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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 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 L^{-1} d^{-1}. The corresponding nitrogen and phosphorus removal rates were 1300 and 150 mg L^{-1} d^{-1}, respectively. The subsequently produced biomass contained 43–53% w/w proteins and 16–25% w/w total fatty acids.

1. Introduction

Innovative, decentralised 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 conducted in several countries such as an office building of 60 employees (Sneek, the Netherlands), the

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Table 1 – Composition of synthetic urine and different batches of human urine.

Composition (g L^{-1})	Synthetic urine[a]	Human urine		
		Batch A	Batch B[b]	Batch C[b]
Total nitrogen (TN)	4326	7167	4358	5310
Phosphate phosphorus (PO_4^{3-}–P)	255	466	200	260
Ammonium nitrogen (NH_4^+-N)[c]	4006	844	393	4660
Chemical oxygen demand (COD)	–	8349	2886	5160
N:P atomic ratio	38:1	34:1	48:1	45:1

[a] Calculated values based on synthetic urine composition.
[b] Urine mixture of males and females from urine diverting toilet.
[c] Ammonium nitrogen concentration was measured at the beginning of the experiments.

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 has human urine 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 than 100 times (Adamsson, 2000; Dao-lun and Zu-cheng 2006; Yang et al., 2008a,b; 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 (unpublished results). 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 optimising the urine N:P molar ratio and by supplementing magnesium.

2. Materials and methods

2.1. Microorganism and urine media

C. 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 25 °C. 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. (2008a,b) 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_2H-$PO_4.2H_2O$, 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 utilisation. 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 1–90; 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 4 °C during all experiments. The composition of the various urine batches is depicted in Table 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 2, the urine with supplemental iron and micronutrients was diluted 50, 20, 10, 5, 3, and 2 times (1

Table 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 and treatment (Times)	Light intensity (μMol 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)[a]	990	0.89
30–55	2	1800	0.97
56–72	2 (2P)[b]	1800	0.92
Experiment 2: Male urine (batch A) (HRT 1.0 ± 0.02 d^{-1})			
Day 1–12	50	1180	0.91
13–19	20	1180	0.92
20–35	10	1180	0.90
36–44	5	1180	0.90
45–66	5	1500	0.91
Experiment 3: Male and female urine (batch B and C) (HRT 1.0 ± 0.02 d^{-1})			
Day 1–8	10	1050	0.97
9–57	5	1050	0.95
58–64	5	1540	0.94
65–76	2	1540	0.97
77–142	3	1540	0.94
143–170	3 (N:P 36:1 + Mg^{2+}[c])	1540	0.97
171–190	3 (N:P 36:1 + Mg^{2+}[c])	2 × 1550[d]	0.91
191–219	3 (N:P 25:1 + Mg^{2+}[c])	2 × 1550[d]	0.91
220–239	3 (N:P 15:1 + Mg^{2+}[c])	2 × 1550[d]	0.93
240–247	3 (N:P 15:1, +Mg^{2+}[e])	2 × 1550[d]	0.91
248–269	3 (N:P 23:1, +Mg^{2+}[f])	2 × 1550[d]	0.94

[a] Phosphorus concentration was halved.
[b] Phosphorus concentration was doubled.
[c] Magnesium concentration with supplemental magnesium in the final diluted urine was 17.3 mg L^{-1}.
[d] Illuminating both sides of the reactor.
[e] Magnesium concentration with supplemental magnesium in the final diluted urine was 28.5 mg L^{-1}.
[f] Magnesium concentration with supplemental magnesium in the final diluted urine was 21.8 mg L^{-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.

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 strenghten this mixing pattern, the riser and downcomer were separated by a baffle. The air was once again enriched with CO_2. PBR 1 and 2 are exhibited in Fig. 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.

Fig. 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 4 °C 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 35 °C for Experiment 1 and 38 °C for Experiments 2 and 3.

The culture pH was controlled at 7.0 ± 0.2 with 2 M HCl and 2 M 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).

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 utilising 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 condensor 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 2 depicts the applied urine dilutions and light intensities (photon flux density; PFD) during each experiment.

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,

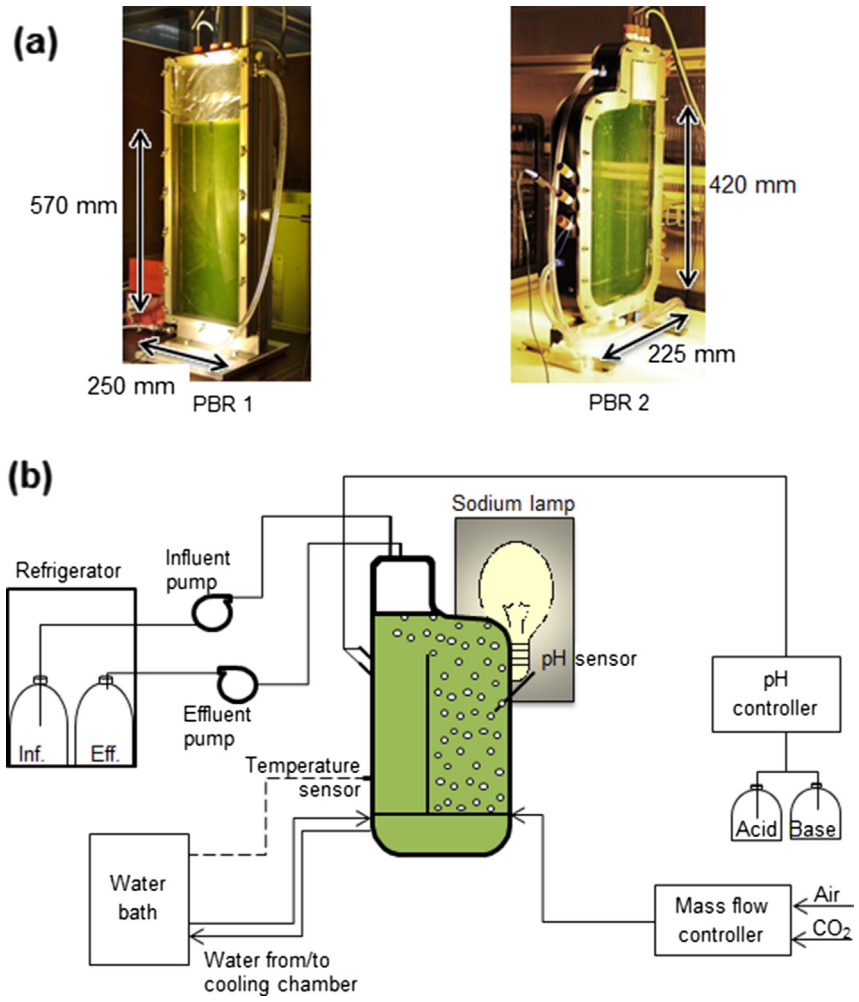


Fig. 1 – (a) Flat panel photobioreactors used for cultivation of *C. sorokiniana* on synthetic urine (PBR 1) and on human urine (PBR 2) and (b) schematic diagram of the complete microalgae culturing system with auxiliary equipment.

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, demineralised water. The diluted samples were subsequently filtered followed by washing with 50 mL of pre-filtered, demineralised water to remove adhering inorganic salts. Filters were dried overnight at 105 °C and cooled in a desiccator for a minimum of 2 h. The dry weight was expressed as the difference in weight of the dry filters and the dry filters containing microalgae.

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 (OD750) was monitored during the settling period. The following equation was employed to calculate the settling efficiency;

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

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 utilising a Mastersizer (Hydro 2000SM, Malvern, AU).

2.5. *Elemental composition of biomass*

Biomass accumulated overnight was harvested by centrifugation at 3620 relative centrifugal force (rcf) for 10 min at 5 °C. The pellet was washed with demineralised 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 analysed in duplicate by combustion at 960 °C using an elemental analyser (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 utilising the ICP-OES

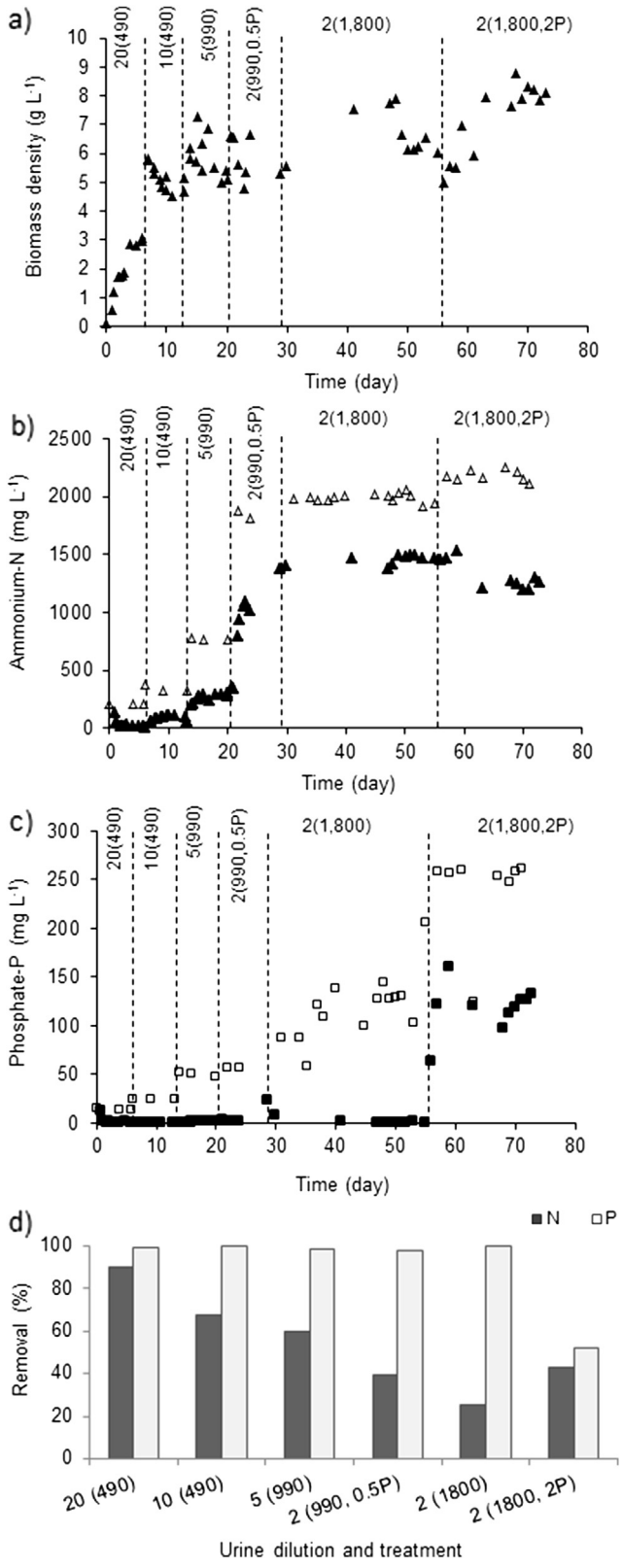


Fig. 2 – Experiment 1: cultivation of *C. sorokiniana* grown on synthetic urine. Time course of (a) biomass density; (b) concentration of influent (△) and effluent (▲) ammonium nitrogen; (c) concentration of influent (□) and effluent (■) phosphate; (d) removal efficiency of nitrogen and phosphorus (*x*-axis shows urine dilution and, in brackets, light intensity, and phosphorus supply).

(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 demineralised water. The O content and dry biomass molar mass were calculated in accordance with Duboc et al. (1999).

2.6. Total fatty acid content of biomass

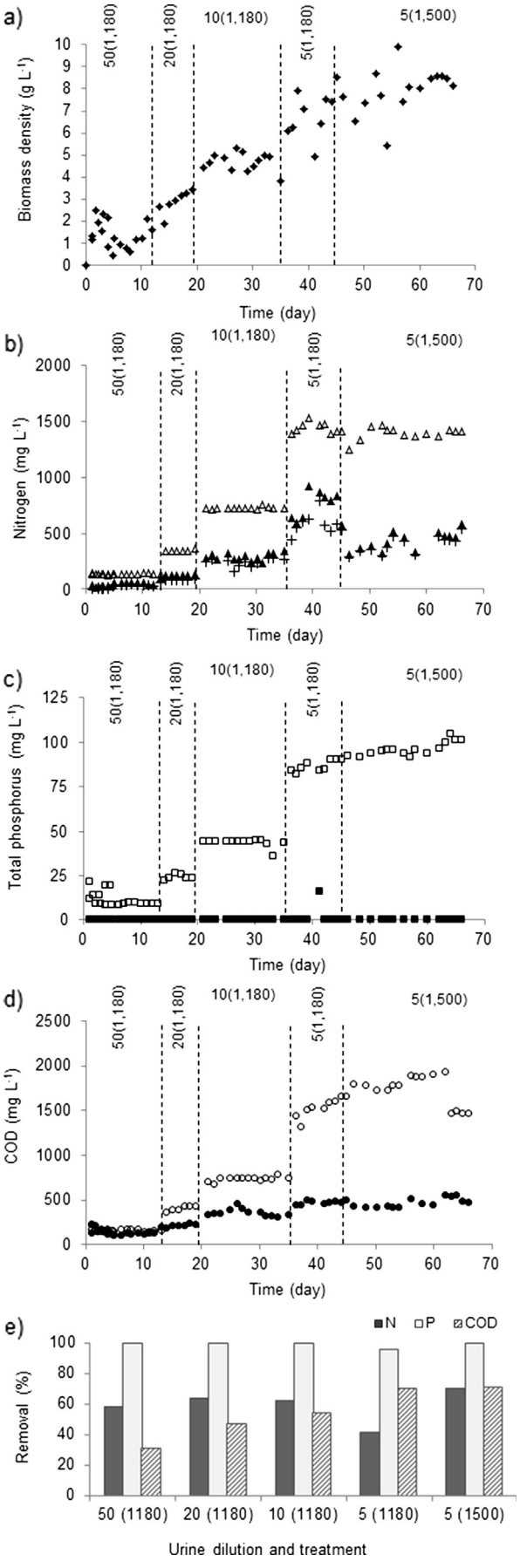
Extraction of total fatty acids was modified in accordance with Lamers et al. (2010). 5–10 mg of ground and freeze-dried biomass was further disrupted by bead beating with a Precellys 24 bead beater (Bertin Technologies, France). Biomass was placed into bead beating tubes (Lysing Matrix E, MP Biomedicals, 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 2500 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).

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 lysisbuffer 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 6500 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 3500 rpm for 10 min. The supernatant was then analysed for protein content utilising 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.

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 4 °C prior to analyses. Synthetic urine samples were analysed for ammonium (NH_4^+–N) and phosphate (PO_4^{3-}–P) according to Standard Methods (APHA, 1998) using a continuous flow analyser (SKALAR). Actual urine samples were analysed 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. Results and discussion

3.1. *pH controling 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 hydrolysed and the urine pH increases up to around pH 9 (Udert et al., 2003b). 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 optimised process conditions and there was still substantial nitrogen not taken up in the effluent. Based on the pH control the alkalinity was directly neutralised requiring acid. But, in an optimised 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.

3.2. *Biomass productivity and nutrient removal*

3.2.1. *Experiment 1:* C. sorokiniana *cultivated in synthetic urine*

In this experiment, *C. 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 2 exhibits the experimental settings during the different experiments, including the applications of urine dilutions and light intensities. Fig. 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

Fig. 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).

density at 20 times diluted synthetic urine and a light intensity of 490 μmol photons m^{-2} s^{-1} was 2.9 g L^{-1} but increased to 6.0 g L^{-1} at 5 times dilution and 990 μmol photons m^{-2} s^{-1}.

During the first time frame when 2 times diluted urine was applied, the phosphorus (as phosphate) concentration was mistakenly halved, resulting in a decrease in biomass density to 5.1 g L^{-1}. With increased light intensity and a corrected phosphate concentration, the biomass density recovered to 6.2 g 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 density increased to 8.1 g 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 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 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 (Fig. 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 phosphorus 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.

3.2.2. *Experiment 2: C. 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, *C. sorokiniana* was cultivated in various dilutions of human urine. Urine concentration is shown in Tables 1 and 2 exhibits the urine dilutions and light intensities that were applied during this experiment. The results in Fig. 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 L^{-1} in 50 times diluted urine at a light intensity of 450 μmol photons m^{-2} s^{-1} to 8.1 g L^{-1} in 5 times diluted urine at 1500 μmol photons m^{-2} s^{-1}. The biomass productivity at 5 times diluted urine was 8.6 g 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 1180 to 1500 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 an intensity of 1180 μmol photons m^{-2} s^{-1} and a biomass density of 6.5 g L^{-1}. With 5 times diluted urine and a light intensity of 1500 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 1125 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 1354 mg COD L^{-1} d^{-1}. Fig. 3(d) and (e) exhibit a development of COD removal during the cultivation period. Although *C. 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 8350 mg COD L^{-1} and the ammonium concentration of 7167 mg N L^{-1} (600 mg N L^{-1} within the reactor) did not negatively effect microalgae growth. The nitrogen removal rate in this study is comparable to that reported by Udert et al. (2003a,b) 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 1000–1300 mg N L^{-1} d^{-1} concomitantly with 82% of COD removal. This study reveals a potential utilisation of microalgae for considerable high nitrogen removal efficiency and complete phosphorus removal. In addition, opportunities remain to enhance the biomass productivity and nutrient removal efficiency.

3.2.3. *Experiment 3: C. 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 maximise the biomass productivity and nutrient removal efficiency. Table 1 shows concentrations of urine batch B and C, and Table 2 presents the urine dilutions and light intensities that were applied. With decreasing urine dilutions

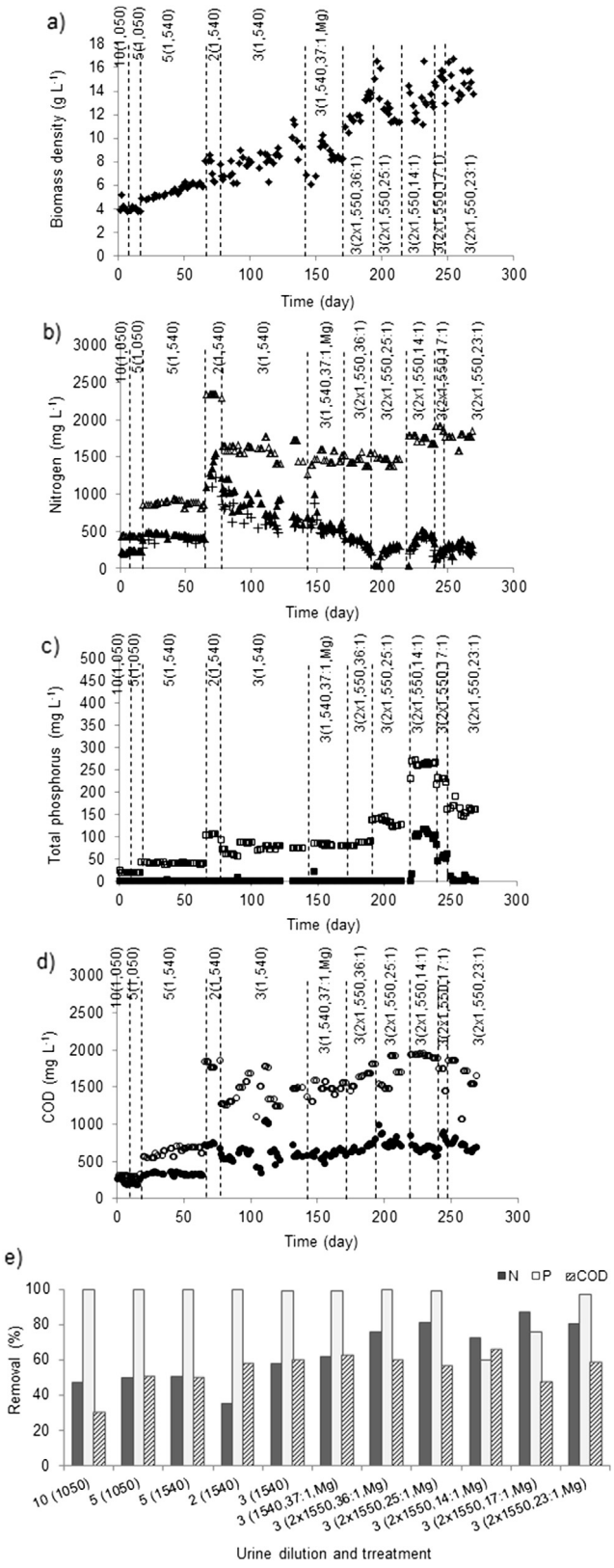


Fig. 4 – Experiment 3: cultivation of *C. sorokiniana* grown on human urine batch B and C. Time course of (a) biomass density; (b) concentration of influent (△) and effluent total nitrogen (▲), and effluent ammonium (+); (c) concentration of influent (□) and effluent (■) total phosphorus; (d) concentration 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).

and increasing light intensities, biomass density increased from 3.8 g L^{-1} in 10 times diluted urine and a light intensity of 1050 μmol photons m^{-2} s^{-1} to 6.6 g L^{-1} in 2 times diluted urine and 1540 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 $NH4^{+}$–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 L^{-1}. The change in dilution was of the urine known as batch B to a new batch C urine.

In the new batch of urine (batch C), urea was already substantially hydrolysed 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 L^{-1}. With batch C and 3 times dilution, several factors were tested to maximise 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 biomass^{-1} to 1.9 and 1.5 mg Mg^{2+} g biomass^{-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 form 9.7 to 9.0 g 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 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 biomass^{-1} raised the biomass density from 12.7 to 15.4 g 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 L^{-1}. The removal of total nitrogen and total phosphorus were 87% and 76%, respectively, corresponding to the removal rates of 1479 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-biomass^{-1}, an increased total phosphorus removal occurred with a relatively high biomass density and

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

Fig. 5 – Nutrient and ionic species balance of microalgae cultivation on hydrolysed urine when the process was optimised.

total nitrogen removal efficiency (Fig. 4(a)and (e)). Although an average N:P ratio of microalgae biomass of 16:1 was mentioned by Ho et al. (2003), the optimum N:P ratio to maximise 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 biomass^{-1} which lies within the range of magnesium required for microalgae in general (0.5–75 mg Mg^{2+} g biomass^{-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 became stable at 3 times diluted urine. Since there was no bacteria 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., 2003a; Kuntke, 2013). The highest COD removal in Experiment 3 was at a N:P ratio of 14:1, i.e. 66% removal or 1137 mg COD L^{-1} d^{-1}. At high biomass densities of 14.5–15.4 g 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. Hydrolysed 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 hydrolysed urine there is the need to neutralise the culture pH. With system optimisation i.e. at a N:P ratio of 23:1, the acid used was minimal. Fig. 5 shows nutrient and ionic species balance of microalgae cultivation on hydrolysed urine when the process was optimised. 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 neutralise the pH in combination with 25.5 mM

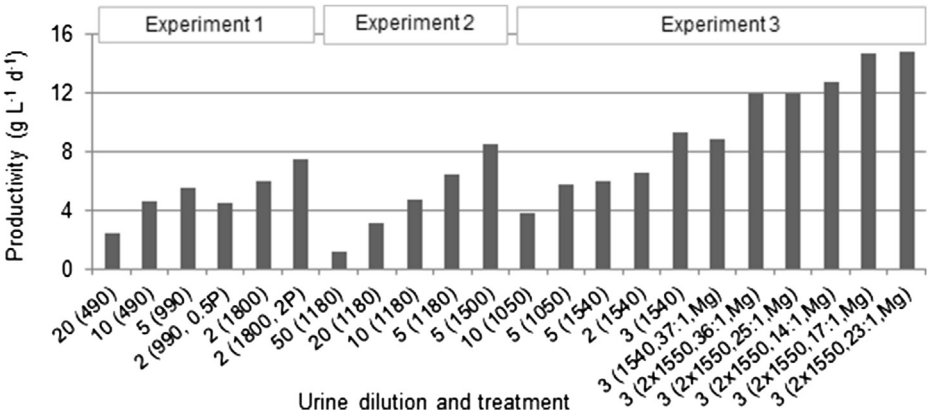


Fig. 6 – Comparison of volumetric biomass productivity of the three experiments (x-axis shows urine dilution and in brackets treatments with different light intensities, phosphorus supply, N:P molar ratio, and additional magnesium).

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) neutralising 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 neutralise the system alkalinity. With optimisation of the urine N:P ratio and enhancing microalgal biomass density, the acid need can be significantly minimised. An additional factor which will help stabilise 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.

3.3. *Volumetric and areal productivity*

Fig. 6 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 L^{-1} d^{-1}, respectively, which corresponds to 74.6 and 93.3 g 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 L^{-1} d^{-1}. The areal productivity with the light path of 5 mm, however, decreased to 74 g m^{-2} d^{-1} due to the doubling of the illuminated area.

Volumetric biomass productivities obtained in other studies employing different types of wastewater were much lower: 0.92 g 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 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 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 L^{-1} d^{-1} for *Spirulina platensis* grown on synthetic urine in a continuous system (Yang et al., 2008b). 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 2100 μmol photon 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 L^{-1} d^{-1} (~120 g 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 L^{-1} d^{-1} (93.3 g m^{-2} d^{-1}) at a light intensity of 1550 μmol photon 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

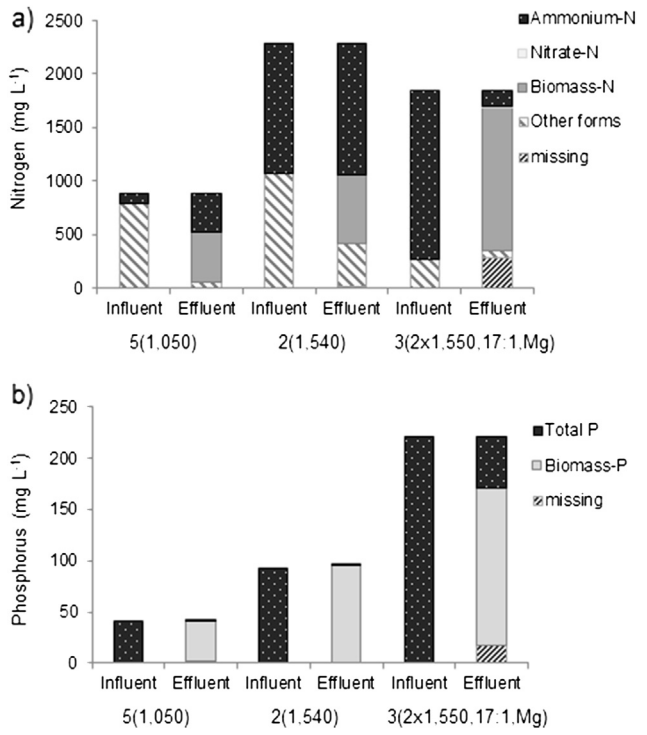


Fig. 7 – Balances of nitrogen (a) and phosphorus (b) at different urine dilutions and treatment of Experiment 3.

under continuous illumination and, hence, a larger illuminated area would be required. Furthermore in the microalgae field, it is well recognised 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).

3.4. *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. Fig. 7 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 algae 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 to 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 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 lipid 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).

3.5. *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 considerred 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 h is required for microalgae grown in urine and 7.7 h 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 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.

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 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. Optimisation by shortening the light path, supplementing magnesium, and optimising the N:P molar ratio enhanced the productivity and nutrient removal to as high as 14.8 g biomass 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.

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