et al. (2009) conducted an experiment with *C.sorokiniana* using modified M-8 medium at a high light intensity of 2,100 µmol-photons m^{-2} s^{-1}, a HRT of 0.83 day and a panel photobioreactor with a light path of 14 mm. This resulted in a volumetric productivity of 7.2 g-dw L^{-1} d^{-1} (~120 g-dw m^{-2} d^{-1}) at a biomass density of 5.7 g L^{-1}. When compared to the study of Cuaresma and co-workers, our results with a HRT of 1 day and a 10 mm light path demonstrated a productivity of 9.3 g-dw L^{-1} d^{-1} (93.3 g-dw m^{-2} d^{-1}) at a light intensity of 1,550 µmol-photons m^{-2} s^{-1}. The shorter light path apparently resulted in increased volumetric productivity.

In this study, the urine was supplemented with iron, micronutrients and magnesium during the last experiment. As a consequence, high volumetric productivity can be maintained. In a previously published study it was shown that iron, magnesium and certain micronutrients are present in low concentrations in urine (Tuantet et al., 2013). When urine was collected and stored, precipitation takes place reducing the availability of these elements. When supplementing urine with these elements again microalgae growth could be enhanced and maintained at high density (Tuantet et al., 2013). The amount of light supplied in this study is relatively elevated and, in practice, it is specific to geographical location and season. Dense cultures can be achieved in areas with high irradiance. In outdoor cultivation, the chronological and seasonal variation of light could result in lower net productivity than what was achieved in this study under continuous illumination and, hence, a larger illuminated area would be required. Furthermore in the microalgae field, it is well recognized that CO_2 supply is a very important aspect for intensive microalgae cultivation (Langley et al., 2012; Acién Fernández et al., 2013). To be able to supply sufficient CO_2 and sufficient rates to rapidly growing high-density microalgae cultures a high driving force for CO_2 transfer from gas to liquid is needed. This means that microalgae photobioreactors must be gassed with gasses rich in CO_2 in the order of 1 -10 % v/v. A carbon source of 5% CO_2 was supplied during Experiment 1 and 2; later, during Experiment 3, the aeration was enriched with 10% CO_2. Therefore, the carbon was supplied in excess, supporting increased volumetric biomass productivity. The supply of CO_2, in practice, can be combined with stripping CO_2 from the exhaust of power plants or from biogas produced by anaerobic digestion. Studies in the field

have demonstrated the possibility of cultivating microalgae on flue gas with CO_2 concentrations ranging from 1% to as high as 40% (Pires et al., 2012).

3.3.4 Nutrient balances and biomass compositions

Analysis of the biomass composition and elemental balance calculations clearly illustrate that nutrients removed from the system were captured within the produced biomass, and microalgae play a major role in removal of these nutrients. Figure 3.6 exhibits an example of the fractions of various forms of nitrogen and phosphorus present in the influent and effluent extracted from Experiment 3. The small fraction of nitrate in the effluent (<1%) indicates that nitrification was insignificant and that nitrogen removal must have been accomplished by fixation in the biomass. The amount of heterotrophic bacterial biomass can be estimated from the COD removal, assuming this was accomplished by biodegradation. Employing a yield of 0.46 g-biomass g-COD removed^{-1} (Metcalf et al., 2003) and the highest COD removal efficiency in Experiment 2, the bacterial biomass accounted for a maximum of 7.7% w/w of the total biomass. With an average composition of bacteria of 12% w/w N and 2% w/w P (Metcalf et al., 2003), the highest fraction of nitrogen and phosphorus removed by bacteria would be 7% and 13% of the total nitrogen and phosphorus that were removed in the photobioreactor. Free ammonia that may have been stripped from the aqueous phase can also be estimated. At 38°C and pH of 7±0.2, the calculated free ammonia, according to Emerson et al. (1975), ranged between 0.9 and 2.1% of the ammonium concentration within the reactor. Taking these processes into consideration, more than 90% of the nitrogen and more than 85% of the phosphorus in urine were assimilated by the microalgae and more than 92% of the produced biomass was calculated to be microalgae.

The average composition of the microalgae biomass grown in human urine is $CH_{1.75\pm0.02}O_{0.42\pm0.04}N_{0.15\pm0.02}P_{0.008\pm0.003}Mg_{0.002\pm0.0003}$. This composition was calculated according to Duboc et al. (1999) and using the results of the elemental analysis of the biomass. This composition of the biomass grown in human urine is identical to that of the *C.sorokiniana* grown in M8a medium from a study of Kliphuis et al. (2010) with respect to C, H, O, and N. In addition, elemental analysis demonstrated that the N:P molar ratio of the biomass ranges between 15:1 and 33:1. The magnesium content lies between 1.2 and 1.9 mg g-biomass^{-1}. The protein content of the produced biomass ranges between 38% and 48 % on a dry weight basis, and the highest protein content was achieved with synthetic urine. The total

fatty acid content is highest in the biomass cultivated in actual urine. The total fatty acid content of the biomass ranges between 16% and 25% w/w. The total fatty acid profile

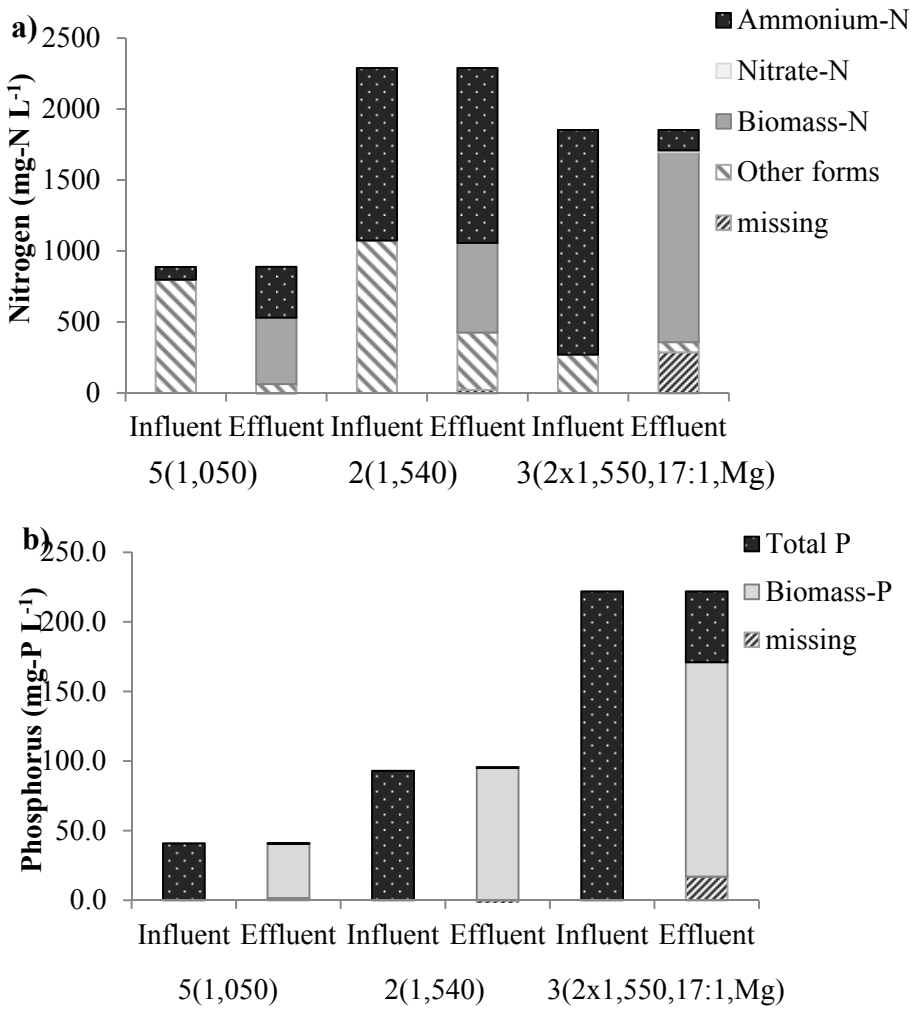

Figure 3.6 Balances of nitrogen (a) and phosphorus (b) at different urine dilutions and treatment of Experiment 3

reveals the major fatty acid fractions of C16:0, C18:3, C18:2, and C16:3. The total fatty acid content and its profile obtained in this study lie within the reported range of *Chlorella* sp. (Dunstan et al., 1992; Přibyl et al., 2012). Although the total fatty acid fraction of the biomass is relatively low, this content is not the only factor determining the oil production ability of microalgae. Together with the oil content, biomass production must be considered as well. Chen et al. (2011) indicated that a more suitable performance index indicating the ability of microalgae regarding oil production is the combined effect of oil content and biomass

production, specifically, lipid productivity. The fast-growing potential and a high productivity demonstrated in this study would allow total fatty acid productivity to become as high as 0.9-3.6 g L^{-1} d^{-1} which is high compared to that of other autotrophic growth of different species and systems (Chen et al., 2011).

3.3.5 Settling efficiency of the biomass

To make use of the biomass, it must be harvested and concentrated. Flocculation combined with sedimentation and subsequently followed by dewatering is considered an energy efficient harvesting process (Schenk et al., 2008). The settling efficiency obtained in a cuvette test according to Salim et al. (2011) (data not shown) has shown that settling times of *C.sorokiniana* grown non-axenically in urine are shorter than that of microalgae grown in M8a medium. To obtain a 50% settling efficiency, 4.3 hours is required for microalgae grown in urine and 7.7 hours for microalgae grown in M8a. Microscopic images and particle size distribution curves revealed a significant fraction of 3-4 μm particle size for microalgae grown in M8a whereas the additional peak of 8-20 μm particle size was observed in urine indicating the presence of flocs. The floc formation could be due to extracellular polymeric substances (EPSs) excreted by bacteria. EPSs have been known to play a role in agglomeration of activated sludge (Sponza, 2002) as well as enhancement of flocculation of unicellular microalgae i.e. *Chlorella vulgaris* (Lee et al., 2013). As a consequence, the non-axenic culture of microalgae demonstrates potential for economical harvesting of biomass.

3.4 Conclusions

This study illustrates the remarkable potential of employing human urine for the production of microalgae biomass coupled with treatment of a concentrated waste stream, specifically, human urine. Although actual urine was applied, an elevated biomass productivity of up to 9.3 g-dw L^{-1} d^{-1} could be achieved which indicates that urine significantly supports microalgae growth and can serve as a major nutrient source for the production of microalgae biomass. A short ligh-path photobioreactor allowed for high cell density and nutrient removal efficiency along with removal of organic substances (COD) from minimally diluted human urine. Optimization by shortening the light path, supplementing magnesium, and optimizing the N:P molar ratio enhanced the productivity and

nutrient removal to as high as 14.8 g-dw L^{-1} d^{-1}, 1.3 g-N L^{-1} d^{-1}, and 0.15 g-P L^{-1} d^{-1}. The system can function continuously and operated for more than 8 consecutive months during this study. There were no indications that the system cannot be operated for an even longer period of time. The elevated volumetric productivity and nutrient removal efficiency obtained in this study was further supported by the application of high light intensities, the supplementation of micronutrients, and sufficient CO_2.

Acknowledgement

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4

Effect of dilution rate on microalgae productivity and nutrient removal in a short light-path photobioreactor treating human urine

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Abstract

Chlorella sorokiniana was cultivated in synthetic and in human urine in a continuously operated flat panel photobioreactor. The width of the reactor was 10 mm and it was illuminated from both sides at 1,530 μmol-photons m^{-2} s^{-1}. The aim of this study was to determine the effect of the reactor dilution rate on biomass productivity, biomass yield on light supplied and on the volumetric nitrogen and phosphorus removal rates. With synthetic urine, these performance parameters were maximal at a dilution rate of 0.10 to 0.15 h^{-1}. With undiluted human urine, biomass density and productivity were highest at a dilution rate of 0.05 h^{-1}. At a dilution rate of 0.10 h^{-1} high ammonium concentrations caused lower biomass productivity. The highest biomass productivities with five times diluted synthetic and undiluted human urine respectively were 1.06 g-dw L^{-1} h^{-1} and 0.79 g-dw L^{-1} h^{-1}. The respective biomass yields on light were 0.98 and 0.74 g-dw mol-photons^{-1}. Maximum nitrogen and phosphorus removal rates were as high as 120 mg-N L^{-1} h^{-1} and 19 mg-P L^{-1} h^{-1} with synthetic urine, and 89 mg-N L^{-1} h^{-1} and 8 mg-P L^{-1} h^{-1} with human urine. The results have shown that biomass productivity and nutrient removal rate can be maximized by optimizing the reactor dilution rate and by minimizing ammonia toxicity i.e. by pre-diluting the urine.

This chapter is submitted for publication.

4.1 Introduction

Coupling microalgae biomass production with wastewater treatment recently has gained a lot of attention (Craggs et al., 2013; Wu et al., 2013; Zhu et al., 2013), mainly because microalgae production costs and water use can be reduced, and at the same time the wastewater is treated (Clarens et al., 2010). Several types of wastewater have been tested for this purpose, for example partially treated municipal wastewaters, (digested) manure, industrial wastewaters and anaerobically digested wastewater (Cai et al., 2013).

Source-separated human urine is rich in nutrients and therefore could serve as growth medium for microalgae cultivation. A previous study (Chapter 2 and 3) demonstrated the feasibility of minimally diluted human urine for microalgae cultivation. It was possible to cultivate the microalga *Chlorella sorokiniana* at a high specific growth rate on urine that was only diluted by a factor of two. Nutrient removal efficiencies were not reported, but a short light path was a prerequisite to obtain high biomass production rates which would lead to high nutrient removal rates.

Algae biomass productivity is largely determined (optimized) by the biomass density, and thus by the dilution rate that is applied during cultivation (Hu et al., 1998; Qiang et al., 1998; Richmond and Zou, 1999; Hoekema et al., 2013). This optimum biomass density will depend strongly on the light intensity at the photobioreactor's surface (i.e. the light supply rate) and on the photobioractor's light-path. The highest productivity ever reported is 1.2 g-dw L^{-1} h^{-1} for *Spirulina platensis* cultivated in a photobioreactor with a light path of 14 mm, operated at a light intensity of 8,000 µmol-photons m^{-2} s^{-1} and at a biomass density of approximately 30 g-dw L^{-1} (Qiang et al., 1998).

A short light-path photobioreactor is prerequisite when concentrated urine is used as a growth medium. In Chapter 3, *C.sorokiniana* was successfully cultivated on human urine minimally diluted for only two times during a period of more than 300 days in a 10 mm light-path photobioreactor (Tuantet et al., 2014). In this study after shortening the light-path to 5 mm, adjusting the N:P ratio of the urine, and supplementing additional magnesium, the nitrogen and phosphorus removal rate and biomass productivity respectively reached 53.6 mg-N L^{-1} h^{-1}, 6.2 mg-P L^{-1} h^{-1} and 617 mg-dw L^{-1} h^{-1}. However, these values reflected only one arbitrarily chosen dilution rate of 0.04 h^{-1}. It was expected that optimization of the dilution rate can result in even higher productivities and nutrient removal rates.

The effect of the dilution rate on microalgal productivity already was determined in earlier studies (using standard media) for microalgae species other than *C.sorokinaina* (Molina Grima et al., 1993; Molina Grima et al., 1994; Cuaresma et al., 2009; Tang et al., 2012; San Pedro et al., 2013). The relation between productivity and dilution rate for *Chlorella sorokiniana* was only partially studied without attention for nutrient removal (Cuaresma et al. 2009). Moreover, until now algae biomass production was only coupled to wastewater treatment for highly diluted wastewaters, and at low dilution rates, typically between 0.01 and 0.08 h^{-1} (Dickinson et al., 2013; Ruiz et al., 2013). As a result, biomass productivities and nutrient removal rates in these studies were relatively low and therefore, a shorter retention time was suggested as a preferable condition for wastewater treatment. No information is available, on microalgae cultivation using concentrated wastewaters, and at higher dilution rates (short hydraulic retention times, HRT), even though this could result in much higher productivities, nutrient removal rates and as a result, smaller reactor volumes.

For these reasons, in this study a wide range of dilution rates (0.04-0.24 h^{-1}) were tested to determine the operational conditions at which microalgae biomass production and nutrient removal rates from urine are maximized. The optimum dilution rate was determined using synthetic urine and this dilution rate was subsequently tested with human urine. *Chlorella sorokiniana*, that had shown to grow well on urine, was selected for continuous cultivation in a short light-path photobioreactor with a light path of 10 mm. This reactor was illuminated from both sides at 1,530 μmol-photons m^{-2} s^{-1}. The biomass productivity, biomass yield on light supplied, nutrient removal rates and biomass content at different dilution rates will be compared and discussed. Also the practical consequences for combined microalgae cultivation and urine treatment will be discussed.

4.2 Materials and Methods

4.2.1 Microorganism and culture media

Chorella sorokiniana CCAP211/8K was obtained from the Culture Collection of Algae and Protozoa, Oban, UK. The microalga was pre-cultivated in M8a medium (Kliphuis et al., 2010) in 250-mL enclosed shake flasks with 100 mL of liquid culture medium. The cultures were kept in a shaking incubator at 25ºC, a light intensity of 20-40 μmol photons m^{-2} s^{-1}, and a 16/8 h day/night cycle. A few days before inoculation, the cultures were placed in

an incubator with continuous illumination of 165 μmol-photons m^{-2} s^{-1} and 2%-CO_2 enriched air in order to reach higher cell density.

Both synthetic urine and real human urine were used. Synthetic urine was employed as a culture medium to determine the relation between biomass productivity and dilution rate and, as such, to maximize productivity. Synthetic urine composition was as follows (amounts per L of urine): 15 g urea, 2.75 g K_2HPO_4, 0.75 g $Na_2HPO_4.2H_2O$, 0.53 g $CaCl_2.2H_2O$, 1.23 g $MgSO_4.7H_2O$, 1.4 g K_2SO_4, and 8 g NaCl. To support dense microalgae cultures, 30 mL Fe-EDTA stock solution, and 15 mL micronutrient stock solution were supplemented. Fe-EDTA stock solution consisted of (amounts per L stock solution) 58 g EDTA ferric sodium salt and 18.6 g $Na_2EDTA.2H_2O$. Micronutrient stock solution consisted of (amounts per L of stock solution) 13 g $MnCl_2.4H_2O$, 3.2 g $ZnSO_4.7H_2O$, 1.83 g $CuSO_4.5H_2O$, and 0.062 g H_3BO_3. The synthetic urine concentration was comparable to averaged urine concentration reported by Kujawa-Roeleveld and Zeeman (2006). The pH of synthetic urine was adjusted to 7 with 3 M HCl prior to use, and 0.032% (v/v) of antifoam B silicone emulsion (J.T.Baker, Germany) was added to synthetic urine to prevent foaming.

Synthetic urine was diluted 1.8 to 10 times to avoid salt precipitation and possible inhibition of microalgae growth at high salt concentrations. Specifically ammonia, which is formed when urea is hydrolyzed, may inhibit microalgae growth. At the same time, dilution was limited to a level expected not to cause any nutrient limitations. Applied synthetic urine dilution factors at different reactor dilution rates are shown in Table 4.1 The dilution factor is defined as the ratio of the volume of diluted urine to the original aliquot volume of non-diluted urine, and hence a dilution factor of 1 is equivalent to non-diluted urine.

Human urine was tested at those reactor dilution rates that gave the highest biomass productivity together with good nutrient removal efficiencies for synthetic urine. The urine was collected from separation toilets (Gustavsberg, Berger Biotechnik GmbH, Germany) receiving urine from male and female employees of Sub-Department of Environmental Technology, Wageningen University, the Netherlands. Because some flush water is used in those toilets, the urine was less concentrated than the synthetic urine that was applied (Table 4.2). The urine was kept at 4°C during the experiment. 1 L of non-diluted urine was supplemented with 30 mL of Fe-EDTA stock solution and 15 mL of micronutrient stock solution with concentrations as indicated above. In addition, 0.032% (v/v) of antifoam B silicone emulsion (J.T.Baker, Germany) was added to prevent foaming.

Magnesium was supplemented to real urine because of its low concentration. This is caused by precipitation as a result of the increasing pH during urea hydrolysis (Tuantet et al., 2014). A stock solution of 8 g L^{-1} of $MgSO_4.7H_2O$ was pumped directly into the reactor at 1.8 mL h^{-1} using a diaphragm metering pump (STEPDOS® 03 RC, Germany).

Table 4.1 Aeration rates, CO_2 concentration, and tested period for *Chlorella sorokiniana* cultivated in synthetic and human urine at different dilution rates

Culture medium	Dilution rate (h^{-1})	Aeration rate (L L^{-1} min^{-1})	CO_2 supplied (%)	Urine dilution factor*	Running time (day)
Synthetic urine	0.04	0.68	20	2.5	7
	0.05	0.69	16	1.8	9
	0.07	0.68	20	3.0	10
	0.10	0.67	11	3.3	4
	0.15	0.70	12	4.8	5
	0.20	0.69	11	7.4	3
	0.24	0.69	8	8.5	3
Human urine**	0.05	0.67	19	Non-diluted	9
	0.11	0.67	19	Non-diluted	6

* Dilution factor is defined as the ratio of final diluted urine volume to an aliquot volume of non-diluted urine i.e. dilution factor of 2.5 means 1 aliquot volume of urine is diluted in 1.5 aliquot volume of demineralized water.

** Human urine was supplemented with trace elements and magnesium.

4.2.2 Photobioreactor

A panel photobioreactor with a light-path of 10 mm was used. The illuminated surface area of each reactor side was 0.0903 m^2 and the working volume of the reactor was 0.92±0.12 L. The cultivation system (Figure 4.1) composed of 6 parts: the photobioreactor, influent and effluent pumps, a temperature control system, a pH control system, an aeration system, and a light source.

Table 4.2 Concentration (in mg L^{-1}) of total nitrogen, ammonium nitrogen, phosphorus as phosphate, and organic compounds as COD of non-diluted synthetic and human urine and the remaining of these compounds in the reactor at different reactor dilution rates

Urine medium/ dilution rate	Total nitrogen	Ammonium-nitrogen	Phosphate-phosphorus	COD[/1]
Synthetic urine	6,990[/2]	-	620[/2]	-
0.04 h^{-1}	1,159	248	<0.05	-
0.05 h^{-1}	2,550	546[/3]	130	-
0.07 h^{-1}	1,353	202	55.5	-
0.10 h^{-1}	720	151[/3]	<0.05	-
0.15 h^{-1}	735	154[/3]	<0.05	-
0.20 h^{-1}	435	90[/3]	2.5	-
0.24 h^{-1}	440	92[/3]	13.3	-
Human urine	2,646	2,130	144	3,262
0.05 h^{-1}	767	625	0.7	825
0.11 h^{-1}	1,858	1,500[/3]	48.7	1,644

[/1] Chemical Oxygen Demand (mg-O_2 L^{-1})

[/2] calculated values of non-diluted synthetic urine and urea is a major form of supplied nitrogen

[/3] calculated values using averaged fractions of ammonia to total ammonium of 0.21(±0.05) and 0.80(±0.04) respectively for synthetic and human urine.

The photobioreactor consisted of a cultivation chamber and a water jacket for temperature control. To prevent evaporation a condenser cooled with water of 4°C was installed at the gas outlet on top of the cultivation chamber. Peristaltic pumps (Watson Marlow 120U, Watson-Marlow pumps, UK) were used to feed and withdraw the influent and effluent. The influent and effluent bottles were kept at 4°C to avoid further urine hydrolysis. The culture temperature was controlled via a temperature sensor connected to a waterbath (Julabo-F25-HE, Julabo Labortechnik GMBH, Germany) circulating water through the water jacket. The culture pH was controlled with concentrated HCl solution. The aeration system was regulated by mass flow controllers. This aeration was done via a gas distribution tube over two third of the reactor width, creating an air-lift mixing resulting in a well-mixed liquid without any stagnant zones.

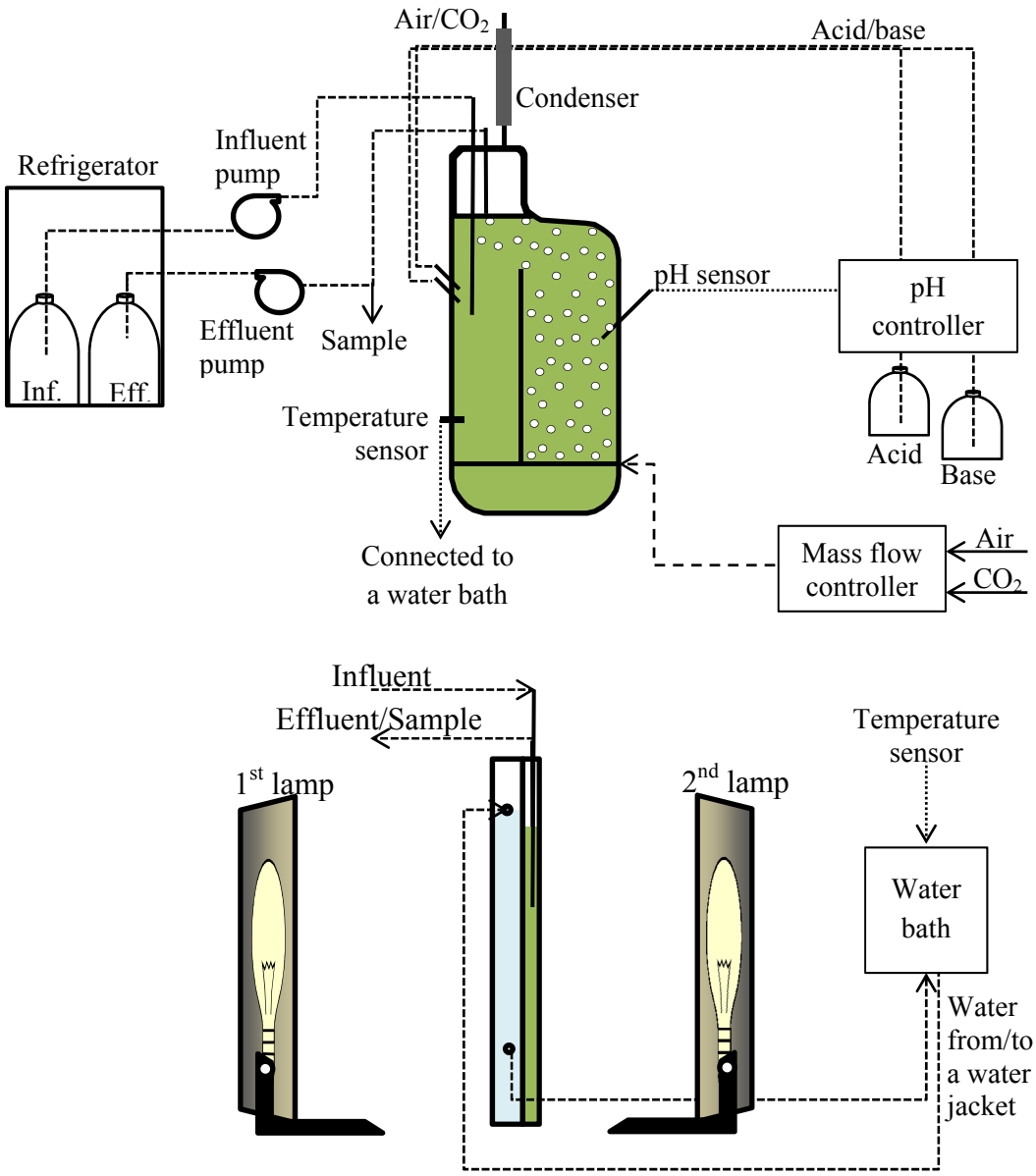

Figure 4.1 Schematic diagram of the microalgae culturing system

The photobioreactor was continuously illuminated by two high-pressure sodium lamps (SON-T Pro, 400W, Phillips, Netherlands) placed on both sides of the reactor surface. Before and after the experiments, the light intensity was measured over 26 spots distributed homogeneously inside the empty growth chamber of the reactor using a 2π PAR quantum sensor (LI-190SA, LiCOR, USA) connected to a Li-COR handheld meter (LI-250A, Li-COR, USA). The reported light intensity was averaged over the reactor surface. The reactor inner

surface was cleaned daily to remove biofilms using a teflon-coated magnetic stirring bar placed within the reactor.

4.2.3 Experimental conditions

During the experiments, temperature was controlled at 38°C and pH was maintained at 7.0±0.2. Air enriched with CO_2 (at different concentrations) was fed into the reactor to supply enough inorganic carbon for microalgae growth and to provide mixing. For each experiment the CO_2 concentration was adjusted to minimize the amount of acid needed for pH control. The actual CO_2 concentrations used at different dilution rates are given in Table 4.1, and ranged between 8 and 20 % v/v. The averaged light intensity over both reactor surfaces was 1,530 μmol-photons PAR m^{-2} s^{-1}. This light intensity represents photons in the so-called PAR (Photosynthetic Active Radiation) ranging from 400 to 700 nm, the wavelength range which can be used by microalgae.

With synthetic urine two experimental series with different microalgae inocula were conducted. Both series were started in batch mode by inoculating microalgae in diluted synthetic urine at low light intensity utilizing one lamp only and shading it with light-diffusing glass plates. As soon as the biomass density started to increase, the light intensity was gradually increased by removing the glass plates and eventually the second lamp was employed illuminating the opposite side of the panel reactor. This was followed by starting continuous chemostat operation at low dilution rate. In total 7 different reactor dilution rates were tested during both experimental series with synthetic urine (see Table 4.1): 0.04, 0.05, 0.07, 0.10, 0.15, 0.20, and 0.24 h^{-1}. During the experiments CO_2 supply and urine pre-dilution were varied but only those results, reflecting conditions where only light was limiting algae growth, will be presented.

After finishing the experiments with synthetic urine, a new experiment with human urine was started with a new inoculum. The same starting-up procedure was performed as with synthetic urine. When the second lamp was started, human urine was pumped to the reactor at a low dilution rate together with synthetic urine so that microalgae could gradually adapt to real urine. During 10 days the ratio between human urine and synthetic urine was gradually increased until solely human urine was supplied. The applied reactor dilution rates of real urine experiment were 0.11 and 0.05 h^{-1} (Table 4.1).

The average dilution rate was checked daily by weighing the influent vessel, and the bottles containing acid, base, and magnesium solutions. Samples were taken during steady state, a period having a constant biomass density for at least duration of 3 times the applied hydraulic retention time (HRT, i.e. the inverse of the dilution rate). At low dilution rates of 0.04-0.10 h^{-1}, samples were taken every 24 hours. At high dilution rates (0.15-0.24 h^{-1}), two samples were taken per day. At least three replicate samples were taken for every dilution rate.

4.2.4 Analytical procedures

The samples taken from the effluent collecting tube were partly used for the analyses of optical density and biomass dry weight concentration. The remaining sample was centrifuged at 10,000 rpm for 10 min to separate algal biomass from the water. The supernatant was used for the following analyses: nitrogen as total nitrogen (TN, for both synthetic and real urine) and ammonium-nitrogen (NH_4^+-N, for real urine), phosphorus as orthophosphate (PO_4^{3-}-P, for synthetic urine), total phosphorus (TP, for real urine) and Chemical Oxygen Demand; COD (for real urine).

Microalgae biomass collected overnight at 4°C was used for analyses of protein, starch and total fatty acid content. Prior to the analyses, the biomass was centrifuged at 3,620 relative centrifugal force (rcf) for 10 min at 5°C. The pellet was washed with demineralized water and centrifuged again. This washing step was repeated twice. The pellets were stored at -20°C before freeze drying overnight. Freeze-dried biomass was manually ground into fine powder and subsequently transferred to glass serum bottles, freeze dried overnight, and sealed with a metal cap under vacuum.

Optical density and biomass dry weight concentration

Optical density (OD) at 680 and 750 nm was measured using a Xion 500 spectrophotometer (Hach Lange, Germany). Biomass dry weight (dw) was determined according to Tuantet et al. (2014).

Nitrogen, phosphorus, and COD concentration

The supernatant after a centrifugation step was kept at 4ºC. For synthetic urine samples, supernatant was analyzed for TN and PO_4^{3-}-P. Supernatant from real urine samples

was analyzed for TN, NH_4^+-N, TP and COD. All of the analyses were done photo-metrically according to Standard Methods (APHA, 1998) using Dr Lange® test kits (Hach Lange GMBH, Germany).

Protein and total fatty acid content

The protein content of the biomass was measured with the BioRad DC protein assay (BioRad Laboratorie Inc., USA) using 10-20 mg of ground and freeze-dried biomass according to Tuantet et al. (2014). Bead beating in bead beating tubes (Lysing Matrix E, MP Biomedicals, Germany) with a Precellys 24 bead beater (Bertin Technologies, France) was employed for cell disruption.

Extraction of fatty acids was modified from Lamers et al. (2010) with preparation procedure as reported by Tuantet et al. (2014). The total fatty acid analysis and quantification procedure were done according to Santos et al. (2012).

4.2.5 Calculations

The volumetric biomass productivity (P_{vol}) is the product of biomass density (C_X, g-dw L^{-1}), and reactor dilution rate (D, h^{-1}), provided that the bioreactor is at steady state and no accumulation of biomass takes place in the reactor, and was calculated according to Eq. 4.1;

$$P_{vol} = C_X \times D \qquad \text{(g-dw } L^{-1} h^{-1}\text{)} \qquad (4.1)$$

The areal productivity (P_{area}) was calculated based on the volumetric productivity and the reactor dimensions (i.e. surface area to volume ratio, SVR, m^{-1}) according to Eq. 4.2;

$$P_{area} = \frac{P_{vol} \times 1000}{SVR} \qquad \text{(g-dw } m^{-2} h^{-1}\text{)} \qquad (4.2)$$

The observed biomass yield on light energy is used to express the efficiency of light use for microalgae photoautotrophic growth. The observed biomass yield on light ($Y_{X,E,obs}$, g-dw mol-photons^{-1}) was calculated based on the biomass density (C_X, g-dw L^{-1}), liquid flow (F, L h^{-1}), supplied light intensity (PFD_{in}, µmol-photons m^{-2} s^{-1}), and reactor surface area (A_R, m^2) according to Eq. 4.3;

$$Y_{X,E,obs} = \frac{C_x \times F}{PFD_{in} \times A_R \times 3600 \times 10^{-6}} \quad \text{(g-dw mol photons}^{-1}\text{)} \quad (4.3)$$

The supplied light intensity averaged over the illuminated surface area was 1,530 µmol-photons m^{-2} s^{-1}. The photobioreactor width was 10 mm. Illuminated from both reactor sides, the photobioreactor *SVR* became 200 m^{-1}. The total reactor surface area (both sides) was 0.181 m^2.

4.3 Results

4.3.1 Biomass density

Biomass density of *C.sorokiniana* grown with synthetic urine decreased with increasing dilution rates (Figure 4.2). The highest biomass density of 15.3 g-dw L^{-1} was achieved at the lowest dilution rate of 0.04 h^{-1}. With increasing dilution rates, biomass density steadily decreased to 3.7 g-dw L^{-1} at a dilution rate of 0.24 h^{-1}, which was also expected because the dilution rate dictates the specific growth rate of the microalgae and, as such, the biomass density. *C.sorokiniana* was able to maintain a specific growth rate of 0.24 h^{-1} at a biomass density of 3.7 g-dw L^{-1}. On human urine, the highest biomass density of 15.7 g-dw L^{-1} was achieved at a dilution rate of 0.05 h^{-1}. At a dilution rate of 0.11 h^{-1}, the biomass density decreased considerably to 6.0 g-dw L^{-1}, significantly lower than that of synthetic urine at a similar dilution rate (10.7 g-dw L^{-1}).

The ratio of the optical density (OD) at 680 nm to 750 nm (OD_{680}/OD_{750}) is used as a relative measure of the chlorophyll content of microalgae cells and is also an indicator for photoinhibition or excessive bacterial growth. At 750 nm, only scattering of light determines the OD and at 680 nm the absorbance of light by microalgal chlorophylls adds to the OD measured. In this study, this ratio was more than 1.30 for dilution rates of 0.04-0.15 h^{-1} (Figure 4.2). At higher dilution rates (0.20-0.24 h^{-1}) the OD_{680}/OD_{750} ratio slightly decreased to 1.18-1.26, indicating a reduction of chlorophyll content at lower biomass densities and less self-shading. With human urine, at low reactor dilution rates, this ratio was between 1.23 and 1.25, which is slightly lower than with synthetic urine. This indicates higher light scattering, possibly due to bacterial cells growing on the organic matter in real urine and contributing to the OD_{750}. In non-axenic cultures in the presence of organic compounds, bacterial growth

cannot be avoided and this is even advantageous as will be discussed later. However, the OD_{680}/OD_{750} ratio was close to that in synthetic urine, showing that microalgae still dominated the culture. This was also confirmed by microscopic observations.

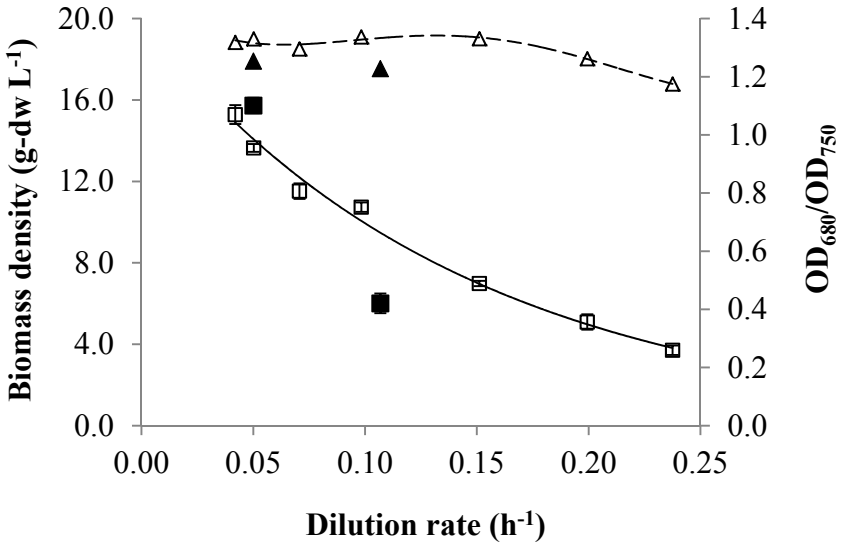

Figure 4.2 The biomass density (C_X) and the ratio between optical density at 680 nm (OD_{680}) and 750 nm (OD_{750}) of Chlorella Sorokiniana grown in synthetic urine and human urine at different dilution rates: [□] C_X in synthetic urine; [■] C_X in human urine; [△] OD_{680}/OD_{750} in synthetic urine; [▲] OD_{680}/OD_{750} in human urine. Error bars represent standard deviation of biomass density (n≥3). The trend lines are given for the biomass density (solid line) and the OD_{680}/OD_{750} ratio (broken line) obtained in synthetic urine.

4.3.2 Biomass productivity

Biomass productivity (Eq. 4.1) was expressed in dry biomass produced per unit reactor volume and per unit illuminated area. Figure 4.2 shows that at low reactor dilution rates biomass densities were higher. However, since the dilution rate was low also biomass productivities were relatively low (Figure 4.3). Biomass productivity increased with increasing reactor dilution rates and reached a maximum at dilution rates between 0.10 and 0.15 h^{-1} with the highest volumetric and areal productivities respectively of 1.06 g-dw L^{-1} h^{-1} and 5.30 g-dw m^{-2} h^{-1}. At higher dilution rates and lower biomass densities biomass productivity declined. Most likely, at these low biomass densities not all incident light could be effectively absorbed within the culture.

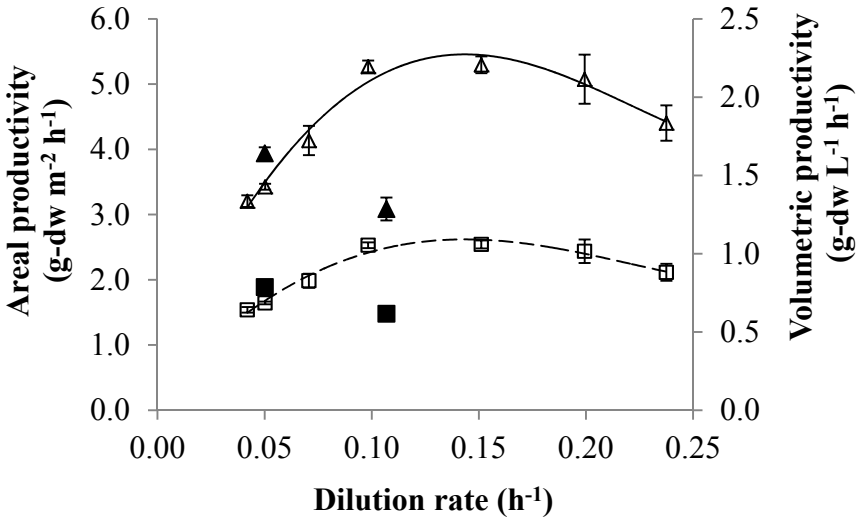

Figure 4.3 Areal and volumetric productivity of *Chlorella Sorokiniana* grown on synthetic urine and human urine at different dilution rates: [△] areal productivity in synthetic urine; [▲] areal productivity in human urine; [□] volumetric productivity in synthetic urine; [■] volumetric productivity in human urine. Error bars represent standard deviation of each parameter (n≥3). The solid trend lines are given for areal productivity (solid line) and volumetric productivity (broken line) in synthetic urine.

With human urine, comparable biomass productivity was observed as with synthetic urine at a reactor dilution rate of 0.05 h^{-1}. However, at a dilution rate of 0.1 h^{-1} a biomass productivity was considerably lower than with synthetic urine. This observation will be discussed later. The highest volumetric and areal productivities with real urine, obtained at a dilution rate of 0.05 h^{-1}, were 0.79 g-dw L^{-1} h^{-1} and 3.94 g-dw m^{-2} h^{-1}.

4.3.3 Biomass yield on light energy

The yield of microalgae biomass (in gram dry weight) on light energy (in mole PAR photons) represents the efficiency of light use in photosynthesis. In this study, the light intensity at the reactor surface was used for calculating the biomass yield on light (Eq. 4.3). Since unused light that could pass through the reactor (especially, at high reactor dilution rates) was not measured, it was assumed that all the supplied light was absorbed by the microalgae. The biomass yield on light followed the same trend as the biomass productivity

and increased with increasing reactor dilution rates, reaching a maximum at dilution rates of 0.10-0.15 h^{-1}, and declining at higher dilution rates (Figure 4.4). Although at low reactor dilution rates all the supplied light is absorbed by the algae, the high biomass density results in a high requirement for maintenance energy and herewith a lower biomass yield on light. At high reactor dilution rates, biomass concentration is relatively low and the supplied light is not completely absorbed by the microalgae and partly passes the reactor. This results in a lower biomass yield on light.

Also with human urine, the biomass yield on light follows the same trend as biomass productivity, with the highest yield recorded at a reactor dilution rate of 0.05 h^{-1} (Figure 4.4). The highest biomass yields on light with synthetic urine and with human urine respectively were 0.98 and 0.74 g-dw mol-photons^{-1}.

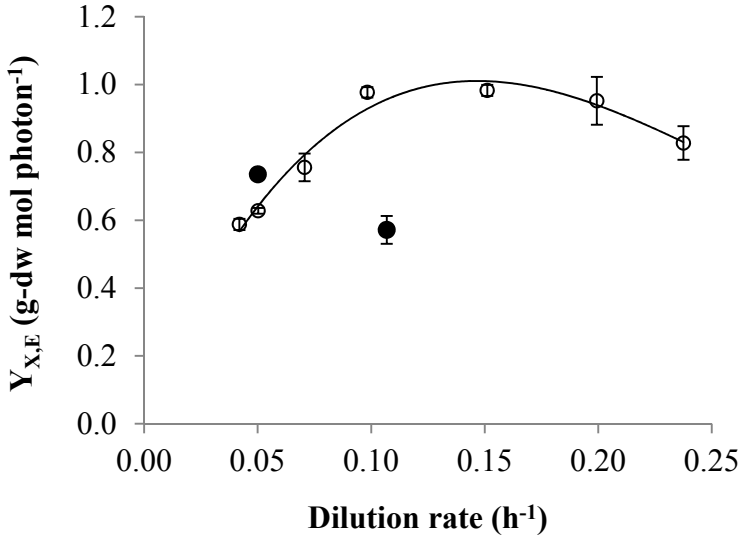

Figure 4.4 Observed biomass yield on light energy ($Y_{X,E}$) of *Chlorella Sorokiniana* grown in synthetic urine and human urine at different dilution rates: [○] biomass yield on light in synthetic urine; [●] biomass yield on light in human urine. Error bars represent standard deviation of $Y_{X,E}$ (n≥3). The trend line is given for the biomass yield on light energy obtained in synthetic urine.

4.3.4 Nutrient removal and biomass composition

Depending on the reactor dilution rate, the synthetic urine was pre-diluted to prevent possible inhibitory effects related to elevated salt and ammonium/ammonia concentrations

(Table 4.1). At the same time urine pre-dilution was minimized to prevent nutrient limitation for microalgae growth. Human urine however, was not diluted due to its low concentration as a result of dilution with flush water (Table 4.2). To ensure that nitrogen and phosphorus were supplied in excess, the nutrient content of the microalgae biomass at different reactor dilution rates was analyzed to confirm that the nutrients supplied always exceeded the assimilated nutrients in the microalgae biomass (data not shown). Figure 4.5 shows the volumetric loading rate and removal rate of nitrogen and phosphorus from synthetic and human urine. In both cases the removal rate of nitrogen increased with increasing reactor dilution rates and volumetric nutrient removal rates thus followed the same trend as the volumetric biomass productivity. The highest nitrogen removal rate of 123 mg-N L^{-1} h^{-1} was achieved on synthetic urine at a reactor dilution rate of 0.10 h^{-1}. At the same dilution rate, also the highest nitrogen removal rate of 88.7 mg-N L^{-1} h^{-1} was observed for human urine. Phosphorus removal from synthetic urine, on the other hand, was maximal at a dilution rate of 0.15 h^{-1} (19.3 mg-P L^{-1} h^{-1}). With human urine, the phosphorus removal rate was 7.8-8.0 mg-P L^{-1} h^{-1} at the two different dilution rates that were applied.

Under light limited condition, it was expected that similar volumetric nutrient removal rates could be obtained on human urine as on synthetic urine and this indeed was the case at a dilution rate of 0.05 h^{-1}. However, at a dilution rate of 0.10 h^{-1} the nutrient removal rates were significantly lower than with synthetic urine. The reason for this will be discussed later.

With human urine, we observed removal of organic compounds (referred to as COD; Chemical Oxygen Demand) because the urine was not sterilized. The system thus was open for bacterial growth and this resulted in partial degradation and removal of these organic compounds. COD removal significantly increased with increasing dilution rate (data not shown). The highest removal rate was 161 mg-COD L^{-1} h^{-1} at a dilution rate of 0.1 h^{-1}. At a dilution rate of 0.05 h^{-1}, the COD removal rate was 110 mg-COD L^{-1} h^{-1}.

Nitrogen removal efficiencies ranged between 36 and 58% with synthetic urine and between 32 and 69% with human urine. Phosphorus removal efficiencies ranged between 60 and ~100% for both synthetic and human urine. Due to high N:P ratio of both synthetic and human urine (25-41:1), the remaining nitrogen in the reactor ranged between 0.40-2.55 g-N L^{-1} and 0.77-1.86 g-N L^{-1} for synthetic urine and real urine respectively (Table 4.2). The issue of remaining nitrogen in the reactor will be discussed later. At a phosphorus removal efficiency of approximately 100% (for three reactor dilution rates with synthetic urine), the

microalgae biomass contained less phosphorus than the amount that was removed (data not shown). The phosphorus content in the biomass was however on a normal level for microalgae biomass of 1.0-1.1% w/w. These observations show that phosphorus did not limit microalgae growth. Other processes that might have played a role in phosphorus removal will be discussed in Section 4.4.

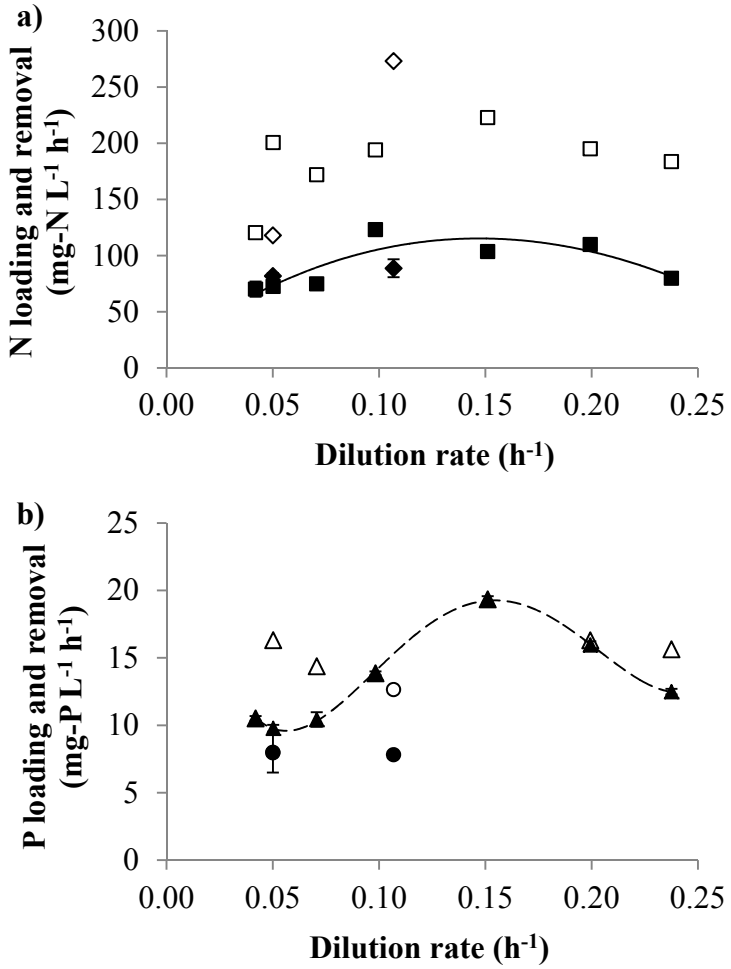

Figure 4.5 a) Nitrogen loading rate from [□] synthetic urine and [◇] human urine and nitrogen removal rate from [■] synthetic urine; [◆] human urine, and b) phosphorus loading rate from [△] synthetic urine and [○] human urine and phosphorus removal rate from [▲] synthetic and [●] human urine. Error bars represent standard deviation of nitrogen or phosphorus removal (n≥3). The trend lines are given for the nitrogen removal rate (solid line) and the phosphorus removal rate (broken line) from synthetic urine.

The biomass grown on synthetic and real urine at different reactor dilution rates was analyzed for protein and fatty acid content (Figure 4.6). The protein content of biomass grown on both synthetic and human urine tended to decrease with increasing reactor dilution rates. The highest protein content of the biomass grown on synthetic urine and human urine respectively were 66% and 61%, both obtained at low reactor dilution rates. The total fatty acid content of the biomass grown on synthetic urine was similar at all reactor dilution rates, i.e 8-9%. With human urine, the total fatty acid content was 10% and the major fatty acids for both biomass grown on synthetic and real urine were C18:3(n-3), C16:0, and C16:3.

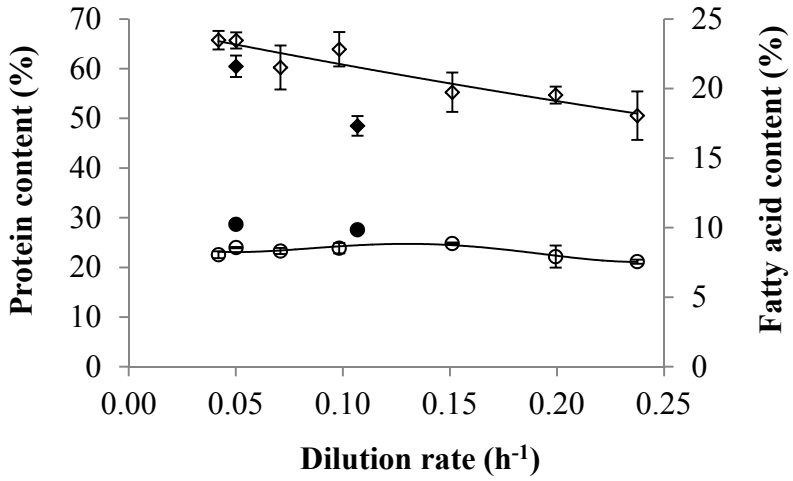

Figure 4.6 Protein content of *Chlorella Sorokiniana* biomass grown in [◇] synthetic urine and [◆] human urine and total fatty acid content of biomass grown in [○] synthetic urine and [●] human urine at different dilution rates. Error bars represent standard deviation of each parameter (n≥2). The trend lines are given for protein content (solid line) and fatty acid content (broken line) of biomass grown in synthetic urine.

4.4 Discussion

4.4.1 Biomass density

This study aimed at determining the relation between the reactor dilution rate and algae biomass productivity and nutrient removal efficiency from urine. For this purpose *Chlorella sorokiniana* was cultivated in a short light-path photobioreactor illuminated from both sides with an average light intensity of 1,530 μmol-photons PAR m^{-2} s^{-1}. *C.sorokiniana* could rapidly grow on synthetic urine and could maintain a high specific growth rate of 0.24

h^{-1}. This is in agreement with other studies reporting specific growth rates of *C.sorokiniana* as high as 0.26 h^{-1} (Cuaresma et al., 2009) and 0.29 h^{-1} (Sorokin, 1967).

With human urine, the biomass density at a dilution rate of 0.1 h^{-1} was considerably lower than with synthetic urine. This may be due to the high ammonium/ammonia concentration (NH_4^++ NH_3) in the reactor of 1.5 g-N L^{-1} (Table 4.2). Although the pH was controlled at 7.0, high temperature and high ammonium concentrations could result in presence of free ammonia up to ~20 mg-N L^{-1}. Azov and Goldman (1982) reported a free ammonia concentration of 17.5 mg-N L^{-1} already reduced carbon uptake rate of *Scenedemus obliquus* by 50%. In addition, the high irradiance was reported to enhance ammonia toxicity due to stress on photosynthetic machinery (Admiraal, 1977). A reduced growth rate of *C.sorokiniana* was also observed at a total ammonium concentration of 1.4 g-N L^{-1} in Chapter 2. At lower dilution rates, where biomass densities were higher, this effect was not observed because of lower reactor ammonium concentrations. This implies that when running photobioreactors at high reactor dilution rates, actions should be taken to avoid ammonia inhibition (without causing phosphorus limitation) i.e. urine pre-dilution or preventing urea hydrolysis. With synthetic urine, free ammonia toxicity was not observed because urea was slightly hydrolyzed and the synthetic urine was pre-diluted. Hydrolysis of the urea in the reactor appeared to be low with total ammonium concentrations lower than 400 mg-N L^{-1} (at pH 7) at all reactor dilution rates.

4.4.2 Biomass productivity

When light is the only limiting factor, it dictates the biomass concentration at any dilution rate (Molina Grima et al., 1994). At low reactor dilution rates the biomass density is high, and because the specific growth rate is low a substantial amount of light energy is needed for maintenance. This results in lower net biomass productivity. At higher reactor dilution rates the biomass density is lower and more light is available for growth. This results in a higher biomass productivity (Molina Grima et al., 1993; Zijffers et al., 2010). At even higher dilution rates, light availability is high and no longer limits growth. However, at such dilution rates the culture density is low and part of the light will pass through the reactor unabsorbed, resulting in lower biomass productivity. Results from this study resemble well the general trend reported in several other studies in this respect (Molina Grima et al., 1993;

Molina Grima et al., 1994; Cuaresma et al., 2009; Tang et al., 2012; San Pedro et al., 2013), although the maximum productivity and optimum dilution rate differ from those studies.

Under nutrient-replete conditions, the volumetric productivity of 1.06 g-dw L^{-1} h^{-1} obtained in this study is higher than values reported by others. Cuaresma and co-workers (2009) obtained a volumetric productivity of 0.5 g-dw L^{-1} h^{-1} for *C.sorokiniana* grown on M8a medium at a dilution rate of 0.24 h^{-1} and light intensity of 2,100 μmol-photons m^{-2} s^{-1} in a photobioreactor with a light path of 14 mm. However, their maximum areal productivity of 7.7 g-dw m^{-2} h^{-1} was higher than in our study (5.3 g-dw m^{-2} h^{-1}) because of the much lower SVR of their reactor (Eq. 4.2). They also observed the same productivity trend in response to the dilution rate. The optimum dilution rate that maximized biomass productivity in their study was 0.24 h^{-1} while in our study this was 0.1-0.15 h^{-1}.

When employing human urine, biomass productivity was lower than with synthetic urine, in particular at the optimum dilution rate of 0.1 h^{-1} as established with synthetic urine. As was discussed earlier, high free ammonia concentration may have inhibited microalgae growth. The biomass productivity obtained from human urine (790 mg-dw L^{-1} h^{-1}) in our study, however, is very high in comparison with other studies on different wastewaters and growth conditions. Biomass productivity from other studies, re-calculated considering microalgae growth during light hours, ranged from 12.5 to 27.1 mg-dw L^{-1} h^{-1} with diluted wastewaters at low light intensity (Molinuevo-Salces et al., 2010; Chang et al., 2013; Dickinson et al., 2013; Ruiz et al., 2013). A short light-path photobioreactor and high light intensity clearly enhanced volumetric biomass productivity in our system.

4.4.3 Biomass yield on light energy

The highest biomass yield on light energy measured in this study (0.98 g-dw mol-photons^{-1}) is similar to the 1 g-dw mol-photons^{-1} achieved by Cuaresma and co-workers (2009) at a dilution rate of 0.24 h^{-1}. Theoretically, the maximum biomass yield on light was reported to be 1.5 g-biomass mol-PAR photons^{-1} excluding maintenance requirements (Blanken et al., 2013). A high photosynthetic efficiency can be expected under low/diluted light conditions where the biomass yield of up to 1.25-1.3 g-dw mol-photons^{-1} was reported (Cuaresma et al., 2011; Kliphuis et al., 2012; Blanken et al., 2013). With human urine, the highest biomass yield was 0.74 g-dw mol-photons^{-1}. The pattern of biomass yield on light was identical to that of the biomass productivity because the unused light was not measured.

4.4.4 Nutrient removal and biomass composition

Volumetric nutrient removal rates of 89 mg-N L^{-1} h^{-1} and 8 mg-P L^{-1} h^{-1} were obtained on human urine. Nutrient removal rates with photoautotrophic processes in other studies (as reviewed by Cai et al. (2013)) were highest at ~40 mg-N L^{-1} h^{-1} and <1 mg-P L^{-1} h^{-1}. Our results clearly show that nutrient removal rates can be even higher since the nutrient removal efficiencies from real human were not yet optimized. Optimization was done only for biomass productivity. However, our results demonstrate the possibility to couple nutrient removal from high-strength wastewaters with microalgal biomass production.

Biomass analysis showed decreasing biomass protein content with increasing reactor dilution rates. A decrease in protein content could have resulted from balancing cell composition with other cell components (such as starch) that were produced when light availability was high i.e. at low biomass densities. Kliphuis et al. (2012) also found a slight increase in starch content with respect to a decrease in biomass protein content of *Chlamydomonas reinhardtii* at high reactor dilution rates. At a reactor dilution rate of 0.1 h^{-1}, the protein content of biomass grown on real urine was significantly lower than that with synthetic urine, even though the nitrogen removed per produced biomass was higher. Since the pH was well controlled at ~7.0, the fraction of a surplus nitrogen and phosphorus removal by chemical processes i.e. precipitation must have been small. Assimilated nitrogen could possibly be used for synthesis of other compounds rather than proteins (i.e. chlorophyll). For phosphorus, luxury uptake of phosphorus, a phenomenon known to occur at increased light intensities and temperatures (Powell et al., 2009) may have supported phosphorus removal i.e. at a dilution rate of 0.15 h^{-1} where biomass density was low but high amount of phosphorus was removed from diluted synthetic urine (18 mg-P g-dw^{-1}). In addition to the luxury uptake, phosphate can precipitate with cations i.e. Mg^{2+} and unused metals (micronutrients) in minimally diluted urine i.e. at a dilution rate of 0.04 h^{-1} where phosphorus removed was higher than phosphorus content of the biomass (data not shown).

Bacterial growth was not prevented in our system because for large-sale practical application this is impossible and because it is beneficial that the bacteria remove organic pollutants from urine while utilizing the oxygen that is produced by the algae. The fraction of bacterial biomass produced can be estimated based on the amount of COD removed using a bacterial biomass yield of 0.46 g-biomass g-COD $removed^{-1}$ (Metcalf et al., 2003). With the maximum COD removal from real urine at a dilution rate of 0.1 h^{-1} of 161 mg-COD L^{-1} h^{-1},

the volumetric bacterial productivity could have reached 0.074 g-dw L^{-1} h^{-1}. This would account for 12% of the total biomass productivity (0.62 g-dw L^{-1} h^{-1}) and hence only for a small fraction of the nutrients that were removed. Clearly, microalgae still dominated the culture and played a dominant role in nutrient removal from real urine.

4.4.5 Application of microalgae cultivation for nutrient removal and microalgae biomass production

The results demonstrate the possibility to optimize microalgae biomass productivities in a short light-path photobioreactor and to treat real urine at high nutrient removal rates with simultaneous removal of organic compounds. With increasing reactor dilution rate, higher biomass productivity and nutrient removal rates (0.8 g-dw L^{-1} h^{-1}, 76 mg-N L^{-1} h^{-1}, and 7 mg-P L^{-1} h^{-1}) could be obtained. This clearly shows an improvement of the system as compared to previously published results at a dilution rate of 0.04 h^{-1} (0.62 g-dw L^{-1} h^{-1}, 53.6 mg-N L^{-1} h^{-1}, and 6.2 mg-P L^{-1} h^{-1}) (Tuantet et al., 2014). If human urine is pre-diluted or pre-hydrolysis is prevented, the system performance on human urine will further improve because ammonia toxicity is avoided. To prevent urine from hydrolysis, methods such as applying urease inhibitors or urine acidification might be applied during urine collection (Maurer et al., 2006).

Coupling urine treatment with production of microalgae biomass implies balancing biomass productivity with nutrient removal efficiency. Meanwhile free ammonia concentration needs to be kept below a toxic level. Decreasing the light path can bring these opposing goals together but a light path lower than 5 mm, such as applied in this study, does not seem to be practically feasible. For this reason, pre-dilution of urine must be adopted when the biomass density required to reach acceptable removal efficiency is too high and/or when free ammonia concentration is higher than the safe level. Partly recycling of the effluent after harvesting microalgae and additional removal of nitrogen can be adopted to dilute the culture.

In the situation that a high nutrient removal efficiency is required without pre-dilution of urine, a low reactor dilution rate must be accepted even though the biomass yield on light (photosynthetic efficiency) and biomass productivity will be lower. This will result in high biomass densities (up to 15 g-dw L^{-1} as compared to 1.5-7 g-dw L^{-1} for typical closed PBRs (Brennan and Owende, 2010)) and, consequently, supplied ammonium will be immediately

consumed mediating a toxic effect. The high biomass density will also minimize the costs for microalgae harvesting (Dillschneider and Posten, 2013; Ruiz et al., 2013).

The remaining amount of nitrogen that needs further treatment will depend on the urine N:P ratio. The urine N:P ratio can largely vary. Kuntke (2013) reported average total N and P contents of respectively 8.6±3.7 g-N L^{-1} and 0.7±0.5 g-P L^{-1} for 106 urine samples showing variation in individual diet and consumption patterns. This variation will also affect microalgae biomass that can be produced and the remaining amount of nitrogen in the effluent.

4.5 Conclusions

The performance of a microalgae cultivation system to couple microalgae biomass production with nutrient removal from urine can be enhanced by optimizing the dilution rate. Increasing reactor dilution rates enhanced volumetric *Chlorella sorokiniana* biomass productivity, biomass yield on light and nutrient removal rates. After reaching the maximal values further increasing reactor dilution rates resulted in a decline of these parameters. The optimum dilution rate giving maximal performance parameters with synthetic urine ranges between 0.10 and 0.15 h^{-1}. On un-diluted human urine most of these parameters were highest at a dilution rate of 0.05 h^{-1} than at 0.10 h^{-1}. Ammonia toxicity probably caused lower productivity with human urine at a dilution rate of 0.1 h^{-1} illustrating the necessity of urine pre-dilution. To couple urine treatment with microalgae production, there is a need to find a balance between maximizing productivity for biomass production and minimizing urine pre-dilution for high nutrient removal efficiency.

Acknowledgement

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5

Microalgae productivity and nutrient removal from synthetic urine under day/night cycles

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Abstract

The microalga, *Chlorella sorokiniana* has been shown to grow well on minimally diluted human urine. In this study, the effects of a simulated day/night cycle on microalgae specific growth rate, areal biomass productivity, nutrient uptake rates, and biomass composition were investigated by applying two hydraulic retention times (HRT; 0.46 and 2.6 days) to create different physiological states. Microalgae were cultivated in short light-path photobioreactors under a sinusoidal day/night cycle (12/12 h) simulating outdoor cultivation with a daily light input of 37 and 41 mol-photons m^{-2} d^{-1}, respectively for a 0.46 and 2.6-day HRT reactor. A daily oscillation of biomass productivity, nutrient uptake rates, and biomass composition was observed in a 0.46-day HRT reactor. In contrast, in a 2.6-day HRT the same parameters remained more constant during the day although measurement uncertainty was higher. The daily averaged areal productivity and nutrient uptake rates for 0.46- and 2.6-day HRT reactors were comparable. The day/night cycle yielded slightly less biomass than cultivation under constant and continuous irradiance of equal daily light flux. High photosynthetic efficiencies could be maintained under day/night cycle but significant nutrient removal during the night could not be demonstrated. For a safe operation in practice the microalgae cultivation system should be operated adopting a short HRT during the day period and a long HRT, or no feeding, during the night to maximize net biomass productivity, as well as nutrient removal efficiency.

This chapter is submitted for publication.

5.1 Introduction

Removal of the nutrients, nitrogen (N) and phosphorus (P), from wastewaters is necessary to avoid polluting surface waters (Rockstrom et al., 2009). In addition, to avoid future shortages of phosphorus, this nutrient should be recovered from wastewaters and reused in agriculture (Wilsenach et al., 2007). Approximately 75% of the nitrogen and 50% of the phosphorus in domestic wastewater originate from human urine, while they are present in just one percent of the total wastewater volume (Kujawa-Roeleveld and Zeeman, 2006). This was one of the driving forces for new sanitation concepts, which emerged in the late 1990s (Larsen and Gujer, 1996; Otterpohl et al., 1997; Zeeman et al., 2008). These concepts are based on separation of wastewater streams at their source and facilitate nutrient recovery from urine and energy production from the organic matter in feces (Maurer et al., 2003; Maurer et al., 2006).

Several studies have demonstrated the feasibility of human urine as a medium to cultivate microalgae (Adamsson, 2000; Feng and Wu, 2006; Tuantet et al., 2013). Diluted human urine, with a dilution factor of 80-120 (Yang et al., 2008; Chang et al., 2013) and even concentrated urine with a dilution factor of 1-20 (Tuantet et al., 2013), were shown to support microalgae growth. A more recent study, with continuous illumination at high light intensity in a short light-path photobioreactor, resulted in a biomass productivity of 15 g-dw L^{-1} d^{-1} and nutrient removal rates as high as 1,300 mg-N L^{-1} d^{-1} and 150 mg-P L^{-1} d^{-1} (Tuantet et al., 2014). The produced algal biomass can be used as a slow release fertilizer (Mulbry et al., 2005; Mulbry et al., 2006) or possibly as a feedstock for the production of proteins, polyunsaturated fatty acids and biodiesel (Bigogno et al., 2002; Becker, 2007; Chen et al., 2011).

In practice, application of (continuous) artificial light is restricted to production of small amounts of high value compounds. Large-scale microalgae cultivation for bulk products should be done outdoors on sunlight (Blanken et al., 2013). However, even outdoor algal cultivation is hampered by economic and operational constraints, mainly caused by daily and seasonal variations of light and temperature (Goldman, 1979). Because light is the energy source for phototrophic algae, growth is slow in the early morning and late afternoon, fast during the middle of the day, and no growth occurs at night. In addition, it was shown that the composition of microalgae changes during day/night cycles, with decreasing carbohydrate and increasing protein content during the night (Cuhel et al., 1984; Ogbonna

and Tanaka, 1996). Cleary, this also influences nutrient removal in outdoor cultivation systems. It has been shown that in the night some diatoms were able to assimilate nitrogen (Clark et al., 2002; Needoba and Harrison, 2004) and take up phosphorus (Nalewajko and Lee, 1983). In agreement with these studies, Craggs and co-workers (Craggs et al., 1995; Craggs et al., 1997) reported a high nutrient removal efficiency (70-100%) at night for marine microalgae grown in corrugated raceways and mini-ponds. Contrarily, a reduced or even absence of nutrient removal was shown in some microalgae reactors at night and during periods of low light (Picot et al., 1993; Boelee et al., 2013). The capability to remove nutrients at night will largely determine the operational strategy for outdoor microalgae cultivation when combined with treatment of urine or any other nutrient containing wastewater.

In this study, we investigated biomass productivity, nutrient removal rate and biomass composition for light-limited growth of *Chlorella sorokiniana* under day/night cycles (12h/12h). Two different hydraulic retention times (HRT) were applied and compared for differences between rapidly and slowly growing microalgae in their response to the changing light intensity. The implications of these results will be discussed with respect to an efficient operational strategy for outdoor microalgae cultivation in combination with nutrient removal from urine.

5.2. Materials and methods

5.2.1 Strain and culture medium

Chlorella sorokiniana (CCAP211/8K) was obtained from the Culture Collection of Algae and Protozoa, Oban, UK. Pre-cultures were maintained in 250 mL shake flasks with 100 mL of modified M8-a medium (Kliphuis et al., 2010) at 25°C, a light intensity of 20-40 µmol photons m^{-2} s^{-1}, and a 16/8 h day/night cycle. Prior to photobioreactor inoculation, the culture density was enriched by incubating at continuous light of 165 µmol-photons m^{-2} s^{-1} and 2% v/v CO_2-enriched air. During the photobioreactor experiments, synthetic urine modified from Yang et al. (2008), was used as a culture medium. Its composition is shown in Table 5.1. The synthetic urine was diluted to provide sufficient nutrients ensuring light-limited condition while avoiding inhibition by high concentrations of salts or free ammonia resulting from urea hydrolysis. As a consequence, at a HRT of 0.46 days, 5 times diluted

synthetic urine was used, and at a HRT of 2.6 days, 3 times diluted synthetic urine. Since urea is easily hydrolyzed at room temperature, the synthetic urine was stored at 4°C in the dark.

Table 5.1 Composition of synthetic urine

Chemical	Amount ($g\ L^{-1}$)	Stock solution/chemical	Amount ($g\ L^{-1}$)
K_2SO_4	1.4	Fe- EDTA	
Urea ($CO(NH_2)_2$)	15	EDTA ferric sodium salt	0.116
$K_2HPO_4.3H_2O$	3.6	$Na_2EDTA.2H_2O$	0.0372
$Na_2HPO_4.2H_2O$	0.75	Micronutrients	
NaCl	8	H_3BO_3	0.0618
$CaCl_2.2H_2O$	0.53	$MnCl_2.4H_2O$	13
$MgSO_4.7H_2O$	1.23	$ZnSO_4.7H_2O$	3.2
Fe-EDTA stock solution (ml)	13	$CuSO_4.5H_2O$	1.83
Micronutrient stock solution (ml)	6.5		

Note: Synthetic urine was diluted three and five times respectively for experiments at a HRT of 2.6 days and 0.46 days

5.2.2 Experimental set-up

The experiments were carried out in short light-path (SLP) flat panel photobioreactors (PBR). A home-made photobioreactor (Type Algaemist, Technical Development Studio, Wageningen UR, NL) (PBR 1) was used for the experiment at a HRT of 0.46 days. This PBR was previously described by Breuer et al. (2013) and has a light path of 14 mm, a working volume of 0.39 L, and an illumination area of 0.029 m^2. To prevent carbon limitation, the pH was controlled at 6.6 by regulating CO_2 addition. The culture was mixed by aeration at a flow rate of 1 L L^{-1} min^{-1}.

Another flat panel photobioreactor (PBR 2) with the same configuration as PBR 1 was operated at a HRT of 2.6 days. This PBR was described in detail by Tuantet et al. (2014) and has a light path of 10 mm, a working volume of 0.94 L, and an illuminated area of 0.093 m^2. The microalgae culture was mixed by aeration at a flow rate of 0.57 L L^{-1} min^{-1}. Extra CO_2 with a concentration of 17.2 % v/v was supplied to prevent carbon limitation. The

culture pH was controlled at 6.9 ± 0.3 by a pH controller with 0.25M HCl and 0.25M NaOH. A condenser was connected to the gas outlet to prevent water evaporation.

In both PBRs culture temperature was monitored with a temperature sensor and maintained at 38^{o}C by a waterbath connected to the PBR cooling jacket. Peristaltic pumps were used to feed synthetic urine and withdraw effluent. All experiments were carried out under nutrient-replete conditions to ensure that light was the only growth limiting factor.

PBR 1 was equipped with 6 integrated warm-white LEDs (Bridgelux BXRA W1200), illuminating one side of the panel reactor. PBR 2 was illuminated with a separate home-made light source (type Reallight 24, Technical Development Studio, Wageningen UR, NL), equipped with 24 of the same warm-white LEDs. Light intensity was controlled according to a sinusoidal day/night regime with pulse-width-modulation (PWM) at a frequency of 10 KHz.

The light intensities inside the empty culture chamber were measured before and after the experiments. A 2π PAR quantum sensor (LI-190SA, Lincoln, Nebraska, USA) was used, connected to a Li-COR handheld meter (LI-250A, Li-COR, USA). The reported light intensities were averaged over 26 homogeneously distributed measuring spots along the surface of the reactor. When day/night cycles were applied, light intensity during the day period was simulated according to the following sinusoidal:

$$PFD_{(t)} = \sin(\frac{t}{p} \times \pi) \times PFD_{max}, \qquad (\mu\text{mol-photons m}^{-2}\text{s}^{-1}) \quad (5.1)$$

with $PFD_{(t)}$ the light intensity at time t, PFD_{max} the maximum light intensity reached at time of $0.5P$ and P the duration of the light period. The maximum light intensity was set at 1,500 and 1,340 μmol-photons $m^{-2}s^{-1}$ for PBR 1 and 2, respectively, which were comparable to average horizontal solar irradiance in the Netherlands in summer around solar noon. Figure 5.1 shows the day/night light curve of PBR 1 used to simulate outdoor light conditions.

The experiments were started in batch mode for approximately 72 h under low light to obtain sufficiently high biomass densities. When the optical density at 750 nm (OD_{750}) reached 10 units (3.4 g-dw L^{-1}), the continuous experiments were initiated and the day/night cycle was simulated. The culture was continuously diluted day and night at a fixed HRT. The influent vessel, effluent vessel, acid, and base bottles were weighed every day to calculate and check the dilution rate. After three consecutive day/night cycles of stable operation, i.e.

with a similar pattern of biomass density in PBR 1, samples were taken for a period of two consecutive days (2 day/night cycles). Reported values are average values of these 2 data sets. For PBR 2, a stable operation was reached after running the reactor for at least seven cycles and afterwards samples of two-hour intervals were taken for a period of 3 days (3 day/night cycles). Reported values are average data from these 3 data sets.

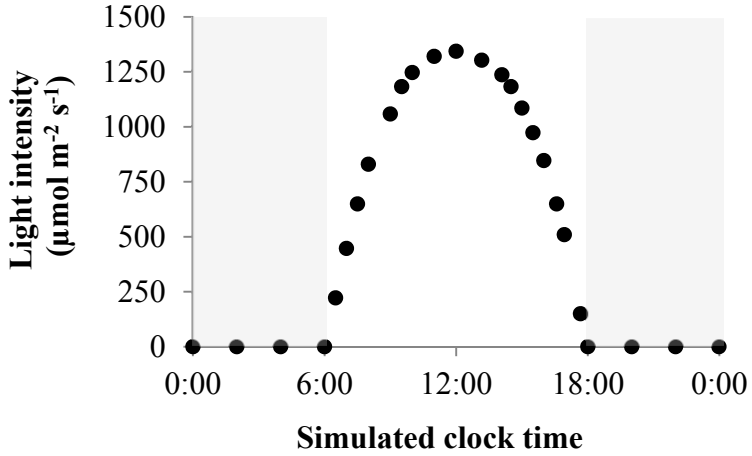

Figure 5.1 Light intensity of the LED lamp under simulated day/night cycle with the day light hours from 06:00 to 18:00. Grey area represents the dark period.

5.2.3 Analytical procedures

The reactor effluent was taken every day for analyses of dry weight (DW) and optical density (OD). Supernatants of the influent and effluent obtained after centrifugation at 10,000 rpm for 10 min were analyzed for nutrients. Additional biomass was collected for analyses of protein, starch and total fatty acids content. The biomass was prepared by centrifugation at 3,620 relative centrifugal force (rcf) at 5°C for 10 min. The pellet was washed with demineralized water and centrifuged once again. This washing step was repeated twice. The pellets were stored at -20°C before freeze drying. Freeze dried biomass was manually ground into a fine powder and put in glass serum bottles, freeze dried again, and sealed with a metal cap under vacuum.

Biomass dry weight and optical density measurements

Biomass dry weight concentration was determined using a calibration factor between OD_{750} and dry weight. Biomass dry weight was measured regularly according to Tuantet et

al. (2014). Optical density was determined at 750 nm (OD_{750}) using a spectrophotometer (Xion 500, Hach Lange, Germany). The relationship between OD_{750} and biomass dry weight was as follows:

$$Biomass\ dry\ weight = 0.3421 \times OD_{750}, \quad R^2=0.9617 \quad (\text{g-dw } L^{-1}) \qquad (5.2).$$

Analysis of nitrogen and phosphorus

Total nitrogen (TN) and orthophosphate-P (PO_4-P) were determined in supernatants by photometric analyses according to Standard Methods (APHA, 1998) using Dr.Lange® test kits.

Analysis of biomass content

Ground freeze-dried biomass was disrupted and analyzed for protein and total fatty acids using the same procedures as reported in Tuantet et al. (2014). Total starch content was quantified enzymatically using a total starch kit of Megazyme (Ireland, 2011). An amount of 10 mg of ground freeze-dried biomass, was dissolved in 1 mL 80% ethanol in a bead beating tube, and was beaten for 3 cycles of 60 s and 6,000 rpm with 120 s breaks in between. The homogenates were transferred to glass tubes with additional 4 mL 80% ethanol for rinsing. After following the procedure of the total starch kit, the absorbance of samples and a D-glucose control were measured at 510 nm in a spectrophotometer against a reagent blank solution consisting 0.1 mL water and 3.0 mL GOPOD Reagent.

5.2.4 Calculations

The net specific microalgae growth rate $\mu(t)$ was calculated using the mass balance for the microalgae:

$$\frac{dC_x(t)}{dt} = (\mu(t) - D)C_x(t) \qquad (\text{g-dw } L^{-1}\ h^{-1}) \qquad (5.3)$$

with $C_x(t)$ the biomass density (g-dw L^{-1}) at time t (h), D the medium dilution rate (h^{-1}) and $\mu(t)$ the net specific growth (h^{-1}). This net specific growth rate is a function of the light intensity and includes the effect of algae respiration. To calculate μ as a function of time, Eq. 5.3 was numerically integrated over consecutive sampling intervals.

Similarly, the nutrient (nitrogen and phosphorus) uptake rates $r_S(t)$ (mg L^{-1} h^{-1}) were calculated from the mass balances for these nutrients:

$$\frac{dS(t)}{dt} = D\big(S_i - S(t)\big) - r_S(t) \qquad \text{(mg } L^{-1} h^{-1}\text{)} \qquad (5.4)$$

with S_i and $S(t)$ being the influent and effluent nutrients concentrations, respectively (mg L^{-1}). Nutrient uptake rates were calculated as a function of time by numerically integrating this equation over consecutive sampling intervals.

Areal biomass production $P_{area}(t)$ as a function of time was calculated from measured biomass densities, the medium dilution rate and reactor surface to volume ratio (SVR in m^{-1}) according to:

$$P_{area}(t) = \frac{D(C_x(t) \times 10^3)}{SVR} \qquad \text{(g-dw } m^{-2} h^{-1}\text{)} \qquad (5.5).$$

These data were also used to calculate the (cumulative) overall areal biomass production during a completed day/night cycle.

5.3 Results and discussion

5.3.1 Biomass density

Figure 5.2 shows biomass densities of *C.sorokiniana* grown during day/night cycling at the two different HRTs that were applied. As expected, the long HRT of 2.6 days gave much higher densities (6.2-8.3 g-dw L^{-1}) than the short HRT of 0.46 day (0.6-1.3 g-dw L^{-1}). At 0.46-day HRT, the biomass density only started to increase approximately 2 hours after starting the light period at 6:00. Between 12:00 and 18:00 the biomass density further increased from approximately 0.6 g-dw L^{-1} to 1.3-1.4 g-dw L^{-1}. Immediately after the light was shut down at 18:00 the biomass density started to decrease as can be expected due wash-out in the absence of growth. At 2.6-day HRT a similar trend was observed. However, it took much longer (5-6 h) before the biomass density started to increase after the light period was initiated. The biomass density reached a peak of 8.4 g-dw L^{-1} at 21:00, i.e. 3 hours after the

light period had ended at 18:00. After this, the biomass density gradually decreased to 6.2 g-dw L^{-1} until the start of the (next) light period.

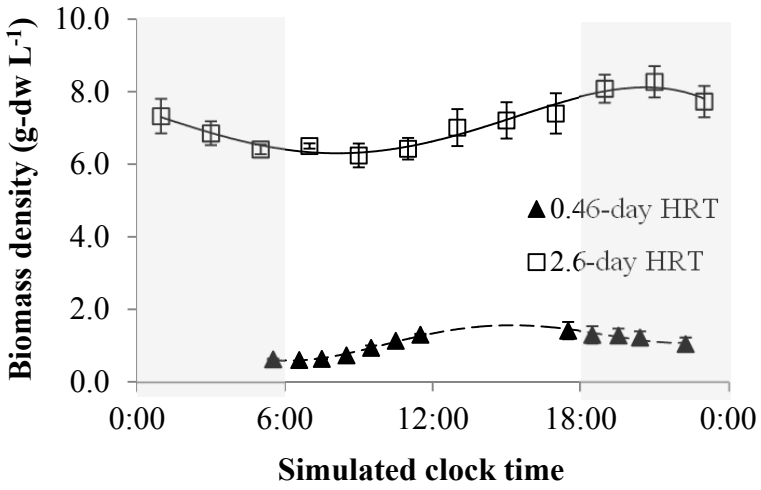

Figure 5.2 Biomass density of *C.sorokiniana* grown at different HRTs under simulated day/night cycle. Grey area represents the dark period. Error bars show standard deviations (0.46-day HRT, n=2; 2.6-day HRT, n≥3), trend lines are given for HRT of 2.6 days (solid line) and 0.46 day (broken line).

The delay of an increase in biomass density in response to the onset of light availability that was observed at both HRTs can be explained by the continuous culture dilution. Compared to 0.46-day HRT, the biomass density in the 2.6-day HRT exhibited a somewhat longer delay. This may have been caused by the lower light supply per microalgae cell at the higher biomass density in this reactor leading to a lower specific growth rate.

After shutting down the light at 18:00, wash-out caused an immediate decrease of the biomass density in the 0.46-day HRT reactor. In the 2.6-day HRT reactor the biomass density continued to increase for another 3 hours until 21:00 before such a decrease could be observed. Although a biological effect cannot be excluded, the nature of the OD_{750} measurement could have affected the estimation of biomass density. Optical density measurement at 750 nm is determined by scattering, in other words, cell numbers. Cell division has been reported to occur during the night (Goto and Johnson, 1995; Suzuki and Johnson, 2001) which may have increased the OD_{750}/dry-weight ratio. As a consequence, true dry weight concentration and biomass productivity during the dark period may have been

overestimated leading to the observation of increasing biomass density in a 2.6-day HRT reactor in darkness.

5.3.2 Microalgae specific growth rate and biomass productivity

Because nutrients were supplied in excess, light was the only growth-limiting factor in the PBRs. Figure 5.3 shows the net specific growth rates, calculated from Eq. 5.3. These growth rates followed the light intensity during the day/night cycle (Figure 5.1). The negative net growth rates during the night to early light period are caused by microalgae respiration.

In the 0.46-day HRT reactor, the highest specific growth rates of 0.23-0.30 h^{-1} were observed between 9:30 and 11:30. These growth rates are in the same range as maximum reported values for the same microalga of approximately 0.25 h^{-1} (Sorokin, 1967; Cuaresma et al., 2009). At 2.6-days HRT, the highest growth rate was significantly lower, i.e. 0.06 h^{-1} (between 13:00 and 19:00). This can be explained by a lower light availability for the microalgae at the higher biomass density in this reactor (Figure 5.2).

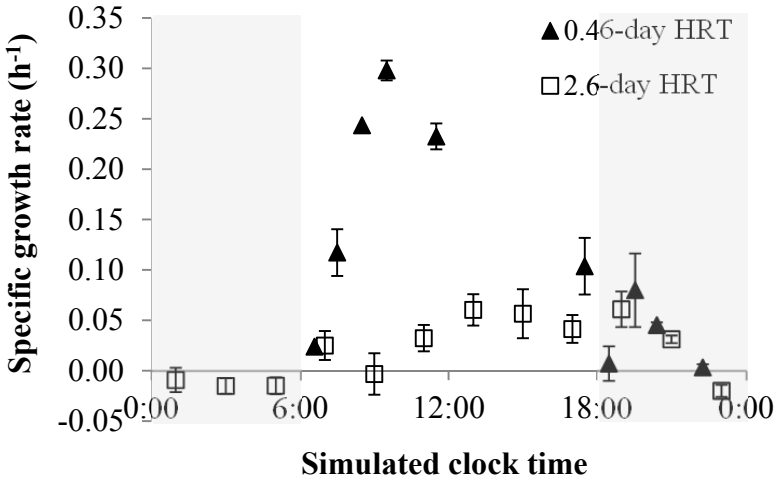

Figure 5.3 Observed specific growth rate of *C.sorokiniana* grown at 0.46-day HRT and 2.6-day HRT under simulated day/night cycle. Grey area represents the dark period. Error bars show standard deviations (0.46-day HRT, n=2; 2.6-day HRT, n≥3).

Figure 5.4 shows areal PBR productivity as a function of biomass density, reactor dilution rate, and PBR dimension (Eq. 5.5) for both reactors. In the 0.46-day HRT reactor, a significant fluctuation of productivity was observed with the highest productivity in the afternoon. The maximum productivity of the 0.46-day HRT reactor approximately was 1.73

g-dw m^{-2} h^{-1} between 17:30 and 18:30. The productivity in the 2.6-day HRT reactor was relatively stable between 1.11 and 1.45 g-dw m^{-2} h^{-1}. The highest productivity of 1.35-1.45 g-dw m^{-2} h^{-1} was obtained at the early night period. Since there was no light available, the areal PBR productivity during the night represented harvested biomass as a result of continuous dilution. In other words, the biomass harvested grew and accumulated in the photobioreactor during the day. Daily integrated areal productivities in the 0.46-day HRT and 2.6-day HRT reactor were 32.9 and 30.0 g-dw m^{-2} d^{-1}, respectively (Table 5.2). This corresponds to average volumetric productivity during light hours of ~200-250 mg-dw L^{-1} h^{-1}. Compared with other studies, these productivities are much higher than those obtained in diluted wastewaters (13-27 mg-dw L^{-1} h^{-1}) (Molinuevo-Salces et al., 2010; Chang et al., 2013; Dickinson et al., 2013; Ruiz et al., 2013).

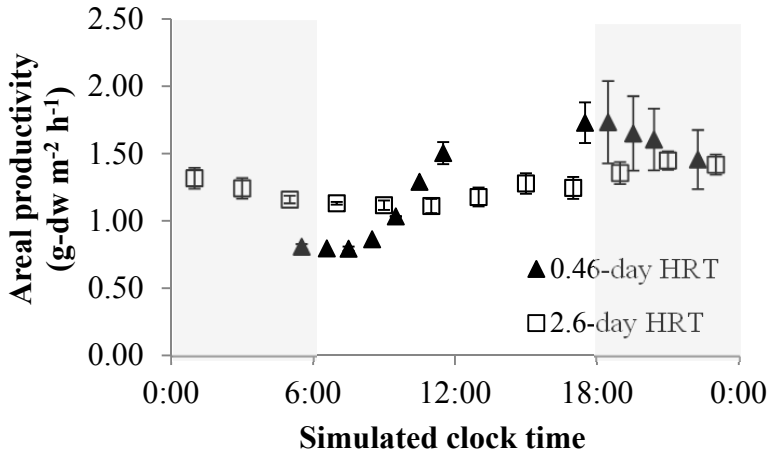

Figure 5.4 Areal productivity of the photobioreactor at HRT of (a) 0.46 day and (b) 2.6 days under simulated day/night cycle. Grey area represents the dark period. Error bars show standard deviations (0.46-day HRT, n=2; 2.6-day HRT, n≥3).

Daily average biomass yields on light in the 0.46-day HRT and 2.6-day HRT reactors were 0.80 and 0.81 g-dw mol-photons^{-1}, respectively These yields are lower than the theoretical yield of 1.5 g-dw mol-photons^{-1} (Blanken et al., 2013), possibly because at high irradiance at noon when light intensities reached 1,340-1,500 μmol-photons m^{-2} s^{-1} a considerable amount of light was dissipated due to oversaturation of microalgae in the surface layers of the PBR. Nevertheless, the yields are relatively high compared to other

observations with several strains in standard media at high and continuous irradiance, i.e. 0.5-1.0 g-dw mol-photons^{-1} as discussed in Zijffers et al. (2010) and Kliphuis et al. (2012).

With the 0.46-day HRT reactor an additional experiment was conducted under continuous illumination at 430 μmol-photons m^{-2} s^{-1} for 24 consecutive hours (Table 5.2). The total amount of light that was supplied (37 mol-photons m^{-2} d^{-1}) was equal to that under the simulated day/night cycles. The areal productivity under continuous illumination was 36.8 g-dw m^{-2} d^{-1}, 23% higher than the net areal productivity under the simulated day/night cycle. The biomass yield on light during continuous illumination (0.99 g-dw mol-photon^{-1}) also was higher than that under day/night cycles. The efficiency of light use at low (average) light intensities is higher than those at high irradiance (Cuaresma et al., 2011; Cuaresma Franco et al., 2012). During the day/night cycles the high light intensity during noon (1,340 and 1,500 μmol-photons m^{-2} s^{-1}) must thus have resulted in more photosaturation for the microalgae than at a constant light level of 430 μmol-photons m^{-2} s^{-1}.

5.3.3 Nutrient uptake rates

Figures 5.5 and 5.6 show the nitrogen and phosphorus uptake rates by the microalgae expressed per illuminated area (according to Eq.5.4). Nutrient uptake in the 0.46-day HRT reactor (Figure 5.5) mainly took place between noon and the end of the light period at 18:00, with uptake rates of 430 mg-N m^{-2} h^{-1} and 58-85 mg-P m^{-2} h^{-1}. During the early light period between 6:00 and 12:00 the uptake rates were marginal and during the night with no growth even negative uptake rates were observed. It was already reported in other studies that nitrogen taken up by microalgae could be excreted in nitrogen-sufficient cultures (Vincent, 1992), potentially explaining the negative uptake rates at night.

In the 2.6-day HRT reactor, nutrient uptake rates by the microalgae fluctuated considerably (Figure 5.6) and this is in contrast with results in a 0.46-day HRT reactor. There is no clear relation between the nitrogen and phosphorus uptake rates and the daily light cycle. Although these results might suggest continuation of nutrient removal during the night it should be noted that the nutrient uptake rates calculated are characterized by large standard deviations. This most likely is the result from the minimal resolution of the applied nutrient analyses in combination with the relatively small absolute difference between influent and effluent nutrient concentrations. For this reason, it remains unclear whether nutrient uptake continues during the night in the 2.6-day HRT reactor. Continuation of nitrogen assimilation

during the night has been reported by others under specific conditions (Clark et al., 2002; Needoba and Harrison, 2004; Sforza et al., In press), while absence of nutrient uptake at night was reported in other studies (Picot et al., 1993; Voltolina et al., 2005; Boelee et al., 2013).

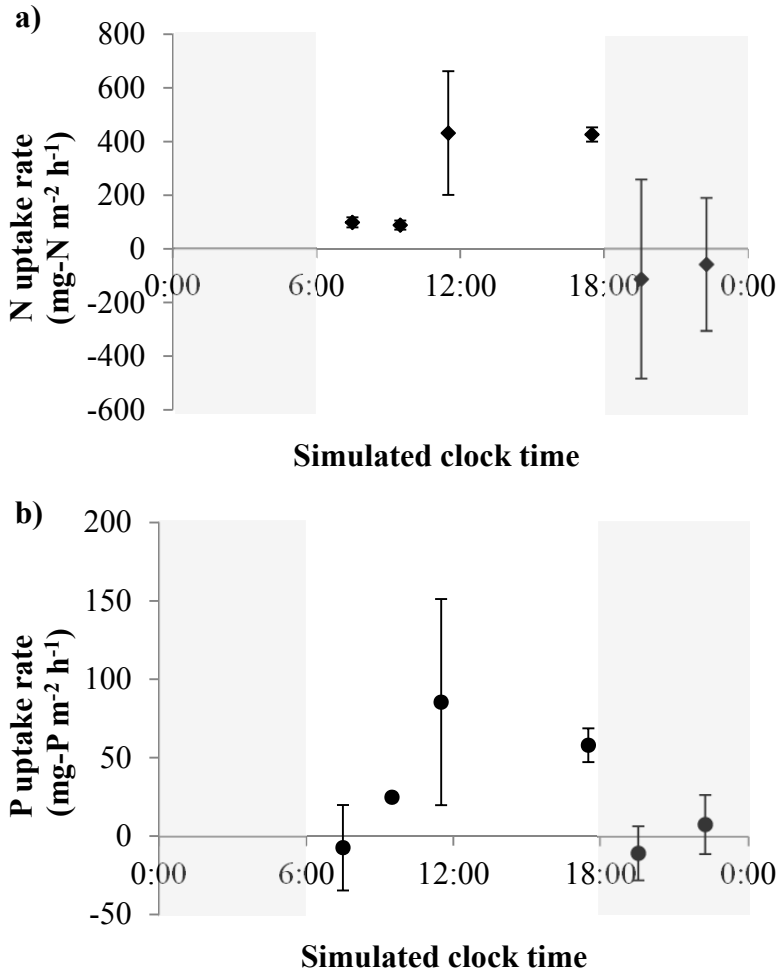

Figure 5.5 (a) Nitrogen and (b) phosphorus uptake rate expressed per illuminated area of *C.sorokiniana* grown on five times diluted synthetic urine at 0.46-day HRT under simulated day/night cycle. Grey area represents the dark period. Error bars show standard deviations (n=2).

The net daily nitrogen and phosphorus uptake rates in the 0.46-day and 2.6-day HRT respectively were 3.42 and 6.12 g-N m^{-2} d^{-1} and 0.65 and 0.56 g-P m^{-2} d^{-1} (Table 2). Under continuous illumination at 2.6-day HRT, the net daily nitrogen and phosphorus uptake were 4.52 g-N m^{-2} d^{-1} and 0.51 g-P m^{-2} d^{-1}, respectively

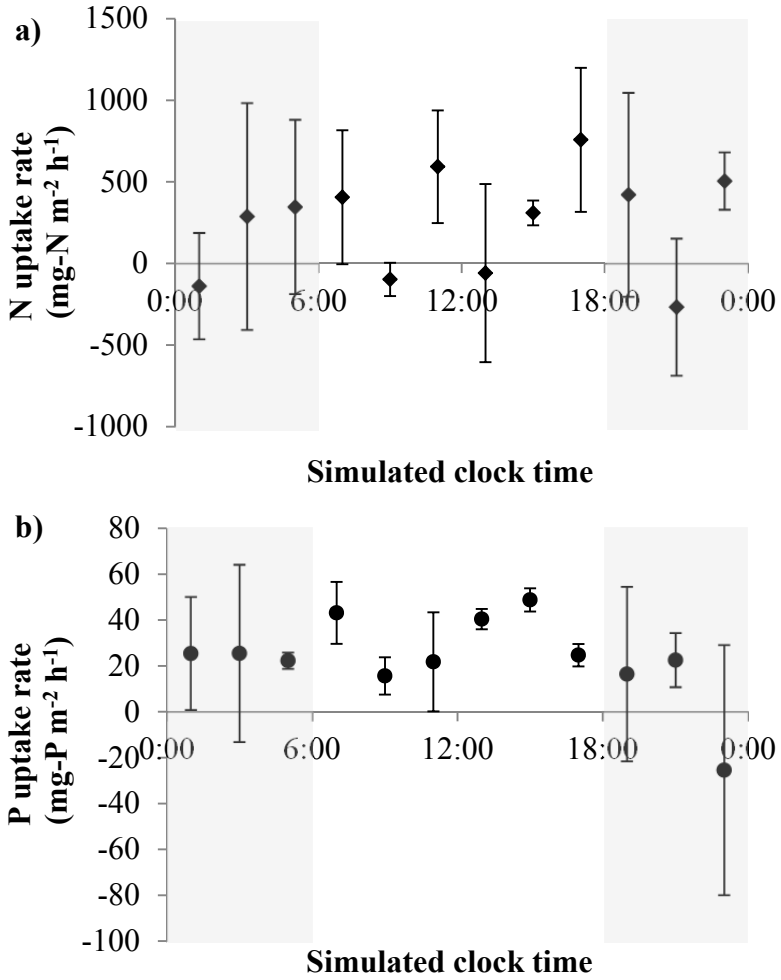

Figure 5.6 (a) Nitrogen and (b) phosphorus uptake rate expressed per illuminated area of *C.sorokiniana* grown on three times diluted synthetic urine at 2.6-day HRT under simulated day/night cycle. Grey area represents the dark period. Error bars show standard deviations (n≥3).

Unexpectedly, the net daily nitrogen uptake in the 2.6-day HRT reactor under simulated day/night cycle was significantly higher than that in the 0.46-day HRT reactor and that under continuous illumination at 2.6-day HRT. For this reason, the nitrogen uptake rates were also calculated based on the biomass productivity and its average protein content using a protein-nitrogen fraction (0.168 g-N g-protein^{-1}) as reported by Kliphuis et al. (2012). As shown in Table 5.2, the estimated nitrogen uptake rate at 0.46-day HRT (3.53 g-N m^{-2} d^{-1}) fits well with the nitrogen uptake rate calculated from the nutrient analyses. In contrast, for 2.6-day HRT under day/night cycle the nitrogen uptake rate estimated from the biomass productivity was much less than that based on the nutrient analyses. Smaller estimated

nitrogen uptake rate was also observed under continuous illumination in a 2.6-day HRT reactor but the difference was much smaller. Possibly the same measurement errors causing the large deviations in the nutrient uptake rates also resulted in this deviation of daily averaged rates and we suggest that the values recalculated from the biomass productivity are the most accurate.

Table 5.2 Net daily areal biomass productivity and biological nutrient uptake rates for both 0.46-day and 2.6-day HRT reactors

Parameter/ Operating condition	0.46-day HRT	2.6-day HRT	
	Day/night cycle	Day/night cycle	Continuous illumination
Daily light input (mol-photons m^{-2} d^{-1})	41	37	37
Areal productivity (g-dw m^{-2} d^{-1})	32.9	30.0	36.8
Daily average biomass yield on light energy (g-dw mol-photons^{-1})	0.80	0.81	0.99
Nitrogen uptake rate (g-N m^{-2} d^{-1})	3.42	6.12	4.52
Phosphorus uptake rate (g-P m^{-2} d^{-1})	0.65	0.56	0.51
Calculated nitrogen uptake rate (g-N m^{-2} d^{-1})*	3.53	3.14	3.60

* Calculated nitrogen uptake rate was estimated from areal productivity and average protein content of the biomass in both reactors. The protein-N content of the biomass of 0.168 g-N g-protein^{-1} was taken from Appendix D of Kliphuis et al. (2012).

5.3.4 Biomass composition

Figure 5.7 shows mass percentages of protein, total fatty acids and starch in the biomass of the 0.46-day and 2.6-day HRT reactors. In the 0.46-day HRT reactor, the protein and starch content both expressed a daily cycle. During the first half of the light period, a decrease of the protein content can be observed followed by a substantial increase in protein content towards the end of the light period at 18:00. This subsequent increase in protein content at the end of the light period possibly is related to the consumption of starch to produce proteins, RNA and other plasmatic material (Prison and Lorenzen, 1966; Eppley et al., 1967; Post et al., 1985; Post et al., 1986; Ogbonna and Tanaka, 1996). The sharp decrease of protein content at the early light period is related to the increase in the relative starch

content; a balancing effect of the different biomass constituents. The starch content of the biomass in this short HRT reactor was low at night to early day period (1.3–2.6 %). It increased to 30 % during the first half of the light period and then decreased again to 2% at the end of the light period. The big difference in starch content between day and night for the 0.46-days HRT reactor can be explained by the fact that under high light supply rates (i.e. at low biomass density) at 0.46-day HRT, microalgae cells make use of their intrinsic (starch) storage capacity. Starch is an energy reserve and it is consumed again to provide ATP and building blocks for cell growth which include proteins but also other components such as lipids and non-starch carbohydrates (Zachleder and Brányiková, 2014).

In the 2.6-day HRT reactor, the protein and starch content was lower and stable due to a low specific light supply rate at high biomass density. The starch content of the biomass in the 2.6-days HRT reactor was within a range of 7.8 to 8.8 %. Brányiková and co-workers (2011) also reported lower biomass starch content under high cell density and average low light intensity for asynchronous culture of *Chlorella vulgaris*.

On the basis of a daily average, the protein and total fatty acid content in the two reactors were not significantly different from each other: 48-73 % proteins and 7.4–9.5 % fatty acids in the 0.46-days HRT reactor and 60-71 % proteins and 8.0-9.2 % fatty acids in the 2.6-days HRT reactor. Similar values were also reported by others for *Chlorella sp.* (Aaronson and Dubinsky, 1982; Matsukawa et al., 2000; Becker, 2007). Total fatty acid profiles showed that in both reactors C16:0, C16:3, C18:2(n-6), and C 18:3(n-3) were the major fatty acids, as is commonly found for *Chlorella* sp. (Zhukova and Aizdaicher, 1995; Petkov and Garcia, 2007).

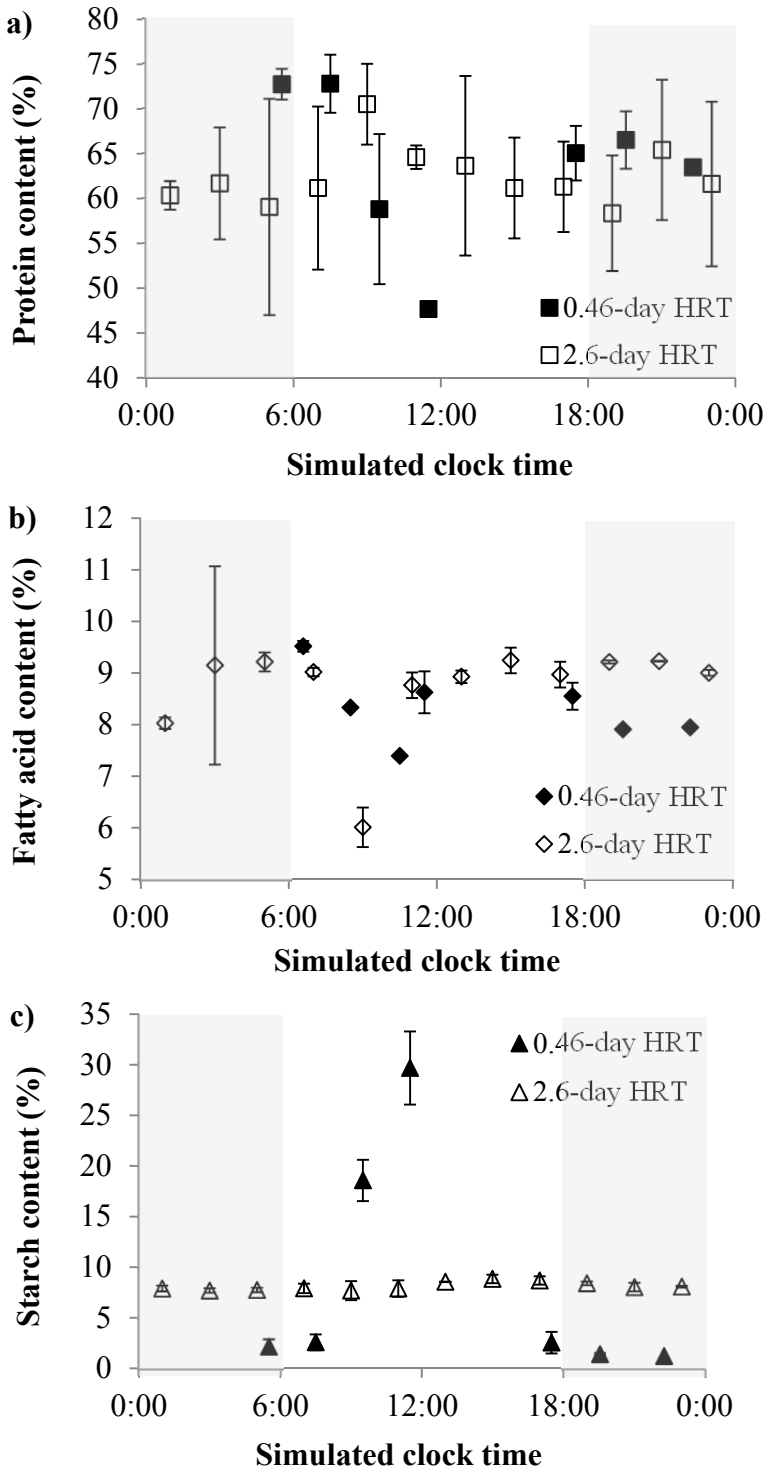

Figure 5.7 Protein (a), total fatty acid (b), and starch (c) contents of *C.sorokiniana* grown in synthetic urine under simulated day/night cycle at different HRTs. Grey area represents the dark period. Error bars show standard deviations (n=2; duplicate samples).

5.3.5 Operation of microalgae cultivation under day/night cycle

The findings of this study show that high biomass productivity can be obtained during simulated day/night cycles with the mesophilic microalgae *Chlorella sorokiniana* reaching more than 30 g-dw m^{-2} d^{-1}. Varying HRTs or reactor dilution rates must be adopted to maximize biomass productivity. For a safe operation only during day time fresh (diluted) urine can be fed to the system. In the night no dilution should be carried out in order to guarantee an effluent with minimal residual nitrogen. During the day time the dilution rate can be as high as 43.5 L m^{-2} d^{-1} down to 7.7 L m^{-2} d^{-1} at a daily solar irradiance of 37-41 mol-photons m^{-2} d^{-1} and 12 hour day length. These values are recalculated from the applied HRTs (0.46 days and 2.6 days) and corrected for the absence of dilution during the night. The longer HRT is favored in case the urine stream is minimally diluted and higher removal efficiency is required. This is related to the higher biomass density supporting higher nutrient removal efficiency at high nutrient concentrations. Considering the variable character of daily irradiance it is safest not to operate the system at fixed dilution rate but at fixed biomass density. This mode of operation is also called turbidostat operation.

Adopting this strategy, a buffer is needed to store urine during the night or during days with low irradiance. The size of the buffer system depends upon the production of urine at night. Urine is mainly produced during the day. Night urine production accounts for approximately 34% of the total daily production (Homma et al., 2000) which, at least, determines the minimum size of the buffer system.

Regarding urine treatment, the effluent quality becomes an important factor for designing the cultivation system. The effluent nutrient concentrations will specify the required nutrient removal efficiency and subsequently, a desired biomass density and dilution rate. Meanwhile the local light conditions and the light-path of a PBR are factors determining the possible biomass concentration and the actual nutrient removal efficiency. If dilution is needed i.e. to decrease a dark zone in a culture, recycling part of the effluent after algae harvesting could be adopted in order to completely remove all nutrients and to avoid using fresh water as diluent.

We cannot draw a general conclusion from our findings with respect to the occurrence of nutrient uptake during the night. Hence, very low dilution rate or no dilution should be executed at night to ensure an effluent with minimal nitrogen or phosphorus concentrations. However, readily-degradable organic compounds containing in urine may support

heterotrophic microalgal growth at night and this may be an option for maintaining biomass production as well as maintaining nutrient removal during the night. There are microalgae species showing the potential to grow mixotrophically (i.e. *Spirulina platensis* and *Chlorella sorokiniana*) with an affinity to remove acetate and propionate from high strength organic wastewater (Ogbonna et al., 2000; Van Wagenen et al., 2014). Furthermore, organic compounds contained in urine can also support heterotrophic bacterial growth which is beneficial in terms of urine treatment. Previously published studies indeed report COD removal of up to 70% in the microalgae cultivation system in human urine (Tuantet et al., 2014).

5.4 Conclusion

A simulated day/night cycle (12/12 h) was applied for *Chlorella sorokiniana* grown in synthetic urine at two different biological states: fast-growing microalgae in a 0.46-day HRT reactor and slow-growing microalgae in a 2.6-day HRT reactor. The microalgae specific growth rate, areal biomass productivity, nutrient uptake rates, and biomass composition were exerted during the day period for fast-growing microalgae. However, net daily performances of both reactors were similar. Under day/night cycle, high photosynthetic efficiency could be maintained and the system performances were slightly inferior to those under continuous illumination. We suggest that the microalgae cultivation system can be running robustly at a short HRT during the day period, and a long HRT or no feeding strategy can be adopted during the night to maximize microalgae productivity and to ensure minimal nutrient discharge for urine treatment in outdoor cultivation.

Acknowledgement

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6

General discussion

6.1 Introduction

Recent interest to improve the sustainability of municipal wastewater treatment systems has provided the impetus for new treatment concepts, addressing issues such as maximizing water and energy recovery and producing inorganic and organic fertilizers (Verstraete and Vlaeminck, 2011). In developed countries such as the Netherlands, 122 liters of water is consumed per capita per day for domestic use and this is later discharged as wastewater (EUREAU, 2009). Household wastewater is discharged from different sources including the toilet (black water), laundry machines, hand basins, the bathroom, and the kitchen. Black water contains large amounts of organic pollutants (mainly from feces) and nutrients (mainly from urine). However, dilution with grey water and storm water makes nutrient and energy recovery more difficult and costly. Although conventional wastewater management has been widely accepted because it offers comfort to people, urine source separation has also been acknowledged as a favorable and acceptable technology (Lienert and Larsen, 2009). Source-separation concepts facilitate energy and nutrient recovery from black water and/or urine while treated grey water can be reused for irrigation or toilet flushing (Abu Ghunmi et al., 2010; Hernández Leal et al., 2011).

Among the techniques applied for urine treatment, struvite precipitation seems to be the only technique for simultaneous recovery of phosphorus and nitrogen. But with the low N:P molar ratio of struvite of 1:1 compared with urine (N:P of >25:1), this technique can only remove a trivial amount of nitrogen. The remaining nitrogen needs further treatment. In this study microalgae were selected to simultaneously remove/recover nitrogen and phosphorus because they can do this at a much higher N:P molar ratio of 16:1 (balance ratio). Microalgae have been introduced for treatment of diluted wastewater in pond systems since the early 20th century (Oswald, 1988). In addition, microalgae biomass makes a versatile feedstock for biofuel and bioenergy, chemicals and fertilizer. The use of nutrient-rich wastewaters, such as urine, as microalgae cultivation media is believed to be an environmentally and economically viable way of producing microalgae biomass (Yang et al., 2011).

In this study, urine source-separation is proposed as an additional step to conventional wastewater management to reduce the nutrient load to central wastewater treatment plants (WWTP) or during a transition phase towards new sanitation concepts. It has been illustrated that urine source separation can improve effluent quality of existing wastewater treatment plant, extend the lifetime of treatment works, and reduce the need for post-denitrification

(Wilsenach et al., 2005). Furthermore, nutrients are expected to be effectively recovered from urine within this concept.

6.2 Microalgae cultivation for human urine treatment and production of microalgae biomass

6.2.1 Microalgae growth in urine

Only a few studies have been conducted towards microalgae and/or cyanobacteria cultivation on human urine. In these studies a few species were used, for example *Scenedesmus acuminatus* (Adamsson, 2000) and *Arthrospira* (*Spirulina*) *platensis* (Feng and Wu, 2006; Yang et al., 2008; Chang et al., 2013), utilizing highly diluted synthetic urine or 50-180 times diluted human urine. In batch mode, *A. platensis* could remove nitrogen and phosphorus up to >85% and 98%, respectively (Yang et al., 2008; Yang et al., 2008; Chang et al., 2013). In Chapter 2, for the first time, the successful growth was demonstrated of *Chlorella sorokiniana* utilizing non-diluted urine in batch mode. Under light-limited conditions, the microalgal specific growth rate was as high as 0.104 h^{-1} as long as iron, magnesium and micronutrients (Cu, Fe, Mn, and Zn) were supplemented at sufficient quantities. Microalgae growth in both synthetic and human urine at different nutrient concentrations ranged between 0.083 and 0.123 h^{-1}.

During chemostat operation, rapid growth of *C.sorokiniana* in synthetic urine was confirmed (Chapter 4). A reactor dilution rate of 0.24 h^{-1} was successfully applied while a high biomass yield on light of 0.83 g-dw mol-photon^{-1} could be maintained under light saturated conditions. This growth rate was similar to the highest rates reported in standard media (0.27-0.29 h^{-1}) (Sorokin, 1967; Cuaresma et al., 2009). At 0.27 h^{-1}, Cuaresma et al. (2009) obtained a biomass yield of ~0.8 g-dw mol-photon^{-1}. Although *C.sorokiniana* has shown superior growth in urine compared to some other species (i.e. *C.vulgaris*, *S.obliquus*, and a mixed culture, unpublished results), also other microalgal strains may grow with urine to produce specific compounds such as triacyl glycerols (TAGs) as a feedstock for biodiesel (Breuer et al., 2013; Münkel et al., 2013) or building blocks for production of bio-plastics. This aspect certainly needs further investigation.

In this thesis, the culture pH was always controlled at ~7. If the culture pH would not be controlled, a high fraction of free ammonia at the high pH of hydrolyzed urine would affect microalgae growth. Urine should therefore be kept fresh and should immediately be fed to the

microalgae. In this way, not only is free ammonia kept at lower concentrations, but the availability of other essential elements i.e. phosphorus, magnesium, iron, and trace elements also is higher.

6.2.2 Microalgal biomass productivity

In a continuous system with artificial light, *C.sorokiniana* has shown rapid growth in concentrated human urine (Chapter 2). In a photobioractor (PBR) with a light-path of 10 mm operated at a hydraulic retention time (HRT) of 1 day (dilution rate of 0.042 h^{-1}), the microalgal biomass productivity ranged from 104 mg-dw L^{-1} h^{-1} at 490 µmol-photons m^{-2} s^{-1} with 20-times diluted urine to 387 mg-dw L^{-1} h^{-1} at 1,540 µmol-photons m^{-2} s^{-1} (24-h illumination) with 3-times diluted urine. When the system was optimized by shortening the PBR light-path from 10 to 5 mm, the urine N:P ratio was reduced, and magnesium was added, the biomass productivity increased to 617 mg-dw L^{-1} h^{-1} with 3-times diluted urine at 1,550 µmol-photons m^{-2} s^{-1} (Table 6.1).

Microalgae biomass productivity can be further stimulated by optimizing the dilution rate (Chapter 4). Under continuous illumination at 1,530 µmol-photons m^{-2} h^{-1}, the optimal dilution rate that gives the highest biomass yield on light of 0.98 g-dw mol-photons^{-1} was between 0.1 and 0.15 h^{-1}. With this dilution rate, the biomass productivity was as high as 1,045 mg-dw L^{-1} h^{-1} with synthetic urine. However, with non-diluted human urine at a dilution rate of 0.1 h^{-1}, the biomass productivity decreased to 620 mg-dw L^{-1} h^{-1}. Apparently, the high ammonia concentrations (up to 1,500 mg of total ammonium-N L^{-1}) inhibited microalgae growth. In Chapter 2, a significantly reduced growth of *C.sorokiniana* was also observed at total ammonium concentrations higher than 1,400 mg NH_4^+-N L^{-1} (60-140 mg of NH_3-N L^{-1} at 35ºC and pH ≤8.2).

A high light intensity and a short light-path PBR support high biomass productivities when using concentrated urine. When day/night cycles are applied at a relatively low light supply, the net productivities in a 0.46- and 2.6-day HRT reactor were 98 and 125 mg-dw L^{-1} h^{-1} at the highest light intensity at noon at 1,500 and 1,340 µmol-photons m^{-2} s^{-1}, respectively. In a 2.6-day HRT reactor, day/night cycles resulted in a ~19% lower biomass productivity than under continuous light supply of 430 µmol-photons m^{-2} s^{-1}. In Table 6.1 microalgae productivities obtained in this study are compared with the results from other studies. Biomass productivities obtained with continuous irradiance are similar to those under

Table 6.1 Comparison of biomass productivity and biomass yield on light of microalgae grown in this study and under different culturing conditions in other studies.

Microalgae strain	Growth medium	Illumination period (h)	Light intensity (μmol-photons m^{-2} s^{-1})	Biomass productivity (mg-dw L^{-1} h^{-1})/1	Biomass yield on light (g-dw mol-photons^{-1})	References
C.sorokiniana	Human urine	24	1,540	387	0.70	Chapter 3
C.sorokiniana	Human urine	24 (with optimization)	1,550	617	0.55	Chapter 3
Chlorella sorokiniana	Synthetic urine	24	1,530	1,042	0.98	Chapter 4
Chlorella sorokiniana	Human urine	24	1,530	617	0.57	Chapter 4
Chlorella sorokiniana	Synthetic urine	12	1,340-1,500/2	196-250	0.80-0.81	Chapter 5
Chlorella sorokiniana	Synthetic urine	24	430	154	1.0	Chapter 5
Chlorococcum littorale	MC medium	24	2,000	383	0.53/2	Hu et al. (1998)
Chlorella sorokiniana	Urea-based modified M-8a	24	2,100	508	1.0	Cuaresma et al. (2009)
Scenedesmus obliquus	Secondary effluent	14	250	26	0.80/3	Ruiz et al. (2013)
Scenedesmus sp.	Secondary effluent	24	200	13	n.r.	Dickinson et al. (2013)
Spirulina platensis	Synthetic human urine	24	300	3	n.r.	Yang et al. (2008)

/1 the biomass productivity is averaged over illumination period.

/2 maximum light intensity at noon.

/3 calculated values from reported data.

continuous illumination at high light supply in standard media (Hu et al., 1998; Cuaresma et al., 2009). Furthermore, the biomass productivities obtained in this study are much higher than other reported values when wastewater was utilized even when applying day/night cycle.

6.2.3 Nutrient removal/recovery in microalgae cultivation system

In a short light-path photobioractor (5-10 mm light-path) *C.sorokiniana* has shown a high capacity to remove nutrients from concentrated human urine (Chapter 3). The highest rates that were observed were 54 mg-N L^{-1} h^{-1} and 6 mg-P L^{-1} h^{-1}. At optimized reactor dilution rates (0.1-0.15 h^{-1}), even higher nutrient removal rates were obtained of 100-125 mg-N L^{-1} h^{-1} and 14-19 mg-P L^{-1} h^{-1} (Chapter 4). Nitrogen removal efficiency in minimally diluted urine (dilution factor <10) ranged between 40% and 70%. Due to the high N:P ratio of urine, the phosphorus removal efficiency could be maintained at >99%. After shortening the PBR light-path from 10 to 5 mm, adjusting the urine N:P ratio to 23-25:1 and after adding magnesium, the nitrogen removal efficiency could be further increased to 80%. Because microalgae produce oxygen they stimulate aerobic heterotrophic bacterial growth on the organic compounds in urine (expressed in COD, Chemical Oxygen Demand). This COD was removed up to 70%, depending on the readily biodegradable fraction of this COD in urine.

Under simulated day/night cycles (Chapter 5) in a 0.46-day HRT reactor, high light availability during the day resulted in high growth rates and hence, high nutrient uptake rates from synthetic urine. The highest nutrient uptake rates were 31 mg-N L^{-1} h^{-1} and 5 mg-P L^{-1} h^{-1} (during 11:30-17:30). The average nitrogen and phosphorus uptake rate during day light hours in a 0.46-day HRT reactor respectively were 22 mg-P L^{-1} h^{-1} and 5 mg-P L^{-1} h^{-1}. At a 2.6-day HRT, a clear relationship between light intensity and nitrogen uptake rate during the day was not observed. Hence, the nitrogen uptake rate during day light hours was estimated based on the biomass production and its protein content to be 27 mg-N L^{-1} h^{-1}. The average phosphorus uptake rate in a 2.6-day HRT reactor was 5 mg-P L^{-1} h^{-1}. In none of the reactors nutrient uptake took place during the night.

Although much lower nutrient uptake rates were obtained under day/night cycles as compared to those under continuous illumination, they are still higher than those reported by others. Reported and calculated nutrient uptake rates of several microalgal strains grown in different wastewaters are within a range of <10 mg-N L^{-1} h^{-1} and <1 mg-P L^{-1} h^{-1} for microalgae grown in less concentrated wastewater (Molinuevo-Salces et al., 2010; Wang et al., 2010; Cai et al., 2013; Ruiz et al., 2013) to ~35 mg-N L^{-1} h^{-1} in 50% of liquid swine

manure (Gantar et al., 1991). Zhang et al. (2014) reported nutrient uptake rates of <5 mg-N L^{-1} h^{-1} and <1 mg-P L^{-1} h^{-1} for *C.sorokiniana* grown in diluted human urine in a semi-continuous system. However, much lower rates were observed for *S.platensis* grown in diluted urine (Yang et al., 2008; Yang et al., 2008; Chang et al., 2013). This clearly shows the high nutrient removal efficiency from minimally diluted urine utilizing a short light-path photobioreactor.

6.3 Pre-design of microalgae cultivation system under Dutch irradiance conditions

The potential of a microalgae based system to treat stored urine and produce microalgae biomass was evaluated for Dutch irradiance conditions. Detailed information about urine characteristics and about the calculation procedure can be found in the supporting information (SI). A panel type of photobioreactor with a light-path of 10 mm was adopted and an (optimal) temperature of 38ºC was assumed. In this system, the microalgae biomass density was dictated by the phosphorus concentration in stored urine as the growth limiting nutrient. Reactor performance and reactor size (i.e. ground area) were mainly influenced by light availability. The actual biological performance of this hypothetical panel photobioreactor was deduced from laboratory scale studies as reported in Chapter 4. Monthly averaged light intensity and averaged day light hours were used to illustrate the effect of seasonal changes in irradiance on biomass productivity, area requirement, nutrient removal rate, and effluent quality of the reactor when receiving urine daily produced by one individual.

6.3.1 Biomass specific growth rate and areal productivity

After collection and storage, the urine associated nitrogen and phosphorus loads to the microalgae cultivation system were 9.0 g-N p^{-1} d^{-1} and 0.54 g-P p^{-1} d^{-1}, respectively Because the N:P ratio of stored urine was 38:1, phosphorus becomes the first depleted nutrient. This amount of phosphorus supports 34.2 g-dw of biomass produced per person per day and the corresponding biomass density ($C_{x,P}$) is 14.2 g-dw L^{-1}. Because of this high biomass density the specific light supply rate to the microalgae is low, and a significant part of the culture suffers from light limitation. A large fraction of the absorbed light energy is needed for cellular maintenance, resulting in a low specific growth rate. During periods with a very low monthly averaged irradiance, i.e from November to January, this implies that no net growth

takes place. High light availability during late spring to summer results in specific growth rates of 0.21 - 0.24 d^{-1} (during May to August).

Figure 6.1 shows the biomass areal productivity. As explained previously, biomass production only is possible from February to October. The areal productivity (P_{area}) predicted for this period varies between 1 and 23 g-dw m^{-2} d^{-1}. Between May and July the productivity predicted is highest at 22 g-dw m^{-2} d^{-1}. However, this predicted value is lower than the productivity that was actually observed at a long HRT under day/night cycle operation of 30 g-dw m^{-2} d^{-1} (Chapter 5) while the amount of daily light input and PBR are similar. Perhaps this can be explained by a higher biomass density in the calculations, viz.14.2 g-dw L^{-1}, compared to an average density of 6.8 g-dw L^{-1} as obtained in the experiments. This higher biomass density gives a lower growth rate and lower productivity (Eq. 6.3 and 6.4 in the SI). This prediction is a worst-case scenario because the biomass yield on light $Y_{x,E}$ used in the calculation is based on experiments performed at high light intensity (1,500 μmol-photon m^{-2} s^{-1}). Under high light intensity, photosaturation will lead to lower photosynthetic efficiency and hence lower biomass yield on light. In outdoor conditions, higher biomass yields on light can be expected because light levels will be lower than 1,500 μmol-photons m^{-2} s^{-1} on many days, and on many hours during a day. In other words, the current approach allows for a conservative estimate of real outdoor production potential.

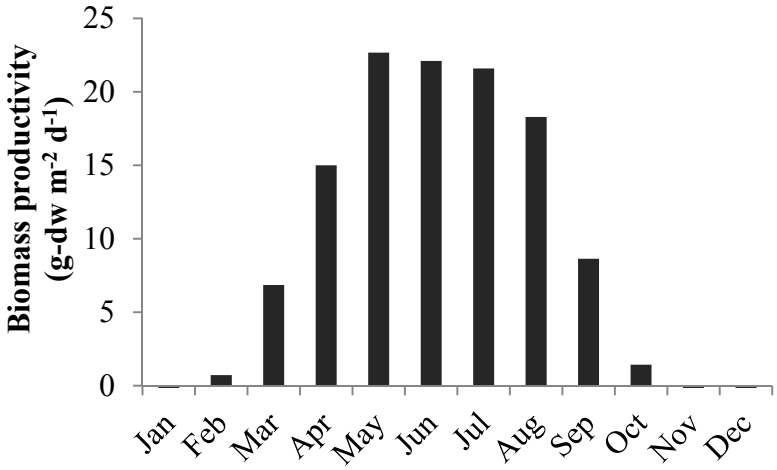

Figure 6.1 Biomass productivity in response to seasonal changes of solar irradiance

6.3.2 Area requirement

In outdoor cultivation systems, the seasonal change in solar irradiance affects the light availability, the microalgae specific growth rate and accordingly, the biomass productivity

and area requirement. Figure 6.2 shows the area requirement related to seasonal irradiance. During late spring and the summer period (May to August) only 1.5 (in May-June) to 1.9 m^2 $person^{-1}$ (in August) is required. During late spring and early autumn, 2.3 (April) to 5.0 m^2 $person^{-1}$ (March) is needed. Much bigger areas are needed in February and October.

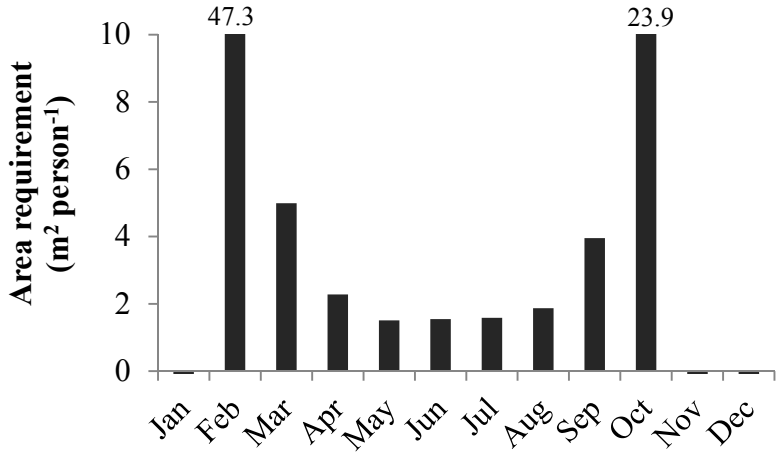

Figure 6.2 Area requirements in relation to seasonal changes of solar irradiance. (Numbers represent area requirements for values higher than presented in Y-axis)

6.3.3 Nutrient recovery rates

Nutrient removal/recovery rates are determined by biomass productivity and biomass nitrogen and phosphorus content. During May to July, the nutrient recovery rates are the highest at 14-15 mg-N m^{-2} h^{-1} and 1.9-2.1 mg-P m^{-2} h^{-1}. In April and August, the nutrient recovery rates are 11-13 g-N m^{-2} h^{-1} and 1.6-1.8 mg-P m^{-2} h^{-1}. As mentioned earlier, the high biomass density results in lower biomass productivities and therefore also the nutrient recovery rates are much lower than those under simulated day/night cycles 22-27 mg-N L^{-1} h^{-1} and 3-5 mg-P L^{-1} h^{-1} (Chapter 5)

6.3.4 Effluent quality

During the period that microalgae growth is feasible, inorganic phosphorus is assumed to be fully taken up by microalgae. Organic-P is assumed not to be consumed by the microalgae and leaves the system with the effluent. In practice, however, organic P might be consumed by bacteria during oxidation of organic compounds in urine, or is broken down into inorganic phosphate that can subsequently be used by the microalgae. As shown in Chapter 2, nearly 100% of the urine's total phosphorus (TP) was removed showing that also organic-P can be removed in the microalgae reactor. The corresponding effluent phosphorus

concentration is expected to be lower than required for discharge: 2 mg-P L^{-1} for urban wastewater effluent from a WWTP treating 10,000-100,000 person equivalent (PE) (European Union, 1991).

The higher N:P ratio of urine compared to the N:P ratio of microalgal biomass of 16:1 results in relatively high effluent nitrogen concentrations of 2.29 g-N L^{-1}. This concentration also is much higher than what is required for discharge; the allowed discharge level is 10-15 mg-N L^{-1} depending on the WWTP size (European Union, 1991). Unless the effluent is discharged to the central sewer, additional nitrogen removal therefore is required. Nitritation-anammox may be a feasible process for further nitrogen removal with a proven nitrogen removal efficiency of 85-89% for anaerobic black water effluent with an ammonium concentration of 1-1.5 g- N L^{-1} (de Graaff et al., 2010). However, further investigation may be needed for the nitritation-anammox process at high ammonium concentration as for microalgae reactor effluent. A recently developed nitrogen recovery technology is a microbial fuel cell (MFC). This can achieve an ammonium recovery rate of 3.3 g-N d^{-1} m^{-2} (membrane surface area) while producing 3.5 kJ $g\text{-}N^{-1}$ of energy (Kuntke et al., 2012). This technique could also be suitable for removing remaining nitrogen and COD from the effluent of a microalgae cultivation system, providing that sufficient amount of biodegradable COD is available.

As was mentioned earlier, urine also contains organic compounds, expressed as COD. Without bacterial oxidation the effluent COD concentration of a microalgae reactor would be as high as 4.58 g L^{-1} However, in Chapter 3 it was shown that readily biodegradable COD can be removed by heterotrophic bacteria, such that 70% of this COD was removed. This results in an effluent COD concentration of 1.38 g L^{-1}. However, this concentration still is far above the discharge guidelines for urban wastewater effluent of 125 mg L^{-1} and further treatment is needed unless the effluent is discharged to the sewer.

Even though the concentrations of nutrients and COD in the effluent from a microalgae cultivation system are higher than the discharge guidelines, the nitrogen, phosphorus and COD loads of toilet water to the central wastewater treatment plant would be reduced significantly, viz. 33%, 38% and 13%, respectively. In this case, the urine fed to microalgae reactor is assumed to be fully hydrolyzed and phosphorus is significantly precipitated. If urea hydrolysis is prevented, more phosphorus is available and microalgae would have reduced the nutrient loads from toilet water by 50% for N and 58% for P.

6.3.5 Productivity enhancement

As compared to productivities obtained under day/night cycles in Chapter 5, the prediction of biomass productivity seems to be too conservative. This is because the biomass yield on light was estimated based on data obtained under high light intensity including a considerable effect of photosaturation. In outdoor practice, there are periods with lower irradiance during a day where higher biomass yields on light can be expected. In this calculation, the high nutrient concentrations in urine and the low light availability in the Netherlands give high biomass densities and low productivities. During late autumn, winter and early spring, light availability is extremely low and microalgae biomass production in a 1-cm light-path reactor in concentrated urine is not possible. Shortening the photobioreactor light path can reduce the dark zone and enhance biomass productivity in areas with low light availability. In this calculation, shortening the light path from 1 cm to 0.5 cm results in a more than 30% higher biomass productivity (Figure 6.3a). In this case, microalgae growth also becomes possible in November and January. The area requirement is reduced by more than 11% (Figure 6.3a and 6.3c). Photobioreactors with a light path of only 6-8 mm are feasible i.e. inclined lane type photobioreactors (flow-through system). The biomass density obtained from this photobioreactor type can be as high as 40-50 g-dw L^{-1}, resulting in biomass productivities of 11-38 g-dw m^{-2} d^{-1} (Doucha et al., 2005; Doucha and Lívanský, 2006; Doucha and Lívanský, 2009), which is comparable or even higher than those obtained in our pre-design during spring to summer period.

In addition to shortening the light-path, dilution of urine also helps to decrease the biomass density because it increases light availability for the microalgae. This can be accomplished by recycling part of the effluent or by applying toilets that use small amounts of flushing water. Figures 6.3b and d show biomass productivities and area requirements of microalgae cultivation systems with 1:1 urine dilution (1 urine volume: 1 diluting water volume). In a 1-cm light-path reactor, dilution of 1:1 increased biomass productivity for more than 31% while the area requirement decreases for more than 62%. A combination of shortening the PBR light-path and urine dilution would result in more than 47% increasing biomass productivity and a more than 66% smaller area requirement. In December, however, the light availability is still too low to grow microalgae in 1:1 diluted urine. Urine needs to be diluted further for December cultivation. During the winter period, not only light availability hinders microalgae growth, but also low temperatures will retard their growth. Unless waste heat is available, microalgae cultivation systems for biomass production and urine treatment

are not attractive for areas with a cold and dim winter period. Furthermore, during this period urine needs to be stored until light intensity allows the system to run robustly such as in summer period.

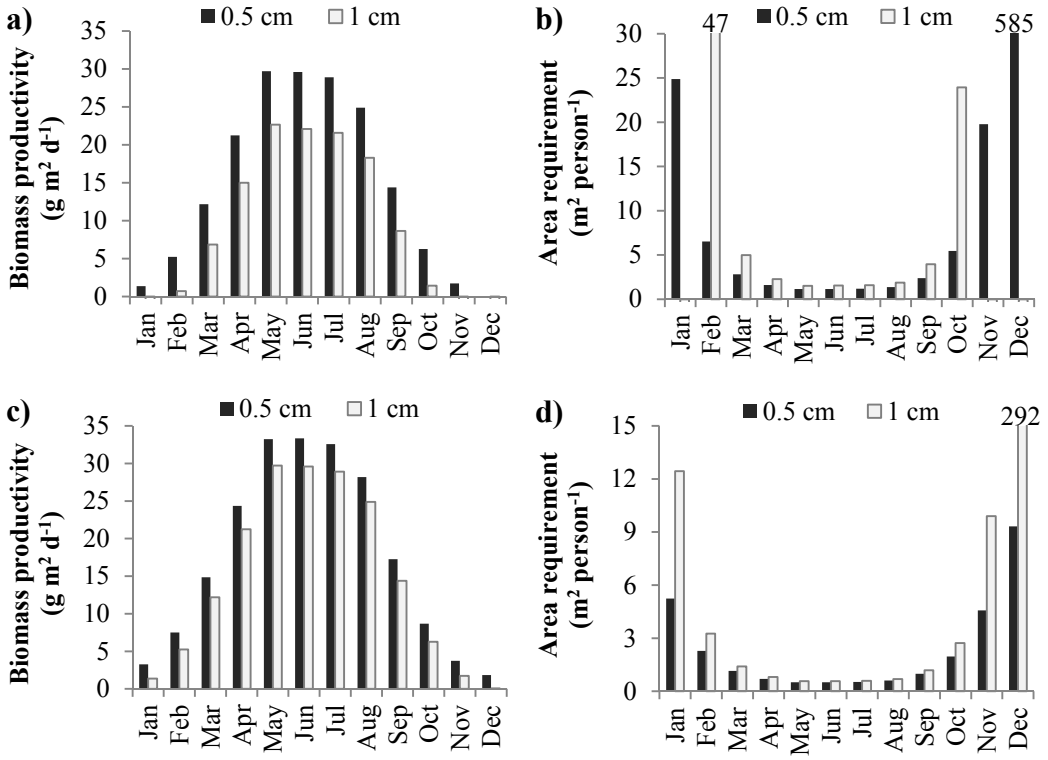

Figure 6.3 Comparison of calculated biomass productivity and area requirement of *C.sorokiniana* cultivated in a 0.5 or 1 cm light-path photobioreactor in non-diluted urine (a and b) and in 1:1 urine dilution (c and d). (Numbers represent area requirements for values higher than presented in Y-axis)

6.4 Comparison with other urine treatment/recovery technology

Table 6.2 shows a comparison between different urine treatment technologies with respect to N, P and COD removal efficiency, additives that are needed and the products that are obtained. Similar to struvite precipitation, microalgae treatment will give almost 100% phosphorus recovery. Nitrogen recovery/removal by microalgae treatment (up to 80% N removal) is much higher than obtained by struvite precipitation (~3% N removal) and a large fraction (up to 80%) of the COD can be removed. Physical-treatment technologies other than struvite precipitation are not energy efficient (Maurer et al., 2006), unless excess energy is available. For example, Saniphos in Zutphen, the Netherlands, applies ammonia stripping and

Table 6.2 Comparison between microalgae technology and other technologies for urine treatment.

Treatment technology	Nitrogen removal	Phosphorus removal	COD removal	Additives	Products	Source
Struvite precipitation	3%	~98%	None	Mg^{2+} with Mg:P of 1-1.2:1	Struvite with N:P ratio of 1:1	Maurer et al. (2006)
Ammonia stripping	Up to >90%	None	None	Pressure/ increased temperature, pH	10% ammonia solution	Başakçilardan-Kabakci et al. (2007)
Partial nitrification/ Anammox	85-90%	None	~70% (during nitrification)	Calcium addition improves biomass granulation	None	de Graaff et al. (2011)
Microbial fuel cell (MFC)	3.3 g-N d^{-1} m^{-2} (membrane surface area), up to 50% recovered	None	Biodegradable COD is expected to be fully removed	Necessary to remove P to prevent scaling (struvite precipitation)	Surplus energy of 3.5 kJ $g\text{-}N^{-1}$ and enriched ammonium liquid	Kuntke et al. (2012)
Microalgae	< 80% (~3 g-N d^{-1} m^{-2}, illuminated area under day/night cycle)	~100%	~ 70%	CO_2, Mg^{2+}, trace elements	Microalgae biomass with N:P ratio of 16:1	Chapter 3

struvite precipitation to recover both N and P from urine using excess heat from sludge composting. Biological nitrogen removal by combined partial nitritation and anammox was proposed for urine treatment (Wilsenach and van Loossdrecht, 2003; de Graaff et al., 2010). With this technology all the nitrogen can be removed but nitrogen and phosphorus are not recovered. Recently, microbial fuel cells (MFC) have been used for ammonium recovery while producing electricity from the COD in the urine (Kuntke et al., 2012). Although high ammonia can be recovered and COD is removed, phosphorus removal is low in the MFC. A combined technique for phosphorus recovery such as pre-struvite precipitation is needed while microalgae technology can recover both compounds at high efficiency in one reactor.

Considering the fraction of nitrogen, phosphorus, and COD simultaneously removed/recovered and the low requirement for additives, microalgae are an attractive technology for urine treatment, but probably only in areas with a high irradiance and temperature such as tropic regions. Given that waste CO_2 is available, microalgae technology will become much more promising since high density culture needs also high CO_2 supply. If urine separation is applied as an additional step to conventional wastewater treatment facilities, the needed surface area for algae growth should be added to the 0.2-0.3 m^2 $person^{-1}$ for conventional activated sludge systems (von Sperling, 1996). This additional area should compensate for the costs and energy requirement associated with nitrification, denitrification and biological or chemical phosphorus removal accompanying activated sludge systems.

6.5 Application of microalgae biomass

Microalgae biomass can be used for several applications: production of the energy carrier methane by anaerobic digestion, biodiesel, and for carbohydrate fermentation to produce butanol or ethanol, etc. (Craggs et al., 2013). To produce 1 kg of biodiesel from *C.vulgaris* biomass, Yang et al. (2011) reported that 3.7 kg water, 0.33 kg nitrogen, 0.71 kg phosphate and 0.58 kg potassium are needed. Using wastewater would considerably reduce the need for these nutrients as well as the need for valuable fresh water. Our study showed that the nutrients in urine can be a potential nutrient source for microalgae and therefore will reduce the costs and consumption of valuable resources. To improve energy recovery efficiency from microalgae biomass, remaining biomass after i.e. extraction and transesterification, can be digested to produce another form of energy, methane (Bohutskyi

and Bouwer, 2013). The nutrients that are released during anaerobic digestion can be reused for agriculture and/or the production of more microalgae as a biodiesel feedstock.

Microalgae biomass can also directly be used as slow-release fertilizer (Mulbry et al., 2005). In this manner it serves as a reservoir for nutrients which are slowly released into the soil while the microalgae biomass is being degraded. However, the required N:P ratio of fertilizers differs among different plants and growth stages. In general high nitrogen fertilizers enhance rapid growth, boost leaf growth and benefit plants in several other ways (Dana, 2013). Using microalgae biomass grown on urine as a fertilizer do partly close the nutrient cycle since nutrients originating from agriculture and subsequent food consumption are recycled to agriculture. Microalgae based fertilizers can be beneficial in some areas because they help to increase the soil organic carbon content and in this manner reduce water runoff, improve soil structure and crop productivity, and mitigate greenhouse effects due to soil carbon sequestration (Lal et al., 2004). If biodiesel would be produced from algae biomass, the low C:N ratio of the remaining biomass would make it a suitable fertilizer for areas with a high soil carbon content.

6.6 Further research

6.6.1 Pharmaceutical residues

Many pharmaceutical residues are excreted via urine (Winker, 2010). Microalgae systems have the potential to remove at least some of these pharmaceuticals by biochemical conversion. For example, fifty percent removal of carbamazepine, generally considered to be a recalcitrant compound, was reported to be removed during a 60-day culture of *Ankistrodesmus braunii* (Andreozzi et al., 2003). Our own results (preliminary data) have shown significant transformation within 2 weeks of ibuprofen, diclofenac, trimethoprim, and carbamazepine with a batch culture of *C.sorokiniana.* Only trace amounts of these pharmaceuticals were detected in the produced biomass. However, the transformation mechanisms were not identified and need further investigation. Micropollutant removal increases the possibility to safely apply the microalgae biomass as a fertilizer. However, the formation of potential hazardous intermediates and the biochemical conversion of other pharmaceuticals need further research. Furthermore, microalgae have shown deactivation of pathogens by changing culture conditions i.e. rising pH, temperature, and dissolved oxygen

(Muñoz and Guieysse, 2006). Source-separated urine itself hardly contains pathogens, but may be contaminated with pathogen during collection. Also this pathogen deactivation potential by microalgae growing on urine needs further investigation.

6.6.2 Application of algae treatment within new sanitation concepts

We have shown the potential of microalgae to recover P and partially recover N from source-separated urine. Urine composition will influence the reactor size, the amount of nutrients that can be recovered and the effluent quality. The final choice of the sanitation concept depends on the local situation. In new buildings, or in case of renovations, the application of new sanitation concept using vacuum collection and separate treatment of black water are attractive options. In existing situations, urine separation can be applied in combination with conventional collection, transport and treatment of the remaining wastewater. The introduction of water-free urinals for men in the Netherlands and elsewhere, is more and more applied in offices, restaurants, etc. The calculated annual load of nutrients (in g per person equivalent per year) that are excreted and can be recovered from urine via algae growth is summarized in Table 6.3.

Table 6.3 Nutrient loads and percentage of nutrients recovered from urine via algae growth

Nutrient load	Nitrogen	Phosphorus
Excreted nutrients (kg pe^{-1} $year^{-1}$)	3.29	0.29
Nutrients recovery by algae	39%	62%
Nutrients in precipitate (via struvite)	~0%	33%
Remaining in the effluent	61%	5%

This shows that microalgae can play an important role in nutrient recovery from urine. However, if urea hydrolysis is allowed, a significant fraction of phosphorus will precipitate and may cause blockage in the collection system. Precipitation also reduces the availability of some trace elements needed for microalgae growth and importantly, availability of phosphorus that can be recovered. As mentioned before, it is better to use fresh urine. More study therefore is needed to find out a proper way to prevent hydrolysis. The pre-design has shown that microalgae cultivation in non-diluted urine under the Dutch climate is possible during late spring to late summer period while during the rest of the year it is not an attractive

option. Although productivity improvements are possible during periods of low irradiance (shorter light-path and urine dilution), the area requirement during the winter period would still be too high. Coupling nutrient recovery from urine to the production of microalgae biomass is, therefore, only feasible in high irradiance areas. In this thesis, the culture pH and temperature were kept optimal. The challenge is to find sufficient CO_2 to support microalgae growth as well as to control pH. In addition to that for *C.sorokiniana* it is important to find ways to work at high (optimal) temperature at minimal (energy) cost. Furthermore, investigations are also needed on (1) the cost-effective and energy-efficient scale up of photobioreactors (2) screening of microalgae that produce high value compounds and can use urine as their growth medium and (3) mixotrophic microalgae growth using organic compounds present in urine to enhance nutrient removal and biomass production during the night.

Supporting Information

Microalgae cultivation system under the Dutch climate

A flat short light-path photobioreactor (PBR) was adopted as a microalgae cultivation reactor because it gives a high biomass productivity and nutrient removal efficiency (Chapters 3 and 4). In these calculations, we assumed a PBR with a light path of 10 mm, which is technically feasible. Based on the results of the day/night experiments of Chapter 5, the urine was fed to the microalgae reactor during day light hours but not during the night.

In Figure 6.4, the monthly averaged light intensity on a horizontal surface in The Netherlands (Amsterdam) is shown, together with the monthly number of daylight hours. The light intensity was averaged over the amount of daylight hours. The seasonal variation is large, ranging from 140 μmol- photons PAR m^{-2} s^{-1} in December to 650-700 μmol- photons PAR m^{-2} s^{-1} in May-August. PAR refers to photons in the so-called Photosynthetic Active Radiation range from 400 to 700 nm, which is the wavelength range of solar irradiance which can be absorbed and used by microalgae.

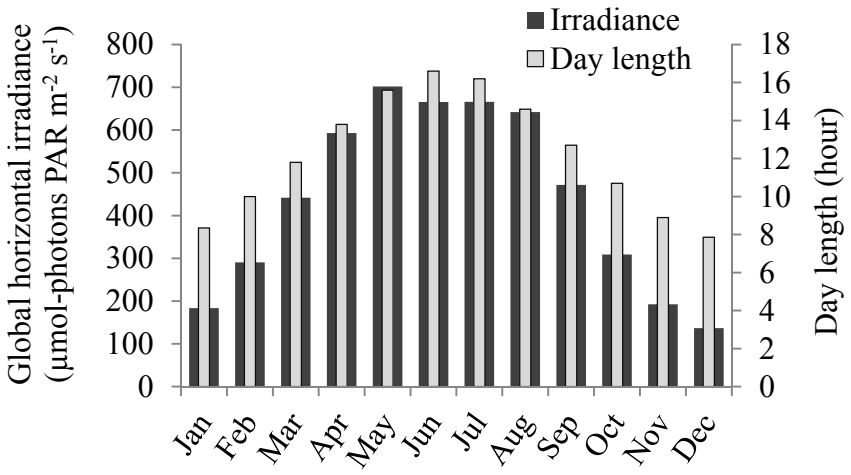

Figure 6.4 Monthly averaged global horizontal irradiance over 22 years (Jul 1983 - Jun 2005 (NASA, 2010))

Urine composition

Concentrations of nutrients (nitrogen and phosphorus) and COD, nutrient loading rates and the N:P ratio in fresh urine and urine after storage are presented in Table 6.4. These concentrations were calculated based on the assumptions that in urine 90-95% of total

nitrogen (TN) is present in the form of urea, ammonia, and uric acid, and <5-10% is amino acid associated N (Kirchmann and Pettersson, 1995). During storage, urea and uric acid are decomposed into ammonia which can be used by microalgae. Given that the storage period is short and storage is such that ammonia volatilization is negligible, 95% of TN was assumed to be available for microalgae growth.

Table 6.4 Composition and nutrient load of fresh and stored urine fed to the microalgae reactor

Parameters	Fresh urine	Stored urine fed to microalgae reactor[/1]
Urine volume (L d^{-1})	2.4[/2]	2.4
COD (g-O_2 L^{-1})	4.6	3.21[/3]
Total nitrogen		
- Loading (g-N p^{-1} d^{-1})	9.0	9.0
- Concentration (g-N L^{-1})	3.75	3.56
Total phosphorus (g-P L^{-1})		
- Loading (g-P p^{-1} d^{-1})	0.80	0.536
- Concentration (g-P L^{-1})	0.33	0.207
N:P atomic ratio	25:1	38:1

[/1] In stored urine, nutrient concentrations shown are available nutrients that can be consumed by microalgae.

[/2] Given that urine diverting toilets are applied with 5 small flushes per day (0.2 L per flush) for urine. The urine produced by an individual per day is 1.4 L.

[/3] This term is available COD is defined as biodegradable COD .

For total phosphorus (TP), about 95-100% of TP in urine is present as soluble phosphate (Udert et al., 2006). Inorganic phosphate, in particular $H_2PO_4^-$ and HPO_4^{2-}, is the desirable form for microalgae. When urine is collected and stored, precipitation of hydroxyapatite, struvite, and calcite readily occurs, particularly when urine is allowed to hydrolyze (Udert et al., 2003). The degree of precipitation depends on the degree of dilution with flush water. With a dilution factor of 1.7 (total volume of 2.4 to urine volume of 1.4), about 35% of inorganic phosphate precipitates (Udert et al., 2003). Taken into account that

95% TP is soluble phosphate of which 35% is precipitated, the remaining 62% of TP is available phosphate for microalgae growth.

In the calculations the nutrient concentrations of stored urine are used. With the imbalanced N:P ratio of stored urine (38:1) compared with a balanced ratio of microalgae (16:1 (Redfield, 1958)), phosphorus becomes the first-depleted nutrient and part of the nitrogen will thus remain in the effluent. As a consequence, the phosphorus concentration in stored urine dictates the microalgae biomass density that can be produced.

Calculations

Light availability influences microalgae growth and herewith the size of the microalgae cultivation system. The relation between microalgae growth and light intensity under constant illumination in dilute cultures is well-described and understood (Falkowski and Raven, 2007). In outdoor production systems, on the other hand, microalgae growth is based on daily and seasonally varying sunlight and growth is less well described by existing productivity models. Moreover, the productivity of cultivation systems with a light gradient going from full sunlight at the surface to almost complete darkness in the inner part of the culture (the usual case) is an area of ongoing research (Cuaresma et al., 2011; Béchet et al., 2013; Slegers et al., 2013; Lee et al., 2014).

In our calculations we adopted the work of Zijffers et al. (2010), who could accurately predict the specific growth rate of microalgae as a function of the biomass density in a short light path photobioreactor (PBR). The work of Zijffers showed that in PBRs, illuminated with over-saturating light (930 μmol-photon m^{-2} s^{-1}), the specific growth rate (μ in h^{-1}) of the microalga *Chlorella sorokiniana* could be described as a function of the specific light supply rate ($r_{E,x}$ in mol-photons g-dw^{-1} h^{-1}) and two constants: the biomass yield on light energy ($Y_{x,E}$ in g-dw mol-$photon^{-1}$) and the maintenance coefficient of the biomass ($m_{E,x}$ in mol-photons g-dw^{-1} h^{-1}) according to Eq. 6.1:

$$r_{E,x} = \frac{\mu}{Y_{x,E}} + m_{E,x} \qquad \text{(mol-photons } g^{-1}\ h^{-1}\text{)} \quad (6.1).$$

The specific light supply rate ($r_{E,x}$) is dependent on the light supply (expressed as PFD_{in}; Photon Flux Density in μmol-photon m^{-2} s^{-1}), the photobioreactor dimension (Surface

Area to Volume ratio; SVR in m^2 m^{-3} or m^{-1}), and the biomass density based on phosphorus as a limiting nutrient in urine ($C_{x,P}$ in g-dw L^{-1}) as:

$$r_{E,x} = \frac{PFD_{in} \times SVR \times 3600 \times 10^{-6}}{C_{x,P} \times 1000} \quad \text{(mol-photons } g^{-1} h^{-1}\text{)} \quad (6.2).$$

Re-calculating the experimental results obtained in Chapter 4, the relationship between $r_{E,x}$ and the reactor dilution rates (D=μ in a chemostat mode) can be plotted (Figure 6.5). Fitting this curve with Eq. 6.1, an inverse of the slope gives a $Y_{x,E}$ of 0.933 g-dw mol-photon^{-1}. The offset represents a $m_{E,x}$ of 0.0159 mol-photon g-dw^{-1} h^{-1}. This maintenance coefficient is much higher than reported values in other studies, possibly due to the high light intensity that was applied in the experiments described in Chapter 4. In addition, based on the data of Chapter 4 the extrapolation to a dilution rate of 0 h^{-1} is not very accurate because the lowest dilution rate applied still was 0.05 h^{-1}. Zijffers et al. (2010) worked at very low dilution rates (i.e. more than half of the data points were between 0 and 0.04 h^{-1}) together with high biomass densities similar to the ones we used for our design scenarios. For this reason, we considered this considerably lower $m_{E,x}$ of 0.0068 mol-photon g-dw^{-1} h^{-1} more suitable to our design condition and we adopted this $m_{E,x}$ for further calculations. Moreover, only with this lower maintenance coefficient we were able to predict the productivity measured under the simulated day/night cycles reported in Chapter 5.

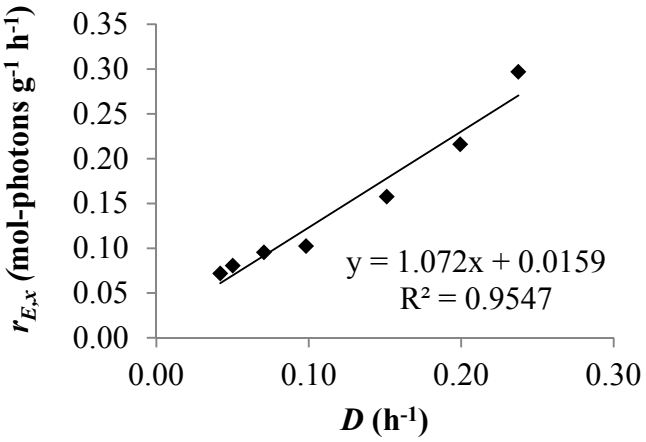

Figure 6.5 Relation between specific light supply rates and reactor dilution rates for estimate of biomass yield on light and maintenance coefficient (data obtained from Chapter 4)

With a phosphorus concentration of 0.207 g-P L^{-1} (Table 6.4) and an average phosphorus content of biomass grown on synthetic urine (Chapter 4) of 14.5 mg-P g-dw^{-1}, $C_{x,P}$ becomes 14.2 g-dw L^{-1}. The microalgae specific growth rate based on varying light availability due to seasonal change now can be calculated based on equation 1 and the estimated $Y_{x,E}$ and $m_{E,x}$. This approach excludes the complicated and detailed dynamic response of microalgae to spatially and dynamically changing light levels in real production systems. This approach also is a worst-case scenario since the biomass yield on light $Y_{x,e}$ is based on experiments performed at 1500 µmol-photon m^{-2} s^{-1} and is thus reduced by the effect of photosaturation. In outdoor conditions on many days, and on many hours during a day, light levels will be lower resulting in higher biomass yields on light. In other words, the current approach allows for a conservative estimate of real outdoor production potential.

Rearranging Eq. 6.1 gives:

$$\mu = Y_{X,E} \times \left(\frac{PFD \times SVR \times 3600 \times 10^{-6}}{C_{x,P} \times 1000} - m_{E,X}\right) \qquad (h^{-1}) \qquad (6.3).$$

The biomass productivity (P_{area} in g-dw m^{-2} h^{-1}) and area requirement of the cultivation system ($Area$ in m^2) (Eq. 6.2) were calculated from the calculated specific growth rate, the $C_{x,P}$, SVR (100 m^{-1} in 10-mm light-path PBR) and urine flow rate (F_W, m^3 h^{-1}) as shown in Eq. 6.4 and Eq. 6.5. The urine flow rate was an average flow over the day light hours (i.e. 2.4 L in 16 hours equals 12.5x10^{-6} m^3 h^{-1} in May).

$$P_{area} = \frac{\mu \times C_{x,P} \times 1000}{SVR} \qquad (g\text{-}dw\ m^{-2}\ h^{-1}) \qquad (6.4)$$

$$Area = \frac{SVR \times F_W}{\mu} \qquad (m^2) \qquad (6.5)$$

The nutrient removal/recovery rate expressed per unit ground area can be calculated from P_{area} and the averaged nutrient content of the produced biomass (103 mg-N g-dw^{-1} and 14.5 mg-P g-dw^{-1}; Chapter 4).

$$N_{removal} = P_{area} \times N_{content} \quad \text{(mg-N m}^{-2}\text{ h}^{-1}\text{)} \quad (6.6)$$

$$P_{removal} = P_{area} \times P_{content} \quad \text{(mg-P m}^{-2}\text{ h}^{-1}\text{)} \quad (6.7)$$

Nomenclature:

COD	Chemical oxygen demand
$C_{x,P}$	Biomass density based on available P in urine, g-dw L^{-1}
D	Dilution rate (h^{-1})
HRT	Hydraulic retention time (hour)
$m_{E,x}$	Maintenance coefficient, mol-photons g-dw^{-1} h^{-1}
N	Nitrogen
$N_{content}$	Nitrogen content of microalgae biomass, g-N g-$biomass^{-1}$
$N_{removal}$	Nitrogen removal rate, mg-N m^{-2} h^{-1}
P	Phosphorus
P_{area}	Productivity per unit ground area, g-dw m^{-2} d^{-1}
PBR	Photobioreactor
$P_{content}$	Phosphorus content of microalgae biomass, g-P g-$biomass^{-1}$
$P_{removal}$	Phosphorus removal rate, g-P m^{-2} h^{-1}
PAR	Photosynthetic active radiation (400-700 nm)
PFD	Photon flux density within PAR range, µmol-photons m^{-2} s^{-1}
PFD_{in}	Supplied light intensity, µmol-photons m^{-2} s^{-1}
F_W	Wastewater flow rate, m^3 h^{-1}
$r_{E,x}$	Specific light supply rate, mol-photons g^{-1} h^{-1}
SVR	Reactor surface area per reactor volume, m^{-1}
TN	Total nitrogen
TP	Total phosphorus
$Y_{x,E}$	Biomass yield on light energy, g-dw mol-$photons^{-1}$
μ	Specific growth rate, h^{-1}

Summary

Discharge of wastewaters and waste streams still containing considerable amounts of the nutrients nitrogen (N) and phosphorus (P) has several negative effects on the environment. At the same time these nutrients are essential elements for all living organisms and thus also required for food production. Because of the growing world population and the related growing demand for food, the coming decades a scarcity in particular of P can be expected. Thus, the global nutrient cycle should become more closed. Nutrient recovery, rather than removal from wastewaters as by conventional sanitation and wastewater treatment processes, presents the most logic and viable option to achieve this objective.

Chapter 1 gives an overview of the importance of N and P and their impact on the environment. Regarding wastewaters as resources, new source separation based sanitation concepts have gained more and more attention and have shown a high potential for recovery, not only of nutrients but also of organic matter, energy and water. Several of such new sanitation concepts projects were implemented in countries such as the Netherlands, Germany, Sweden and China. The different wastewater streams produced in household are characterized by different resource concentrations. Source separated black water generated from toilets contains high concentrations of organic compounds and nutrients, but also pathogens, pharmaceuticals and estrogens. Urine, the major source of nutrients in black water, can be further separated from toilet water. This is considered important as the N, P and potassium (K) content in urine alone could already cover 24, 20, and 32% of the world's artificial fertilizer consumption for these respective nutrients. Among the available nutrient recovery techniques from urine, no single technique can effectively recover both N and P. In contrast, because of the high N:P ratio of microalgae biomass, microalgae cultivation could be a promising technique for their simultaneous recovery from urine. Until now, only a few studies utilizing concentrated urine for microalgae cultivation were conducted. In particular,

because of the high N concentrations and a change in urine composition during hydrolysis of urea to ammonia, the microalgae cultivation system needs to be carefully designed. We selected a short light-path photobioreactor as a suitable system to investigate and further optimize microalgal biomass production and nutrient recovery from human urine.

In **Chapter 2**, microalgae growth was determined in small microplates under light-limited conditions, using minimally diluted urine (dilution factors of 1-20). *Chlorella sorokiniana* was chosen as a microalgal species with it's a high growth rate in urine. The highest specific growth rate of *C.sorokiniana* of 0.158 h^{-1} was obtained with male urine and with supplemental amounts of several trace elements (B, Cu, Fe, Mn, and Zn). These trace elements usually are present at low concentrations in urine and/or precipitate as a result of an increasing pH during urea hydrolysis. Also magnesium (Mg^{2+}) precipitates (as struvite) during urine hydrolysis. Addition of magnesium, an important element for microalgae growth, therefore was found to be necessary to stimulate microalgae growth. For fresh urine addition of Mg^{2+} was not required and it supported high specific growth rates (0.80-0.116 h^{-1}). Although both urea and ammonium can be utilized by microalgae as nutrient sources, growth with ammonium (in hydrolyzed urine) was inhibited at concentrations above 1,400 mg-NH_4^+-N L^{-1}. In addition, a successful microalgal growth in non-diluted urine was achieved in a simulated short light-path system confirming that this system was an appropriate system for microalgae cultivation in concentrated urine

Successful continuous cultivation of *C.sorokiniana* in minimally diluted urine is described in **Chapter 3**. Synthetic urine and different batches of human urine were utilized for microalgae cultivation coupling biomass production and urine treatment in a photobioreactor with a light-path of 5-10 mm. This reactor was operated for more than 250 days. The culture was continuously illuminated and operated at an optimal temperature of 38°C and neutral pH. The system was operated at a fixed hydraulic retention time (HRT) of 1 day. With decreasing urine dilutions and concomitantly increasing light intensity, the biomass density and biomass productivity could be enhanced. In urine with a low degree of hydrolysis, the highest biomass productivity at 5 times diluted urine was 8.6 g-dw L^{-1} d^{-1} at a light intensity of 1500 μmol-photons m^{-2} s^{-1} and with supplemental trace elements. The total N and P removal efficiencies were 70% and more than 99%, respectively. This corresponds

to removal rates of 1,125 mg-N L^{-1} d^{-1} and 99 mg-P L^{-1} d^{-1}. In hydrolyzed urine, supplemental magnesium is needed. The optimum magnesium supply was 1.5 - 1.8 mg-Mg^{2+} g-biomass^{-1}. The optimal urine N:P ratio was found to be 23:1. Under these conditions, the biomass productivity was 14.8 g L^{-1} d^{-1} and 81% total N and 97%-total P could be removed. This corresponds with removal rates of 1,300 mg-N L^{-1} d^{-1} and 150 mg-P L^{-1} d^{-1}.

During operation it was observed that microalgae growth on human urine resulted in a production of aerobic bacterial growth on the organic pollutants contained in urine. The removal efficiency for these compounds (expressed as COD; Chemical Oxygen Demand) depended upon the readily biodegradable COD fraction. The highest COD removal obtained in a 1-day HRT reactor was approximately 70%, corresponding to a removal rate of 1.35 g-COD L^{-1} d^{-1}. In spite of this aerobic heterotrophic bacterial growth, calculations showed that microalgae still dominated the microbial culture and that nutrient removal was mainly realized by their uptake by the microalgae.

Microalgae biomass productivity can be maximized by optimizing the reactor dilution rate or HRT (**Chapter 4**). Biomass productivity, biomass yield on light and nutrient removal rates of *C.sorokiniana* were determined at various dilution rates ranging from 0.04 to 0.24 h^{-1}. A photobioreactor with a light-path of 10 mm was exploited with 2-sided illumination at 1,530 µmol-photons m^{-2} s^{-1} to simulate a 5-mm light-path photobioreactor. With synthetic urine all of these performance parameters increased with increasing dilution rates. A maximum performance was obtained at dilution rates between 0.10 to 0.15 h^{-1}. At higher dilution rates system performance decreased. At the optimum dilution rates a high biomass yield on light of 0.98 g-dw mol-PAR photons^{-1} was achieved. This illustrates a good photosynthetic efficiency, even at high light intensities by which photoinhibiton could affect microalgae growth. The highest biomass productivity and nutrient removal rates were 1.1 g-dw L^{-1} h^{-1} (corresponding to 5.3 g-dw m^{-2} h^{-1}), 104-123 mg-N L^{-1} h^{-1}, and 14-19 mg-P L^{-1} h^{-1}, respectively Utilizing non-diluted human urine at these dilution rates resulted in considerably lower biomass productivities and nutrient removal rates, most likely because of the higher ammonia concentrations in the reactor. At a dilution rate of 0.05 h^{-1}, all the performance parameters with human urine were higher than those with synthetic urine. This clearly shows that minimizing ammonia toxicity is one of the key issues for optimizing

microalgae cultivation on concentrated human urine. Urine pre-dilution might be adopted to reduce this ammonia toxicity.

Utilizing artificial light for microalgae is energy consuming and only worthwhile for production of high value compounds. Economical microalgae biomass production with a lower value is only possible with outdoor utilizing sun light. In **Chapter 5**, *C.sorokiniana* therefore was cultivated under simulated outdoor conditions by applying day/night cycles at two different HRTs representing fast-growing (0.46-day HRT) and slow-growing microalgae (2.6-day HRT). In the 0.46-day HRT reactor, microalgae specific growth rate, areal biomass productivity and biological nutrient uptake rates were high during the daylight period. A low biomass density at this HRT resulted in a high light supply per microalgae cell and high microalgae specific growth rate of 0.23-0.30 h^{-1} at midday. Microalgae nutrient uptake rates during this period were as high as 430 mg-N m^{-2} h^{-1} and 85 mg-P m^{-2} h^{-1}. Significant fluctuations of the protein and starch content of the biomass were also observed. Starch content, known as an energy reserve, significantly increased during high light period and it decreased at the second half of the day period concomitantly with a substantial increase in protein content. In the 2.6-day HRT reactor the highest specific growth rate was 0.06 h^{-1}. A stable areal biomass productivity and P uptake rate were observed over the day, but the trend of microalgae the N uptake rate was not very clear. Starch, protein and total fatty acids were mostly stable in this reactor. In none of the reactors, growth and nutrient uptake took place during the night. The net daily areal productivities in the 0.46- and 2.6-day HRT reactors were 33.5 and 30.0 g-dw m^{-2} d^{-1}, respectively. At 2.6-day HRT, the performance under continuous illumination was also determined at a daily light supply equivalent to that under the simulated day/night cycle. The biomass yield on light with continuous illumination gave a 23% higher biomass productivity than with the simulated day/night cycles. To maximize the net biomass productivity and the nutrient removal efficiencies the results with a short and long HRT reactor indicate that a microalgae cultivation system should be operated at a short HRT (high dilution rate) during the day, whereas a long HRT or even batch operation should be applied during the night .

The performance and feasibility of a microalgae cultivation system utilizing human urine as the growth medium are discussed in **Chapter 6**. Microalgae cultivation has shown a

strong potential for urine treatment in comparison to most other techniques that already are commercially available. A model system was evaluated for Dutch conditions using parameter values form our own experiments and literature parameter values. This demonstrated that in the Netherlands microalgae growth is possible from late spring to late summer, but that during the rest of the year light availability is too low to support microalgae growth. This implies that unless the light-path of photobioreactors can be further shortened and urine dilution is applied, combined microalgae cultivation and urine treatment will be restricted to areas with high irradiances. Alternatively, urine storage could be applied for the winter period. Other challenges for urine treatment by microalgae are finding a cheap CO_2 supply and the cost-effective and energy-efficient scale up of photobioreactors.

Samenvatting

Afvalwater en afvalstromen bevatten meestal een aanzienlijke hoeveelheid van de nutriënten stikstof (N) en fosfaat (P), hetgeen verschillende negatieve effecten op het milieu kan hebben. Tegelijkertijd zijn deze nutriënten essentieel voor alle levende organismen en dus ook onmisbaar voor de productie van voedsel. Vanwege de groeiende wereldbevolking in de komende decennia, en de daaraan gerelateerde stijgende vraag naar voedsel, wordt een schaarste van vooral fosfaat verwacht. Het is daarom erg belangrijk om de mondiale nutriënten kringloop te sluiten. Het terugwinnen van nutriënten, in plaats van de verwijdering met afvalwaterbehandelingsprocessen, is de meest logische en realistische manier om dit doel te bereiken.

Hoofdstuk 1 geef een overzicht van het belang van stikstof en fosfaat en hun effect op het milieu. Nieuwe, brongerichte sanitatieconcepten krijgen meer en meer aandacht en laten een groot potentieel zien voor het terugwinnen van nutriënten, organische stof en water, en voor de productie van energie. Verschillende van deze nieuwe sanitatieconcepten zijn geïmplementeerd in landen zoals Nederland, Duitsland, Zweden en China. De verschillende afvalwaterstromen die huishoudens produceren worden gekarakteriseerd door verschillende concentraties van vervuilende stoffen waaronder de nutriënten stikstof en fosfor. Zwart water van toiletten bevat hoge concentraties aan organische bestanddelen en nutriënten, maar ook pathogenen, geneesmiddelen en oestrogenen. Urine, de voornaamste bron van nutriënten in zwart water, kan gescheiden worden van de vaste bestanddelen in toilet water. Dit is van belang omdat het aandeel van stikstof, fosfor en Kalium in urine alleen al respectievelijk 24, 20, en 32% van de wereldwijde consumptie van kunstmest voor zijn rekening neemt.

Van de beschikbare terugwinningstechnieken voor urine is er geen één die zowel stikstof als fosfor kan terugwinnen. Dat is wel mogelijk door algen te kweken op urine, vanwege de hoge N:P ratio in de algen biomassa. Tot nu toe zijn er maar een beperkt aantal studies uitgevoerd gericht op het gebruik van urine voor het kweken van microalgen. Dit kan vooral verklaard worden doordat de hoge stikstof concentraties en de hydrolyse van ureum naar ammonia in de urine. Dit maakt het ontwerp en de procesvoering van een algen kweeksysteem erg lastig. Wij selecteerden een fotobioreactor met een korte lichtweg als een geschikt systeem voor de productie van microalgen op urine om zo de biomassaproductie als wel het terugwinnen van nutriënten uit urine te kunnen maximaliseren.

In **Hoofdstuk 2** is de groei van microalgen bepaald in zogenaamde microtiterplaten onder licht gelimiteerde omstandigheden, gebruik makend van minimaal verdunde urine (verdunningsfactoren van 1-20). De groene microalg *Chlorella sorokiniana* werd geselecteerd omdat deze de hoogste groei ratio op urine liet zien. De hoogste groeisnelheid van *C.sorokiniana* was 0.158 uur^{-1} en werd verkregen met mannelijk urine waaraan een hoeveelheid micronutriënten (B, Cu, Fe, Mn en Zn) was toegevoegd. Deze micronutriënten zijn gewoonlijk al in lage concentraties aanwezig in urine en/of in het bezinksel dat ontstaat door de toename van de pH tijdens de hydrolyse van ureum. Tijdens dit hydrolyseproces slaat ook magnesium (Mg) neer als struviet. Toevoegen van extra magnesium, een belangrijk element voor de groei van microalgen, bleek noodzakelijk te zijn voor een snelle groei van de microalgen. Bij verse, niet gehydrolyseerde urine was de toevoeging van Mg niet vereist en werden hoge groeisnelheden waargenomen (0.80-0.116 uur^{-1}). Alhoewel zowel ureum en ammonium door microalgen kunnen worden gebruikt als bron van nutriënten, werd de groei op ammonium (in gehydrolyseerde urine) geremd door concentraties boven de 1400 mg-N L^{-1} aan ammonium. Daarnaast is aangetoond dat microalgen kunnen groeien op onverdunde urine in een microtiterplaat waarin een korte lichtweg werd nagebootst. Dit bevestigde dat een fotobioreactor met een korte lichtweg een zeer geschikt systeem is voor het kweken van microalgen op geconcentreerde urine.

De succesvolle, continue kweek van *C.sorokiniana* op minimaal verdunde urine is beschreven in **Hoofdstuk 3**. Synthetische urine en verschillende batches menselijke urine

werden gebruikt voor de combinatie van microalgenkweek en behandeling van urine. Hiervoor werd een fotobioreactor gebruikt met een lichtweg van 10 mm. Deze reactor was meer dan 250 dagen in bedrijf. De algen cultuur werd continu belicht, bij een optimale temperatuur van 38°C en een neutrale zuurgraad (pH). Het systeem werkte met een vaste hydraulische verblijftijd van één dag. Met een afnemende verdunning van de urine en een toenemende lichtintensiteit kon de biomassadichtheid en biomassaproductie worden verhoogd. In 5 keer verdunde urine met een lage mate van hydrolyse werd een biomassa productiviteit van 8.6 g-dw L^{-1} d^{-1} bereikt bij een lichtintensiteit van 1500 µmol-fotonen m^{-2} s^{-1}. De totale stikstof- en fosfor verwijderingsefficiënties waren respectievelijk 70%, en meer dan 99%. Dit correspondeerde met verwijderingssnelheden van 1125 mg-N L^{-1} d^{-1} en 99 mg-P L^{-1} d^{-1}. Met gehydrolyseerde urine was extra magnesium nodig. De optimale magnesium toediening was 1.5 tot 1.8 mg-Mg^{2+} g-$biomass^{-1}$. De optimale N:P ratio was 23:1. Onder deze omstandigheden was de biomassaproductie 14.8 g L^{-1} d^{-1} waarbij 81% van de stikstof, en 97% van de fosfor kon worden verwijderd. Dit komt overeen met verwijderingssnelheden van 1300 mg-N L^{-1} d^{-1} en 150 mg-P L^{-1} d^{-1}.

Tijdens de productie van de microalgen vond ook groei plaats van aërobe bacteriën op de organische verontreinigingen aanwezig in de urine. De verwijderingsefficientie voor deze verontreinigingen (uitgedrukt als CZV; Chemisch Zuurstof Verbruik) is afhankelijk van hun biologisch afbreekbaarheid. De hoogste CZV verwijdering, bereikt in een reactor met een verblijftijd van 1 dag was ongeveer 70%, hetgeen overeenkomt met een verwijderingssnelheid 1.35 g-CZV L^{-1} d^{-1}. Berekeningen toonden aan dat, ondanks de aanwezigheid van aërobe chemoheterofe groei, de fotoautrofe microalgen nog steeds domineerden en de nutrietenverwijdering voornamelijk door de algen werd gerealiseerd.

De biomassa productiviteit van de fotobioreactor voor de algenkweek kon worden gemaximaliseerd door het optimaliseren van de verdunningssnelheid (**Hoofdstuk 4**). Biomassaproductiviteit en biomassaopbrengst op licht van *C.sorokiniana,* en de hoeveelheden verwijderde nutriënten zijn bepaald bij verschillende reactor verdunningssnelheden, variërend van 0.04 tot 0.24 1/uur. Hiertoe werd een fotobioreactor met een lichtweg van 10 mm gebruikt met een belichting aan 2 kanten met 1530 µmol-fotonen m^{-2} s^{-1}. Op deze manier werd een fotobioreactor met een 5-mm lichtweg gesimuleerd. Met synthetische urine namen alle eerder genoemde prestatieparameters toe bij toenemende

verdunning. De beste prestatie werd bereikt bij een verdunningsfactor tussen de 0.10 en 0.15 1/uur. Bij hogere verdunningssnelheden namen de prestaties weer af. Bij de optimale verdunningssnelheid werd een hoge biomassaopbrengst op licht verwezenlijkt van 0.98 g-dw mol-PAR fotonen^{-1}. Dit geeft aan dat de efficiëntie van de fotosynthese hoog was, zelfs bij hoge lichtintensiteiten die de microalgengroei negatief kunnen beïnvloeden. De hoogste biomassaproductiviteit en nutriënten verwijderingssnelheden waren 1.1 g-dw L^{-1} uur^{-1} (overeenkomend met 5.3 g-dw m^{-2} uur^{-1}), 104-123 mg-N L^{-1} uur^{-1}, en 14-19 mg-P L^{-1} uur^{-1}. Niet verdunde humane urine resulteerde in aanzienlijk lagere biomassaproductiviteit en verwijderingssnelheden bij een verdunningssnelheid van 0.1 1/uur, hoogstwaarschijnlijk veroorzaakt door de hogere ammonia concentraties in de fotobioreactor. Bij een verdunningssnelheid van 0.05 uur^{-1}, waren alle prestatieparameters met humane urine hoger dan die met synthetische urine. Dit laat duidelijk zien dat het vermijden van ammoniatoxiciteit een van de voornaamste punten is bij het optimaliseren van microalgenkweek op geconcentreerde menselijke urine. Vooraf verdunnen van urine is hiervoor een van de mogelijkheden.

Gebruik van kunstlicht voor de kweek van microalgen is energie intensief en alleen geschikt voor de productie van waardevolle componenten. Biomassa productie met een lage waarde is economisch alleen mogelijk door gebruik te maken van zonlicht. Daarom is in **Hoofdstuk 5** *C.sorokiniana* gekweekt onder gesimuleerde buitencondities door dag/nacht cycli toe te passen bij twee verschillende hydraulische verblijftijden die snel groeiende algen (0.46-dagen verblijftijd) en langzaam groeiende algen (2.6-dagen verblijftijd) opleveren. Bij 0.46-dagen verblijftijd waren de specifieke groeisnelheid van de microalgen, de biomassaproductiviteit en de nutriëntenopname hoog gedurende de dag. De relatief lage biomassadichtheid bij deze verblijftijd resulteerde in een hoge lichtdosering per microalgencel en een hoge specifieke groeisnelheid van 0.23-0.30 uur^{-1} rond het middaguur. De nutriënten opnamesnelheden gedurende deze periode waren hoog, tot wel 430 mg-N m^{-2} uur^{-1} en 85 mg-P m^{-2} uur^{-1}. Er werden significante fluctuaties van het eiwit- en zetmeel -gehalte in de biomassa waargenomen. Het zetmeel-gehalte, de energiereserve van de algen, nam significant toe tijdens perioden met een hoge lichtintensiteit en nam af gedurende het tweede deel van de dag. Gelijktijdig nam het eiwit-gehalte substantieel af. Bij 2.6-dagen verblijftijd was de hoogste specifieke groeisnelheid 0.06 uur^{-1}. Een stabiele biomassa

productiviteit en fosfor opname werden waargenomen gedurende de dag, maar de stikstof-opname door de microalgen vertoonde geen duidelijke trend. Zetmeel, eiwit en het totaal aan vetzuren waren stabiel in deze fotobioreactor. In geen van de reactoren vond er groei en nutriënten opname plaats gedurende de nacht. De netto dagelijkse productiviteit bij korte en lange verblijftijd waren respectievelijk 33.5 en 30.0 g-dw m^{-2} d^{-1}.

Bij 2.6-dagen verblijftijd werd tevens het rendement bepaald onder continue belichting met een gelijkwaardige dagelijkse lichtdosis in vergelijking tot de gesimuleerde dag/nacht cyclus. De biomassaopbrengst met continue lichttoevoer gaf een 23% hogere biomassaproductiviteit dan de dag/nacht cycli. De resultaten met de korte en lange verblijftijd wijzen erop dat om de biomassa productiviteit te maximaliseren, en om een hoge nutriënten verwijderingsefficiëntie te realiseren, een microalgen kweeksysteem moet werken bij een korte hydraulische verblijftijd (hoge verdunningssnelheid) gedurende de dag. Gedurende de nacht daarentegen moet een lange verblijftijd, of zelfs een batchgewijs proces worden toegepast .

De uiteindelijke prestaties en haalbaarheid van een microalgenkweeksysteem dat gebruik maakt van menselijke urine als groei medium worden besproken in **Hoofdstuk 6**. Microalgenkweek heeft een groot potentieel voor de behandeling van urine in vergelijking met de meeste andere technieken die op dit moment al commercieel beschikbaar zijn. Een model system voor Nederlandse omstandigheden werd geëvalueerd, gebruik makend van parameterwaarden uit de experimenten beschreven in dit proefschrift als wel uit de literatuur. Dit heeft aangetoond dat in Nederland microalgenkweek mogelijk is van de late lente tot laat in de zomer. Maar gedurende de rest van het jaar is de hoeveelheid beschikbaar licht te laag om de groei van microalgen mogelijk te maken. Dit impliceert dat, tenzij de lichtweg van fotobioreactoren verder verkort kan worden en verdunning van urine wordt toegepast, de combinatie van microalgenkweek en urinebehandeling beperkt zal zijn tot gebieden met hogere lichtinstraling. Een alternatief is om de urine op te slaan gedurende de winterperiode. Andere uitdagingen voor de behandeling van urine door microalgen zijn het vinden van een goedkope bron van CO_2 en een kosteneffectieve en energie-efficiënte opschaling van fotobioreactoren.

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Kanjana Tuantet

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About the author

Kanjana Tuantet was born on July 20, 1974 in Nakhonratchasima province, Thailand. She completed high school at Suranaree Wittaya in Nakhonratchasima in 1992. In 1996, she obtained her bachelor's degree in environmental engineering, Khon Kaen University. She started working as a consultant in a private company before starting her master's degree in environmental engineering, Chulalongkorn University. Her master thesis was focusing on comparison between nickel barring sludge stabilization using lime and solidification using lime and lignite fly ash with the aim to encapsulate Ni-waste meanwhile produce usable materials. She completed her master's degree in 2000.

After finishing her master's degree, she worked at Thailand Environment Institute before starting her new job as a researcher in Thailand Institute of Scientific and Technological Research (TISTR) in 2001. While working at TISTR, she was granted a Royal Thai Government scholarship from the Ministry of Science and Technology to study in the Netherlands. In 2009 she completed her second master's degree in environmental technology, Wageningen University. Her master thesis entitled 'Electricity generation by *Glyceria maxima* in a P-MFC applying different nutrient strategies' focused on enhancing electricity production from plant microbial fuel cell. Soon after finishing her master study, she started a PhD program in Sub-Department of Environmental Technology, Wageningen University. At the end of 2013, she went back to Thailand to continue her work at TISTR before returning to Wageningen to finish her PhD in 2015.

Publications

Tuantet, K., M. Janssen, H. Temmink, G. Zeeman, R. Wijffels and C. N. Buisman (2013). "Microalgae growth on concentrated human urine." J Appl Phycol: 1-11.

Tuantet, K., H. Temmink, G. Zeeman, M. Janssen, R. H. Wijffels and C. J. N. Buisman (2014). "Nutrient removal and microalgal biomass production on urine in a short light-path photobioreactor." Water Res 55(0): 162-174.

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The SENSE Research School declares that **Ms Kanjana Tuantet** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 43.3 EC, including the following activities:

SENSE PhD Courses

- Basic statistics (2010)
- Environmental research in context (2010)
- Advanced course on environmental biotechnology (2012)
- Research in Context Activity: Organising Mini-symposium 'A role of microalgae in nutrient removal within new sanitation concepts', Wageningen (2015)

Other PhD and Advanced MSc Courses

- Techniques for Writing and Presenting a Scientific Paper (2009)
- 5th International advanced course 'Bioreactor Design and Operation' (2010)
- Scientific Writing (2011)
- Presentation Skills (2011)
- 1st International course 'Microalgae Process Design: from Cells to Photobioreactors' (2013)
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- Supervising five MSc students with thesis entitled 'possibilities to apply algae treatment technology for domestic waste water treatment in Bandung, Indonesia' (2010); 'Nutrient recovery from separated waste water streams by algae' (2010), 'Hydrolysed-urine pre-treatments for microalgae cultivation' (2011); 'Nutrient recovery and microalgal biomass productivity in a continuous system' (2012); 'Effect of day-night cycles on microalgal productivity and nutrient removal from human urine' (2013)
- Organising the water group meeting within the Sub-department of Environmental Technology, Wageningen University (2011)

Oral Presentations

- *Microalgae cultivation for treatment of human urine within new sanitation concepts.* 8th Asia-Pacific Conference on Algal Biotechnology, 9-12 July 2012, Adelaide, Australia
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